Studies on the Purification of Chitin Synthase from *Coprinus cinereus*

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Chitin synthase has been characterized from the stipes of *Coprinus cinereus* by a number of techniques. In the absence of digitonin, on gel filtration columns and during electrophoresis the enzyme showed properties consistent with having molecular weights from $1.5 \times 10^5$ to several million, suggesting its reversible aggregation into large multimolecular units. Gel chromatography in buffers containing digitonin gave highly reproducible results, and when followed by anion-exchange chromatography gave preparations with very high activity [e.g. $3.4 \mu$mol substrate incorporated min$^{-1}$ (mg protein)$^{-1}$] and apparent molecular weight $8.0 \times 10^5$. The best purification (140-fold) was achieved by gel chromatography followed by copper chelate affinity chromatography, giving a nearly pure enzyme preparation of activity $4.7 \mu$mol substrate incorporated min$^{-1}$ (mg protein)$^{-1}$, which showed only one band of molecular weight $6.7 \times 10^4$ on SDS-polyacrylamide electrophoresis. These purified preparations were free of the nucleoside diphosphatase and protease activities that were present in the early stages of purification.

**INTRODUCTION**

The membrane-bound enzyme chitin synthase (uridine diphosphate-\textit{N}-acetylglucosamine:chitin \textit{N}-acetylglucosaminyl transferase, EC 2.4.1.16) has been detected in preparations from a wide range of fungi, where it plays a major role in the biosynthesis of the cell wall (Gooday & Trinci, 1980; Bartnicki-Garcia et al., 1979; Cabib et al., 1979). To date it has not been fully characterized from any source. We present here results of studies aimed at its purification from stipes of the toadstool *Coprinus cinereus* (Schaaff. ex Fries) S. F. Gray. This source was chosen for four reasons: (a) the initial specific activity is very high (Adams & Gooday, 1980); (b) there are kinetic data for the activity of this enzyme (de Rousset-Hall & Gooday, 1975; Gooday, 1977, 1979); (c) the stipe is a specialized tissue, and its elongation involves substantial chitin synthesis (Gooday, 1979); (d) preparations from this source do not require activation by exogenous proteases, in contrast to those from other sources (Cabib et al., 1979).

Characteristics are included of two other types of enzyme activity in the preparations which may play regulatory roles in fungal chitin synthesis: proteases, which may be responsible for endogenous activation of chitin synthasezymogen, and nucleoside diphosphatases which may modulate activity by removing the inhibitory product UDP (de Rousset-Hall & Gooday, 1975; Gooday, 1979).

**METHODS**

*Enzyme preparations.* Growth of the *C. cinereus* and the preparation of the two digitonin-solubilized enzyme extracts were as described before: a solubilized microsomal preparation (Gooday & de Rousset-Hall, 1975); and an initially solubilized preparation (Adams & Gooday, 1980).

*Enzyme assays.* Assays for chitin synthase were as described before (Adams & Gooday, 1980; Gooday & de Rousset-Hall, 1975), except that incubations of column eluates consisted of an 80 \mu$l sample in a total volume of 100 \mu$l containing final concentrations of 50 mM-Tris/HCl pH 7.5, 25 mM-MgCl$_2$, 1 mM-EDTA, 25 mM-\textit{N}-acetylglucosamine, 1 mM-UDP-\textit{N}-acetylglucosamine (10 nCi UDP-[U-\textsuperscript{14}C]GlcNAc), incubated for between 30 and 60 min according to activity. Assays for nucleoside diphosphatase, with UDP as substrate, were as described before (de Rousset-Hall & Gooday, 1975). Assays for proteolytic activities were by measuring rates of increase in absorbance of 405 nm caused by the release of 4-nitroaniline from solutions of chromogenic model peptide sub-

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strates (Kabi Diagnostica, Stockholm, Sweden), as described by Achstetter et al. (1981). Incubation volumes were 100 μl at 37 °C for 30 min, during which time the rate of release of 4-nitroaniline was linear. Ten peptide sequences were tested: S-2484, S-2337, S-2238, S-2302, S-2160, S-2586, S-2288, S-2444, S-2222 and S-2527. Protein contents were measured according to Bradford (1976).

**Electrophoresis.** Polyacrylamide gradient gel electrophoresis used prepared gels PAA 2/16 (Pharmacia) in a Uniscil Gradiapore system (Universal Scientific Ltd, London, UK) at 4 °C. Upper and lower reservoirs were filled with buffer (90 mM-Tris, 80 mM-boric acid, 3 mM-EDTA, pH 8.35), and the system was equilibrated at 125 V for 15 min, and 70 V for 20 min. Solubilized microsomal enzyme preparation (500 μl) plus bromophenol blue (0.05%, w/v)/glycerol (1:1, v/v) (100 μl) was layered on the gel and electrophoresed at 125 V for 1 h. In order to locate enzyme activity a central 4 mm longitudinal section was removed from the gel and sliced into 1 mm portions, each of which was incubated in 100 μl 50 mM-Tris/HCl pH 7.5, 40 mM-MgCl₂, 1 mM-EDTA, 5 mM-N-acetylglucosamine, 4 mM-UDP-GlcNAc (50 mM-UDP-[U-14C]GlcNAc), 0.02% NaN₃, at 25 °C for 24 h. Gel slices were dissolved by immersion for 3 h at 95 °C in hydrogen peroxide (1 ml, 30%, w/v), filtered by Millipore GS filters (pore size 0.22 μm) and washed through with 5 × 1 ml 1% (v/v) Triton X-100 and 15 ml water. Filters were dried and counted for radioactivity (Adams & Gooday, 1980). The remainder of the gel was stained for protein by immersion in 0.2% (w/v) Coomassie Brilliant Blue R250, 10% (v/v) acetic acid, 40% (v/v) ethanol for 60 min at 55 °C, and destained in 15% (v/v) acetic acid, 15% (v/v) ethanol for 30 min at 55 °C followed by washing in water and storage in 7% (v/v) acetic acid. An identical gel was calibrated with the markers catalase, apoferritin, thyroglobulin and α₂-macroglobulin.

SDS-polyacrylamide disc gel electrophoresis was as described by Weber & Osborn (1969), run at 5 mA per gel for 5 h and stained as above. SDS-polyacrylamide slab gel electrophoresis was by the method of Laemmli (1970), modified by silver staining as described by Merril et al. (1981).

**Column chromatography.** Five types of gel matrix were used, all from Pharmacia: Sepharose 4B, Sepharose 6B, Sepharose CL-6B, Sephacryl S-200 Superfine, and Sephacryl S-300 Superfine. Buffers were based on 25 mM-Tris/HCl pH 7.5, with additions where stated. All procedures were at 4 °C. Buffers for gel filtration experiments with Sephacryl columns (86 × 1.6 cm and 56 × 1.6 cm) contained 0-1% (w/v) digitonin (Sigma). The columns were calibrated with a series of molecular weight markers, usually Blue Dextran (Pharmacia), ferritin, catalase, albumin, haemoglobin and RNAase A.

**Anion-exchange chromatography.** A column (10 × 0.8 cm) of DEAE-Sepharose CL-6B was equilibrated with 25 mM-Tris/HCl pH 7.5, 0.01% (w/v) digitonin. Active fractions from the eluate of Sephacryl columns were applied and eluted under gravity flow with 1 ml Tris/digitonin, followed by 20 ml of a linear gradient of 0-0.4 M-NaCl in this buffer.

**Metal chelate affinity chromatography.** The synthesis of the gel matrix was based on the procedure of Porath et al. (1975). Epoxy-activated Sepharose 6B (3 g) was washed, reswollen and incubated for 24 h with constant agitation at 65 °C with 2 g iminodiacetic acid sodium carbonate. A column (1-0 × 10-0 cm) of the product, bis-carboxymethylaminoagarose, was loaded to only two-thirds its length by the passage of a solution of copper sulphate (1 mg ml⁻³). This was to ensure that no Cu²⁺ eluted from the column, as this ion is an inhibitor of enzyme activity (Gooday, 1979). The column was equilibrated with 50 mM-Tris/HCl pH 9.0, 0.1% (w/v) digitonin. A 2 ml sample of a pool of active fractions from a Sephacryl column, concentrated by ultrafiltration, was applied and eluted with the same buffer, at a flow rate of 9 ml h⁻¹. Fractions of 2 ml were collected and those containing chitin synthase activity were dialysed at 4°C against 1% (w/v) ammonium bicarbonate and lyophilized.

**Affinity chromatography.** Agarose-p-aminophenyl-[β-D-N-acetylglucosamine was supplied by P-L Biochemicals, Milwaukee, USA. Sepharose-hexanolamine-UDP was generously donated by Dr B. Burchell, University of Dundee, UK. Sepharose-adipic acid dihydrazide-UDP was prepared by the method of Lamed et al. (1973). Sepharose-nikkomycin was prepared by incubating 350 mg nikkomycin dissolved in 0.1 M-NaOH with 5 ml swollen epoxy-activated Sepharose 6B for 18 h at 40 °C with constant agitation. Any epoxide reactive groups remaining were blocked by soaking the gel in 1 M-ethanolamine for 4 h, and excess ligand and contaminants were removed by washing with several volumes of 50 mM-Tris/HCl 0.5 M-NaCl pH 8.0 and 200 mM-acetate buffer. Nikkomycin was a gift from Bayer AG, Leverkusen, FRG.

A column (0.7 × 6.0 cm) of nikkomycin-Sepharose was equilibrated with several volumes of saline solution [0-9% (w/v) NaCl, 0.1% (w/v) digitonin] and a 5 ml sample of initially solubilized enzyme (dialysed against saline solution) was loaded and eluted with 30 ml of this solution. Affinity elution was then attempted by including 5 ml 2.5% (w/v) UDP-GlcNAc in the eluant.

The other three affinity gel types were stored at 4 °C in equilibrating buffer (100 mM-Tris/HCl pH 7.5, 25 mM-MgCl₂, 1 mM-EDTA) containing 0.02% (w/v) sodium azide. Chromatography was at 4 °C, on 3 ml columns, with 1 ml initially solubilized enzyme preparation applied and washed on with 15 ml of the Tris buffer. The columns were washed through with 9 ml 1 M-UDP, 1 M-UDP-GlcNAc or 1 M-GlcNAc as appropriate, followed by 9 ml 10 mM-UDP, 10 mM-UDP-GlcNAc or 10 mM-GlcNAc. The UDP was removed from eluates by ultrafiltration with a Diaflo DM5 membrane (Amicon Corp., Lexington, USA) followed by chromatography on Sephadex G-25 (Pharmacia).
Chitin synthase from Coprinus cinereus

RESULTS

Purification of chitin synthase

Two major peaks of activity were obtained from electrophoresis of solubilized microsomal chitin synthase on gradient polyacrylamide gel, corresponding to apparent molecular weights of $7 \times 10^5$ and $1.1 \times 10^6$ (Fig. 1). Gel permeation chromatography of this enzyme preparation with eluting buffers which did not contain digitonin produced a high recovery of enzyme activity, but poor reproducibility of elution profile. With Sepharose 4B there were often two poorly separated peaks of activity, the first overlapping with the void volume, but sometimes the second peak was missing. This behaviour was not affected by storing or freeze-thawing. The activity usually eluted in the void volume of Sepharose 6B columns, indicating a molecular weight $> 5 \times 10^6$, but became increasingly included in the gel matrix in the presence of increasing concentrations of sodium chloride. Thus in buffer containing 0.2 M-NaCl the activity eluted as a single peak with a mobility between those of lactose dehydrogenase and catalase, corresponding to an apparent molecular weight of $1.5 \times 10^5$.

However, gel chromatography using buffers containing digitonin gave highly reproducible elution profiles. Chitin synthase activity eluted as a single peak from columns of Sephacryl S-200 and Sephacryl S-300 (Fig. 2). The highest activity in the pooled fractions was $1.3 \mu$mol GlcNAc incorporated min$^{-1}$ (mg protein)$^{-1}$, representing a 28-fold purification (Table 1). The enzyme activity eluted after the void volume, but with a mobility greater than ferritin, behaving with an apparent molecular weight of approximately $8.0 \times 10^5$. Further purification was achieved by anion-exchange chromatography using DEAE-Sephacryl to give a preparation with an activity of $3.4 \mu$mol GlcNAc incorporated min$^{-1}$ (mg protein)$^{-1}$ representing a 73-fold purification (Fig. 3; Table 1). SDS-polyacrylamide gel electrophoresis of the most active fractions detected more than one band of staining material, but one band was common to all fractions.

The most successful purification was obtained by chromatography of active fractions from a Sephacryl S-300 Superfine column by metal chelate affinity matrices. Gels with manganese and zinc as the attached cation both gave purifications but the best resolution was obtained with copper when eluted at high pH. The most active fraction from a copper-chelate column was $4.7 \mu$mol GlcNAc incorporated min$^{-1}$ (mg protein)$^{-1}$, representing a 140-fold purification of...
Fraction number

Fig. 2. Typical elution pattern for gel filtration of initially solubilized chitin synthase preparation on Sephacryl S-300 Superfine. ●, Chitin synthase activity; ▲, A$_{280}$. Column size was 86 x 1-6 cm, with 2-2 ml sample applied, 75-drop fractions collected. Eluting buffer was 25 mM-Tris/HCl, pH 7-5, 25 mM-MgCl$_2$, 0-5 M-NaCl, 1 mM-EDTA, 0-01% digitonin.

Fig. 3. Anion exchange chromatography of chitin synthase from peak activity from a gel filtration column. ●, Chitin synthase activity; ———, A$_{280}$ (Isco UA5 absorbance monitor). Column was DEAE-Sepharose CL-6B, 10 x 0-8 cm; a-b loading sample in 25 mM-Tris/HCl, pH 7-5, 0-01% digitonin, b-c washing with 10 ml buffer, c-d elution with 20 ml linear gradient of 0-0-4 M-NaCl in buffer, d-e washing with 5 ml buffer; 25-drop fractions collected.

Table 1. Typical results of purification of chitin synthase from Coprinus cinereus

<table>
<thead>
<tr>
<th>Fractionation step</th>
<th>Protein applied (µg)</th>
<th>Protein collected (µg)</th>
<th>Specific activity [nmol min$^{-1}$ (mg protein)$^{-1}$]</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Initially solubilized preparation</td>
<td>-</td>
<td>-</td>
<td>46</td>
<td>-</td>
</tr>
<tr>
<td>Sephacryl S-300 chromatography</td>
<td>-</td>
<td>-</td>
<td>1300</td>
<td>28</td>
</tr>
<tr>
<td>DEAE-Sepharose chromatography</td>
<td>-</td>
<td>-</td>
<td>3400</td>
<td>73</td>
</tr>
<tr>
<td>(b) Initially solubilized preparation</td>
<td>5200</td>
<td>590</td>
<td>33-5</td>
<td>-</td>
</tr>
<tr>
<td>Sephacryl S-300 chromatography</td>
<td>350</td>
<td>25</td>
<td>4700</td>
<td>140</td>
</tr>
<tr>
<td>Copper-chelate chromatography</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>
Chitin synthase from Coprinus cinereus

Fig. 4. Copper-chelate chromatography of chitin synthase from peak activity from a gel filtration column. ■, Chitin synthase activity; —, $A_{280}$.

Fig. 5. SDS-polyacrylamide gel electrophoresis of chitin synthase preparations, illustrated by drawings of photographs of representative gels. (a–c) Disc gels, stained with Coomassie Brilliant Blue R, with bromophenol blue marking the front. (a) Initially solubilized enzyme preparations (major components only); (b) peak fraction of chitin synthase activity from a Sephacryl S-300 Superfine column; (c) peak fraction of chitin synthase activity from a copper-chelate column. (d) Slab gel, stained with silver nitrate, of peak fraction of chitin synthase activity from a copper-chelate column. Estimates of molecular weights ($\times 10^4$) of each gel are indicated. The position of the front ($R_f = 1$) is indicated by 'f'.

the initially solubilized enzyme (Fig. 4; Table 1). The purification was monitored by SDS-polyacrylamide disc gel electrophoresis (Fig. 5), which showed only one band, corresponding to a molecular weight of approximately $6.7 \times 10^4$. This band ran in the same position in reducing conditions in the presence of 1% (v/v) mercaptoethanol. The product of this combined purification sequence was also analysed by silver-staining an SDS-polyacrylamide slab gel, which also showed the presence of traces of proteins of molecular weights $4.2 \times 10^4$, $3.4 \times 10^4$ and $2.4 \times 10^4$ (Fig. 5). However, as this is a very sensitive technique it should be emphasized that these components are present in only nanogram quantities, undetectable by more conventional staining methods, whereas the major $6.7 \times 10^4$ molecular weight band represents several micrograms of protein.

The interactions between the enzyme and the four affinity chromatographic supports were as follows: with Sepharose-adipic acid dihydrazide-UDP (linked through $-\text{OH}$ groups of ribose), agarose-aminophenyl-GlcNAc (linked through C1) and Sepharose-nikkomycin (epoxide linked via a short hydrophilic spacer arm, probably through phenolic $-\text{OH}$ group), virtually all activity eluted through in the void volume, and no further activity could be washed off; with Sepharose-hexanolamine-UDP (linked through pyrophosphate) over 95% of the activity appeared in the void volume, and the remaining activity was eluted through as a broad peak by buffer containing UDP but not by buffer containing UDP-GlcNAc.
Table 2. Protease activities associated with chitin synthase preparations

<table>
<thead>
<tr>
<th>Peptide substrate</th>
<th>Initially solubilized preparation</th>
<th>Sephacryl S-300 eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-2586</td>
<td>1.67</td>
<td>12.1*</td>
</tr>
<tr>
<td>S-2160</td>
<td>0.93</td>
<td>0</td>
</tr>
<tr>
<td>S-2337</td>
<td>0.81</td>
<td>0</td>
</tr>
<tr>
<td>S-2484</td>
<td>0.33</td>
<td>0</td>
</tr>
<tr>
<td>S-2288</td>
<td>0.17</td>
<td>0</td>
</tr>
<tr>
<td>S-2302</td>
<td>0.10</td>
<td>0</td>
</tr>
<tr>
<td>S-2444</td>
<td>0.10</td>
<td>0</td>
</tr>
<tr>
<td>S-2238</td>
<td>0.07</td>
<td>0</td>
</tr>
<tr>
<td>S-2251</td>
<td>0.07</td>
<td>0</td>
</tr>
</tbody>
</table>

* This activity was removed from chitin synthase activity by copper-chelate chromatography. The structure of S-2586 is 3-carbomethoxypropionyl-L-arginyl-L-prolyl-L-tyrosinyl-p-nitroanilide hydrochloride.

Table 3. Nucleoside diphosphatase activity associated with chitin synthase preparations

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity [nmol min⁻¹ (mg protein)⁻¹]*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal preparation</td>
<td>120</td>
</tr>
<tr>
<td>Solubilized microsomal</td>
<td>131</td>
</tr>
<tr>
<td>preparation</td>
<td></td>
</tr>
<tr>
<td>Sephacryl S-300 preparation</td>
<td>0</td>
</tr>
</tbody>
</table>

* With UDP as substrate; IDP, ADP and GDP were also hydrolysed.

Protease and nucleoside diphosphatase activities

The initially solubilized preparation contained appreciable activity towards three of the ten synthetic protease substrates (Table 2). The peak with chitin synthase activity resulting from chromatography on Sephacryl S-300 retained activity to one of these, S-2586, which is a model substrate for chymotrypsin. This activity was removed from the chitin synthase activity by copper chelate chromatography (Table 2). As described before (de Rousset-Hall & Gooday, 1975; Gooday, 1979) nucleoside diphosphatase activity was present in solubilized microsomal preparations, but this was lost from the chitin synthase activity by chromatography on Sephacryl S-300 (Table 3).

DISCUSSION

The most purified preparation described here gives an apparent molecular weight of 6.7 x 10⁴ in the dissociating conditions on SDS-polyacrylamide gel electrophoresis, in contrast to the molecular weights of several hundred thousand shown by active enzyme preparations eluting from gel filtration columns. This suggests that the active enzyme exists as a multimeric complex. In our preparations this complex is also certainly associated with lipids which are essential for activity (unpublished results).

The estimated molecular weight of the activity recovered by gel filtration is similar to values obtained by Braun & Calderone (1979) and Duran & Cabib (1978) for chitin synthase from the yeasts Candida albicans and Saccharomyces cerevisiae respectively, and by Ruiz-Herrera et al. (1980) and Hänseler et al. (1983) for subunits with a sedimentation coefficient of 16S obtained by treatment of chitosomes of Mucor rouxii and Agaricus bisporus respectively with digitonin.

Some of the increase in specific activity recorded here as purification will be the result of the removal of digitonin, an inhibitor of chitin synthase (Duran & Cabib, 1978; Ruiz-Herrera et al.,...
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1980). The 140-fold increase in activity, however, to give an activity of 4.7 nmol GlcNAc incorporated into chitin min⁻¹ (mg protein)⁻¹, does represent the highest level of purity of this enzyme yet recorded, and demonstrates the suitability of the stipe of Coprinus cinereus as a source of the enzyme.

The purification of the chitin synthase from its accompanying nucleoside diphosphatase and protease activities suggests that these are not present in the cell as a multifunctional protein complex. The chymotrypsin-like proteolytic activity which co-purified sevenfold in the chitin complex. The chymotrypsin-like proteolytic activity which co-purified sevenfold in the chitin synthase fractions requires comment, as serine proteases are usually of low molecular weight (Morihara, 1974), and this had an elution volume on gel filtration columns corresponding to a molecular weight greater than 2.5 × 10⁵. It may be complexing with the membrane lipids in the preparation, and may represent a membrane-bound proteolytic activity in vivo.

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REFERENCES


