Isolation of Chitin Synthetase from *Saccharomyces cerevisiae*

PURIFICATION OF AN ENZYME BY ENTRAPMENT IN THE REACTION PRODUCT*

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Chitin synthetase, in the zymogen form, was extracted with digitonin from a particulate fraction from *Saccharomyces cerevisiae* and converted into active form by treatment with immobilized trypsin. When the activated enzyme was incubated with UDP-GlcNAc and other components of an assay mixture, a chitin precipitate formed, trapping a large portion of the synthetase. The enzyme was easily extracted from the chitin gel with a recovery of approximately 50% and an enrichment of ~100-fold. Further purification was obtained by repeating the chitin step. After polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the purified synthetase showed a major band corresponding to an *M*₀ of 570,000, a weaker band at *M*₀ 74,000, and some other minor bands. Under non-denaturing conditions, an *M*₀ of 570,000 was calculated for the enzyme from Stokes radius and sedimentation coefficient determinations. After electrophoresis in a nondenaturing gel and incubation with the components of the standard assay, chitin was formed and precipitated in the gel, yielding an opaque band.

Soluble oligosaccharides were not precursors for insoluble chitin, suggesting that synthesis of chitin chains takes place by a processive mechanism. N-Acetylglucosamine stimulated the purified synthetase only slightly and did not participate as a primer in the reaction. The same chain length, somewhat more than 100 units of GlcNAc, was determined in samples of chitin that had been synthesized either *in vivo* or with a membrane preparation or with purified synthetase. These results suggest that chitin synthetase itself is capable both of initiating chitin chains without a primer and of determining their length.

Polysaccharides are major components of the cell wall in many procaryotic and eucaryotic organisms. Several enzymatic activities which participate in the synthesis of structural polysaccharides have been detected but little progress has been made in the purification of the enzymes (1). Consequently, the molecular mechanisms for the synthesis of these important biological products are not as well known as those of other cellular components. Among cell-wall polysaccharides, chitin is one of the most common because of its widespread occurrence in fungi (2). In *Saccharomyces cerevisiae* it has a special role as the major or sole component of the primary septum (3, 4). This structural component of the cell has been used in our laboratory as a model for the study of morphogenesis (4). In the course of those investigations it was discovered that chitin synthetase, a plasmalemma-bound enzyme, is present in the cell as a zymogen and can be converted into an active form by partial proteolysis (5, 6). Recently, we found that chitin synthetase catalyzes a unidirectional synthesis of chitin in the plasma membrane (7). To elucidate the relationship between zymogen and active enzyme and to understand the functioning of the synthetase in the membrane, it was necessary to purify the enzyme. Although chitin synthetase was solubilized with digitonin from both *Coprinus* (8) and *Saccharomyces* (6) several years ago, efforts in our laboratory to further purify the enzyme with many conventional procedures met with failure. Finally, we were able to take advantage of the fact that the solubilized enzyme gives rise to an insoluble product (6). A considerable amount of synthetase is trapped in the chitin formed in the reaction and can be easily eluted from it. This is the crucial step in the procedure for isolation of the enzyme that we describe in this report.

**EXPERIMENTAL PROCEDURES**

**Materials**

Digitonin, trypsin, soybean trypsin inhibitor, bovine liver catalase, urease, apoferritin, phosphorylase α, phosphatidylycerine, and UDP-GlcNAc were purchased from Sigma. Bio-Gel P-2 and P-4 (200–400 mesh) and low-molecular-weight electrophoresis standards were from Bio-Rad. Sephadex G-75, Sephacryl S-300, and activated CH-Sepharose 4B were obtained from Pharmacia. Fluorescein isothiocyanate, wheat germ agglutinin and Zymolyase 60,000 were from Miles Laboratories, whereas diacetylchitobiose and triacetylchitotriose were purchased from V-Labs, Inc., Covington, LA. Cytohelicase (snail intestinal juice) was obtained from Industrie Biologique Francaise, Clichy, France.

UDP-N-acetyl-[14C]glucosamine (291 mCi/mmol) was from Amersham and sodium borotritide (341 mCi/mmol) from New England Nuclear. Chitinase from *Serratia marcescens* (9) and α-macroglobulin (10) were prepared as described previously.

**Preparation of Immobilized Trypsin**—Trypsin (400 mg) was coupled to activated CH-Sepharose 4B according to the manufacturer's directions. After coupling, no trypsin could be detected in the supernatant with the Hide Powder Azure assay (11). The gel was stored at 5°C in 50 mM Tris-chloride, pH 7.5, containing 0.02% sodium azide. Before and after use, the gel was washed twice with 50 mM Tris-chloride, pH 7.5. The same preparation of trypsin-Sepharose could be used at least 10 times for activation of chitin synthetase zymogen without apparent loss of activity. In fact, aged preparations often performed better than fresh ones.
Yeast Chitin Synthetase

**Methods**

**Yeast Strain and Culture Conditions**—The organism was a diploid strain of *S. cerevisiae*, strain *Mato/Mata prbl-1122/prbl-1122* (12), that lacks proteinase B. Cells were grown to a density of 200 liters of minimal medium (2% glucose, 0.7% yeast nitrogen base, Difco) in a fermentor at the pilot plant facility of the National Institutes of Health. Cells were harvested in late logarithmic to early stationary phase, when the absorbance was 0.5. The yield was about 5 g of yeast, wet weight, per liter of culture.

**Cell Disruption and Initial Purification Steps—Operations** were performed at 0–5 °C, unless otherwise indicated. Yeast cells were processed in batches of approximately 0.5 kg. In a typical preparation, 557 g of yeast cells were suspended in Buffer A (20 mM Tris-chloride, pH 7.5, containing 2 mM magnesium acetate) to a final volume of 1200 ml. A 200-ml portion of the suspension was added to 306 g of glass beads (0.5-mm diameter, B. Braun, Melunagen, West Germany) in the vessel of a Bead-Buster (Biospec Products, Bartlesville, OH), with ice cooling. The Bead-Buster was operated for 4 min periods, with a 1-min cooling period in between. Cell breakage was usually about 90%. The extract was aspirated from the glass beads with a long-tipped Pasteur pipette connected to an evacuated filter flask, and the beads were washed several times with small portions of Buffer A. Each portion of beads was used to disrupt three 200-ml batches of cell suspension. The final volume of the extract was 1570 ml.

The crude extract was centrifuged at 100,000 *g* for 30 min. The supernatant liquid was discarded and the pellet was suspended with a Dounce homogenizer in Buffer A to a final volume of 1 liter. After centrifugation, the supernatant liquid was discarded and the pellet was suspended in a solution containing 1% digitonin (w/v), 20 mM Tris-chloride, pH 7.5, 5 mM magnesium acetate, and 0.2 M NaCl, to a final volume of 1600 ml. After homogenization with a Dounce homogenizer, the suspension was incubated at 30 °C for 45 min with shaking, then centrifuged as above. The supernatant liquid, 1480 ml, containing the solubilized chitin synthetase zymogen was stored at −80 °C.

The next step, filtration through Sephadex G-75, was performed with 150 ml of solubilized zymogen each time. The column, 4 × 85 cm, was equilibrated with 20 mM Tris-chloride, pH 7.5, containing 0.1% digitonin; the same buffer was used for elution. The absorbance of the effluent at 280 nm was recorded with an LKB UV-monitor and the large peak emerging at the void volume was collected in a total volume of 220–250 ml.

**Activation of Chitin Synthetase Zymogen with Immobilized Trypsin—** The Sephadex G-75 eluate was mixed with 20–30 ml (settled gel volume) of trypsin-Sepharose 4B and the suspension was incubated with shaking for 30 min at 30 °C. The trypsin-Sepharose was pelleted in a clinical centrifuge and washed with 15 ml of 0.05 M Tris-chloride, pH 7.5. The wash fluid was added to the first supernatant liquid and the crude enzyme was concentrated to 1 ml in an ultrafiltration cell fitted with an Amicon XM-100 membrane.

The amount of immobilized trypsin required for optimal activation was ascertained in scale trials for each batch of zymogen. With some batches, it was necessary to add more digitonin to the zymogen to obtain maximal activation with immobilized trypsin. No such requirement was found for activation with soluble trypsin, suggesting that the zymogen molecules had aggregated and had become partly inaccessible to the trypsin-Sepharose beads. The additional detergent may have been required to break up the aggregates. When additional digitonin was needed, the final concentration was −0.5%.

**Chitin-entrapment Step—** To the bottom of each of four centrifuge tubes for the Beckman SW27 rotor were added 6 ml of a mixture containing 12% glycerol, 6 mM UDP-N-acetylglucosamine, and 4 mM magnesium acetate. To 20 ml of concentrated enzyme from the previous step was added in a 1:1, 3 ml of 50 mM UDP-GlcNac, 1.2 ml of 0.8 M N-acetylglucosamine, and 0.1 ml of 1 M magnesium acetate. After mixing, the solution was immediately layered on the glycerol-containing cushion. The tubes were incubated for 15 min in a 30 °C bath, before being placed on ice for 2.5 h. A preweighed amount of glass beads was added immediately after shaking, and the components of the reaction and became heavy and flocculent subsequently. The tubes were centrifuged in an SW27 Beckman rotor for 25 min at 20,000 rpm (53,000 × *g*). The supernatant fluid was carefully aspirated and the pellets were stored overnight at −80 °C. For extraction of the enzyme from the pellets, the suspension was subjected to 10 min of 200 ml of 20 mM Tris-chloride, pH 7.5, containing 0.1% digitonin and homogenized with a Dounce homogenizer. After incubation for 5 min at 30 °C, the suspension was centrifuged for 10 min at 16,000 × *g* and the supernatant fluid was saved.

When so dispersed, the chitin-purification step was repeated immediately in the same manner as the first one, but halving all quantities and using tubes for the Beckman SW40 rotor. The purified enzyme was stored in polypropylene tubes at −80 °C.

**Chitin Synthetase Assay—** The standard assay mixture contained 30 mM Tris-chloride, pH 7.5, or imidazole acetate, pH 7.7, 3.2 mM N-acetylgalactosamine, 4 mM magnesium acetate, 0.18 mg/ml of phosphatidylerine, 1 mM UDP-N-acetyl['³⁵]C]glucosamine (4 × 10⁶ cpm/μg), and 0.2 mg/ml of bovine serum albumin. The enzyme, purified beyond the chitin-entrapment step, the mixture also contained 0.3 mg/ml of bovine serum albumin and 0.3 mg/ml of digitonin, and incubation was carried out in 1.5-mL Eppendorf polypropylene tubes. Incubation was at 30 °C for 30 min. Usually, the reaction was stopped with 10% trichloroacetic acid and the radioactivity in insoluble material was counted after filtration through glass-fiber filters, as described for β(1→3)glucan synthetase (13). With preparations that contained very little protein, activity was often determined by counting the material that remained at the origin after chromatography of the reaction mixture on Gelman silica-impregnated glass fiber strips (14).

For determination of activity in zymogen preparations, the synthetase was activated with trypsin prior to assay (6).

The formation of soluble products (oligosaccharides) in chitin synthetase zymogen mixtures first adsorbing the reaction substrate on an anion-exchange resin and then determining the soluble radioactivity after filtration through glass-fiber filters (15).

**Polyacrylamide Gel Electrophoresis—** For SDS gels, the procedure of Leemlin (16) was used. The concentration of acrylamide was 4% for the stacking gel and 13.5% for the running gel. The thickness of the gel was 0.75 mm for silver staining and 1.5 mm for Coomasie staining. Electrophoresis in non-denaturing gels was carried out at pH 8.9, with an acrylamide concentration of 3% for the stacking gel and 5% for the running gel, as described by Matzey (17). Gels were stained overnight with a solution containing 0.5% Coomasie Brilliant Blue R, 35% methanol, and 10% acetic acid and destained with 35% methanol containing 10% acetic acid. For silver staining, the method of Merril et al. (18) was used (Silver Stain Kit from Bio-Rad). The Kodak (Eastman Kodak) staining procedure was carried out according to the manufacturer's directions.

**Preparation of Chitin in Vivo and in Vitro—** For the isolation of chitin formed in vivo, yeast cells were disrupted with glass beads in a Bead-Buster as described for enzyme purification but with a 30-ml adapter. From 7.5 g of yeast, wet weight, 40 ml of broken cell walls was obtained. In 50 mM Tris-chloride, pH 7.5, the walls were obtained. The cell walls were sedimented by centrifugation at 3000 × *g* for 10 min and washed four times with water. To an aqueous suspension containing cell walls from 7 g of cells, wet weight, in a total volume of 7 ml, 340 ml of 50 mM Tris-chloride (pH 7.5), and 3 ml of Zymolyase 60,000 (7.5 mg/ml in 0.2 M potassium phosphate at pH 7) were added. The suspension was incubated at 37 °C with shaking and the absorbance at 660 nm was monitored. After 45 min, the absorbance had decreased to about 2% of the original value. Inspection under the phase-contrast microscope showed the presence of only septa and some cell-wall fragments. The suspension was centrifuged at 12,000 × *g* for 10 min. The pellet was washed twice with 40 ml of 1% SDS and three times with 40 ml of water, and finally suspended in water.

For the preparation of chitin by membranes, 5.6 g of yeast cells, wet weight, were disrupted with glass beads in the Bead-Buster (30-ml adapter) as for the purification of chitin synthetase. Cell walls were obtained by centrifugation of the crude extract for 10 min at 3000 × *g*, and membranes were sedimented from the supernatant fluid for 30 min at 100,000 × *g*. The membrane pellet was washed once in 20 mM Tris-chloride, pH 7.5, containing 2 mM magnesium acetate and resuspended in the same buffer to a final volume of 9.5 ml. Of this suspension, 2 ml were treated with trypsin to activate chitin synthetase (6) and then incubated for 15 h at 30 °C with UDP-[¹⁴]C]GlcNAc in a scaled-up chitin synthetase assay mixture (see above; total volume, 40 ml, with sodium azide added to a final concentration of 0.02%). From a parallel incubation with UDP-[¹⁴]C]GlcNAc, 6.4 pmol of chitin, as N-acetylglucosamine, was calculated to have been synthesized. After centrifuging for 10 min at 16,000 × *g*, the pellet was washed twice with 40 ml of 1% SDS and three times with 40 ml of water, and finally suspended in water.

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
was suspended in 20 ml of 1% SDS and the suspension was placed for 5 min in a boiling water bath, followed by centrifugation. The pellet was washed three times with water and resuspended in water.

Chitin synthesized by purified enzyme was that formed in the second chitin purification step of the synthetase. The polysaccharide was used after extraction of chitin synthetase and further washing with water. Each fraction from the column was taken for measurement of radioactivity. For determination of N-acetylglucosamine, 15 μl of NaHPO₄ (1 M) was added to 100 μl of each fraction, followed by 5 μl of Cytohelicase (diluted 3-fold) as a source of β-N-acetylglucosaminidase. The mixture was incubated for 45 min at 30 °C and N-acetylglucosamine was measured colorimetrically (19). Since the reduced trisaccharide and reduced disaccharide by Cytohelicase (data not shown), the specific activity of the reduced trisaccharide, and, thus, the specific activity of N-acetylglucosaminyl could be determined directly. Chain lengths were calculated from the N-acetylglucosaminyl specific activity and a specific activity obtained by dividing total recovered radioactivity by total recovered N-acetylglucosamine. The latter was the sum of the free monosaccharide peak plus the N-acetylglucosamine liberated by Cytohelicase from the disaccharide peak.

The same chain-length values could be obtained by direct measurement of radioactivity and N-acetylglucosamine (after Cytohelicase digestion) in the chitinase digest. In this case, it was necessary to determine the correct specific activity of sodium borotritide, which is known to contain radioactive impurities. For this purpose, triacetylchitotriose was used with excess borotritide. Following Bio-Gel P-2 chromatography, the specific activity of pure reduced trisaccharide was determined by measuring radioactivity and N-acetylglucosamine following Cytohelicase digestion. Since the same lot of borotritide was used for each experiment, the specific activity was found in each case to be the same.

RESULTS

Purification of Chitin Synthetase—In the present study, yeast cells were disrupted with glass beads, because conversion of cells to protoplasts, as used in previous experiments prior to lysis (6), is not suitable for large-scale preparations. The yield of enzymatic activity in a crude extract was somewhat greater than in protoplast lysates. Soluble proteins were removed by centrifugation and the particulate material was extracted with digitonin to solubilize chitin synthetase, (for details, see "Methods"). In early preparations, the crude extract was first centrifuged at low speed to remove cell walls, but this step was later discontinued because in some batches a considerable amount of membrane and of chitin synthetase sedimented with the walls. The digitonin extract was filtered through a Sephadex G-75 column, and the enzyme was recovered in the void volume fraction.

Activation of thezymogen form of the enzyme had usually been performed with trypsin solution followed by addition of soybean trypsin inhibitor. Recently, it was found possible to use immobilized trypsin attached to Sepharose 4B in the activation step. In this way, the activation is terminated by centrifuging the immobilized trypsin, thus avoiding addition of inhibitor. It seems also probable that the use of immobilized trypsin, because of steric constraints, will be less injurious to the enzyme molecules. Activation, however, takes place readily with the immobilized protease, presumably because the bond(s) broken in this process are in an exposed portion of the polypeptide chain.

When the activated enzyme was incubated with UDP-GlcNAc in a standard assay mixture, insoluble chitin appeared as a flocculent precipitate (6). On centrifugation, about half of the chitin synthetase activity sedimented with the chitin and could be recovered after extraction of the precipitate with Triton buffer. Sedimentation of the enzyme was not simply caused by affinity for chitin. Addition to the synthetase preparation of either "regenerated" chitin (20) or enzymatically prepared chitin, followed by centrifugation, did not lead to sedimentation of the enzyme (Table I).

We could not find conditions for washing the chitin pellet without extracting the synthetase. Therefore, the reaction mixture was layered, in a centrifuge tube, on a cushion containing 12% glycerol. During incubation, the flocculated chitin accumulated in the upper part of the tube. During subsequent centrifugation it was partially washed by passage through the glycerol layer. The best incubation conditions for maximal yield were found to be 15 min at 30 °C followed by storage for 2-3 h on ice, which corresponded to a conversion of about two-thirds of the substrate into product.

The chitin pellet obtained after centrifugation had a gel-like appearance. After freezing at ~80 °C overnight, thawing, suspending in buffer, and centrifuging, the pellet appeared to be much smaller. Freezing and thawing of the pellet before extraction increased the yield of chitin synthetase from ~35% to ~50% of the initial activity. The total recovery of activity (extracted pellet + supernatant) was usually around 75%.
Nevertheless, it was difficult to measure accurately chitin synthetase in the supernatant fluid, because of the presence of UDP, an inhibitor (21), and of remaining UDP-GlcNAc which diluted the radioactive substrate added.

Although 0.1% digitonin was routinely included in the buffer used to extract the enzyme from chitin, omission of the digitonin in some experiments did not lead to a substantial change either in the yield or in the purity of the synthetase.

A summary of one of the best preparations is given in Table II. As shown in the table, the chitin-entrapment step can be repeated for further purification. This should be done immediately after extracting the enzyme from chitin in the first step. Storage of the enzyme at -80 °C followed by repetition of the chitin step resulted in very poor recoveries, although the activity had not decreased during storage. In 29 preparations, the average purification in the first chitin-entrapment step was 97-fold. In the 12 preparations in which the second chitin step was carried out, the average purification in that step was 5-fold. Therefore, the average overall purification in both steps combined was about 500-fold, as compared with 630-fold for the preparation of Table 1.

The activity of purified enzyme was quite stable for several months at -80 °C. Upon lyophilization, about 70% of the activity was recovered.

Polyacrylamide Gel Electrophoresis of the Purified Enzyme

**Table I**

| Source of chitin | % of original chitin synthetase activity |
|-----------------|-----------------------------------------|
|                 | Extract from pellet | Supernatant |
| 1. Formed during incubation | 35 | 28 |
| 2. Regenerated chitin (7.5 mg) | 0.5 | 79 |
| 3. Enzymatically synthesized chitin (7.5 mg) | 0.5 | 71 |

**Effect of chitin source on chitin synthetase precipitation**

The procedure was the same as described for the chitin-purification step under "Methods," except that the volume of activated and concentrated G-75-peak enzyme added was 7 ml (total activity, 500 milliunits) and all the other volumes were reduced in proportion. UDP-N-acetylglucosamine was omitted in tubes 2 and 3, which received preformed chitin. The amount of "regenerated" (20) or enzymatically obtained chitin (7.5 mg) was approximately that expected to be formed in reaction mixture 1. The "supernatant" resulted from centrifugation of the synthesized or preformed chitin. The "extract from pellet" was obtained after freezing the chitin pellets overnight, as outlined under "Methods."

**Table II**

| Step                                    | Volume | Total protein | Total activity* | Specific activity | Purification | Recovery |
|-----------------------------------------|--------|---------------|-----------------|-------------------|--------------|----------|
|                                         | ml     | mg            | units/mg        | units/mg protein | -fold       | %        |
| Crude extract                           | 1570   | 69,640        | 292             | 0.004             | 1            | 100      |
| Particulate fraction*                   | 1480   | 18,500        | 274             | 0.015             | 3.5          | 94       |
| Solubilized zymogen                     |        | 10,800        | 164             | 0.015             | 3.5          | 56       |
| Activated and concentrated              | (800)* | (2,500)       | (171)           | 0.068             | 17           | 58       |
| Sephadex G-75 eluate                    |        |               |                 |                   |              |          |
| Chitin entrapment                       |        |               |                 |                   |              |          |
| Step 1                                  | (400)  | (19.5)        | (102)           | 5.2               | 1,300        | 35       |
| Step 2                                  | (200)  | (1.08)        | (46)            | 42.7              | 10,700       | 16       |

*In the first three steps, chitin synthetase activity was measured after activation with optimal amounts of trypsin (6).

*An aliquot of the crude extract was separately centrifuged and the pellet was suspended in Tris buffer for assay.

*Values in parentheses have been recalculated as if the whole preparation had been used in the chitin-entrapment steps. In fact, only about 10% of the total was used each time.

Nevertheless, it was difficult to measure accurately chitin synthetase in the supernatant fluid, because of the presence of UDP, an inhibitor (21), and of remaining UDP-GlcNAc which diluted the radioactive substrate added.

Although 0.1% digitonin was routinely included in the buffer used to extract the enzyme from chitin, omission of the digitonin in some experiments did not lead to a substantial change either in the yield or in the purity of the synthetase.

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| Step 1                                  | (400)  | (19.5)        | (102)           | 5.2               | 1,300        | 35       |
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*In the first three steps, chitin synthetase activity was measured after activation with optimal amounts of trypsin (6).

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Synthetase. The bands: either until the stronger bands appeared (short) silver reagent were run on separate days. The material applied to the gel was an activated and concentrated Sephadex G-75 filtrate staining, is artifactual because it also appeared in a lane where only no more bands become visible (long). The two gels revealed with the weight markers are shown for the first and the third gels. The same gel seem to be caused by the presence of digitonin. Molecular weight markers are shown for the first and the third gels. 92K, 92,000, for example.

**Kinetic Properties of Purified Chitin Synthetase**—The apparent $K_m$ for UDP-GlcNAc was 0.7 mM, compared to values between 0.6 and 1.5 mM, as obtained earlier with crude preparations of the enzyme (6, 21). Both phosphatidylserine and digitonin were required for maximal activity. In the presence of digitonin, lower concentration of phosphatidylserine (0.05 mg/ml) was required than when the phospholipid was added alone; higher concentrations of phosphatidylserine were inhibitory. The dependence on these compounds varied from preparation to preparation. Usually, the enzymatic activity was increased about 3-fold in the presence of both phosphatidylserine and digitonin. In some cases, the enzyme showed practically no activity in the absence of both substances (data not shown).

A somewhat surprising result was that the purified enzyme had lost its dependence on addition of free $N$-acetylglucosamine almost completely (21). Omission of the acetylamino sugar from the reaction mixture decreased the activity by only 13%.

**Formation of Oligosaccharides**—At low concentrations of UDP-GlcNAc, (less than 30 μM) no insoluble chitin was formed (Fig. 4, and inset). Instead, $N$-acetylglucosamine was incorporated into water-soluble products. Chromatography on a Bio-Gel P-4 column and incubation with purified chitinase showed these products to be a series of β(1→4)-linked chito-oligosaccharides ranging from 2 to 8 or 9 sugar units (data not shown). At higher concentrations of substrate, the amount of oligosaccharides sharply decreased and only represented a few per cent of the total reaction product. The formation of oligosaccharides may result from the presence of chitinase activity, either as a contaminant or intrinsic to chitin synthetase itself. Because the hydrolytic enzyme is especially effec-

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**FIG. 2.** SDS-polyacrylamide gel electrophoresis of chitin synthetase. Left, stained with Coomassie Blue; right, stained with silver. Short and long refer to the time allowed for development of the bands: either until the stronger bands appeared (short) or until no more bands become visible (long). The two gels revealed with the silver reagent were run on separate days. The material applied to the gel was an activated and concentrated Sephadex G-75 filtrate staining, is artifactual because it also appeared in a lane where only no more bands become visible (long). The two gels revealed with the weight markers are shown for the first and the third gels. The same gel seem to be caused by the presence of digitonin. Molecular weight markers are shown for the first and the third gels. 92K, 92,000, for example.

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**FIG. 3.** Electrophoresis of purified enzyme on nondenaturing gels. The amount of protein applied to each gel was 3.9 μg (92 milliunits). After electrophoresis, gels were incubated overnight at 30°C in 1.5 ml of standard assay mixture except that for gels A and B, 5 mM unlabeled UDP-GlcNAc was substituted for UDP-[3H] GlcNAc. After incubation, gels A and B were soaked in water for several hours, gel C for 72 h, with several changes. Gel A was photographed directly. Gel B was soaked for 3 h in a solution containing 50 mM phosphate buffer, pH 7.5, and 0.3 mg/ml of fluorescein isothiocyanate-wheat germ agglutinin, then washed in water overnight. The gel was photographed under UV light with a short-wave (maximum at 254 nm) Mineralight R51 lamp. Gel C was cut into 0.15-mm slices with an electrical slicer. To each slice, in a test tube, 1 ml of 30% hydrogen peroxide was added and the tubes were incubated 16 h at 60°C to solubilize the gel. The solution was transferred to a scintillation vial, followed by 10 ml of Hydrofluor (National Diagnostics). The distribution of radioactivity in the gel is shown in C. The radioactivity present at the origin may correspond to enzyme that had aggregated and did not penetrate the gel.

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**TABLE III**

| Hydrodynamic properties of undenatured chitin synthetase |
|---------------------------------|-----------------|-----------------|
| Stokes radius*                   | 75              | 18.4            |
| Sedimentation coefficient*       | 570,000         |

*Measured by chromatography on a Sephacyr-S-300 column (1 x 90 cm). Standards, with Stokes radius in parentheses, were: bovine liver catalase (53), glycogen phosphorylase a dimer (53) and tetramer (66), apo-ferritin (64), and $α$2-macroglobulin (96). The elution profile of chitin synthetase was determined by measuring the enzymatic activity in the column fractions.

† Determined by sedimentation in sucrose gradients (22). Standards, with sedimentation coefficient in parentheses, were: bovine liver catalase (11.2), apo-ferritin (17.6), urease (18.6), and $α$2-macroglobulin (19.5). The position of chitin synthetase was ascertained by measuring the enzymatic activity in the fractionated gradients.

‡ Estimated from the Stokes radius and the sedimentation coefficient with Svedberg's equation (23). The partial specific volume of chitin synthetase was assumed to be 0.725.
tive on the nascent chitin chains manufactured by the synthetase (15, 24) a very small amount might have been sufficient for the observed effect. Alternatively, the oligosaccharides could represent prematurely terminated chitin chains. Addition of chitin to the enzymatic preparation in order to adsorb any putative chitinase (24) had no effect on the production of oligosaccharides. Nevertheless, when a mixture of the labeled oligosaccharides was incubated again with the chitin synthetase preparation in the absence of UDP-GlcNAc, some degradation appeared to occur, as judged from a shift in amount from the higher to the lower members of the series (data not shown). Therefore, a final conclusion about the origin of oligosaccharides cannot be made.

There is no indication that the oligosaccharides are capable of further elongation. Thus, incubation of radioactive tetraacetylchitotetraose with chitin synthetase and unlabeled UDP-GlcNAc did not result in a change in the amount or position of the oligosaccharide upon subsequent column chromatography. Furthermore, preliminary pulse-chase experiments failed to show incorporation of the oligosaccharides into larger compounds.

Length of Chitin Chains—Since preliminary experiments suggested that chitin chains made by purified enzyme carry a free reducing end group, chain lengths were estimated by reduction with borotritide, followed by digestion with purified Serratia chitinase (9) and separation of the products on a P-2 column. Because of the specificity of the chitinase, the end groups were converted into a mixture of reduced disaccharide and reduced trisaccharide. The latter was hydrolyzed by β-N-acetylglucosaminidase to reduced disaccharide and free N-acetylglucosamine that could be measured colorimetrically, thereby allowing determination of the specific activity of the terminal N-acetylglucosaminyl residue with which the enzyme coprecipitates. Simple binding of the synthetase to chitin does not explain the precipitation of the enzyme, because external addition of the polysaccharide was without effect. Rather, it appears that the synthetase remains physically trapped in the highly hydrated and hydrogen-bonded network of chitin chains that it has spun, much as a silkworm is enclosed in its cocoon. This view is supported by the increase in enzyme recovery if the gel is disrupted and partially dehydrated by freezing and thawing. This methodology may be applicable to other polysaccharides when solubilized preparations of these enzymes become available.

Our purified preparations still contain contaminants, notably the band of 74,000 daltons that has been seen in all preparations subjected to electrophoresis. Some heterogeneity may be expected because of partial degradation of the enzyme by the trypsin used in the activation step. Conversely, some of the enzyme subunits may have escaped trypsin action and still be in the zymogen form. These subunits would remain together with those in the active form in the high-molecular-weight aggregate found in nondenatured preparations and would accompany them during further purification. It is indeed possible that the 74,000-dalton band corresponds to enzyme subunits in the zymogen form. Another possibility, that chitin synthetase consists of different subunits, cannot be excluded at the present time.

Our tentative conclusion, that the 63,000-dalton band represents active chitin synthetase, is based on the fact that it is the major band and that it clearly shows enrichment when comparing first and second chitin steps. A final answer on this point will have to await the availability of antibodies to chitin synthetase. Recently, purification of chitin synthetase from Coprinus cinereus, a basidiomycete, was reported by

**Fig. 4. Formation of soluble product at low concentrations of UDP-GlcNAc.** The reaction mixtures were those of the standard assay except for the concentration of substrate that was varied as shown. The formation of chitin or of soluble products was measured as described under "Methods." Notice that the oligosaccharides are represented in a 16-fold expanded scale. The empty circles indicate the values obtained by adding together soluble and insoluble products.

**Table IV**

| Origin             | N-Acetylglucosaminyl residues per end group |
|--------------------|-------------------------------------------|
| In vivo            | 106                                        |
| Membrane-bound synthetase | 139                                      |
| Solubilized and purified synthetase | 116                                      |

two other samples of chitin, one obtained from yeast cell septa and the other synthesized with membrane-bound synthetase. Surprisingly, the same chain length, within experimental error, was measured in all three cases (Table IV). The relative shortness of the chains might be artifactual, if chitin chains were broken under reduction conditions, i.e. 0.01 N alkali. This seems quite unlikely, because of the high stability of chitin to alkali. Furthermore, reduction of tetraacetylchitotriose with borotritide under the same conditions gave rise to a single product with the expected elution position in a Bio-Gel P-4 column. On the other hand, we cannot exclude the possibility that the chains are even shorter than determined. The calculated values of the chain length are based on the assumption that sodium borohydride can react with all the reducing ends in insoluble chitin. This assumption has not been tested.

**DISCUSSION**

Chitin synthetase is the first enzyme, that has been extensively purified, capable of catalyzing the formation of a structural polysaccharide. Our main purification step takes advantage of a unique property of this type of enzyme, that of catalyzing the synthesis of an insoluble product with which the enzyme coprecipitates. Simple binding of the synthetase to chitin does not explain the precipitation of the enzyme, because external addition of the polysaccharide was without effect. Rather, it appears that the synthetase remains physically trapped in the highly hydrated and hydrogen-bonded network of chitin chains that it has spun, much as a silkworm is enclosed in its cocoon. This view is supported by the increase in enzyme recovery if the gel is disrupted and partially dehydrated by freezing and thawing. This methodology may be applicable to other polysaccharides when solubilized preparations of these enzymes become available.

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Montgomery et al. (25). Their preparation also shows some heterogeneity, but the main band corresponds to a molecular weight of ~67,000, which is not very different from our value. There is no evidence that the enzyme from Coprinus can exist in a zymogen form (8).

It is of interest that the purified synthetase had almost completely lost dependence on free N-acetylglucosamine, which stimulated the activity severalfold in membrane preparations and might have been thought of as a possible primer. The only requirements of the purified enzyme were a divalent cation, phosphatidylserine, and digitonin. The last two may be favorable in that they tend to reconstruct an environment similar to that of the natural habitat of the enzyme, the plasma membrane. The synthetase was able to catalyze the formation of chitin after electrophoresis in nondenaturing gels and while still embedded in the gel. This provides a striking illustration of the autonomy of the enzyme. If a primer or a lipid intermediate, for which we have been unable to find evidence (6), were necessary for the reaction, they would have to be very strongly bound to the enzyme for the association to survive after electrophoresis.

The formation of oligosaccharides that takes place at low concentrations of substrate may be due to a trace of chitinase activity present in the preparation but it could also result from premature termination of growing chitin chains. If the first interpretation is correct, the decrease in production of oligosaccharide as the formation of insoluble chitin increases might be due to binding of the higher oligosaccharides to chitin by hydrogen bonding and consequent protection against chitinase action. Alternatively, premature termination of the chains may be prevented at higher concentrations of substrate by an increase in elongation rate. Whatever the interpretation, pulse-chase experiments proved conclusively that the free oligosaccharides are not intermediates in the synthesis of insoluble chitin. This suggests that each chitin chain is synthesized by a processive mechanism (26), i.e. it is not released from the enzyme until completed.

It is remarkable that samples of chitin synthesized in vivo, by a membrane preparation, and by purified synthetase, all have the same chain length. It appears that the enzyme itself, and not other factors or organizational elements present in the membrane or in the intact cell, determines at what point a chitin chain should be terminated and a new one started.

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