Chitin Synthase Activity from *Neocallimastix frontalis*, an Anaerobic Rumen Fungus

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Chitin synthase activity was detected in actively growing mycelium of *Neocallimastix frontalis* after mechanical disruption of the cells. Chitin formation in fungal extracts at 32 °C was linear with respect to time for at least 60 min, and with respect to protein concentration up to 750 µg ml⁻¹. The optimum pH for enzyme activity was 8.5 using 10 mM-Tris/HCl buffer. Mg²⁺ was necessary for maximum activity and 10 mM-MgCl₂ was routinely used during the assays. The apparent K_m for the substrate UDP-GlcNAc was 2 mM. Polyoxin D was a competitive inhibitor of chitin synthesis with an apparent K_i of 4 µM. Following treatment with trypsin (12.5 µg ml⁻¹), the chitin synthase activity of the fungal extract increased by six-fold, indicating that most of the chitin synthase activity was zymogenic. The reaction product was insoluble in 1 M-KOH or 1 M-acetic acid, but it was solubilized by heating in 6 M-HCl at 120 °C for 2.5 h and was hydrolysed by chitinase into diacetylchitobiose.

INTRODUCTION

The membrane-bound enzyme chitin synthase (EC 2.4.1.16) has been isolated from a wide range of fungi (Cabib, 1981), where it plays a major role in cell-wall biosynthesis (Cabib *et al.*, 1979; Gooday & Trinci, 1980). Studies of chitin synthase in higher fungi or yeasts have contributed to an understanding of the regulation of chitin synthesis, but little information is available about the obligate anaerobic fungus *Neocallimastix frontalis*, which inhabits the gut of sheep. This chitinous fungus (Orpin, 1977) and related species appear unique in many respects (morphology, life cycle, ability to grow under conditions of low redox potential and in the absence of oxygen). The aim of this study was the characterization of chitin synthase from a laboratory-grown strain of *N. frontalis*.

METHODS

Preparation of extracts. *Neocallimastix frontalis* (isolated from sheep rumen) was maintained on the synthetic liquid medium B of Lowe *et al.* (1985), modified as follows: Na₂CO₃ 8 g l⁻¹ instead of 80 g l⁻¹; cellobiose 10 g l⁻¹ instead of 3.75%; lysozyme was omitted; the reducing agent solution contained L-cysteine 1 g l⁻¹ instead of 2.5 g l⁻¹ and NaHCO₃ 6 g l⁻¹ instead of Na₂S.9H₂O 2.5 g l⁻¹. Cultures were grown for 72 h at 39 °C and harvested by centrifugation (8000 g for 10 min). Cells were broken in 10 mM-Tris/HCl buffer pH 7.5 plus 2 mM-Na₂EDTA, with a Virtis blade homogenizer (20000 r.p.m. for 3 × 30 s) at 4 °C, and the resulting crude homogenate (CH) was centrifuged at 1000 g for 10 min. The pellet consisted mainly of large cell wall debris (cell-wall fraction: CWF) and was retained. The supernatant fluid was centrifuged at 48000 g for 30 min; the pellet (mixed-membrane fraction: MMF) and the supernatant fluid (supernatant fraction: SF) were used as sources of enzyme.

Abbreviations: CH, crude homogenate; CWF, cell-wall fraction; MMF, mixed-membrane fraction; SF, supernatant fraction; GlcNAc, *N*-acetylglucosamine; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine.

Chitin synthase assay. A modification of the method of Raeven (1981) was used. The cell extracts (CWF, MMF) were suspended in 20 mM-Tris/HCl buffer pH 7.5 containing 10 mM-MgCl₂. The standard reaction mixture consisted of 0.5 mM-UDP-GlcNAc [25 nCi (925 Bq) UDP-[U-¹⁴C]GlcNAc, sp. act. 302 Ci mol⁻¹ (11.2 TBq mmol⁻¹)], 20 mM-GlcNAc, 20 mM-Tris/HCl buffer pH 8.5, 10 mM-MgCl₂, 12.5 µg trypsin ml⁻¹ (bovine pancreas, Boehringer), and 50 µl of the enzyme extract, in a final volume of 200 µl; incubation was for 40 min at 32 °C. The reaction was stopped with 2 ml 95% (v/v) ethanol and the reaction product was filtered on glass-fibre filters (GF/C, Whatman) and washed successively with 5 ml 0.5 M-NaOH, 2 × 10 ml 95% ethanol/1 M-acetic acid (2:8, v/v), and 2 × 5 ml 95% ethanol.

Specific activity of the enzyme was expressed as nmol GlcNAc incorporated in 1 min by 1 mg protein (measured by the Lowry method).

Characterization of the reaction product. The reaction mixture was made up to 2 ml and incubated for 2.5 h. The reaction was stopped with 6 ml 95% ethanol and the material was then centrifuged at 48000 g for 30 min to yield a radioactive pellet and a clear supernatant. The pellet was suspended in 2 ml distilled water and samples of the suspension were used for the identification of the product, by either enzyme or acid hydrolysis.

For enzyme hydrolysis, 0.8 mg chitinase (Sigma) in 800 µl 2 mM-sodium phosphate buffer pH 6, containing 80 U penicillin and 24 µg streptomycin, was added to 400 µl of the radioactive suspension. The mixture was incubated for 20 h at 30 °C and the reaction was terminated by the addition of 95% ethanol. The mixture was either filtered through a glass-fibre filter and its radioactivity measured, or it was subjected to chromatography on cellulose F pre-coated TLC plates (Merck). The chromatograms were developed in 95% ethanol/1 M-ammonium acetate (7:3, v/v), butanol/pyridine/water (6:4:3, by vol.) or ethyl acetate/pyridine/water (2:2:1, by vol.). The spots were visualized by spraying with silver reagent or ninhydrin (for glucosamine) and were identified by comparing the *R_F* values with those obtained using authentic compounds. The spots were excised and their radioactivity was measured.

For acid hydrolysis, 400 µl of the radioactive suspension was hydrolysed with 800 µl 6 M-HCl at 120 °C for 2.5 h and then chromatographed as described above. Other samples were treated with 1 M-KOH for 1 h at 100 °C or with 1 M-acetic acid for 30 min at 50 °C.

RESULTS

Distribution of chitin synthase activity. The fractions (CH, CWF, MMF and SF) obtained by differential centrifugation were assayed for their chitin synthase activity (Table 1). Chitin synthase was located mainly in the cell wall fraction (1000 g pellet) and the mixed membrane fraction (48000 g pellet): the supernatant fraction (48000 g supernatant) yielded very little activity. The distribution of the enzyme activity between CWF and MMF varied according to the degree of homogenization: CWF activity was lowered in heavily disrupted extracts (3 × 2 min homogenization) but MMF activity was not concomitantly increased, showing that the extra membranes released from CWF were disorganized. On average, 60–65% of the total activity was recovered in CWF, and 30–35% in MMF. This distribution is consistent with the hypothesis (Vermeulen *et al.*, 1979) that the enzyme is attached to the plasma membrane which can, depending on the homogenization procedure, remain more or less attached to the cell wall. The low specific activity of the crude extract (about half that of CWF or MMF) could be related to inhibitory substances released during homogenization, but it could also be explained by the presence of EDTA in the homogenization buffer, which could interfere with the divalent cations required for the reaction (Orlean, 1987). In control experiments in which MMF was incubated in the presence of 2 mM-Na₂EDTA, specific activity was lowered to 10.0 nmol min⁻¹ (mg protein)⁻¹.

The MMF was used for enzyme characterization due to its greater homogeneity and higher specific activity. Only data from experiments using MMF are presented here, but properties of chitin synthase in CWF resembled those of MMF.

Proteolytic activation of the enzyme. When fungal extracts were pretreated for 30 min with trypsin (bovine pancreas, Boehringer) (1–50 µg ml⁻¹) then with trypsin inhibitor (soybean, Boehringer) (2–200 µg ml⁻¹) prior to the chitin synthase assay, enzyme activity was inhibited. Subsequently, trypsin was incorporated in the assay mixture throughout the incubation. The enzyme activity was strongly stimulated (5- to 10-fold) by such protease treatment (Fig. 1), with maximum activity at 12.5 µg trypsin ml⁻¹. At higher trypsin concentrations, chitin synthase activity was lower, presumably because of enzyme inactivation.

Fig. 1. Effect of trypsin on chitin synthase activity [$\text{nmol GlcNAc min}^{-1} (\text{mg protein})^{-1}$] of MMF ($420 \mu\text{g protein ml}^{-1}$; pH 7.5; 24°C for 40 min).

Table 1. Distribution of chitin synthase activity in cell-free extracts of *N. frontalis*

The fractions were prepared and assayed as described in Methods. For each fraction, the results show the specific activity [$\text{nmol GlcNAc min}^{-1} (\text{mg protein})^{-1}$, $\pm\text{SD}$] and the total activity ($\text{nmol GlcNAc min}^{-1}$). Activity is also expressed as a percentage of the total activity in all four fractions. All observations were repeated five times.

Fraction	Protein content (mg)	Percentage of total protein	Total activity	Percentage of total activity	Specific activity
CH	4.65	100	30.2	100	6.5 ± 2.4
CWF	1.72	37	19.6	60–65	11.4 ± 2.3
MMF	0.60	13	9.9	30–35	16.5 ± 1.0
SF	2.33	50	0.7	0–5	0.3 ± 0.2

The formation of chitin as a function of time. At a UDP-GlcNAc concentration of 0.5 mM, the rate of incorporation of GlcNAc into chitin at 32°C was almost linear up to 60 min. The subsequent decline in activity may have been due to the depletion of substrate or to the accumulation of UDP, which may be inhibitory (Raeven, 1981), and perhaps to the destruction of enzyme by trypsin. For further experiments, the reaction was thus always stopped after 40 min incubation.

Dependence of chitin synthesis on temperature and pH. The optimal temperature for enzyme activity in 40 min assays was about 32°C , with a sharp decrease in activity at low (14°C) or high (50°C) temperatures. This high temperature optimum might relate to the natural environment of the fungus, but this value would also represent a balance between a high initial stimulation of the enzyme and its degradation by trypsin.

The enzyme was assayed at 24°C under standard conditions at pH values ranging from 6 to 9, using 20 mM-MES or Tris/HCl buffers. The synthesis of chitin was very low at pH 6 [$1.6 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$], and maximal activity was observed at pH 8.5 [$9 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$].

Kinetics and competitive inhibition. Lineweaver-Burk plots of reaction velocity against substrate concentration (0.1–5 mM-UDP-GlcNAc) (Fig. 2a) were linear at high concentration but became sigmoidal at low concentration, as observed by de Rousset-Hall & Gooday (1975) for chitin synthase from *Coprinus cinereus*. The apparent K_m value for UDP-GlcNAc was about 2 mM. With different concentrations of substrate (0.5 and 1 mM-GlcNAc), polyoxin D, a specific inhibitor of chitin synthase activity (Endo *et al.*, 1970), acted as a competitive inhibitor; plots of activity vs. inhibitor concentration (ranging from 1 to $100 \mu\text{g ml}^{-1}$) were linear and revealed a K_i of about $4 \mu\text{M}$ (Fig. 2b). On the other hand, the enzyme activity was stimulated by 20 mM-GlcNAc, which acts as an allosteric activator of other chitin synthases (Gooday, 1978). Divalent cations were needed for maximum activity, and Mg^{2+} was the most efficient of those tested: a

Fig. 2. Effect of substrate concentration and polyoxin D on chitin synthase activity. (a) Lineweaver-Burk plots of the velocity (v , nmol GlcNAc incorporated min^{-1}) of chitin synthase activity against substrate concentration (S , mM GlcNAc). (b) Effect of polyoxin D concentration on enzyme activity at UDP-GlcNAc concentrations of 1 mM (○) or 0.5 mM (●).

change of Mg^{2+} to Co^{2+} or Mn^{2+} lowered the specific activity of MMF from 14.6 to 1.7 or 1.2 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$, respectively.

Identification of the reaction product. Most (97%) of the radioactivity in the incubated mixture was retained on the glass-fibre filter, indicating that a polymer was produced. Following digestion with chitinase, only 8% of the radioactivity remained insoluble and the chromatographic analysis of the solubilized fraction (92% of the radioactivity) revealed mainly diacetylchitobiose. On strong acid hydrolysis (6 M-HCl), 95% of the radioactivity was solubilized and chromatograms revealed glucosamine as the only component of the hydrolysed fraction.

The product of the reaction in the incubation mixture was insoluble in 1 M-acetic acid or in 1 M-KOH, as respectively 96% and 93% of the radioactivity was recovered on glass-fibre filters. Essentially all of the polymer synthesized can thus be accounted for as chitin because the major products obtained from chitinase action or acid hydrolysis were diacetylchitobiose and glucosamine respectively.

DISCUSSION

This study indicates that most of the properties of chitin synthase extracted from the anaerobic rumen fungus *N. frontalis* are similar to what is known from other fungi (Gooday & Trinci, 1980). The sugar donor was UDP-GlcNAc with an apparent K_m of 2 mM but the biphasic nature of the Lineweaver-Burk plots suggests that UDP-GlcNAc is an activator as well as a substrate to the *N. frontalis* chitin synthase and could act as an allosteric effector of the enzyme. The considerable degree of activation of chitin synthase by GlcNAc and Mg^{2+} observed with membrane fractions supports their roles as allosteric activator and cofactor, respectively. The enzyme was largely present in a zymogenic form (presumably the native form: Cabib, 1981) that was activated by limited proteolysis with trypsin (12.5 $\mu\text{g ml}^{-1}$) and UDP-GlcNAc incorporation was competitively inhibited by polyoxin with a K_i of 4 μM . In contrast to the chitin synthases extracted from aerobic fungi, which are generally active at low temperature (24 °C) and at pH < 7.5 [except for *Candida albicans* (Hardy & Gooday, 1978) and *Coprinus cinereus* (Gooday & de Rousset-Hall, 1975)], *N. frontalis* chitin synthase was more active at 32–39 °C and its optimum pH was more alkaline than optima generally reported for this enzyme in other fungi. These properties resemble those of chitin synthase-2 extracted from *Saccharomyces cerevisiae* (Sburlati & Cabib, 1986; Orlean, 1987), but the *N. frontalis* enzyme was far more active and was inhibited by Co^{2+} .

Since chitin is synthesized during the growth cycle of the fungus, chitin synthase activity could be a useful physiological parameter to estimate fungal growth. Further experiments will determine whether chitin synthase activity could be a reliable method to quantify *N. frontalis* biomass.

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