Chitin Synthase in *Mortierella vinacea*: Properties, Cellular Location and Synthesis in Growing Cultures

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SUMMARY

Chitin synthase of *Mortierella vinacea* was present in the 'microsomal' fraction (100 000 g precipitate), the 'cell-wall' fraction (2000 g precipitate) and the 'mitochondrial' fraction (10000 g precipitate). The properties of the 'microsomal' enzyme were investigated. The pH optimum was between 5.8 and 6.2, and the temperature optimum was between 31 and 33 °C. The K_m for UDP *N*-acetyl-D-glucosamine was 1.8 mM. The enzyme was stimulated by Mg^{2+} and a slight stimulation was also effected by *N*-acetyl-D-glucosamine. Soluble chitodextrins were inhibitory. A pH-dependent, heat-stable inhibitor of chitin synthase activity was present in the soluble cytoplasm from the mycelium. The effects of aeration and glucose concentration on enzyme production in growing cultures were also investigated; maximum specific activity of chitin synthase was associated with the cessation of exponential growth.

INTRODUCTION

Chitin synthase (chitin-UDP acetylglucosaminyltransferase, EC. 2.4.1.16), which catalyses the formation of chitin from UDP *N*-acetyl-D-glucosamine (UDP-GlcNAc), has been reported in many fungi (Glaser & Brown, 1957; Jaworski, Wang & Carpenter, 1965; Porter & Jaworski, 1966; Camargo *et al.* 1967; Keller & Cabib, 1971; McMurrough, Flores-Carreón & Bartnicki-Garcia, 1971; Gooday, 1972; Jan, 1974). The enzyme exhibits varying properties and activities in the different taxonomic groups, but there have been few attempts to relate the synthesis of the enzyme to the patterns of mycelial growth. Activities of chitin synthase were shown to depend on culture age in *Venturia inequalis* (Jaworski *et al.* 1965) and *Mucor rouxii* (McMurrough *et al.* 1971). Similarly, Gooday (1973) demonstrated that the specific activity of chitin synthase in the fruiting bodies of *Coprinus cinereus* was dependent on their size and age.

This paper describes some properties of the enzyme chitin synthase of *Mortierella vinacea*, including its inhibition, cellular location and synthesis in growing cultures.

METHODS

Organism and culture. *Mortierella vinacea* strain NF133 (University of Nottingham collection) was maintained on malt extract agar at 30 °C. Spore suspensions for inoculation of liquid cultures were prepared in distilled water using four-day-old slopes. Mycelium, for enzyme preparation, was grown in 5 l Erlenmeyer flasks containing 500 ml of Vogel's

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salts medium N (Vogel, 1956). This solution contained (g/l): sodium citrate.5H₂O, 3; KH₂PO₄, 5; NH₄NO₃, 2; MgSO₄.7H₂O, 0.2; and 5 ml of trace element solution. The latter contained (g/100 ml): citric acid, 5; ZnSO₄.7H₂O, 5; Fe(NH₄)₂(SO₄)₂.6H₂O, 1; CuSO₄.5H₂O, 0.25; MnSO₄.1H₂O, 0.05; H₃BO₃, 0.05; Na₂MoO₄.2H₂O, 0.05. Glucose was added at 1 g/100 ml and the medium adjusted to pH 6.5. Spores were added at 10⁶ ml. The flasks were incubated at 30 °C on a reciprocal shaker at 100 strokes/min. The mycelium was harvested after 24 h by filtration through a sintered glass filter (porosity 0) and washed three times with ice-cold 50 mM-KH₂PO₄-KOH buffer, pH 6.0, containing 10 mM-MgCl₂ (McMurrough *et al.* 1971).

In some experiments the fungus was grown in a simple fermenter. Spores were prepared and inoculated as described above. For these experiments cultures were grown on modified Vogel's salts medium containing NH₄Cl as the nitrogen source and glucose was added at different concentrations. The fermenter contained 3 l medium and was maintained at 30 °C. The cultures were agitated by a magnetic stirrer and aerated through a sparger.

Preparation of enzyme fractions. Washed mycelium was suspended in an equal volume of 0.5 M-KH₂PO₄-KOH buffer, pH 6.0, containing 10 mM-MgCl₂ and rapidly disrupted for 45 s in a Braun MSK homogenizer (4000 rev./min) cooled with CO₂. Microscopic examination showed that at least 99 % of the cells were ruptured and the contents released. After centrifuging at 2000 g (*g*_{av}, with swing-out rotors) for 10 min, the pellet ('cell-wall' fraction) was resuspended in 50 mM-KH₂PO₄-KOH buffer, pH 6.0, containing 1 mM-MgCl₂. The supernatant was centrifuged at 10000 g for 10 min and the pellet ('mitochondrial' fraction) resuspended in buffer. The 10000 g supernatant was then centrifuged at 100000 g for 30 min to prepare the 'microsomal' fraction, which was also suspended in buffer. The supernatant from this centrifugation was dialysed for 4 h against 50 mM-KH₂PO₄-KOH buffer, pH 6.0. The other fractions were recentrifuged and resuspended twice in 50 mM buffer, before finally storing at -20 °C together with the supernatant fraction.

Chitin synthase assay was based on the method described by Glaser & Brown (1957). For most experiments the reaction mixture contained 1 mM-UDP-GlcNAc, 5 to 10 µCi UDP-[U-¹⁴C]GlcNAc, 20 mM-MgCl₂, 20 mM-*N*-acetylglucosamine (GlcNAc), 25 mM-KH₂PO₄-KOH (pH 6.0) and 'microsomal' fraction enzyme (1 to 5 mg protein), in a total volume of 50 µl. Mixtures were incubated at 31 °C, usually for 15 min, and the reactions were terminated by the addition of 10 µl glacial acetic acid. A sample (50 µl) was removed from each mixture for descending chromatography using Whatman No. 1 paper strips. The chromatograms were usually developed for 6 h in 95 % ethanol-1 M-acetic acid (7:3, v/v). Chitin formed during the assay was not eluted and remained at the origin of the chromatogram. This region was excised and the radioactivity measured. All assays were carried out in triplicate, with controls prepared by adding the acetic acid before the enzyme preparation. In some experiments the chromatograms were developed in 1-butanol-pyridine-water (6:4:3, by vol.). A Panex RTL 5-1A thin-layer scanner was used to locate the components of the mixtures. Isotope counts were determined using a Packard Tri-Carb liquid scintillation counter. Pieces of chromatography paper were suspended in 5 ml scintillation fluid [0.1 g 1,4-bis-(5-phenyloxazol-2-yl) benzene and 2 g 2,5-diphenyloxazole in 1 l scintillation grade toluene]; quench corrections were made. Chitin synthase activity was expressed as nmol GlcNAc incorporated/min/mg protein, except where stated.

Protein determinations. Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Analyses on fermenter cultures. Residual glucose in the culture medium was determined by the anthrone method (Trevelyan & Harrison, 1952) as modified by Yemm & Willis

(1954). Residual nitrogen was determined as ammonia using Nessler's reagent. The pH was measured using an E.I.L. meter. Dry weight determinations were made on small volumes of the cultures by vacuum filtration through previously-tared filter papers, which were then dried for 24 h at 105 °C before reweighing.

Preparation of soluble chitodextrins. Soluble chitodextrins were prepared by hydrolysis of pure chitin for 1 h with 5 M-HCl at 100 °C (Zechmeister & Toth, 1931). Excess acid was removed by precipitation with excess Ag_2CO_3 . The soluble chitodextrins were precipitated from solution with ethanol. Two fractions (F_1 and F_2) were obtained using 48 and 89 % (v/v) ethanol.

Chemicals. UDP-[U- ^{14}C]GlcNAc, specific activity 200 mCi/nmol, was obtained from the Radiochemical Centre, Amersham, Buckinghamshire. Chitinase (fungal) was purchased from Mann Research Laboratories Inc., New York, U.S.A.; other biochemicals were from Sigma. General chemicals, of analytical grade where possible, were from BDH.

RESULTS

Distribution of chitin synthase activity in the cellular fractions

Almost half the total chitin synthase was found in the 'cell-wall' fraction (Table 1), with most of the remainder in the 'microsomal' and 'mitochondrial' fractions. However, the three particulate fractions showed similar specific activities. Further disruptions of the 'cell-wall' fraction followed by washings showed a very small decrease in the enzyme activity of the walls.

Effects of storage on enzyme stability

The activity of the 'microsomal' fraction had a half-life of 8 to 9 days, 24 h and 12 h, respectively, when stored at -20 °C, 0 °C or 18 °C in 25 mM- KH_2PO_4 -KOH buffer (pH 6.0) containing 1 mM- MgCl_2 . Storage at 20 °C, with subsequent thawing and refreezing every 24 h, resulted in a reduction of the enzyme half-life to 4 to 5 days.

Reaction kinetics

From a slightly curved Lineweaver-Burk plot (Fig. 1), the apparent K_m for UDP-GlcNAc was estimated to be about 1.8 mM. In further experiments the concentration of UDP-GlcNAc was maintained at 1 mM whilst the GlcNAc concentration was varied between 0 and 20 mM. The addition of increasing concentrations of GlcNAc caused a slight stimulation of enzyme activity. Inclusion of the soluble chitodextrins F_1 and F_{29} in the assay mixtures had an inhibitory effect (Table 2).

Identification of assay product

Reactions similar to those described in Methods were scaled up to give a final working volume of 500 μl , and terminated after 2 h. Chromatography (ethanol:acetic acid) of samples from the reaction mixtures demonstrated the presence of two peaks; the first remained at the origin and the second had an R_F value (0.42) similar to the UDP-GlcNAc standard. After dialysis of the reaction mixtures against distilled water for 24 h at 4 °C, to remove the substrate, the non-dialysable material was centrifuged at 2000 g for 20 min and finally resuspended in 0.5 ml distilled water. Hydrolysis with concentrated HCl for 2 h at 100 °C, followed by neutralization, resulted in a single product with an R_F value (0.59) equivalent to glucosamine. The effect of chitinase was tested by mixing equal volumes of the 'chitin' material with a solution of the enzyme (1 mg/ml) in 50 mM- KH_2PO_4 -KOH buffer, pH 6.5, for 2 h at 30 °C. The reaction was terminated with glacial acetic acid and

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Effect of substrate concentration on the chitin synthase activity of the 'microsomal' fraction. Results are presented as a double reciprocal plot of rate of incorporation of substrate into chitin against UDP-GlcNAc concentration. The protein concentration was 1.94 mg/ml and the assays were terminated after 30 min.

Fig. 2. Effect of incubation time on chitin formed by various fractions. Assay conditions were as described for the standard method except that the incubation time was varied. Substrate incorporation is expressed as nmol/0.1 mg protein and as a percentage of substrate released as product. Protein concentrations (mg/ml): 'microsomal' fraction (○), 1.94; 'mitochondrial' fraction (●), 2.1.

Fig. 3. Effect of buffer concentration on chitin formation by the 'microsomal' fraction. The reaction conditions were as described for the standard assay except that the concentration of KH_2PO_4 -KOH buffer, pH 6.5, was varied. The protein concentration was 1.94 mg/ml.

Table 1. *Distribution of chitin synthase in the different cellular fractions of M. vinacea*

The fractions were prepared by centrifugation as described in Methods; each fraction was diluted to give 1 to 5 mg protein/ml. Total activity is based on the activity of enzyme and amount of protein in each fraction.

Fraction	Specific activity (nmol/min/mg protein)	Total activity (%)
'Cell-wall'	10.5	48.7
'Mitochondrial'	11.4	16.3
'Microsomal'	8.4	28.1
Supernatant	2.3	6.9

the neutralized mixture was chromatographed, using butanol-pyridine-water (6:4:3, by vol.) as solvent. Only 3 % of the activity remained at the origin, the remainder being located as a single peak with an R_F value (0.73) similar to that of GlcNAc.

Time course of chitin formation

After 20 min incubation, 18.9 and 16.2 % of the substrate was converted to chitin by the 'mitochondrial' and 'microsomal' material, respectively. The initial rates of activity in this experiment were 6.33 and 5.2 nmol GlcNAc incorporated/min/mg protein for the 'mitochondrial' and 'microsomal' fractions, respectively (Fig. 2).

Effect of buffer concentration and pH

The pH of the incubation mixture was varied using 25 mM- KH_2PO_4 -KOH buffer and 50 mM-tris-HCl. Maximum activity was found with KH_2PO_4 -KOH, the optimum pH value being between 5.8 and 6.2. Increasing the concentration of phosphate buffer (pH 6.5,

Table 2. *The effect of soluble chitodextrins on chitin synthase*

'Microsomal' fractions were used in the assays, which were carried out as described in Methods.

Chitodextrin concn (mg/ml)	Chitin synthase activity* (nmol GlcNAc incorporated/min/mg protein)	
	Chitodextrin F ₁	Chitodextrin F ₂
0	2.7 ± 0.11	2.7 ± 0.11
2.5	2.0 ± 0.15	1.9 ± 0.13
5.0	1.4 ± 0.20	0.9 ± 0.41

* Standard deviations from three replicate assays are given.

Table 3. *Effect of cations on chitin synthase activity*

Assays were carried out using the standard procedure described in Methods; the 'microsomal' fraction was used as the source of enzyme. The reaction mixture contained 0.4 mM-MgCl₂ and other cations, added as chlorides, at a final concentration of 10 mM.

Cation	Enzyme activity	
	GlcNAc incorporation (nmol/min/mg protein)	Percentage of control level
None (control)	0.90	100
Fe ³⁺	0.26	28.5
Mn ²⁺	0.80	88.0
Mg ²⁺	1.88	206.6
Ca ²⁺	0.36	39.6
Na ⁺	0.89	97.8
K ⁺	0.78	85.7
Co ²⁺	1.02	112.1
Cu ²⁺	0.48	52.7
Zn ²⁺	0.15	16.5
NH ₄ ⁺	0.85	93.4
Ba ²⁺	0.35	38.5

containing 20 mM-MgCl₂) from 1 to 500 mM, caused an inhibition of the chitin synthase activity of the 'microsomal' fraction (Fig. 3). The pH of the final reaction mixture was corrected in each case. The 25 mM buffer was selected for use, despite a slight inhibitory effect, because at lower concentrations buffering capacity was severely reduced.

Effect of different cations on chitin synthase activity

The influence of several metallic ions was investigated and only Mg²⁺ showed a marked stimulation in activity over the control (Table 3). Co²⁺ showed a slight increase in activity, but Zn²⁺, Fe²⁺, Ba²⁺, and Ca²⁺ caused the greatest inhibition of chitin formation. Variation in MgCl₂ concentration between 0.6 and 100 mM showed an increase in activity up to 20 mM, after which there was a progressive inhibition of the enzyme.

Effect of temperature

Standard reaction mixtures were incubated at various temperatures for 15 min. Chitin synthase activity was maximal between 31 and 33 °C.

Chitin synthase inhibitor in soluble cytoplasm

The presence of a soluble cytoplasmic inhibitor of chitin synthase activity *in vitro* was observed. Mycelium was homogenized without buffer and submitted to differential centri-

Table 4. *The effect of pH on the inhibition of chitin synthase activity by soluble cytoplasm*

Enzyme assays using the 'microsomal' fraction were carried out according to the procedure described in Methods. The 100000 g supernatant was added at a final concentration, as protein, of 1 mg/ml.

pH value	Enzyme activity (nmol/min/mg protein)		Relative activity (B/A)
	Without cytoplasm (A)	With cytoplasm (B)	
5.5	4.0	4.4	1.10
5.75	4.0	4.2	1.05
6.0	4.2	3.5	0.83
6.2	3.1	2.5	0.81
6.55	1.9	1.5	0.79
6.75	1.3	1.7	0.76
7.0	1.2	0.7	0.58
7.25	0.7	0.3	0.43
7.5	0.6	0.2	0.33

fugation as described in Methods. The 100000 g supernatant was added to the standard assay mixture containing the 'microsomal'-fraction enzyme. The inhibitor activity was not lost by heating to 100 °C or by dialysis against cold buffer. Scans of assay chromatograms showed only two peaks, corresponding to substrate and chitin, indicating that the inhibitor prevented product formation and did not cause product breakdown. The inhibitory effect of the cytoplasmic component was found to be pH-dependent. Assay mixtures containing 25 mM-KH₂PO₄-KOH buffer over the range pH 5.5 to 7.5 were set up and the 100000 g supernatant was added at a concentration of 1 mg protein/ml. Inhibition was found to increase linearly up to pH 7.5 (Table 4).

*Chitin synthase activities of M. vinacea in a fermenter
under different growth conditions*

In an attempt to relate chitin synthase production to growth of *M. vinacea*, the fungus was grown in a batch fermenter with different rates of aeration and with different medium compositions. The results are shown in Fig. 4. Halving the aeration rate (Fig. 4*a* compared with Fig. 4*b*) markedly affected the utilization of glucose and about 45 % of the substrate remained in the medium when the culture entered the stationary phase. At the higher rate of aeration the glucose was rapidly consumed and appeared to be the nutrient limiting extent of growth. The substrate was rapidly removed from the low-glucose medium (Fig. 4*c*), again indicating limitation of growth. The specific growth rates during exponential growth, calculated from logarithmic plots, were: high glucose-high aeration, 0.28 h⁻¹; high glucose-low aeration, 0.18 h⁻¹; low glucose, 0.25 h⁻¹. Biomass, at the maximum population phase, was greatest with high glucose and high aeration; reduced aeration halved the mycelial yield and low glucose reduced it to about 25 % of that on the high-glucose medium. Chitin synthase, expressed as specific activity or as total activity, showed broadly similar patterns under all conditions of growth. Maximum specific activity of the enzyme was observed at the end of exponential growth.

Fig. 4. Chitin synthase production by *M. vinacea* in batch fermenter culture. ○, Mycelium dry weight (mg/ml); ●, chitin synthase, total activity; □, chitin synthase, specific activity; ■, residual glucose in culture medium; △, residual ammonia in culture medium. (a) The culture medium was supplemented with glucose (10 g/l) and NH_4Cl (2.6 g/l) and the culture was aerated at 3 l/min. (b) The medium was as in (a), but aeration was 6 l/min. (c) The medium was supplemented with glucose (2.5 g/l) and NH_4Cl (2.6 g/l) and the culture was aerated at 6 l/min.

DISCUSSION

The high activity of chitin synthase in the 2000 g 'cell-wall' fraction of *M. vinacea* corresponds with the findings in the Mucorales, *Mucor rouxii* (McMurrough *et al.* 1971), *Phycomyces blakesleeanus* (Jan, 1974) and *Cunninghamella elegans* (Moore & Peberdy, 1975). In other organisms studied (Glaser & Brown, 1957; Jaworski *et al.* 1965; Porter & Jaworski, 1966; Camargo *et al.* 1967) the enzyme was said to be located mainly in the mitochondrial and 'microsomal' fractions, but no reference to the examination of 'cell-wall' fractions was made by these authors.

On the basis of their findings with *M. rouxii*, McMurrough *et al.* (1971) proposed that the 100000 g 'microsomal' fraction is the site of chitin synthase formation before its migration to its site of action in the cell wall. The difficulty of removing chitin synthase from the 'cell-wall' fractions of *M. vinacea* and *M. rouxii* (McMurrough *et al.* 1971) suggests that the enzyme is bound to the wall. However, electron micrographs of wall fractions of *Phycomyces blakesleeanus* (Jan, 1974) show high contamination of the walls with attached membranous material. A membranous location for chitin synthase in the yeast-form of *M. rouxii* is now indicated by the work of Ruiz-Herrera & Bartnicki-Garcia (1974). It is hoped that future studies on chitin synthase in protoplasts of *M. vinacea* will also help to elucidate the nature of the relationship between the enzyme and the hyphal wall.

Porter & Jaworski (1966) suggested that freezing and thawing of chitin synthase fractions may release inhibitor or hydrolytic enzymes which act upon the substrate or the chitin formed. The presence of activators and inhibitors of chitin synthase have subsequently been demonstrated in yeast cells (Cabib & Keller, 1971; Cabib & Ulane, 1973*a, b*). The presence of a heat-stable, pH-dependent inhibitor of chitin synthase, similar to that found in *M. vinacea*, has been reported in *M. rouxii* (McMurrough & Bartnicki-Garcia, 1973) and it has been purified as a protein of molecular weight 8500 from *Saccharomyces cerevisiae* (Ulane & Cabib, 1974). Activators of chitin synthase have not as yet been isolated from filamentous fungi, although activation by the addition of proteases has been demonstrated in *M. rouxii* (McMurrough & Bartnicki-Garcia, 1973) and *Aspergillus nidulans* (N. R. Ryder, personal communication).

The formation of diacetylchitobiose as an intermediate in chitin synthesis has been reported in *Blastocladiella emersonii* (Camargo *et al.* 1967), and diacetylchitobiose was formed in place of chitin in the presence of low UDP-GlcNAc concentrations. In *M. vinacea* we did not detect diacetylchitobiose but this may have been due to the relatively large amounts of UDP-GlcNAc used (50 to 100 nmol/assay, compared with approximately 2 nmol used with *B. emersonii*). On the other hand, with *M. rouxii*, diacetylchitobiose was detected only with high concentrations of UDP-GlcNAc (McMurrough *et al.* 1971).

Identification of the chromatographically-immobile substance as chitin was confirmed by its hydrolysis with concentrated HCl to glucosamine, and by the action of chitinase upon it, which yielded GlcNAc as the sole product.

In chitin synthesis, GlcNAc and soluble chitodextrins have been described as activators (Glaser & Brown, 1957; Jaworski *et al.* 1965; Porter & Jaworski, 1966). Activation was also found to be induced by *N*-acetyl chitodextrins in *M. rouxii* (McMurrough & Bartnicki-Garcia, 1971) and by mannose, cellobiose, glucose and glycerol (Keller & Cabib, 1971) in *S. cerevisiae*. GlcNAc is thought to bear the role of an allosteric effector (Glaser & Brown, 1957), although Camargo *et al.* (1967) proposed a second role as that of chitin initiator. Activation of *M. vinacea* chitin synthetase by GlcNAc was low compared with other organisms (Glaser & Brown, 1957; Porter & Jaworski, 1966; McMurrough & Bartnicki-Garcia, 1971). Soluble chitodextrins are thought to behave as primers (Glaser & Brown, 1957). In our investigations soluble chitodextrins were found to behave as inhibitors of chitin synthesis, leading to large reductions in net product formation. Similar results were reported with *S. cerevisiae* (Keller & Cabib, 1971) and *C. elegans* (Moore & Peberdy, 1975).

In growing hyphae the tips are the sites of wall synthesis (Bartnicki-Garcia & Lippman, 1969; Grove & Bracker, 1970; Gooday, 1971), and the increase in specific activity of chitin synthase during exponential growth may correlate with the increase of growing points due to branching, which occurs during this phase. The decrease in specific activity during the later stages of growth may be due to protein turnover or the formation of new enzyme coupled with a decline in the number of new growth points. The involvement of the cytoplasmic inhibitor and of proteases in releasing activated chitin synthase in growing cultures is not yet known; it is likely they play a very complex regulatory role.

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