Chitin Synthase in Aspergillus nidulans: Properties and Proteolytic Activation

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SUMMARY

Chitin synthase (EC 2.4.1.16) from membrane preparations of Aspergillus nidulans was characterized and the optimum conditions for enzyme activity were determined. A reaction velocity–substrate concentration plot was sigmoidal, but was hyperbolic in the presence of N-acetylglucosamine when the \( K_m \) for UDP-N-acetylglucosamine was 3.1 mmol l\(^{-1}\). Chitin synthase activity could be increased sixfold by digestion of enzyme preparations with trypsin for short periods. The trypsin-activated enzyme had altered kinetic and storage properties.

INTRODUCTION

Chitin synthase (UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-\( \beta \)-acetamidodeoxyglucosyltransferase, EC 2.4.1.16) forms chitin from UDP-N-acetylglucosamine (UDP-GlcNAc). The enzyme was first characterized by Glaser & Brown (1957) in Neurospora crassa and has subsequently been described in various fungi (Jaworski, Wang & Carpenter, 1965; Porter & Jaworski, 1966; Camargo et al., 1967; Keller & Cabib, 1971; McMurrough, Flores-Carreon & Bartnicki-Garcia, 1971; Jan, 1974; Moore & Peberdy, 1975; Peberdy & Moore, 1975; Goody & de Rousset-Hall, 1975; Moore & Peberdy, 1976). Chitin is a structural polymer of many fungal walls and the control of chitin synthesis must be an important aspect of fungal growth. The regulation of chitin synthesis in bud-septum development in yeast has been described in a model involving the proteolytic activation of a zymogen form of chitin synthase (Cabib & Farkas, 1971; Cabib, Ulane & Bowers, 1974). Little is known about the regulation of wall synthesis in filamentous fungi and Aspergillus nidulans was chosen as a suitable organism in which to study this problem. This paper describes the properties of chitin synthase in A. nidulans with particular reference to potential regulatory mechanisms.

METHODS

Organism and culture. Aspergillus nidulans wild-type strain NF33 (University of Nottingham collection) was maintained on malt agar. Spore suspensions were obtained from cultures grown at 37 °C for 3 days, by shaking with glass beads and 0.01% (v/v) Tween 80. The spores, after washing in distilled water, were inoculated into liquid cultures at a final concentration of 2 x 10⁶ ml⁻¹. Mycelium was grown in 2 l Erlenmeyer flasks containing 500 ml minimal salts medium (Peberdy & Moore, 1975) with glucose at 10 g l⁻¹. Cultures were incubated for 20 h at 30 °C on a rotary shaker at 200 rev. min⁻¹.

Preparation of enzyme. Cultures were harvested by filtration through nylon gauze, washed twice in distilled water and once in cold 200 mM-Tris/HCl buffer, pH 7.5. Subsequent operations were performed at 0 to 4 °C. Mycelium was disrupted for 30 s in a Braun MSK...
homogenizer (4000 rev. min⁻¹) cooled with CO₂. The homogenate was centrifuged at 2500 g max. for 10 min to remove unbroken hyphae and cell walls. The supernatant was then centrifuged at 20000 g for 60 min using a Beckman L2 65B ultracentrifuge. The pellet was washed once in 200 mM-Tris/HCl buffer, pH 7.5, resuspended in buffer and used as the crude enzyme preparation. Preparations were used immediately or stored at −20 °C.

Chitin synthase assay. The assay procedure was similar to that described by Jan (1974). The standard reaction mixture contained: 1 mM-UDP-GlcNAc, including 10 nCi UDP-N-acetyl-d-[U-¹⁴C]glucosamine; 50 mM-GlcNAc; 2.5 mM-MgSO₄; 100 mM-Tris/HCl, pH 7.5; and enzyme preparation (up to 0.9 mg protein) in a total volume of 100 µl. Mixtures were incubated at 25 °C for 10 to 15 min and the assay was terminated by adding 1 ml ice-cold 5% (w/v) trichloroacetic acid. Each assay tube was left in ice for at least 30 min and the contents were then washed out with distilled water and filtered under vacuum on to a glass-fibre disc (Whatman GF/A, diam. 2.4 cm) held in a Millipore filtration unit. Discs were washed twice with about 20 ml distilled water and dried. Chitin formed during the reaction was retained on the filter discs, possibly bound to the precipitated protein, and the soluble assay components were removed. Further radioactive material could not be removed from the discs by repeated washing with water.

The filter discs were placed in 3 ml scintillation fluid (5 g 2,5-diphenyloxazole and 0.25 g 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene in 11 toluene) and radioactivity was counted in a Packard Tri-Carb liquid scintillation counter. The counting efficiency was typically 80% using radioactive standards. Corrections were made for background counts and quenching. Chitin synthase activity was determined from the percentage incorporation of radioactivity into insoluble products and expressed as nmol GlcNAc incorporated min⁻¹ (mg protein)⁻¹. All assays were performed in triplicate.

Protein determination. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard, and calibrated with correction for interference by Tris buffer (Rej & Richards, 1974).

Characterization of product. Reaction mixtures were scaled up to 1 ml and incubated for 1 h. The precipitate was washed four times with distilled water and stored at −20 °C. A sample (43000 c.p.m.) was incubated with 5 mg chitinase for 8 h at 30 °C. Other samples were treated with 1 M-acetic acid at 55 °C for 30 min or hydrolysed in 6 M-HCl at 120 °C under nitrogen for 4.5 h, dried in vacuo and the residue dissolved in distilled water. Similar samples, with 4 mg purified chitin as carrier, were partially hydrolysed in HCl and selectively N-acetylated with acetic anhydride using methods described by McMurrough et al. (1971). Descending chromatography was performed on Whatman no. 1 paper, developed in 95% ethanol/1 M-acetic acid (7:3, by vol.). Radioactive spots were located using a Panax RTL 5-IA scanner and chromatogram origins were excised and counted.

Trypsin digestion of enzyme preparation. A sample of enzyme preparation (150 µl) was incubated at 25 °C with 10 µg trypsin (crystalline from bovine pancreas) in a total volume of 195 µl containing 200 mM-Tris/HCl, pH 7.5, and 1 mM-MgSO₄. Digestion was terminated by adding excess soybean trypsin inhibitor. In controls, the inhibitor was added first.

Materials. UDP-N-acetyl-d-[U-¹⁴C]glucosamine (269 mCi mmol⁻¹) and N-acetyl-d-[¹⁴C]glucosamine (60 mCi mmol⁻¹) were from The Radiochemical Centre, Amersham. Fungal chitinase was from Koch-Light. Biochemicals were from Sigma and other chemicals, of analytical grade, from BDH.
Table 1. Distribution of chitin synthase activity in different cellular fractions, and the effects of freezing and trypsin-digestion

Fractions were prepared as described in Methods except that centrifugation was at 10000 g for 15 min, 100000 g for 60 min and 270000 g for 60 min. Specific activities are expressed as nmol GlcNAc incorporated min\(^{-1}\) (mg protein\(^{-1}\)).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fraction (sedimentation force, g)</th>
<th>Specific activity</th>
<th>Percentage of total activity</th>
<th>Percentage of untreated activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly prepared</td>
<td>10000</td>
<td>1·33</td>
<td>19·8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100000</td>
<td>1·39</td>
<td>77·9</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>270000</td>
<td>0·40</td>
<td>2·3</td>
<td>100</td>
</tr>
<tr>
<td>Trypsin digest*</td>
<td>10000</td>
<td>4·19</td>
<td>18·1</td>
<td>421</td>
</tr>
<tr>
<td></td>
<td>100000</td>
<td>6·64</td>
<td>80·9</td>
<td>480</td>
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<td></td>
<td>270000</td>
<td>0·79</td>
<td>1·0</td>
<td>198</td>
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<tr>
<td>Stored frozen†</td>
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<td>1·28</td>
<td>18·8</td>
<td>96</td>
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<td></td>
<td>100000</td>
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<td>78·0</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>270000</td>
<td>0·78</td>
<td>3·2</td>
<td>194</td>
</tr>
</tbody>
</table>

* Digested with trypsin by the standard method for 10 min.
† Stored at -20 °C for 24 h.

RESULTS

Enzyme preparations and properties

The assay method was rapid and reproducible, the standard deviation for triplicate assays being routinely less than 10% of the mean value. The method was compared with one involving paper chromatography of the reaction mixture (Peberdy & Moore, 1975) and identical results were obtained, confirming that the product was completely retained on the filters. The substrate did not adhere to the filters and controls, using previously inactivated enzyme, generally had counts about 10% above the background.

Freshly prepared enzyme had an average chitin synthase activity of about 1·0 (±0·5) nmol GlcNAc incorporated min\(^{-1}\) (mg protein\(^{-1}\)). The activity depended on the length of time of homogenization; maximum enzyme yield was achieved after 30 s, and a further 30 s treatment reduced the yield by 50%. The distribution of chitin synthase activity in cellular fractions is shown in Table 1. Values for wall fractions are not presented because some hyphae were not broken and the homogenizing treatment extracted only about 30% of the total protein in the mycelium. Activity in the 200000 g supernatant was negligible.

When enzyme preparations were stored at -20 °C, the resultant chitin synthase activity was increased by up to 100%. The activity was constant for any period of storage between 2 h and 4 days, but declined slowly over longer periods. A second freezing and thawing within this period caused a further marginal increase in activity. The increase in activity after storage of different cellular fractions is shown in Table 1. In one experiment the activity of an enzyme preparation increased by 70% during storage at 0 °C for 5 h, and by 50% during storage at 25 °C for 2 h, thereafter declining. Increases during the assay period were negligible.

Chromatograms of reaction mixtures showed only two radioactive spots, one at the origin and one corresponding to UDP-GlcNAc standards (R\(_f\) 0·40), indicating the absence of soluble glycosyl products. Chitinase treatment solubilized 98% of the reaction product giving a single radioactive product identified as GlcNAc (R\(_f\) 0·75). The reaction product was insoluble in 1 M-acetic acid, but was completely hydrolysed by 6 M-HCl forming glucosamine (R\(_f\) 0·61) and a small quantity of an unidentified product (R\(_f\) 0·86) which was also obtained.
when GlcNAc was hydrolysed by the same method. Therefore the product was a polymer of GlcNAc. Partial hydrolysis yielded one immobile and three mobile peaks ($R_p$ 0.22, 0.56, 0.74) which co-chromatographed with those obtained from purified chitin, thus confirming that the reaction product was chitin.

The rate of incorporation of GlcNAc into chitin [in a preparation of activity 1.46 nmol GlcNAc incorporated min$^{-1}$ (mg protein)$^{-1}$] was linear for the first 25 min of assay, when approximately 20% of the substrate had been used, and then declined. This may have been due to depletion of substrate or accumulation of inhibitory UDP. Enzyme activity also varied linearly with enzyme concentration in the range 2 to 9 mg protein (ml reaction mixture)$^{-1}$.

Tris/HCl and KH$_2$PO$_4$/Na$_2$HPO$_4$ buffers, final concentrations 100 mM, were used to vary the pH of reaction mixtures. In both cases the optimum was pH 7.5, with half-maximum activity at pH 7.05 (phosphate buffer) and pH 8.25 (Tris buffer). Concentrations of up to 500 mM-Tris/HCl buffer, pH 7.5, had no effect on chitin synthase activity. To investigate the effect of temperature, reaction mixtures were buffered with 50 mM-HEPES buffer, pH 7.5 ($N$-2-hydroxyethylpiperazine-$N'$-2-ethanesulphonic acid) because the $pK_a$ of this buffer does not change appreciably with temperature (Good et al., 1966). The optimum temperature was 30.5 °C with half-maximum activity at 17.5 and 41 °C. Activity was 90% of the maximum at the standard assay temperature of 25 °C.

To investigate requirements for metal ions, enzyme preparations were dialysed against 100 mM-Tris/HCl, pH 7.5, for 4 h at 4 °C using two changes of buffer. Chitin synthase activity was greatly stimulated by the presence of Mg$^{2+}$ ions, the optimum concentration being 2.5 mM, but higher concentrations were inhibitory with half-maximum activity at 17 mM-MgSO$_4$. Enzyme activity in the absence of added Mg$^{2+}$ was 11% of the maximum. Various other divalent cations, added as their chlorides or sulphates, were tested but none produced a similar degree of stimulation. KCl or K$_2$SO$_4$ at final concentrations of 20 mM had no significant effect. The chelating agent EDTA (10 mM) caused total inhibition of chitin synthase activity suggesting that the enzyme had an absolute requirement for a metal ion.

Addition of GlcNAc to reaction mixtures stimulated chitin synthase activity and this effect was more pronounced at a lower concentration of UDP-GlcNAc (Fig. 1). Addition of 50 mM-glucose or 50 mM-$N$-acetylgalactosamine also caused significant stimulation.

The effect of substrate concentration on reaction velocity was investigated in the presence and absence of 50 mM-GlcNAc (Fig. 2). In the absence of GlcNAc, a sigmoidal plot was obtained, and the corresponding Lineweaver–Burk plot curved steeply (Fig. 3). When activity was stimulated by GlcNAc, a linear Lineweaver–Burk plot was obtained (Fig. 3) and the $K_m$ for UDP-GlcNAc was 3.1 mmol l$^{-1}$. This plot was upward-curving below 0.5 mM-UDP-GlcNAc. A Hill plot of these data was constructed (Fig. 4) by the method of Atkinson, Hathaway & Smith (1965). In the absence of GlcNAc, the slope ($h$) above 4 mM-UDP-GlcNAc was 1.0 and below this concentration the plot was non-linear. In the presence of 50 mM-GlcNAc, the plot was linear above 0.5 mM-UDP-GlcNAc and $h = 1$.

The kinetic properties of preparations before and after storage at −20 °C were identical.

**Proteolytic activation of chitin synthase**

When chitin synthase preparations were digested with trypsin for short periods, their activity increased up to 500%. The extent of this activation depended on the trypsin concentration and the length of treatment although the optimum values of these factors varied between different enzyme preparations. This effect was partially dependent on the presence
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of Mg$^{2+}$ ions as the inclusion of 1 mM-MgSO$_4$ in the trypsin-digestion mixture increased the degree of activation by up to 25% compared with controls in which MgSO$_4$ was added after the digestion. The absence of MgSO$_4$ did not affect the optimum period of treatment and it is possible that Mg$^{2+}$ ions partially protect the enzyme from degradation by the trypsin. Trypsin digestion of dilutions of a chitin synthase preparation gave samples in which the activity was linear with protein concentration above 6 mg protein (ml digestion mixture)$^{-1}$. Below this value, activity was lower than expected, probably due to destruction of enzyme by the excess trypsin. The degree of activation of different cellular fractions is shown in Table I. The stimulation of enzyme activity following storage at $-20$ °C was eliminated by trypsin digestion either before or after the storage period.

Trypsin digestion modified the kinetic properties of the enzyme. Plots of reaction velocity
Table 2. Effect of trypsin digestion on stimulation of chitin synthase activity by GlcNAc

Enzyme preparations were assayed without GlcNAc (controls) or at a final concentration of 100 mM-GlcNAc: 1 mM-UDP-GlcNAc was present in all assays. Specific activities are expressed as nmol GlcNAc incorporated min⁻¹ (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>Control</th>
<th>With GlcNAc</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated*</td>
<td>0.06</td>
<td>1.90</td>
<td>3272</td>
</tr>
<tr>
<td>Trypsin digest†</td>
<td>0.54</td>
<td>3.01</td>
<td>556</td>
</tr>
</tbody>
</table>

* Results from Fig. 3.
† Digested with trypsin by the standard method for 5 min.

against substrate concentration in the presence of 50 mM-GlcNAc were similar to those shown in Figs 2 to 4 except that the $K_m$ for UDP-GlcNAc was $2.2 \pm 0.05 \text{ mmol l}^{-1}$, compared with $3.1 \pm 0.08$ for the untreated enzyme (mean values and standard deviations for three separate experiments). Stimulation by GlcNAc was less marked after trypsin-activation (Table 2) and there was little increase in activity above 30 mM-GlcNAc (compare with Fig. 1 for untreated enzyme).

**DISCUSSION**

The general properties of chitin synthase in *A. nidulans* are similar to those in other fungi. The absolute requirements for chitin synthesis were UDP-GlcNAc and a divalent metal cation, preferably Mg²⁺.

Chitin synthase of *A. nidulans* displays sigmoidal reaction kinetics in the absence of GlcNAc. A similar pattern has been described for chitin synthase preparations from *Mucor rouxii* (McMurrough & Bartnicki-Garcia, 1971), *Blastocladiella emersonii* (Camargo et al., 1967) and *Coprinus cinereus* (de Rousset-Hall & Gooday, 1975). These results indicate that chitin synthase may be an allosteric enzyme, in which case there must be more than one UDP-GlcNAc binding site per enzyme molecule (Monod, Changeux & Jacob, 1963). The value of the Hill number $h$ has been taken as indicating the number of substrate binding sites when there is strong interaction between them (Atkinson et al., 1965). As the interaction or the number of subunits per enzyme molecule decreases, so $h$ tends toward 1.0. By this method, de Rousset-Hall & Gooday (1975) have deduced the presence of four UDP-GlcNAc binding sites per chitin synthase molecule in *C. cinereus*. The Hill plot for our data was curved below 4 mM-UDP-GlcNAc but above this value $h$ was 1, indicating that Michaelis–Menten kinetics were operative.

GlcNAc stimulates chitin synthase activity in all fungi studied, and has been suggested as a positive allosteric effector of the enzyme (Carmargo et al., 1967; McMurrough & Bartnicki-Garcia, 1971; de Rousset-Hall & Gooday, 1975). In *A. nidulans* the effect of adding GlcNAc was similar to that of high substrate concentrations, giving Michaelis–Menten kinetics. GlcNAc also caused considerable stimulation of activity, even at near maximum concentrations of UDP-GlcNAc, so there may be more than one stimulatory action involved.

Irrespective of the molecular mechanism, the stimulation of chitin synthase by substrate and effectors is clearly an important potential regulatory system for the fine control of chitin synthesis *in vivo*.

Our results suggest that a high proportion of the chitin synthase in growing cultures of *A. nidulans* exists as an inactive zymogen which can be converted to active enzyme by proteo-
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lytic digestion. Proteolytic activation of chitin synthase has been described in yeast (Cabib & Farkas, 1971) and in the dimorphic fungus M. rouxii (Ruiz-Herrera & Bartnicki-Garcia, 1974; Ruiz-Herrera et al., 1975) but has not previously been reported in a purely filamentous fungus. Trypsin-digested chitin synthase from yeast (Cabib & Keller, 1971) showed less stimulation by GlcNAc, as do similar preparations from A. nidulans. In contrast, the yeast enzyme did not have a reduced $K_m$ for UDP-GlcNAc.

In yeast, an endogenous activating factor was shown to be located in the vacuole (Cabib, Ulane & Bowers, 1973) and was later identified as yeast proteinase B (Ulane & Cabib, 1976). Increases in chitin synthase activity during storage of M. rouxii extracts at $-20^\circ$C have been taken as evidence of proteolytic activation (McMurrough & Bartnicki-Garcia, 1973). A similar effect occurred in our preparations but may have been at least partly due to exposure of further enzyme sites by the freeze/thaw process.

Growing hyphae synthesize new wall material at their apices (Bartnicki-Garcia & Lippman, 1969; Gooday, 1971) and chitin synthase is almost certainly active at these sites. Subapical wall synthesis must occur during branch initiation and Katz & Rosenberger (1971) have shown that hyphae of A. nidulans have the potential to synthesize chitin along their entire length. One explanation for this was the activation of pre-existing enzyme sites (Sternlicht, Katz & Rosenberger, 1973) and it has been suggested that proteolytic activation of a zymogen could account for this (McMurrough & Bartnicki-Garcia, 1973). However, there are other possible explanations, including regulation by availability of substrate and allosteric effectors, and proteolytic activation of chitin synthase has not yet been demonstrated in vivo.

Note added in proof. Proteolytic activation of chitin synthase from Aspergillus flavus has also been reported recently [E. Lopez-Romero & J. Ruiz-Herrera (1976). Antonie van Leeuwenhoek 42, 261-276].

REFERENCES


