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Effect of tunicamycin on *N*-acetyl-β-D-glucosaminidase produced by *Trichoderma harzianum*

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Abstract

The effect of tunicamycin, an inhibitor of protein *N*-glycosylation, was studied in non-growing mycelium of *Trichoderma harzianum* induced to secrete *N*-acetyl-β-D-glucosaminidase by the addition of *N*-acetylglucosamine. Tunicamycin (30 μg ml^{-1}) had no significant effect on growth of the fungus, or on the total protein secreted or specific activity of *N*-acetyl-β-D-glucosaminidase. However, in the presence of the inhibitor an underglycosylated form of the enzyme was produced. The apparent molecular masses for this and the native enzyme were 110 and 124 kDa, respectively. Both forms of the enzyme showed the same optimum pH and temperature, but the underglycosylated form was more sensitive to inactivation by both high temperature (60°C) and the proteolytic enzyme trypsin.

Keywords: *N*-Acetyl-β-D-glucosaminidase; Glycosylation; Tunicamycin; *Trichoderma harzianum*

1. Introduction

The chitinolytic system of the filamentous fungus *Trichoderma harzianum* consists of two basic types of enzymes: chitinases (EC 3.2.1.14) and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30). These enzymes are involved in the growth and differentiation of all chitin-containing fungi, and may also play an important nutritional role [1]. Recent work indicates that the production of these enzymes by species of *Trichoderma* might be associated with their antagonistic behaviour against other soil fungi [2]. Chitinase and *N*-acetyl-β-D-glucosaminidase from many isolates of *T. harzianum* have been purified and characterised [3] and the elucidation of the complete gene sequence of three chitinases and one *N*-acetyl-β-D-glucosaminidase from *T. harzianum* has been reported. [4–7]. This has led to many studies on the molecular biology of the chitinase and *N*-acetyl-β-D-glucosaminidase genes, opening new fields of investigation such as the organisation and regulation of these genes during mycoparasitism.

The *N*-acetyl-β-D-glucosaminidase produced by *T. harzianum* during growth on chitin or *N*-acetylglucosamine is a glycoprotein secreted into the culture medium [8]. We have examined the effect of tunicamycin on the growth and secretion of this enzyme by non-growing mycelium of *T. harzianum* in an effort to elucidate the role of *N*-glycosylation in enzyme secretion. Treatment with tunicamycin in vivo produces under- or non-glycosylated proteins in both animal cells and in eucaryotic microorganisms, such as fungi [9,10]. Tunicamycin is an antibiotic that blocks the synthesis of dolichol pyrophosphate-*N*-acetylglucosamine, a key intermediate in the biosynthetic pathway of *N*-glycosylated glycoproteins [11,12]. The results suggested that *N*-glycosylation is not necessary for secretion of *N*-acetyl-β-D-glucosaminidase by *T. harzianum*, but is important for the enzyme's thermal stability and resistance against proteolytic attack.

2. Materials and methods

2.1. Strain and culture methods

T. harzianum (strain 39.1, University of Nottingham collection) was maintained on MYG medium (yeast extract, 0.2%; malt extract, 0.2%; glucose, 2%; agar, 2%).

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The fungus was grown routinely in 500 ml conical flasks containing 50 ml TM medium, containing (g l^{-1}): bactopeptone, 1.0; urea, 0.3; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 6H_2O$, 0.3; glucose, 10.0 and 0.1% (v/v) of the trace element solution containing Fe^{2+} Mn^{2+}, Zn^{2+} and Co^{2+}. The cultures were further incubated at 28°C on a rotary shaker at 180 rpm.

2.2. Conditions for enzyme induction

For induction experiments, mycelium was harvested after 24 h incubation on TM medium, washed three times with 10 mM phosphate buffer pH 6.0 and transferred to conical flasks containing 25 ml of MM medium containing (g l^{-1}): KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 6H_2O$, 0.3, supplemented with 1.0% (w/v) *N*-acetylglucosamine. Mycelial suspensions were further incubated at 28°C on a rotary shaker at 180 rpm for up to 24 h, and harvested by filtration onto Whatman No. 1 filter paper. The culture filtrate obtained was dialysed overnight at 4°C, against 10 mM phosphate buffer pH 6.0, and concentrated by lyophilisation. When tunicamycin (Sigma) was added to the MM medium, this was done by using an appropriately concentrated (1 mg/ml) solution in 20 mM NaOH. An equivalent volume of 20 mM NaOH was added to control flasks.

2.3. Analytical procedures

N-Acetyl-β-D-glucosaminidase activity was assayed by monitoring the rate of formation of ρ-nitrophenol from ρ-nitrophenol-β-*N*-acetylglucosamine (ρNPGlcNAc; Sigma) [13]. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of ρ-nitrophenol in 1 min at 37°C. Protein was determined by the method of Bradford [14] using bovine albumin as the standard. Gel filtration was carried out on a column (2.5×85 cm) packed with Sephacryl S-200 (Pharmacia) and equilibrated with 50 mM phosphate buffer pH 6.0, containing 0.15 M NaCl. The column was eluted with the same buffer, at a flow rate of 18 ml h^{-1}. Fractions of about 1 ml were collected and monitored at 280 nm and their enzyme activity was determined. The column was previously calibrated with the following molecular weight markers (Sigma): catalase (232 kDa), aldolase (158 kDa), bovine albumin (66 kDa) and chymotrypsinogen A (25 kDa).

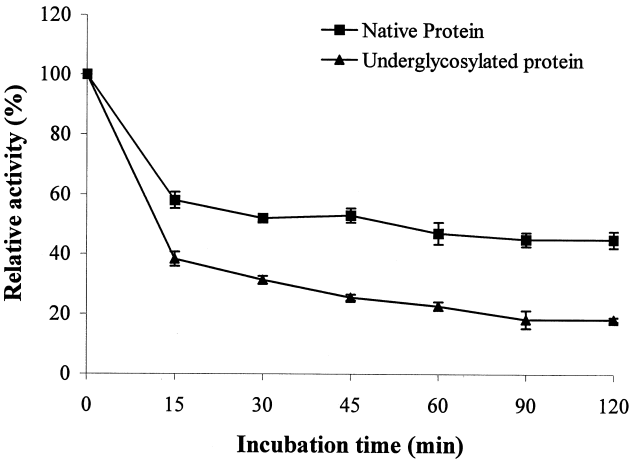

Fig. 1. Effect of temperature on the stability of *N*-acetyl-D-glucosaminidase produced by *T. harzianum* in the absence and presence of tunicamycin. Stability of the enzyme was examined by maintaining solutions at 60°C up to 120 min followed by the assessment of activity under standard conditions. Maximum activity was taken as 100%.

3. Results and discussion

Previously we reported the purification of a *N*-acetyl-β-D-glucosaminidase produced and secreted by *T. harzianum*, when grown in the presence of chitin or *N*-acetylglucosamine as a carbon source [15]. The enzyme was characterised as a glycoprotein with a molecular mass of 64 and 118 kDa as determined by SDS–PAGE and gel filtration on Sephacryl S-300, respectively. Peterbauer et al. [7] reported the cloning and characterisation of a *N*-acetyl-β-D-glucosaminidase gene of *T. harzianum* encoding a mature protein of 561 amino acids with a molecular mass of approx. 62.7 kDa. They also reported the presence of seven consensus amino acid sequences (N-X-S/T-X) as potential sites for glycosylation of the protein. In an effort to understand the role of the *N*-glycosylation mechanisms involved in the secretion of *N*-acetyl-β-D-glucosaminidase by *T. harzianum*, studies to determine the effect of tunicamycin on growth, enzyme secretion and stability were performed. The effect of tunicamycin on the synthesis and secretion of glycoproteins has been evaluated in several fungal systems [9,10,16,17].

The growth of *T. harzianum* in the presence of tunicamycin was measured using radial growth (MYG medium) and dry weight methods (TM medium). Tunicamycin at

Table 1
Effects of tunicamycin on growth, extracellular protein and secretion of *N*-acetyl-β-D-glucosaminidase by *T. harzianum*

Tunicamycin (μg/ml)	Radial growth[a] (cm)	Dry weight[b] (μg)	Protein (μg/ml)	Specific activity (U mg^{-1})
0	4.0 ± 0.1	67.5 ± 3.3	14.6 ± 0.6	19.1 ± 0.6
30	3.8 ± 0.2	64.2 ± 3.2	13.8 ± 0.5	19.6 ± 0.4

[a,b]Radial growth and dry weight were measured after 48 h incubation at 28°C on MYG and TM medium, respectively. All values are means of three replicates.

Table 2
Characterisation of *N*-acetyl-β-D-glucosaminidase secreted by *T. harzianum* in the absence or presence of tunicamycin

Tunicamycin (μg/ml)	Molecular mass (kDa)[a]	K_m (μmol)	Optimum pH	Optimum temperature (°C)	Activity after trypsin (%)[b]
0	124	1.27	5.6	50	71
30	110	1.10	5.6	50	48

[a]Molecular mass determined by gel filtration on Sephacryl S-200.
[b]*N*-Acetyl-β-D-glucosaminidase activity was determined after incubation of the enzyme solution with trypsin (300 μg ml^{-1}) at 30°C for 4 h. These experiments were conducted three times.

30 μg ml^{-1} had no effect on growth (Table 1), but at a concentration above 30 μg ml^{-1} growth was significantly reduced (data no shown). However, the reduction of molecular mass observed for the enzyme produced in the presence of tunicamycin (Table 2) is circumstantial evidence for a reduction in the glycan content of the glycoprotein. Other unpublished observations on the reduced binding of the enzyme, produced in the presence of the antibiotic, to a concanavalin A column also support this view. The electrophoretic data also suggest that there is no effect on the dimerisation of the protein at 30 μg ml^{-1} tunicamycin. This correlates with the fact that the presence of the inhibitor also had no significant effect on the total protein secreted or the specific activity of *N*-acetyl-β-D-glucosaminidase (Table 1). Enzyme secreted in the presence of tunicamycin binds only weakly to concanavalin A, implying the absence of N-linked glycans on the protein. These results suggested that *N*-glycosylation is not necessary for the secretion of *N*-acetyl-β-D-glucosaminidase by *T. harzianum*, since the presence of tunicamycin in the medium had no effect on total extracellular protein (Table 1). A similar result has been described on secretion of endoglucanase (I and II) and cellobiohydrolase by *T. reesei*, as their synthesis was not affected by the presence of tunicamycin [9,10]. However, the synthesis of endoglucanases I and II was found to be strongly inhibited by 2-deoxyglucose, a compound used as an inhibitor of O-linked glycosylation [10]. The presence of O-linked glycans on *N*-acetyl-β-D-glucosaminidase has not been investigated.

The concentrated *N*-acetyl-β-D-glucosaminidase present in the culture filtrate of mycelium, which had been incubated in the presence or absence of tunicamycin, was applied to a Sephacryl S-200 column. Two *N*-acetyl-β-D-glucosaminidases were identified, the native and underglycosylated forms with molecular masses of 124 and 110 kDa, respectively (Table 2). If a similar distribution of N-linked glycosyl chains of *N*-acetylglucosaminidase described by Peterbauer et al. [7] occurs in the enzyme used in this study, this difference was probably due to the absence of *N*-glycosylation after tunicamycin treatment.

To establish the efficacy of the native and the underglycosylated enzymes for substrate hydrolysis under different conditions, some kinetic parameters were determined (Table 2). Both forms of the enzyme showed a comparable K_m and the same optimum pH (5.6) and temperature (50°C). These results are in agreement with the purified enzyme described by Ulhoa and Peberdy [15]. However, the underglycosylated form was more sensitive to inactivation by both the proteolytic enzyme trypsin and high temperature. The native and underglycosylated forms of the enzyme, when incubated for 4 h in the presence of trypsin, retained 71% and 48% activity, respectively. The native enzyme treated at 60°C for 120 min retained 45% of its maximum activity, whereas the activity of the underglycosylated form under the same conditions was lowered to about 18% (Fig. 1). The difference in behaviour of the enzyme in terms of activity and stability after the addition of tunicamycin during synthesis may be due to the decreased carbohydrate content, as previously reported in the literature [9,10,16,17]. The results of this work suggested that the major role of the N-linked carbohydrate may be to stabilise the structure of the *N*-acetyl-β-D-glucosaminidase produced by *T. harzianum* and so protect it from physical and proteolytic attack after secretion.

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