Purification and characterization of two forms of *N*-acetylglucosaminidase from *Candida albicans* showing widely different outer chain glycosylation

Chris Molloy,¹ Richard D. Cannon,² Patrick A. Sullivan³
and Maxwell G. Shepherd²

Author for correspondence: Richard D. Cannon. Tel: +64 3 479 7081. Fax: +64 3 479 7063.
e-mail: cannon@otago.ac.nz

¹ Animal and Veterinary Sciences, PO Box 84, Lincoln University, Canterbury, New Zealand

^{2,3} Experimental Oral Biology Laboratory, School of Dentistry², and Department of Biochemistry³, University of Otago, PO Box 647 Dunedin, New Zealand

Two forms of *N*-acetylglucosaminidase were purified to homogeneity by ion exchange (TSK DEAE-3SW, Aquapore CX-300) and gel filtration (TSK G4000 SW) HPLC of *Candida albicans* ATCC 10261 culture filtrates. Synthesis and secretion of *N*-acetylglucosaminidase were induced by incubating starved yeast cells at 37 °C in medium containing *N*-acetylglucosamine (GlcNAc). The form of the enzyme depended on the cell growth and starvation conditions before GlcNAc induction. *N*-Acetylglucosaminidase A (32 % total carbohydrate, M_r 85 000 subunit) was isolated from cells grown in glucose/salts/biotin medium, and *N*-acetylglucosaminidase B (56 % carbohydrate, M_r 132 000 subunit) was isolated from cells grown in yeast extract/peptone/dextrose. The estimated relative molecular masses of the native enzymes, based on Sephacryl S-300 gel filtration were: A form, 350 000; B form, 600 000; A and B forms after endoglycosidase H (endo H) treatment, 180 000. The purified enzymes migrated on SDS polyacrylamide gels as heterogeneous glycoproteins of M_r centred at ~100 000 (A) and ~150 000 (B) but were reduced to a single 58 000 band after denaturation with SDS and cleavage of asparagine-linked sidechains by endo H. When the native glycoproteins were treated with endo H, both enzyme forms had three oligosaccharide sidechains of M_r ~3000 that were endo H resistant. Therefore the difference in the size of *N*-acetylglucosaminidase A and B was due to variations in outer chain glycosylation of endo H-sensitive inner core structures. *N*-Acetylglucosaminidase was active and stable over a broad pH range with maximum activity against both *p*-nitrophenylGlcNAc (pNPGlcNAc) and pNPGalNAc at pH 4.0. The kinetic parameters k_{cat} (s⁻¹) and K_m (mM) of *N*-acetylglucosaminidase A using the following substrates were, respectively: pNPGlcNAc, 740, 0.77; pNPGalNAc, 910, 1.26; *N,N'*-diacetylchitobiose 620, 0.20; and *N,N',N''*-triacetylchitotriose, 170, 0.044. The enzyme showed substrate inhibition with all substrates above 0.5 mM except for pNPGalNAc.

Keywords: *Candida albicans*, *N*-acetylglucosaminidase, *N*-acetylhexosaminidase, glycosylation

INTRODUCTION

Candida albicans is an opportunistic human pathogen which can undergo a reversible transition from the yeast

Abbreviations: endo H, endo- β -*N*-acetylglucosaminidase H; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; pNPGlcNAc, *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide; pNPGalNAc, *p*-nitrophenyl-*N*-acetyl- β -D-galactosaminide.

to mycelial morphological form (reviewed by Odds, 1988). Germ-tube emergence, the initial phase of mycelial growth, may be induced in preconditioned yeast cells at 37 °C by the addition of *N*-acetyl-D-glucosamine (GlcNAc), or related *N*-acetylglucosamine derivatives, to the growth medium (Shepherd *et al.*, 1985). GlcNAc induces the synthesis of enzymes necessary for its metabolism (Gopal *et al.*, 1982), as well as a secreted *N*-acetylglucosaminidase (Sullivan *et al.*, 1984). The im-

portance of *N*-acetylglucosaminidase to *C. albicans* was illustrated by a mutant strain (EOB4), deficient in this enzyme, which displayed normal growth and GlcNAc-induced morphogenesis, but was an order of magnitude less pathogenic than the wild-type strain (Jenkinson & Shepherd, 1987).

N-Acetylglucosaminidase was first localized at the *C. albicans* cell periphery by cytohistochemistry (Pugh & Cawson, 1977). Sullivan *et al.* (1984) demonstrated GlcNAc-induced *N*-acetylglucosaminidase secretion during yeast growth at 28 °C and germ-tube formation at 37 °C. At 28 °C the enzyme was largely retained within the cell envelope but at 37 °C nearly 50% of the total activity was exported into the medium, which is an ideal source for purification. In this study we demonstrate that secreted *N*-acetylglucosaminidase is a mannoprotein with many similarities to *Saccharomyces cerevisiae* invertase. The enzyme was purified from cells grown under two different conditions and showed a remarkable difference in associated carbohydrate, which can be attributed to variation in outer chain glycosylation.

METHODS

Growth conditions and *N*-acetylglucosaminidase induction.

N-Acetylglucosaminidase was purified from two different preparations of *Candida albicans* ATCC 10261. In the first, yeast cells were grown for 20 h at 28 °C in sixty 1 litre shake cultures containing glucose/salts/biotin (GSB: Shepherd *et al.*, 1980), harvested (500 g, 5 min), washed in distilled water, aerated for 24 h at room temperature in distilled water, then stored as a thick suspension in distilled water at 4 °C for 3–13 d. *N*-Acetylglucosaminidase was induced by incubating the starved cells (21 mg dry wt ml⁻¹) at 37 °C in a fermenter containing 22 litres GlcNAc/salts/biotin medium (per litre: 4 g K₂HPO₄, 0.05 g CaCl₂·2H₂O, 0.05 g MgSO₄·7H₂O, 1 µg biotin, 25 mM *N*-acetylglucosamine, pH adjusted to 6.0 with KOH) with aeration (12 litres min⁻¹) and agitation (200 r.p.m.). After 12 h the suspension was cooled to 10 °C, centrifuged (6000 g, 5 min) and the medium was filtered (GA55, Advantec). All of the cells formed germ-tubes within the first 4–6 h. The enzyme purified from the spent medium in this experiment was called *N*-acetylglucosaminidase A.

For the second enzyme purification, *C. albicans* ATCC 10261 yeast cells were grown for 17 h at 28 °C in a fermenter containing 20 litres YEPD (per litre: 10 g yeast extract, 20 g peptone, 10 g glucose, 1 µg biotin) to a final OD₆₄₀ of 11 (approximately 11 mg dry wt ml⁻¹). The cells were harvested, aerated in distilled water for 24 h at room temperature, washed in distilled water then incubated in a fermenter in 33 litres GlcNAc/salts/biotin at 37 °C. The cells (6.5 mg dry wt ml⁻¹) were aerated (12 litres min⁻¹) and agitated at 200 r.p.m. At the time of harvesting (6 h) < 1% of the cells had formed germ-tubes, the remainder showed yeast-phase morphology. The enzyme purified from the spent medium in this experiment was called *N*-acetylglucosaminidase B.

Purification of *N*-acetylglucosaminidase

Both enzyme forms were purified by the same procedure apart from an additional final step for *N*-acetylglucosaminidase A. All purification steps apart from HPLC were carried out at 4 °C.

The secreted enzyme was concentrated overnight by passing the medium (final pH 7.8) through a DEAE-cellulose column (10 cm × 8 cm diameter, Indion HA3, Life Technologies) pre-equilibrated with 20 mM KH₂PO₄/KOH, pH 7.0 (phosphate buffer). After washing the column with 5 litres of phosphate buffer containing 0.5 mM phenylmethylsulfonyl fluoride, *N*-acetylglucosaminidase was eluted with 0.3 M NaCl, dialysed against phosphate buffer and filtered (0.45 µm HA, Millipore).

DEAE-HPLC. The filtrate was pumped (3 ml min⁻¹) through a TSK DEAE-3SW column (150 mm × 21 mm, Toyo Soda, Japan) equilibrated with phosphate buffer. After loading, the column was washed with 100 ml phosphate buffer, then *N*-acetylglucosaminidase was eluted with a linear phosphate gradient (20–250 mM, 300 ml, 1.5 ml min⁻¹).

CM-HPLC. Enzyme-containing DEAE-HPLC fractions were dialysed against 20 mM acetic acid/Ba(OH)₂, pH 4.0 (acetate buffer), filtered (0.45 µm) and pumped (2 ml min⁻¹) through an Aquapore CX-300 column (220 mm × 4.6 mm, Brownlee Labs) equilibrated with acetate buffer. After washing the column with 200 ml acetate buffer, *N*-acetylglucosaminidase was eluted with a linear NaCl gradient (0–300 mM, 180 ml, 1 ml min⁻¹) in acetate buffer, dialysed against deionized water and lyophilized.

G4000 SW gel filtration. The lyophilized material was dissolved in 2 ml deionized water, centrifuged (50000 g, 20 min) and the supernatant fractionated (1.5 ml min⁻¹) on a TSK G4000 SW column (600 mm × 21 mm, Toyo Soda, Japan) equilibrated with 0.1 M KH₂PO₄/KOH buffer, pH 7.0 containing 0.3 M NaCl. Active fractions of *N*-acetylglucosaminidase B were pooled, dialysed against deionized water, lyophilized and stored at –20 °C. *N*-Acetylglucosaminidase A fractions were lyophilized as described above then refractionated on the G4000 SW column at 1.0 ml min⁻¹ before storage at –20 °C.

The G4000 SW column was calibrated with the following *M_r* standards (Sigma): thyroglobulin (669000), apoferritin (443000), β-amylase (200000), alcohol dehydrogenase (150000), bovine serum albumin (66000) and carbonic anhydrase (29000). The void volume (*V₀*) was identified as a small peak eluting at 95 ml in samples and standards (Fig. 1d).

Sephacryl S-300 gel filtration. *M_r* values were estimated at 4 °C using a Sephacryl S-300 superfine (Pharmacia) column (99 cm × 1.5 cm). Material was eluted with sodium acetate buffer (0.2 M, pH 5.6, 10 ml h⁻¹) and 2 ml fractions were collected. The *M_r* calibration standards (Pharmacia) were: Blue Dextran (*V₀*), thyroglobulin (669000), ferritin (440000), aldolase (158000), bovine serum albumin (66000), ovalbumin (45000) and ribonuclease A (13700).

SDS-polyacrylamide gel electrophoresis. Protein samples were heated in SDS sample buffer (Laemmli, 1970) for 3–5 min at 100 °C and fractionated by electrophoresis at 200 V on 5–15% or 7.5–15% (w/v) gradient gels with a 4% stacking gel. Proteins were visualized by staining with Coomassie Brilliant Blue (R-250, Sigma) or by silver staining (Morrissey, 1981). The *M_r* standards (Sigma) were: myosin (205000), β-galactosidase (116000), phosphorylase b (97400), bovine serum albumin (66000), ovalbumin (45000) and carbonic anhydrase (29000).

Deglycosylation with endoglycosidase H. Conditions for endoglycosidase H (endo H) treatment were based on recommendations by Trimble & Maley (1984). For analysis by electrophoresis, samples of purified *N*-acetylglucosaminidase (5 µg) were incubated for 24 h at 37 °C with Sigma endo H (20,

100 or 500 mU ml⁻¹, see text) in 20 µl 0.1 M citric acid/NaOH, pH 5.0 containing 1 mM phenylmethylsulfonyl fluoride. One 5 µg sample of each N-acetylglucosaminidase preparation was denatured by heating for 5 min at 100 °C in the presence of 6.4 µg SDS and 50 mM 2-mercaptoethanol, before incubating with endo H (100 mU ml⁻¹) as described above.

For analysis by gel filtration, 4 µg N-acetylglucosaminidase was incubated with 500 mU endo H ml⁻¹ in 40 µl 50 mM citric acid/NaOH, pH 5.0 at 37 °C. After 24 h the incubation mixture was diluted to 1 ml with 0.2 M sodium acetate/NaOH, pH 5.6, then chromatographed.

Enzyme assays. All enzymes were assayed at 37 °C. One enzyme unit catalyses the formation of 1 µmol product min⁻¹. The standard assay to detect N-acetylglucosaminidase (EC 3.2.1.52) involved fixed time (10–30 min) incubations of sample plus 1 mM pNPGlcNAc in 0.1 M citric acid/KOH, pH 4.0 (0.5 ml final volume). Reactions were stopped by the addition of 0.5 ml 10% (w/v) trichloroacetic acid followed by 2 ml 4% (w/v) Na₂CO₃. The p-nitrophenol produced was measured spectrophotometrically at A_{420} ($\epsilon = 16.0 \times 10^3$ l mol⁻¹ cm⁻¹). Substrate controls (no enzymes) were incubated in parallel for absorbance blanks. The assay was linear with time up to $A_{420} = 0.8$. N-Acetylgalactosaminidase activity was assayed in the same manner using 1 mM pNPGalNAc as substrate. pH-activity profiles for N-acetylglucosaminidase and N-acetylgalactosaminidase activities were determined with 1 mM substrates in a universal buffer (Johnson & Lindsey, 1939) over the pH range 1.5–10, with substrate controls at each point. A pH-stability curve was obtained by preincubation of 100 µl enzyme samples with 50 µl universal buffer (pH 2–10) for 21 h at 37 °C which were then diluted with 0.35 ml 143 mM citric acid/KOH, pH 4.0 for N-acetylglucosaminidase assays.

Trehalase (EC 3.2.1.28), β -1,3-glucanase (EC 3.2.1.58), β -glucosidase (EC 3.2.1.21) and acid phosphatase (EC 3.1.3.2) were assayed as described by Ram *et al.* (1983, 1984). α -Glucosidase (EC 3.2.1.20, pH 7.0), β -galactosidase (EC 3.2.1.23, pH 4.0) and α -xylosidase (EC 3.2.1.27, pH 4.0) were assayed using the appropriate p-nitrophenyl-linked glycosides (6 mM final concentration), in 0.1 M potassium phosphate buffer (pH 7.0) or 0.1 M citric acid/KOH (pH 4.0), essentially as described for the standard N-acetylglucosaminidase assay.

Kinetic analysis. For enzyme kinetic analysis, velocity measurements using 2.5×10^{-3} U enzyme were performed in triplicate at 0.1–10 mM pNPGlcNAc and 0.1–5 mM pNPGalNAc with substrate controls at each concentration. K_m and V_{max} values were calculated by the direct linear method (Eisenthal & Cornish-Bowden, 1974) using (S, v) co-ordinates shown in Lineweaver–Burk plots in the text. Substrate inhibition constants (K_i values) were determined graphically from different co-ordinates which are also shown in the text. Kinetic data for hydrolysis of N,N'-diacetylchitobiose and N,N',N''-triacetylchitotriose (Sigma) were obtained by measuring GlcNAc production. Triplicate fixed time (15–20 min) incubations containing 0.04–4.0 mM substrate plus 5×10^{-4} U enzyme were conducted in a final volume of 150 µl 0.1 M citric acid/KOH, pH 4.0. The enzyme reactions were stopped and adjusted to the optimum pH (8.9) for colour development in the GlcNAc assay (Reissig *et al.*, 1955) by the addition of 30 µl 0.8 M boric acid/1.25 M KOH, then heated for 3 min at 100 °C. The GlcNAc colour reaction was completed using 0.9 ml freshly prepared Ehrlich reagent as described by Reissig *et al.* (1955). Controls, at each substrate concentration, contained 30 µl 0.8 M boric acid/1.25 M KOH added prior to the enzyme solution.

Enzyme units are defined as the formation of 2 µmole GlcNAc min⁻¹ for N,N'-diacetylchitobiose and 1 µmole min⁻¹ for N,N',N''-triacetylchitotriose.

Analytical methods. Protein was assayed by a modified Lowry method (Eggstein & Kreutz, 1967) using bovine serum albumin standards. Total hexose was assayed by the phenol/H₂SO₄ method (Dubois *et al.*, 1956) with glucose standards. For amino acid analysis, 5 µg samples of N-acetylglucosaminidase protein were hydrolysed *in vacuo* in 6 M HCl for 24 h at 100 °C and separated on a Waters Millipore amino acid analyser. Separate samples were pretreated with performic acid (Moore, 1963) for cysteine determinations. N-terminal protein sequence analysis was carried out using an Applied Biosystems 470A sequencer and on-line 120A HPLC phenylthiohydantoin amino acid detector.

RESULTS

N-Acetylglucosaminidase secretion

In order to maximize N-acetylglucosaminidase secretion, cells were grown in large quantities using glucose as a carbon source, starved for 24 h in distilled water then incubated at high cell concentrations with GlcNAc at 37 °C to induce the enzyme. Cells grown in glucose/salts/biotin (GSB) shake cultures began N-acetylglucosaminidase secretion 4 h after GlcNAc addition, which corresponded to the time of germ-tube emergence (data not shown). After 10–12 h incubation, N-acetylglucosaminidase activity in the medium reached a plateau and, after harvesting 900 g (final wet wt) of germ-tube forming cells, 380 U enzyme were recovered from the spent medium. Cells grown in YEPD medium in a fermenter prior to starvation grew in the yeast phase rather than as germ-tubes when incubated with GlcNAc at 37 °C. The GlcNAc still induced N-acetylglucosaminidase secretion and after 6 h incubation, 230 U N-acetylglucosaminidase were recovered from the culture (1 kg wet wt yeast cells) supernatant. The secreted N-acetylglucosaminidase from GSB-grown cells is subsequently referred to as N-acetylglucosaminidase A and enzyme from YEPD-grown cells is referred to as N-acetylglucosaminidase B.

Purification of N-acetylglucosaminidase A and B

N-Acetylglucosaminidase A and B were purified by the same scheme, as summarized in Table 1. Fig. 1 shows the elution profiles for three chromatography steps in the purification of N-acetylglucosaminidase A and the final gel filtration step for N-acetylglucosaminidase B. In the first purification step both enzyme forms were purified threefold and concentrated 30-fold by overnight passage of the media through a short DEAE cellulose column followed by elution with 0.3 M NaCl (Table 1). The concentrated enzymes were applied to a preparative TSK DEAE HPLC column and eluted with a gradient of phosphate buffer. N-Acetylglucosaminidase A elution peaked at 90 mM phosphate and 95% of the activity eluted in 63 ml (Fig. 1a). By contrast N-acetylglucosaminidase B peaked at 140 mM phosphate, 95% of the activity eluted in 108 ml, and activity was still detectable at the end of the gradient (250 mM phosphate, data not

Table 1. Purification of *N*-acetylglucosaminidase A

Fraction	Volume (ml)	Enzyme units ($\mu\text{mol min}^{-1}$)	Protein (mg)	Specific activity (U mg^{-1})	Purification (fold)	Recovery (%)	$p\text{NPGalNAc}/p\text{NPGlcNAc}^*$
Medium	25400	381	656	0.58	1	100	1.57
DEAE cellulose	775	345	213	1.62	3	91	1.47
DEAE HPLC	85	324	9.2	35.2	61	85	1.43
CM HPLC	2	207	0.92	225	387	54	1.51
G4000 SW (first)	2	144	0.52	277	473	38	1.48
G4000 SW (second)	1	114	0.38	300	518	30	1.50

* $p\text{NPGalNAc}/p\text{NPGlcNAc}$ is the ratio of enzyme activity with *N*-acetylgalactosaminide and *N*-acetylglucosaminide as substrate.

Fig. 1. *N*-Acetylglucosaminidase purification. (a) DEAE HPLC of *N*-acetylglucosaminidase A. (b) CM HPLC of *N*-acetylglucosaminidase A. (c) The second G4000 SW gel filtration of *N*-acetylglucosaminidase A. (d) G4000 SW gel filtration of *N*-acetylglucosaminidase B. Open bars indicate pooled fractions. Elution volumes of M_r standards are indicated in (c) and (d).

shown). In the next step DEAE fractions were applied to a semi-preparative CM HPLC column and eluted with a salt gradient. *N*-Acetylglucosaminidase A eluted at 200 mM NaCl (Fig. 1b) and *N*-acetylglucosaminidase B at 160 mM NaCl (data not shown). In the final G 4000 SW gel filtration step the most notable difference between the two enzyme preparations became apparent (Fig. 1c, d). *N*-Acetylglucosaminidase A eluted at 141 ml ($M_r \sim 600\,000$) while *N*-acetylglucosaminidase B eluted at

128 ml ($M_r \sim 1\,200\,000$). Since *N*-acetylglucosaminidase A was not completely resolved from other high- M_r components after one gel filtration step (data not shown), active fractions were rechromatographed on the same column (Fig. 1c) giving a final specific activity of $300 \text{ U (mg protein)}^{-1}$ (Table 1). *N*-Acetylglucosaminidase B, on the other hand, showed simpler A_{280} profiles during the ion-exchange steps than *N*-acetylglucosaminidase A (data not shown) and, accordingly, this enzyme was purified

Table 2. Amino acid analysis

Amino acid	N-Acetylglucosaminidase A		N-Acetylglucosaminidase B	
	pmol	mol %	pmol	mol %
Cys	144	1.5	133	1.1
Asx	1312	13.6	1577	13.6
Thr	524	5.4	734	6.4
Ser	667	6.9	925	8.0
Glx	866	9.0	1037	9.0
Pro	433	4.5	582	5.0
Gly	527	5.5	691	6.0
Ala	631	6.6	723	6.2
Val	677	7.0	803	6.9
Met	74	0.8	72	0.6
Ile	487	5.1	576	5.0
Leu	759	7.9	873	7.5
Tyr	297	3.1	386	3.3
Phe	275	2.9	301	2.6
His	800	8.3	837	7.2
Lys	409	4.3	474	4.1
Arg	733	7.6	768	6.6
Total	9615	100.0	11492	99.1

after only one gel filtration step (Fig. 1d) to a specific activity of 318 U (mg protein)⁻¹. The overall recovery of N-acetylglucosaminidase B was 52%.

Both enzyme forms were judged to be pure, based on purification to similar specific activities, co-elution of protein and enzyme activity during the final purification step (Fig. 1c, d), similar amino acid compositions (Table 2) and the absence of contaminant bands from earlier purification steps in silver-stained SDS-polyacrylamide gels. For example, the densitometer scan in Fig. 2 shows the purity of N-acetylglucosaminidase A. Furthermore, none of the following enzymes, which were present in the starting material (data not shown), were detected after exhaustive assay incubations with purified N-acetylglucosaminidase A: β -1,3-glucanase; trehalase; acid phosphatase; α - and β -glucosidase; β -galactosidase; and α -xylosidase. N-Acetylgalactosaminidase was present in the final preparation but this activity co-purified with N-acetylglucosaminidase (Table 1) indicating that one enzyme is responsible for both activities (see below).

Glycosylation, subunit structure and composition

A multisubunit structure was indicated by the low elution volumes of the two N-acetylglucosaminidases during preparative gel filtration (Fig. 1c, d). Both enzyme forms were therefore dissociated and analysed on SDS-polyacrylamide gels before and after cleavage of Asn-linked mannose side chains by endo H. Without endo H treatment, N-acetylglucosaminidase A migrated as a broad band centred at $\sim 100\,000$ (Fig. 2 and Fig. 3, lane 6) while the N-acetylglucosaminidase B band was centred at $\sim 150\,000$ (Fig. 3, lane 1). The faint bands migrating at

Fig. 2. Purity and M_r of N-acetylglucosaminidase A. The profile shows a densitometer scan of 10 μ g of purified protein after SDS-polyacrylamide gel electrophoresis on a 5–15% gradient gel and silver staining. The migration of M_r standards is shown above the trace.

approximately twice the M_r of the main bands are believed to be subunit dimers since they were not present after prolonged sample dissociation at 100 °C (for example see Fig. 2).

Both N-acetylglucosaminidases were converted to a single 58 000 band when denatured in SDS then incubated

Fig. 3. Deglycosylation of *N*-acetylglucosaminidase A and B. Samples were treated with endo H, separated by electrophoresis on a 7.5–15% SDS polyacrylamide gel and stained with Coomassie Blue as described in Methods. M, M_r standards; lanes 1–5, *N*-acetylglucosaminidase B; lanes 6–10, *N*-acetylglucosaminidase A. Enzyme (10 μ g) not treated with endo H (lanes 1, 6), 5 μ g enzyme incubated with endo H at: 20 mU ml⁻¹ (lanes 2, 7); 100 mU ml⁻¹ (lanes 3, 8); 500 mU ml⁻¹ (lanes 4, 9), and 5 μ g enzyme denatured by heating in SDS and 2-mercaptoethanol before incubation with 100 mU endo H ml⁻¹ (lanes 5, 10).

with endo H (Fig. 3, lanes 5 and 10). The faint band just ahead of the 58 000 band is the major component in the endo H preparation. Endo H concentrations as low as 20 mU ml⁻¹ are sufficient for complete deglycosylation of denatured *N*-acetylglucosaminidase (data not shown). On the other hand, without prior denaturation, both *N*-acetylglucosaminidases were converted to three intermediates separated from each other and the final product by approximately 3000 (Fig. 3, lanes 2–4 and 7–9). Silver staining of gels showed complete conversion of the large diffuse bands in these samples (data not shown), but the deglycosylation intermediates persisted at endo H concentrations as high as 500 mU ml⁻¹ (Fig. 3, lanes 4 and 9). Inclusion of 10 mM 2-mercaptoethanol without SDS did not promote further deglycosylation of native *N*-acetylglucosaminidase by endo H (data not shown).

The ratio of protein (Lowry assay) to total hexose was 1:0.467 for *N*-acetylglucosaminidase A and 1:1.28 for *N*-acetylglucosaminidase B. Therefore based on a polypeptide M_r of 58 000, the calculated M_r values for the fully glycosylated enzymes were 85 000 (A form) and 132 000 (B form). Both enzymes contained three endo H-resistant oligosaccharides of M_r 3000. The carbohydrate side chains that cleaved readily under non-denaturing conditions

Fig. 4. *N*-Acetylglucosaminidase M_r determination using Sephacryl S-300. Δ , *N*-Acetylglucosaminidase A; $\circ$, *N*-acetylglucosaminidase B; $\blacktriangle$, endo H treated A; $\bullet$, endo H treated B. The inset shows sample elution volumes in relation to the following M_r standards ($\blacktriangledown$): T, thyroglobulin (669 000); F, ferritin (440 000); A, aldolase (158 000); B, bovine serum albumin (66 000); O, ovalbumin (45 000); and R, ribonuclease A (13 700).

therefore account for 18 000 in *N*-acetylglucosaminidase A and 65 000 in *N*-acetylglucosaminidase B.

Analytical Sephacryl S-300 gel filtration under non-dissociating conditions was used to assess the subunit structure of native and partially deglycosylated enzymes (Fig. 4). The M_r values of *N*-acetylglucosaminidase A (350 000) and *N*-acetylglucosaminidase B (640 000) were less than those observed on the G4000 SW column (600 000 and 1 200 000 respectively) but showed similar relative magnitudes. As shown in Fig. 3, treatment of both non-denatured *N*-acetylglucosaminidases with 500 mU endo H ml⁻¹ produced a 58 000 polypeptide plus 61 000, 64 000 and 67 000 intermediates. After this treatment the enzymes chromatographed on Sephacryl S-300 as single peaks at 185 000 (*N*-acetylglucosaminidase A) and 175 000 (*N*-acetylglucosaminidase B). Endo H treatment did not reduce *N*-acetylglucosaminidase activity, so it seems unlikely that other, enzymically inactive deglycosylation products eluted undetected. Endo H itself does not cleave *p*-nitrophenyl GlcNAc. In another experiment it was shown that pre-incubation in 25 mM dithiothreitol for 1 h at 37 °C followed by chromatography in the presence of 10 mM 2-mercaptoethanol did not change the elution behaviour of *N*-acetylglucosaminidase A or its partially deglycosylated form.

The two enzyme forms had very similar amino acid compositions (Table 2) with 65% polar and charged

amino acids, of which Asn and Asp made up more than a fifth. Cys and Met were only minor components. The N-terminal amino acid sequence of N-acetylglucosaminidase A was: (K)(K)(V)EILPAPQSVI(W)E(W)-DTAIIINPRLQA, (amino acids in parentheses represent ambiguous assignments). The N-terminal sequence of N-acetylglucosaminidase purified from another strain of *C. albicans*, A72, was: AKVEILPAPQSVTWE(S)DTAI (R. D. Cannon, unpublished data). This N-acetylglucosaminidase preparation had a similar M_r to N-acetylglucosaminidase B (120 000–150 000 on an SDS-polyacrylamide gel).

Enzyme stability and kinetics

Both pNPGlcNAc and pNPGalNAc were hydrolysed over a broad pH range (2–9), typical of fungal N-acetylglucosaminidases (Bahl & Agrawal, 1969; Oktakara *et al.*, 1981; Yamamoto *et al.*, 1985), with maximum activity at pH 4.0 (data not shown). Standard N-acetylglucosaminidase assays were conducted at pH 4.0 using citrate/KOH buffer (see Methods) which gave 95 % of the activity in the more complex universal buffer used for the pH-activity measurements.

N-Acetylglucosaminidase is a stable enzyme. Only minor inactivation occurred after a 21 h incubation at 37 °C in buffer at pH 4–8 with 50 % inactivation at pH 2.5 and pH 10. Activity decreased by 7 % after 10 weeks storage in distilled water at room temperature. Four freeze–thaw cycles produced a similar decrease in activity. Untreated N-acetylglucosaminidase and samples deglycosylated with endo H at 20 mU ml⁻¹ or 100 mU ml⁻¹ remained active over a 5 d incubation in water at 37 °C (data not shown). Inclusion of 20 mM 2-mercaptoethanol during deglycosylation and subsequent incubations had no effect on enzyme stability. N-Acetylglucosaminidase was active in the presence of 0.1 % SDS.

Kinetics for utilization of pNPGlcNAc, pNPGalNAc, N,N'-diacetylchitobiose and N,N',N''-triacetylchitotriose as substrates were examined using N-acetylglucosaminidase A (Fig. 5, Table 3). Substrate pNPGalNAc evoked classical Michaelis–Menten kinetics and the greatest $V_{\max}$, whereas all three GlcNAc-containing compounds displayed strong substrate inhibition above 0.5 mM. The chitotriose reactions were allowed to proceed up to 30 % conversion of substrate in order to obtain measurable concentrations of the single GlcNAc produced. Since chitotriose cleavage yields chitobiose, which in turn produces two GlcNAc molecules when hydrolysed, the chitotriose velocity data should be regarded as slight overestimates. No activity was detected with substrates p-nitrophenyl-N-acetyl- α -D-glucosaminide or p-nitrophenyl-N-acetyl- α -D-galactosaminide.

DISCUSSION

Yeast mannoproteins have been categorized as (a) structural, containing up to 90 % mannose, (b) soluble secreted enzymes such as invertase and acid phosphatase, con-

Fig. 5. Relationship between substrate concentration and reaction velocity. Substrates: (a) pNPGlcNAc (○, ●), pNPGalNAc (◆); (b) N,N'-diacetylchitobiose (△, ▲), N,N',N''-triacetylchitotriose (□, ■); (c) pNPGlcNAc (○, ●), N,N'-diacetylchitobiose (△, ▲), N,N',N''-triacetylchitotriose (□, ■). The filled symbols in (a) and (b) indicate (1/S, 1/v) co-ordinates used for K_m and $V_{\max}$ calculations, and in (c) indicate (S, 1/v) co-ordinates used for K_i calculations.

taining about 50 % mannose, and (c) vacuolar enzymes such as carboxypeptidase Y, containing about 15 % mannose (Cohen & Ballou, 1981). *C. albicans* N-acetylglucosaminidase clearly falls into the second class but is notable in having quite variable glycosylation. The two forms of N-acetylglucosaminidase described here appear to contain the same polypeptide as judged by (a) enzyme specific activity, (b) amino acid composition, (c) N-terminal amino acid sequence, and (d) their common M_r .

Table 3. Kinetic constants for *N*-acetylglucosaminidase substrates

Reaction velocity measurements over a range of substrate concentrations were carried out in triplicate as described in Methods.

Compound	k_{cat}^* (s^{-1})	$K_m^\dagger$ (mM)	k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)	$K_i^\ddagger$ (mM)
pNPGlcNAc	740	0.77	960	0.95
pNPGalNAc	910	1.26	720	—
<i>N,N'</i> -Diacetylchitobiose	620	0.20	3100	0.98
<i>N,N',N''</i> -Triacetylchitotriose	170	0.044	3800	1.89

* k_{cat} values were calculated from V_{max} determinations assuming one active site per 58000 protein subunit.

† K_m and V_{max} values were calculated by the direct linear method using the data points indicated in the Lineweaver-Burk plots (Fig. 5).

‡ Substrate inhibition constants were calculated from plots of $1/v$ against S (Fig. 5).

(58000) after endo H deglycosylation. In a similar study, Hayashi & Nakamura (1981) fractionated six discrete forms of *Aspergillus niger* glucose oxidase with the same peptide moiety but different associated carbohydrates.

N-Acetylglucosaminidase A (32% carbohydrate, calculated M_r 85000, see Results) was purified from cells grown in carbon-limiting medium (glucose/salts/biotin, Shepherd *et al.*, 1980) before starvation and GlcNAc-induction of germ-tube formation at 37 °C. *N*-Acetylglucosaminidase B (56% carbohydrate, M_r 132000) was isolated from cells grown in nitrogen-limiting medium (YEPA, see Methods) before starvation and GlcNAc induction. Thus *N*-acetylglucosaminidase A was purified from mycelial cells and *N*-acetylglucosaminidase B was purified from yeast cells. However, Western blot studies of enzyme produced from yeast or mycelia grown under a variety of conditions, using an anti-*N*-acetylglucosaminidase antibody, indicated that the size, and hence glycosylation, of the enzyme was dependent on the nutritional history of the cells (whether carbon- or nitrogen-starved) rather than the cell morphology during enzyme secretion (R. D. Cannon, unpublished results). Other glycoprotein enzymes from *C. albicans* include acid phosphatase (85% carbohydrate; Odds & Hierholzer, 1973), and secreted aspartic proteinase (2% carbohydrate; Wright *et al.*, 1992) but to our knowledge, such differently glycosylated forms of the same enzyme have not been purified from this organism.

Like *Saccharomyces cerevisiae* invertase (Lehle *et al.*, 1979; Trimble & Maley, 1984), both forms of *N*-acetylglucosaminidase contain carbohydrate side chains that are readily removed by endo H as well as a family of three oligosaccharides which are resistant to endo H cleavage under non-denaturing conditions and are only completely removed after boiling the protein in SDS. These oligosaccharides of $M_r \sim 3000$ have the same size as the 'inner core' component of higher order polysaccharide side-chains of yeast mannoproteins (Ballou, 1982). Based on their resistance to endo H treatment, Trimble *et al.* (1983)

proposed that the smaller oligosaccharides on invertase and carboxypeptidase Y reside on glycosylated protein domains which become inaccessible to further glycosyltransferases as the enzymes fold into their mature conformation during biosynthesis. Indeed, as a result of premature termination of biosynthesis, the invertase oligosaccharides contain a complete set of structures ranging from $(\text{GlcNAc})_2(\text{Man})_8$ to $(\text{GlcNAc})_2(\text{Man})_{14}$ which define a biosynthetic pathway initiated by co-translational transfer of the dolichol-linked $(\text{GlcNAc})_2(\text{Man})_9(\text{Glc})_3$ precursor to asparagine acceptor sites on the nascent polypeptide and subsequent trimming reactions in the endoplasmic reticulum (Trimble & Atkinson, 1986). The *N*-acetylglucosaminidase oligosaccharides are likely to contain a similar store of biosynthetic information.

Since the two forms of *N*-acetylglucosaminidase share common endo H-resistant core oligosaccharides, the difference in M_r is attributable to outer chain glycosylation. The *N*-acetylglucosaminidase gene, which has been cloned and sequenced, contains seven potential Asn-X-Ser/Thr glycosylation sites (R. D. Cannon, unpublished data). The enzyme could therefore contain up to four endo H-sensitive sidechains, each of which would contain about 28 mannose residues in the A form and 100 mannose residues in the B form, which is within the range for Asn-linked polysaccharides from *S. cerevisiae* mannoproteins (Ballou *et al.*, 1980). The outer chain residues are built up by a reiterative process in the Golgi apparatus during secretion (Ballou, 1982). Differences in the outer chain extension could reflect variations in Golgi transit time, substrate availability and glycosyltransferase activities – processes which may be affected by the nutritional status of the cells.

During purification it was noted that *N*-acetylglucosaminidase B had a higher affinity for the anion-exchange column than *N*-acetylglucosaminidase A, and a lower affinity for the cation-exchange column. This is most likely due to the presence of additional phosphate groups in the B form since *C. albicans* mannoproteins contain

outer chain phosphomannoses (Kobayashi *et al.*, 1990) as do those from *S. cerevisiae* (Ballou, 1982).

A dimer of both forms of N-acetylglucosaminidase seen on SDS-polyacrylamide gels (Fig. 3) was only fully dissociated after prolonged boiling in SDS (Fig. 2), indicating a high degree of dimer stability. Dimers were not apparent on SDS polyacrylamide gels after endo H treatment although both N-acetylglucosaminidase A and B eluted from Sephacryl S-300 as proteins of $M_r \sim 180\,000$ after removal of all but the three resistant oligosaccharide sidechains by endo H. Thus subunit association probably involves both protein-protein and carbohydrate-sidechain interactions. Underglycosylated mutants of *S. cerevisiae* invertase also display reduced oligomer stability compared to the wild-type enzyme (Tammi *et al.*, 1987). Endo H treatment of native N-acetylglucosaminidase produced no change in enzyme activity or stability. Glycosylation may however play an important role in protein folding during biosynthesis, as was demonstrated for human β -hexosaminidase by site-directed mutagenesis (Weitz and Proia, 1992).

In general, N-acetylglucosaminidases in eukaryotes, ranging from yeasts and fungi (Oktakara *et al.*, 1981; Yamamoto *et al.*, 1985) to mammals (Sandhoff *et al.*, 1989), exist as dimers. An important question is whether dimerization is required for N-acetylglucosaminidase activity, as is the case for *S. cerevisiae* invertase (Chu *et al.*, 1983) and human β -hexosaminidase. In the latter case two different subunits, α and β , combine to form β -hexosaminidase A ($\alpha\beta$), β -hexosaminidase B ($\beta\beta$) and β -hexosaminidase S ($\alpha\alpha$) which show quite different substrate specificities (reviewed by Sandhoff *et al.*, 1989).

We detected only one oligomeric form of each N-acetylglucosaminidase by gel filtration in contrast to invertase, which chromatographs as a mixture of dimers, tetramers, hexamers and octamers (Chu *et al.*, 1983; Tammi *et al.*, 1987). Are the N-acetylglucosaminidase peaks dimers or higher oligomers? M_r estimates from Sephacryl S-300 and TSK G4000 SW chromatography are approximately four and eight times, respectively, the calculated subunit M_r values (see Results). This sort of comparison is complicated by different methods of analysis, column matrices and buffer conditions, as well as the anomalous chromatography of glycoproteins (Andrews, 1970). On the other hand, invertase which is present as an octamer throughout the secretory pathway and periplasmic space (Esmon *et al.*, 1987), dissociates into lower oligomers *in vitro* in a manner affected by pH, concentration, freeze-thawing and time (Tammi *et al.*, 1987). The same may apply to *C. albicans* N-acetylglucosaminidase.

We found no evidence that disulphide bonding influences enzyme activity, stability, chromatographic behaviour or susceptibility to endo H. Indeed the protein contained only a trace of cysteine and the amino acid sequence of the mature protein deduced from the cloned gene contained seven cysteine residues (R. D. Cannon, unpublished data).

In contrast human β -hexosaminidase activity is dependent on correct disulphide bonding which is in turn influenced by glycosylation during biosynthesis (Weitz & Proia, 1992).

C. albicans N-acetylglucosaminidase is a true hexosaminidase since it hydrolyses pNPGalNAc as well as pNPGlcNAc and chitin oligosaccharides. Yeast and fungal N-acetylglucosaminidases typically display very broad substrate specificities, hydrolysing oligosaccharides of chitin, a range of aryl-substituted N-acetylhexosaminides, as well as terminal GlcNAc and GalNAc residues on glycoprotein sidechains (Bahl & Agrawal, 1969; Mega *et al.*, 1970; Jones & Kosman, 1980; Oktakara *et al.*, 1981; Yamamoto *et al.*, 1985). In mammals, hexosaminidases play a critical role in lysosomal degradation of glycoproteins (reviewed by Aronson & Kuranda, 1989) and glycolipids (Sandhoff *et al.*, 1989). N-Acetylglucosaminidases are non-reducing end exoglycosidases as opposed to di-N-acetylchitobiase, which cleaves chitin oligosaccharides but not pNPGlcNAc or borohydride-reduced chitin oligosaccharides, and is a reducing end exoglycosidase (Aronson & Kuranda, 1989; Fisher & Aronson, 1992).

K_m and k_{cat} values for pNPGlcNAc and pNPGalNAc were very similar to those of other fungal N-acetylglucosaminidases (Jones & Kosman, 1980; Mega *et al.*, 1970; Yamamoto *et al.*, 1985) and human hexosaminidases (Sandhoff *et al.*, 1989). All three GlcNAc-containing substrates displayed marked substrate inhibition, whereas pNPGalNAc did not, in common with other studies (Mega *et al.*, 1970; Yamamoto *et al.*, 1985). For a single substrate reaction, inhibition at high substrate concentrations results from ineffective enzyme/substrate complexes involving one or more substrate molecules (Dixon & Webb, 1979). Interestingly the axial hydroxyl group of GalNAc is sufficient to prevent non-productive binding of pNPGalNAc, and other fungal N-acetylglucosaminidases are inhibited by GlcNAc but not GalNAc (Oktakara *et al.*, 1981; Yamamoto *et al.*, 1985). Based on the k_{cat}/K_m criterion, N,N'-diacetylchitobiose and N,N',N''-triacetylchitotriose clearly are the preferred substrates for *C. albicans* N-acetylglucosaminidase. It should be noted that the k_{cat}/K_m ratio is not affected by nonproductive substrate binding (Fersht, 1985).

A nutritional role for N-acetylglucosaminidase is evident. In the N-acetylglucosaminidase-deficient mutant *C. albicans* EOB4 the absence of the enzyme did not affect cell growth on glucose or GlcNAc, and did not affect GlcNAc-induced morphogenesis, but as expected cells could not grow on N,N'-diacetylchitobiose (Jenkinson & Shepherd, 1987). The N-acetylglucosaminidase catalysed degradation of chitin oligosaccharides produced by chitinase action would provide the cell with both carbon and nitrogen.

Secreted hydrolytic enzymes of *C. albicans*, including proteinases (Kwon-Chung *et al.*, 1985; Ross *et al.*, 1990) and phospholipases (Barrett-Bee *et al.*, 1985; Ghannoum

& Abu-Elteen, 1990), have been implicated in pathogenesis. The importance of *N*-acetylglucosaminidase in this respect was highlighted by the reduced pathogenicity of *C. albicans* EOB4 compared to the parent strain (Jenkinson & Shepherd, 1987). An essential early step in tissue colonization is adherence to epithelial cells (Douglas, 1991), *N*-Acetylglucosaminidase is a hexosaminidase that is active over a broad pH range and therefore has the potential to modify host cell surface glycoproteins which could in turn regulate the adhesion process.

REFERENCES

- Andrews, P. (1970). Estimation of molecular size and molecular weights of biological compounds by gel filtration. *Methods Biochem Anal* **18**, 1–53.
- Aronson, N. N. & Kuranda, M. J. (1989). Lysosomal degradation of Asn-linked glycoproteins. *FASEB J* **3**, 2615–2622.
- Bahl, O. P. & Agrawal, K. M. L. (1969). Glycosidases of *Aspergillus niger*. I. Purification and characterization of α and β -galactosidases and β -*N*-acetylglucosaminidase. *J Biol Chem* **244**, 2970–2978.
- Ballou, C. E. (1982). Yeast cell wall and cell surface. In *Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression*, pp. 335–360. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- Ballou, L., Cohen, R. E. & Ballou, C. E. (1980). *Saccharomyces cerevisiae* mutants that make mannoproteins with a truncated outer chain. *J Biol Chem* **255**, 5986–5991.
- Barrett-Bee, K., Hayes, Y., Wilson, R. G. & Ryley, J. F. (1985). A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. *J Gen Microbiol* **131**, 1217–1221.
- Chu, F. K., Watorek, W. & Maley, F. (1983). Factors affecting the oligomeric structure of yeast external invertase. *Arch Biochem Biophys* **223**, 543–555.
- Cohen, R. E. & Ballou, C. E. (1981). Mannoproteins: structure. In *Encyclopedia of Plant Physiology: New Series*, vol. 13B, pp. 441–483. New York: Springer-Verlag.
- Dixon, M. & Webb, E. C. (1979). *Enzymes*. London: Longman.
- Douglas, L. J. (1991). Adhesion of *Candida albicans* to host surfaces. In *Candida and Candidamyces*, pp. 43–47. Edited by E. Timbay, H. P. R. Seeliger & Ö. Ang. New York: Plenum Press.
- Dubois, M., Gilles, R. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for the determination of sugars and related substances. *Anal Chem* **28**, 350–356.
- Eggstein, M. & Kreutz, F. H. (1967). In *Techniques in Protein Chemistry*, p. 340. London: Elsevier.
- Eisenthal, R. & Cornish-Bowden, A. (1974). The direct linear plot. *Biochem J* **138**, 715–720.
- Esmon, P. C., Esmon, B. E., Schauer, L. E., Taylor, A. & Schekman, R. (1987). Structure, assembly, and secretion of octomeric invertase. *J Biol Chem* **262**, 4387–4394.
- Fersht, A. (1985). *Enzyme Structure and Mechanism*. New York: W. H. Freeman.
- Fisher, K. J. & Aronson, N. N. (1992). Cloning and expression of the cDNA sequence encoding the lysosomal glycosidase di-*N*-acetylchitinase. *J Biol Chem* **267**, 19607–19616.
- Ghannoum, M. A. & Abu-Elteen, K. H. (1990). Pathogenicity determinants of *Candida*. *Mycoses* **33**, 265–282.
- Gopal, P., Sullivan, P. A. & Shepherd, M. G. (1982). Enzymes of *N*-acetylglucosamine metabolism during germ-tube formation in *Candida albicans*. *J Gen Microbiol* **128**, 2319–2326.
- Hayashi, S. & Nakamura, S. (1981). Multiple forms of glucose oxidase with different carbohydrate compositions. *Biochim Biophys Acta* **657**, 40–51.
- Jenkinson, H. F. & Shepherd, M. G. (1987). A mutant of *Candida albicans* deficient in β -*N*-acetylglucosaminidase (chitinase). *J Gen Microbiol* **133**, 2097–2106.
- Johnson, W. C. & Lindsey, A. J. (1939). An improved universal buffer. *Analyst* **64**, 490–492.
- Jones, C. S. & Kosman, D. J. (1980). Purification, properties, kinetics, and mechanism of β -*N*-acetylglucosaminidase from *Aspergillus niger*. *J Biol Chem* **255**, 11861–11869.
- Kobayashi, H., Shibata, N., Nakada, M., Chaki, S., Mizugami, K., Ohkubo, Y. & Suzuki, S. (1990). Structural study of cell wall phosphomannan of *Candida albicans* NIH B-792 (serotype B) strain, with special reference to ^1H and ^{13}C NMR analyses of acid-labile oligomannosyl residues. *Arch Biochem Biophys* **278**, 195–204.
- Kwon-Chung, K. J., Lehman, D., Good, C. & Magee, P. T. (1985). Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans*. *Infect Immun* **49**, 571–575.
- Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lehle, L., Cohen, R. E. & Ballou, C. E. (1979). Carbohydrate structure of yeast invertase. Demonstration of a form with only core oligosaccharides and a form with completed polysaccharide chains. *J Biol Chem* **254**, 12209–12218.
- Mega, T., Ikenaka, T. & Matsushima, Y. (1970). Studies on *N*-acetyl- β -D-glucosaminidase of *Aspergillus oryzae*. I. Purification and characterization of *N*-acetyl- β -D-glucosaminidase obtained from Takadiastase. *J Biochem* **68**, 109–117.
- Moore, S. (1963). On the determination of cysteine as cysteic acid. *J Biol Chem* **238**, 235–237.
- Morrissey, J. H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal Biochem* **117**, 307–310.
- Odds, F. C. (1988). *Candida and Candidosis*, 2nd edn. London: Baillière Tindall.
- Odds, F. C. & Hierholzer, J. C. (1973). Purification and properties of a glycoprotein acid phosphatase from *Candida albicans*. *J Bacteriol* **114**, 257–266.
- Oktakara, A., Yoshida, M., Murakami, M. & Izumi, T. (1981). Purification and characterization of β -*N*-acetylhexosaminidase from *Picnoporus cinnabarinus*. *Agric Biol Chem* **45**, 239–247.
- Pugh, D. & Cawson, R. A. (1977). The cytochemical localization of acid hydrolases in four common fungi. *Cell Mol Biol* **22**, 125–132.
- Ram, S. P., Sullivan, P. A. & Shepherd, M. G. (1983). The *in situ* assay of *Candida albicans* enzymes during yeast growth and germ-tube formation. *J Gen Microbiol* **129**, 2367–2378.
- Ram, S. P., Romana, L. K., Shepherd, M. G. & Sullivan, P. A. (1984). Exo-(1 $\rightarrow$ 3)- β -glucanase, autolysin and trehalase activities during yeast growth and germ-tube formation in *Candida albicans*. *J Gen Microbiol* **130**, 1227–1236.
- Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). A modified colorimetric method for the estimation of *N*-acetyl amino sugars. *J Biol Chem* **217**, 959–966.
- Ross, I. K., De Bernardis, F., Emerson, G. W., Cassone, A. & Sullivan, P. A. (1990). The secreted aspartate proteinase of *Candida*

albicans: physiology of secretion and virulence of a proteinase-deficient strain. *J Gen Microbiol* **136**, 687–694.

Sandhoff, K., Conzelmann, E., Neufeld, E. F., Kaback, M. M. & Suzuki, K. (1989). The G_{M2} gangliosides. In *The Molecular Basis of Inherited Disease*, vol. 11, pp. 1807–1839. New York: McGraw Hill.

Shepherd, M. G., Chiew, Y. Y., Ram, S. P. & Sullivan, P. A. (1980). Germ-tube induction in *Candida albicans*. *Can J Microbiol* **26**, 21–26.

Shepherd, M. G., Poulter, R. T. M. & Sullivan, P. A. (1985). *Candida albicans*: biology, genetics, and pathogenicity. *Annu Rev Microbiol* **39**, 579–614.

Sullivan, P. A., McHugh, N. J., Romara, L. K. & Shepherd, M. G. (1984). The secretion of N-acetylglucosaminidase during germ-tube formation in *Candida albicans*. *J Gen Microbiol* **130**, 2213–2218.

Tammi, M., Ballou, L., Taylor, A. & Ballou, C. E. (1987). Effect of glycosylation on yeast invertase oligomer stability. *J Biol Chem* **262**, 4395–4401.

Trimble, R. B. & Atkinson, P. H. (1986). Structure of yeast external invertase Man_{8–14} GlcNAc processing intermediates by 500-megahertz ¹H NMR spectroscopy. *J Biol Chem* **261**, 9815–9824.

Trimble, R. B. & Maley, F. (1984). Optimising hydrolysis of N-linked high mannose oligosaccharides by endo-β-N-acetylglucosaminidase H. *Anal Biochem* **141**, 515–522.

Trimble, R. B., Maley, F. & Chu, F. K. (1983). Glycoprotein biosynthesis in yeast. Protein conformation affects processing of high mannose oligosaccharides on carboxypeptidase Y and invertase. *J Biol Chem* **258**, 2562–2567.

Weitz, G. & Proia, R. L. (1992). Analysis of the glycosylation and phosphorylation of the α-subunit of the lysosomal enzyme β-hexosaminidase A by site-directed mutagenesis. *J Biol Chem* **267**, 10039–10044.

Wright, R. J., Carne, A., Hieber, A. D., Lamont, I. L., Emerson, G. W. & Sullivan, P. A. (1992). A second gene for a secreted aspartate proteinase in *Candida albicans*. *J Bacteriol* **174**, 7848–7853.

Yamamoto, K., Lee, K. M., Kumagai, M. & Tochikura, T. (1985). Purification and characterization of β-N-acetylhexosaminidase from *Penicillium oxalicum*. *Agric Biol Chem* **49**, 611–619.

Received 7 December 1993; revised 11 February 1994; accepted 14 February 1994.