The Secretion of *N*-Acetylglucosaminidase during Germ-tube Formation in *Candida albicans*

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N-Acetylglucosaminidase was induced by either *N*-acetylglucosamine or *N*-acetylmannosamine in several strains of *Candida albicans*. Enzyme activity was not induced in a *N*-acetylglucosamine non-utilizing mutant which is unable to express the first three steps in the *N*-acetylglucosamine catabolic pathway. The enzyme, purified 500-fold, had a specific activity of 36.8 units (mg protein)⁻¹ and catalysed the hydrolysis of *p*-nitrophenyl- β -*n*-acetylglucosamine, *N,N'*-diacetylchitobiose and *N,N',N''*-triacetylchitotriose. No activity was observed toward colloidal chitin, hyaluronic acid or mucin. The cellular distribution of *N*-acetylglucosaminidase was determined by measuring *in situ* enzyme activity before and after acid treatment of intact cells. *N*-Acetylglucosaminidase (80–88% of the total cellular activity) was rapidly secreted to the periplasm when the enzyme was induced either during yeast growth at 28 °C or germ-tube formation at 37 °C. Export of the enzyme from the periplasm into the medium was fourfold greater during germ-tube formation, and after 6 h incubation the amount of enzyme released into the medium represented 70% of cell-associated enzyme activity.

INTRODUCTION

N-Acetylglucosamine (GlcNAc) (Simonetti *et al.*, 1974) and some related *N*-acetylhexosamine derivatives (Sullivan & Shepherd, 1982; Shepherd & Sullivan, 1983) induce germ-tube formation when yeast cells of *C. albicans* are incubated at 37 °C. Further, the enzymes of the GlcNAc pathway are induced at both 28 °C, without the dimorphic transition, and at 37 °C (Shepherd *et al.*, 1980*b*; Gopal *et al.*, 1982). In this paper we describe the properties of a secreted *N*-acetyl- β -glucosaminidase which is induced in *C. albicans* by GlcNAc. This enzyme is exported into the medium during germ-tube formation.

METHODS

Organisms. The following strains of *Candida albicans* were used in this work: ATCC 10261; CA2 and A72, kindly provided by Dr A. Cassone, Istituto Superiore di Sanita, Rome; hOG301, a mycelial mutant, and hOG298, an adenine-requiring GlcNAc non-utilizing mutant. The hOG strains were isolated from strain ATCC 10261 and kindly provided by Dr R. T. M. Poulter, Biochemistry Department, University of Otago.

Growth, germ-tube formation and enzyme induction. The maintenance of yeast cultures, growth of yeast batch-cultures (Shepherd & Sullivan, 1976), preparation of starved yeast cells, conditions for germ-tube formation (Shepherd *et al.*, 1980*a*) and enzyme induction (Gopal *et al.*, 1982) have been described previously. Adenine (3.0 mM) was included in the media used for strain hOG298. Unless otherwise stated inducers were added to the medium at a final concentration of 2.5 mM. For germ-tube formation starved cells (4×10^7 cells ml⁻¹) and inducer were incubated at 37 °C in 10 mM-imidazole/HCl buffer, pH 6.6 containing 0.1 mM-MnCl₂. In one experiment (illustrated in Fig. 1) starved yeast cells were incubated in salts/biotin medium minus the glucose and (NH₄)₂SO₄

Abbreviations: GlcNAc, *N*-acetylglucosamine; pNP-GlcNAc, *p*-nitrophenyl- β -*N*-acetylglucosamine.

(Shepherd *et al.*, 1980a) but containing 10 mM-GlcNAc at 28 °C for yeast growth and 37 °C for germ-tube formation. Yeast-budding and germ-tube formation were assessed by phase-contrast microscopy (Shepherd *et al.*, 1980a). Yeast-phase growth was determined from the OD₅₄₀ (Sullivan & Shepherd, 1982).

Enzyme preparations. Cell-free extracts were prepared in either 0.01 M- or 0.1 M-potassium phosphate buffer, pH 6.95, as indicated, with a Braun homogenizer (B. Braun, Melsungen, FRG) (Gopal *et al.*, 1982). Permeabilized cells were prepared for *in situ* assays essentially as described by Ram *et al.* (1983); cell suspensions ($1.2\text{--}1.6 \times 10^9$ cells ml⁻¹ in 0.1 M-potassium phosphate buffer, containing 0.01 M-MgCl₂) were shaken with 10% (v/v) toluene/ethanol/Triton X-100 (1:4:0.2, by vol.) for 5 min at 4 °C and washed twice with 0.1 M-potassium phosphate buffer, pH 6.95.

The cellular distribution of *N*-acetylglucosaminidase was determined by measuring *in situ* enzyme activity before and after treatment of intact cells with 0.1 M-HCl (Ram *et al.*, 1984). Washed cells were resuspended in 0.1 M-HCl at 4 °C, incubated for 15 min, neutralized with NaOH and washed three times with the potassium phosphate buffer. Exported *N*-acetylglucosaminidase was measured in concentrated culture supernatants. Each culture sample was centrifuged (3000 g, 10 min), the supernatant was concentrated 50-fold by dialysis against polyethylene glycol (20000) and finally dialysed against distilled water.

Enzyme assay. The assay system for β -*N*-acetyl-D-glucosaminidase (EC 3.2.1.30) contained in 0.5 ml: 30 μ mol potassium phosphate buffer, pH 6.95, 3 μ mol *p*-nitrophenyl- β -*N*-acetylglucosamine (pNP-GlcNAc) and 0.02–0.2 ml enzyme solution or permeabilized cells suspension. The assays were incubated for a fixed time (up to 30 min) at 37 °C, 2 ml 4% Na₂CO₃ was added, the tubes were centrifuged and the A₄₂₀ was read against a blank which contained buffer and pNP-GlcNAc. A unit of enzyme activity catalyses the formation of 1 μ mol *p*-nitrophenol per min ($\approx 16.0 \times 10^3$ l mol⁻¹ cm⁻¹). Protein was estimated by the modified Lowry method (Eggstein & Kreutz, 1967) with crystalline bovine serum albumin as a standard. Enzyme activity is expressed either as units (mg protein)⁻¹ or units (10¹⁰ cells)⁻¹.

Estimation of GlcNAc. This was done by the method of Reissig *et al.* (1955).

HPLC gel permeation chromatography. The HPLC gel permeation chromatography columns used in the enzyme purification were a GSWP pre-column (7.5 × 75 mm) and a G3000 SW fractionation column (7.5 × 1200 mm) obtained from the Toya Soda Manufacturing Co., Nan-Yo, Yamaguchi, Japan.

Purification of the enzyme. Strain CA2 was grown for 16 h at 28 °C, harvested and incubated with GlcNAc for 2 h to induce *N*-acetylglucosaminidase. These cells (11 g, wet wt) were disrupted in 0.01 M-potassium phosphate buffer, pH 6.95, and the enzyme was adsorbed batchwise onto 120 ml bed volume of DEAE-Sephacel equilibrated with 10 mM-potassium phosphate, pH 6.95. The DEAE-Sephacel was washed three times with 80 ml buffer and the enzyme was eluted with five washes of buffer containing 100 mM-NaCl. The combined eluate (200 ml) was dialysed overnight against 1 mM-potassium phosphate buffer, pH 6.95 (2 × 2 l), concentrated to 10 ml with a Millipore Pellicon membrane (exclusion limit 10000 Dal) and lyophilized. The freeze-dried preparation was dissolved in distilled water (3 ml) and two 1.3 ml samples were fractionated on a TSK GSW3000 HPLC gel permeation column (0.7 cm × 1.2 m) equilibrated with 10 mM-potassium phosphate buffer, pH 6.95. The flow rate was 0.6 ml min⁻¹. Fractions (0.6 ml) containing enzyme activity were pooled, dialysed against distilled water, lyophilized and rerun through the column. The final preparation contained insufficient protein for a Lowry estimation but the protein content determined by the dye-binding method (Bradford, 1976) indicated that the specific activity was 36.8 units (mg protein)⁻¹. This represented a 500-fold purification with a 12% recovery. *N*-Acetylglucosaminidase partially purified by the same procedure from strain ATCC 10261 had a specific activity of 2 units (mg protein)⁻¹.

Materials. *p*-Nitrophenyl derivatives, *N*-acetyl amino sugars, hyaluronic acid and naphthol AS-LC *N*-acetyl- β -glucosamine were obtained from Sigma. Colloidal chitin was prepared by the method of Jeuniaux (1966) from crab shells. Mucin, purified from pig intestine, was kindly provided by Dr A. M. Roberton, Biochemistry Department, University of Auckland, New Zealand. Naphthyl- β -*N*-acetylglucosamine was obtained from Serva, Heidelberg, FRG.

RESULTS

Induction of N-acetylglucosaminidase

During studies on GlcNAc-induced germ-tube formation in *C. albicans* it was noted that permeabilized cells contained *N*-acetylglucosaminidase activity. The enzyme was induced by either GlcNAc or *N*-acetylmannosamine and at 28 °C (yeast growth) or 37 °C (germ-tube formation). After 3 h incubation at 28 °C the specific activity in ATCC 10261 was 0.6 units (10¹⁰ cells)⁻¹ with GlcNAc and 0.5 units (10¹⁰ cells)⁻¹ with *N*-acetylmannosamine. The enzyme was not induced by glucose, hyaluronic acid (0.2 mg ml⁻¹), chitin (0.2 mg ml⁻¹), chitin plus glucose, glucose plus glutamine, mucin (1 mg ml⁻¹), peptone (0.2%, w/v) or serum (1%, v/v). All the

Table 1. *N*-Acetylglucosaminidase activity in strains of *C. albicans*

Enzyme activity was induced with GlcNAc and measured in the *in situ* assay system. The values in parentheses were obtained with cells incubated in buffer without GlcNAc. The values shown are the means of duplicate determinations.

Strain	Activity [units (10 ¹⁰ cells) ⁻¹]
ATCC 10261	0.85 (0.05)
A72	2.5 (0.03)
CA2	5.5 (0.05)
hOG298	0 (NT)
hOG301	0.65 (0.03)

NT, Not tested.

Table 2. *Purification of N*-acetylglucosaminidase

N-Acetylglucosaminidase was purified from strain CA2 yeast cells as described in the text.

Fraction	Vol. (ml)	Total enzyme (units)	Total protein (mg)	Specific activity [units (mg protein) ⁻¹]
Cell-free extract	20	12.1	168	0.072
After DEAE-Sephacel step	200	5.2	20	0.26
After lyophilization	3	3.3	6	0.55
Concentrate from gel permeation chromatography	2	1.8	0.4	4.5
After the second gel permeation step	2.8	1.40	0.038	36.8

components listed except glucose and peptone induced germ-tube formation at 37 °C; hyaluronic acid, chitin and mucin did not support growth. *N*-Acetylglucosaminidase was induced by GlcNAc in all strains tested except hOG298, the GlcNAc non-utilizing mutant (Table 1). The data given represent typical examples of experiments which were repeated with duplicate assays (SE <10%). The variability between experiments was less than 20%.

Purification and properties

A rapid and effective purification of *N*-acetylglucosaminidase was achieved with ion-exchange chromatography and gel permeation HPLC (Table 2). After the second passage through the HPLC column a single symmetrical peak was obtained. *N*-Acetylglucosaminidase was stable for several weeks at 4 °C and indefinitely at -20 °C. In potassium phosphate buffers the enzyme exhibited a broad pH-activity curve with a maximum at pH 6.5. The *K_m* for pNP-GlcNAc under standard assay conditions was 0.45 mM. No activity was observed with the following compounds each at 5 mM: *p*-nitrophenyl- α - or - β -glucoside, *p*-nitrophenyl- α - or - β -mannoside and *p*-nitrophenyl- β -diacetylchitobiose. The enzyme did not produce GlcNAc from chitin (0.04 mg), hyaluronic acid (0.05 mg) or mucin (0.4 mg) when these compounds were added to the standard system. However, as shown in Table 3, the enzyme hydrolysed diacetylchitobiose and triacetylchitotriose. The elution volume for *N*-acetylglucosaminidase from the gel permeation column was 26.4 ml, similar to that for ovalbumin (26 ml) which has a molecular weight of 44 000. In SDS-polyacrylamide gel electrophoresis however, the purified enzyme from strain CA2 showed two overlapping bands which migrated as a protein of molecular weight 66 000.

Secretion of N-acetylglucosaminidase

Cell viability and the capacity to form germ-tubes is unaffected when *C. albicans* cells are suspended in 0.1 M-HCl at 4 °C for 15 min. However, enzymes associated with the cell wall and periplasmic space are inactivated by this treatment (Ram *et al.*, 1984). A preliminary

Fig. 1. Induction and secretion of *N*-acetylglucosaminidase. Starved yeast cells of strain ATCC 10261 were incubated at 8×10^7 cells ml^{-1} in salts/biotin medium containing 10 mM-GlcNAc. Yeast growth at 28 °C (■) was measured at 540 nm, and germ-tube formation (□) at 37 °C was assessed by phase-contrast microscopy. At the times indicated total *in situ* activity (●), acid-resistant *in situ* activity (○) and enzyme exported to the medium (▲) were measured during yeast growth (a) and germ-tube formation (b). The values shown are the means of at least two determinations. Experiments were reproducible with variation of <20%.

Table 3. *Substrate specificity of N-acetylglucosaminidase*

Purified *N*-acetylglucosaminidase from strain CA2 was incubated with the compounds listed (5 mM final concentration) under standard assay conditions. Activity towards each of the *p*-nitrophenyl derivatives was measured by the release of *p*-nitrophenol. Activity towards the other substrates was determined by the formation of GlcNAc as summarized in Methods.

Substrate	Activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
<i>p</i> -Nitrophenyl- β - <i>N</i> -acetylglucosamine	17
<i>p</i> -Nitrophenyl- β - <i>N</i> -diacetylchitobiose	0
Diacetylchitobiose	11.5
Triacetylchitotriose	3.3
Naphthyl- β - <i>N</i> -acetylglucosamine	0
Naphthol AS-LC <i>N</i> -acetyl- β -D-glucosamine	0

experiment showed that more than 80% of the *in situ* *N*-acetylglucosaminidase was inactivated by acid treatment. Therefore the rates of induction, secretion and the release of the enzyme into the medium were measured during yeast growth at 28 °C and germ-tube formation at 37 °C. In the GlcNAc/salts/biotin medium at 28 °C more than 80% of the cells formed buds and daughter cells. After 6 h incubation the cell density had doubled. At 37 °C more than 80% of the cells formed germ-tubes and after 6 h hyphae were four to five times the mother cell diameter in length (Fig. 1). The apparent rate of enzyme synthesis (total *in situ* activity) was faster at 37 °C during the first hour but beyond 2 h the total *in situ* activity in the yeast cells (28 °C) was greater. *N*-Acetylglucosaminidase was secreted to the periplasmic space during yeast growth and germ-tube formation as judged by the effect of acid treatment prior to an *in situ* assay; after 3 h incubation 88% and 80%, respectively, of the total *in situ* activity was acid-labile (i.e. associated with the wall and periplasmic space). The enzyme was released into the medium at a much higher rate during germ-tube formation. After 3 h incubation no enzyme was detected in the yeast growth medium but the enzyme exported by the germ-tube forming cells represented 29%

of the total *in situ* activity. At 6 h the exported enzyme was 14% and 70% of the total cellular enzyme for the yeast and hyphal cultures, respectively.

DISCUSSION

N-Acetylglucosaminidase was induced gratuitously by either GlcNAc or *N*-acetylmannosamine. Since the enzyme hydrolysed diacetylchitobiose and to a lesser extent triacetylchitotriose it could participate in the degradation of small oligomers arising from chitin. Further, it is possible that the enzyme can remove terminal GlcNAc residues from glycopeptides and glycans although it exhibited no activity towards chitin, mucin or hyaluronic acid. The SDS-PAGE and gel permeation chromatography data suggest that the native enzyme is a monomer with a molecular weight of 66000, although it has been found that a number of proteins of known molecular weights do not exhibit predicted elution volumes in the HPLC gel permeation columns (P. A. Sullivan, unpublished results).

All the strains of *C. albicans* tested except hOG298 possessed the inducible *N*-acetylglucosaminidase. The mutant hOG298 is unable to induce the GlcNAc uptake system, GlcNAc-kinase and GlcNAc-6-phosphate deacetylase (B. E. Corner, R. T. M. Poulter, M. G. Shepherd & P. A. Sullivan, unpublished results). The present results suggest that induction of *N*-acetylglucosaminidase may be co-ordinated with induction of the permease and catabolic enzymes. Consistent with this suggestion, it was previously found that *N*-acetylmannosamine is not taken up by *C. albicans* but that it is a gratuitous inducer for the permease and the catabolic enzymes (Sullivan & Shepherd, 1982).

Pugh & Cawson (1977) reported a cytochemical detection of *N*-acetylglucosaminidase in *C. albicans*. The enzyme was reported to be secreted around the entire yeast cell, associated with intracellular vesicles and was secreted by germ-tube forming cells. In the present study more than 80% of the enzyme was secreted as judged by the acid lability in intact cells. It was not, however, tightly associated with the cell wall since all (>95%) of the total activity was recovered in the soluble fraction after cell disruption (data not shown). Following enzyme induction *N*-acetylglucosaminidase was rapidly secreted to the periplasm of cells grown at both 28 °C and 37 °C but export of the enzyme to the medium was fourfold greater during hyphal formation. Recently we found that exo-(1→3)- β -glucanase was secreted and exported in *C. albicans* during germ-tube formation but negligible enzyme was released into the yeast growth medium (Ram *et al.*, 1984). It is suggested that the germ-tube wall is more porous than that of yeast cells and consequently export of enzymes is facilitated by the formation of hyphae. The secretion of hydrolases and production of hyphae have frequently been cited as determinants of virulence in *C. albicans* (Odds, 1979). Recently MacDonald & Odds (1983) reported the isolation of a protease-secretion-deficient mutant of *C. albicans* which was less virulent towards mice. Although mycelium is usually encountered in infected tissue the importance of hyphae in the development of candidosis has remained controversial (Odds, 1979). The results reported in this paper suggest that hyphal formation and the export of hydrolytic enzymes should be considered as related virulence determinants since the former facilitates the latter. Finally, it should also be noted that the appearance of an electron-transparent innermost layer in the cell wall with the onset of germ-tube formation (Cassone *et al.*, 1973) may reflect the export of cell wall-associated proteins.

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