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INVOLVEMENT OF G PROTEINS AND cAMP IN THE PRODUCTION OF CHITINOLYTIC ENZYMES BY *TRICHODERMA HARZIANUM*

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ABSTRACT

The effect of G protein modulators and cyclic AMP (cAMP) on N-acetylglucosaminidase (NAGase) production was investigated during 84 h of growth of a *Trichoderma harzianum* strain in chitin-containing medium. Caffeine (5 mM), N[6]–2'-*O*-dibutyryladenosine 3'5'-cyclic monophosphate sodium salt (dBcAMP) (1 mM) and 3-isobutyl-1-methylxanthine (IBMX) (2 mM) decreased extracellular NAGase activity by 80%, 77% and 37%, respectively. $AlCl_3$/KF (100 µM/10 mM and 200 µM/ 20 mM) decreased the activity by 85% and 95%, respectively. Cholera (10 µ/mL) and pertussis (20 µ/mL) toxins also affected NAGase activity, causing a decrease of approximately 75%. Upon all treatments, protein bands of approximately 73 kDa, 68 kDa and 45 kDa had their signals diminished whilst a 50 kDa band was enhanced only by treatment with cholera and pertussis toxins. N-terminal sequencing analysis identified the 73 kDa and 68 kDa proteins as being *T. harzianum* NAGase in two different truncated forms whereas the 45 kDa band comprised a *T. harzianum* endochitinase. The 50 kDa protein showed sequence similarity to *Coriolus vesicolor* cellobiohydrolase. The above results suggest that a signaling pathway comprising G-proteins, adenylate cyclase and cAMP may be involved in the synthesis of *T. harzianum* chitinases.

Key words: *Trichoderma*, N-acetylglucosaminidase, chitinase, cAMP, G protein.

INTRODUCTION

The mycoparasite *Trichoderma harzianum* is a filamentous fungus described as a biocontrol agent against several phytopathogenic fungi. One of its antagonistic mechanisms is the production of hydrolytic enzymes responsible for the breakdown of the host cell walls (4). Since chitin, an unbranched homopolymer of 1,4-β-linked N-acetylglucosamine (GlcNAc), is the major component of most fungal cell walls, a principal role has been attributed to enzymes from the chitinolytic system. A considerable amount of research has been aimed at elucidating the chitinolytic system of *T. harzianum* during growth in different carbon sources (11,14,15). It has been found to be a complex system consisting of two N-acetylglucosaminidases (73 and 102 kDa), which split diacetylchitobiose, chitotriose and chitotetraose into GlcNAc monomers, four endochitinases (31, 33, 42 and 52 kDa), which cleave chitin randomly in internal sites and one exochitinase (40 kDa) that catalyses the progressive release of diacetylchitobiose in a stepwise fashion (4).

The purification and characterization of a 102 kDa N-acetylglucosaminidase (NAGase) produced by *T. harzianum* was previously described (14). This enzyme is a glycoprotein secreted in chitin-containing medium and repressed when the mycelium is provided with an easily metabolized carbon source, such as glucose (11,15). The reason for this could be explained by the lack of a substrate for NAGase induction, since synthesis of endo and exochitinases was repressed by glucose in chitin-containing media. However, when GlcNAc was used as carbon source, glucose did not repress NAGase synthesis (11,15).

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Expression of the chitinolytic system from *T. harzianum* appears to be coordinated, suggesting a regulatory mechanism involving substrate induction and catabolic repression. Several proposals have been made to explain how induction may occur in this situation, since chitin is an insoluble substrate. Some authors suggest that the most probable inducers of NAGase in *T. harzianum* are soluble oligomers, released from the chitin by chitinases (7). On the other hand, enzyme regulation could result from the physical contact between the insoluble substrate and receptors present on the cell surface, activating a transmembrane signal transduction pathway. Here we show that the production of *T. harzianum* NAGase and endochitinase in response to chitin can be affected by modulators of G proteins and cyclic cAMP, indicating the participation of a G-protein-linked signal transduction pathway in the process.

MATERIALS AND METHODS

Organism and culture conditions

Spores from *Trichoderma harzianum* Rifai strain 39.1 (University of Nottingham collection) were maintained in MYG agar medium (0.5% malt extract, 0.25% yeast extract, 1% glucose and 2% agar). The fungus (1 x 10^7 spores mL^{-1} of culture medium) was grown routinely in 2 L conical flasks containing 500 mL of medium with constant shaking at 28ºC for 3-4 days. The medium contained: 0.1% bactopeptone, 0.03 % urea, 0.2 % KH_2PO_4, 1.4% $(NH_4)_2SO_4$, 0.03 % $MgSO_4.7H_2O$, 0.015% $CaCl_2$, 0.1 % trace elements solution containing Fe^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and 2% glucose.

Conditions for enzyme induction

For induction experiments cultures were harvested after 24 h growth in the conditions described above. The mycelia were washed with 500 mL sterile 0.9% NaCl solution, and 250 mg of mycelia were transferred to 10 mL minimal medium (0.2% KH_2PO_4, 1.4% $(NH_4)_2SO_4$, 0.03% $MgSO_4.7H_2O$, 0.015% $CaCl_2$, 0.1% trace elements solution) supplemented with 1% chitin, and the signal transduction modulator to be tested. In controls no modulator was added. The culture was incubated in rotatory shaker (120 rpm) at 28ºC. Aliquots of 50 µL were collected from the culture filtrate every 12 h and used for NAGase activity. After 84 h of growth 60 µL aliquots from the culture filtrate were subjected to SDS-PAGE. Modulators of signal transduction pathways were added to minimal medium at the following concentrations: $AlCl_3$/ KF (100 µM/10 mM and 200 µM/20 mM), cholera toxin (10 µg.mL^{-1}), pertussis toxin (20 mg.mL^{-1}), caffeine (5 mM), 3-isobutyl-1-methylxanthine (IBMX) (2 mM); N^6–2'-*O*-dibutyryladenosine 3'5'-cyclic monophosphate sodium salt (dBcAMP) (1 mM); N-

(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide (W7) (100 µM) and trifluoroperazine dimaleate (TFP) (50 µM).

N-acetylglucosaminidase assay

Enzyme activity was measured by monitoring the rate of formation of p-nitrophenol from p-nitrophenyl-β-N-acetylglucosamine (pNglcNAc) as described by Yabuki *et al.* (16). One unit of enzyme activity was defined as the amount of enzyme which releases 1 µmol of p-nitrophenol in 1 min at 37ºC.

SDS-PAGE and electrotransfer

The protein profile of *T. harzianum* secreted proteins after 84 h of treatment with different modulators was determined by SDS-PAGE (6) system, using 12% polyacrylamide separating gel. Equal volumes of extracellular media were analysed from each sample. After electrophoresis, gels were either silver stained according to Blum *et al.* (2) or submitted to electrotransfer to PVDF membranes using a Multiphor II semidry electroblotting system (Amersham Pharmacia Biotech) and visualized by staining with Coomassie Blue.

Protein sequencing

Amino terminal sequencing of the blotted protein was carried out by automated Edman degradation using a 477/120A Protein Sequencer (Applied Biosystems). Amino acid sequence homology searches were performed against a non-redundant database (GenBank CDS translations + PDB + SwissProt + PIR + PRF) through the BLAST program.

RESULTS AND DISCUSSION

The initial aim of this work was to investigate the involvement of G proteins, cAMP and Ca^{2+} on the production of NAGase by *T. harzianum*. G-proteins act as transducing signals from G protein coupled receptors (GPCRs) to effectors (e.g. adenylate cyclase and ion channels) which modulate levels of second messengers such as cAMP and Ca^{2+} (9). There is evidence that filamentous fungi possess G-proteins similar to the ones found in yeast and animal cells (1,8,13). In addition, cAMP was shown to be involved in the control of a wide variety of functions, including the utilization of endogenous and exogenous carbon source, the synthesis and degradation of enzymes, conidiation, and hyphae coiling (3,5,10,12).

The effect of dBcAMP, G-protein modulators, phosphodiesterase and calmodulin inhibitors on the extracellular activity of *T. harzianum* NAGase, grown in presence of 1% chitin, was analyzed for a period of 84 hours. Thus, dBcAMP (1 mM), a permeable analogue of cAMP, decreased the enzyme activity 77% after 84


Abbreviations: cAMP: cyclic AMP; NAGase: N-acetylglucosaminidase; IBMX: 3-isobutyl-1-methylxanthine; dBcAMP: N^6–2'-*O*-dibutyryladenosine 3'5'-cyclic monophosphate; W7: N-(6-Aminohexyl)-5-chloro-1-naphtalenesulfonamide; TFP: trifluoroperazine dimaleate; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF: polyvinylidene difluoride.

hours incubation (Fig. 1). Inhibitors of cAMP phosphodiesterase such as IBMX and caffeine are known to increase the intracellular levels of cAMP in eukaryotic cells including fungi (5). In our experiments IBMX (2 mM) and caffeine (5 mM) decreased NAGase activity by 40% and 80%, respectively (Fig. 1).

The ion AlF_4^-, an activator of G-proteins, was also tested by addition of $AlCl_3$ and KF to the medium. AlF_4^- forms a complex with GDP in which it mimics the γ phosphate group of GTP and keeps the G proteins in the active state (9). Two concentrations of $AlCl_3$/KF (100 μM/10 mM and 200 μM/20 mM) were used and decreased NAGase activity in 85% and 95%, respectively (Fig. 2).
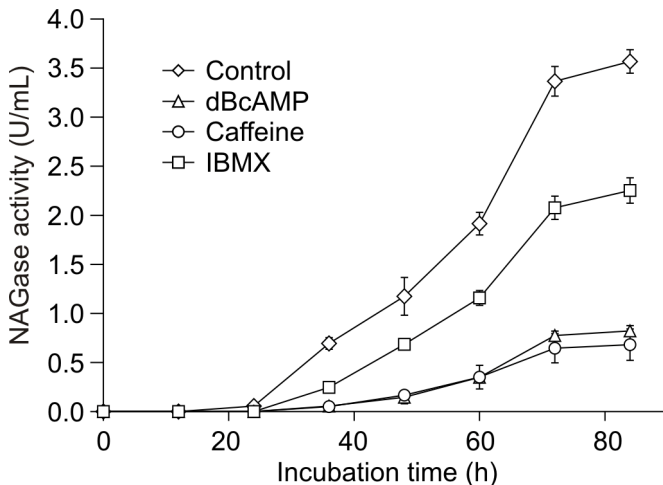
Figure 1. Effect of IBMX, caffeine and dBcAMP on *T. harzianum* NAGase extracellular activity. All values are means of three replicates.
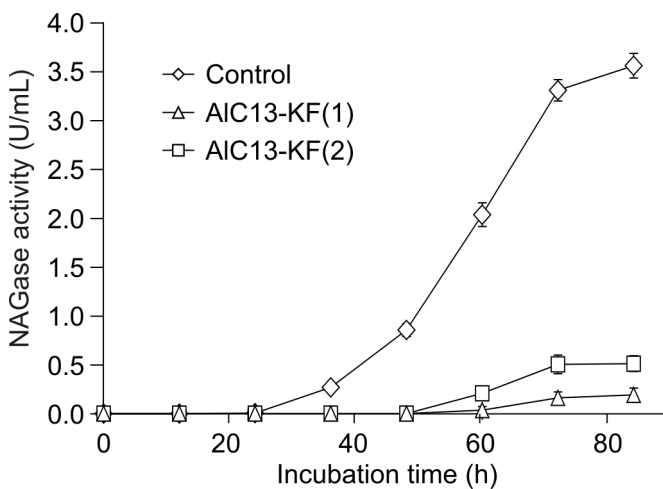

Figure 2. Effect of AlF_4^- on *T. harzianum* NAGase extracellular activity. All values are means of three replicates.
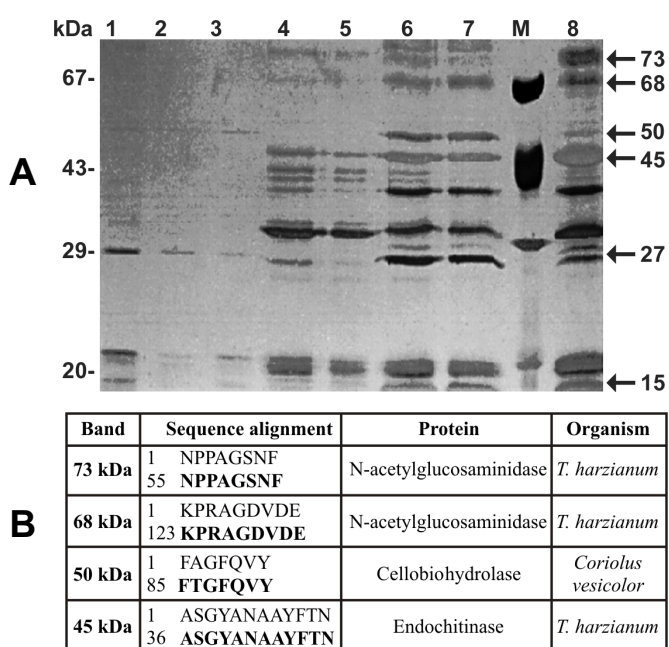
The bacterial exotoxins, cholera and pertussis, can also modulate the activity of G-proteins from the subfamilies Gs (stimulatory of adenylate cyclase) and Gi (inhibitory of adenylate cyclase) (9). Cholera toxin catalyses the ADP-ribosylation of Gα subunits from the Gs subfamily, keeping them permanently activated. Pertussis toxin catalyses the ADP-ribosylation of G proteins from the Gi subfamily causing a permanent inactivation. Consequently, both toxins increase the concentration of intracellular cAMP. Cholera and pertussis toxins affected the synthesis of NAGase in *T. harzianum* negatively causing a decrease of approximately 75% of the enzyme activity (Fig. 3). Inhibitors of calmodulin (W7 and TFP) were also tested to verify the involvement of calcium signalling in NAGase synthesis, but no effect was observed (data not shown).

In order to obtain a more complete picture of *T. harzianum* response to the different modulators, the proteins secreted in the different media after 84 hours of growth were analysed by SDS-PAGE as shown in Fig. 4A. Samples treated with dBcAMP, caffeine and IBMX (lanes 1, 2 and 3 respectively) presented much less intense protein bands than the control (lane 8) indicating that *T. harzianum* protein secretion decreased in response to the rise in intracellular cAMP levels. A possible explanation for this result could be a non-specific toxic effect of the reagents used. However it was previously reported that concentrations of 5 mM IBMX and 1 mM cAMP were not toxic for *T. harzianum* (10). Furthermore, when we tested the effect of dBcAMP, caffeine and IBMX on NAGase extracellular activity using a medium containing N-acetylglucosamine (GlcNAc) as a carbon source instead of chitin, the extracellular enzyme activity was not affected indicating that the reagents used were not toxic to the fungus (data not shown).

More specific differences in protein profiles were observed when AlF_4^- and bacterial toxins were tested. When AlF_4^- was used as modulator (lanes 4 and 5), bands of 73 kDa, 68 kDa, 45
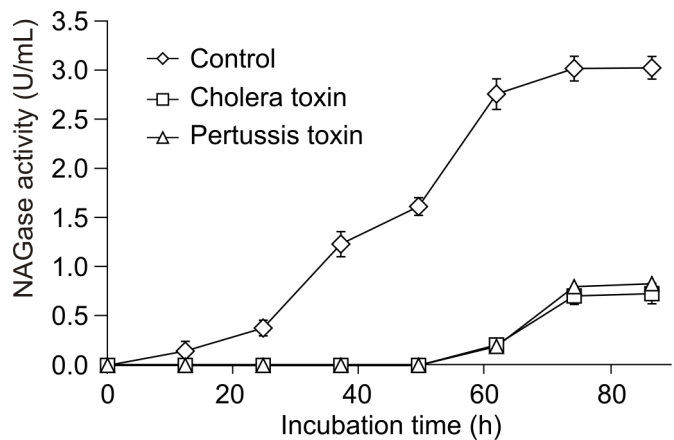
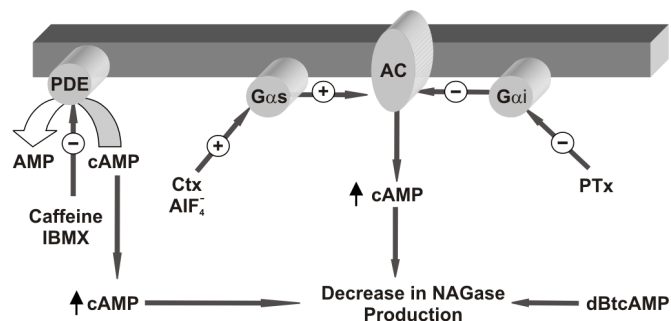**Figure 3.** Effect of cholera and pertussis toxins on NAGase activity. All values are means of three replicates.

Figure 5. Model for NAGase repression by signal transduction modulators. See explanation in text. PDE-cAMP phosphodiesterase; AC-adenylate cyclase; CTx-cholera toxin; PTx-pertussis toxin; (+)-activation; (-)-inhibition; ↑-increase in concentration.

Figure 4. SDS-PAGE and N-terminal sequence of *T. harzianum* secreted proteins. **A**, *T. harzianum* was grown in minimal medium containing 1% chitin and supplemented with: **1**, 1 mM dBcAMP; **2**, 5 mM caffeine; **3**, 2 mM IBMX; **4**, 100 μM/10 mM AlCl$_3$/KF; **5**, 200 μM/20 mM AlCl$_3$/KF; **6**, 10 μg.mL^{-1} cholera toxin; **7**, 20 μg.mL^{-1} pertussis toxin; **8**, control. A volume of 60 μL of each culture filtrate after 84 h of growth was applied to the gel. **M**, molecular mass markers. **B**, Table presenting the alignment of the 73 kDa, 66 kDa, 50 kDa and 45 kDa polypeptides with matched sequences from protein databases (bold type).

kDa, 27 kDa and 15 kDa showed decrease compared to the control (lane 8). Cholera and pertussis toxin gave identical protein profiles (lanes 6 and 7) with a decline in staining of protein bands corresponding to 73 kDa, 68 kDa and 45 kDa, whereas an enhance in a 50 kDa polypeptide was observed. These four polypeptides were subjected to Edman degradation and the resulting sequences are shown in Fig 4B. The N-terminus of the 73 kDa and 68 kDa proteins showed 100% identity to different regions of *T. harzianum* NAGase. Probably both polypeptides correspond to two truncated forms of NAGase that suffered different post-translational processing. The 45 kDa protein was identified as being *T. harzianum* 42 kDa endochitinase (4). The N-terminal sequence of the 50 kDa showed high sequence identity to a cellobiohydrolase from the fungus *Coriolus vesicolor*.

The results indicate that when *T. harzianum* is grown in liquid shake culture containing chitin, the extracellular concentration of NAGase is affected by G protein modulators and cAMP as summarized in Fig. 5. Therefore, the rise in the intracellular levels

of cAMP would cause a reduction in NAGase production. This rise would derive from the inhibition of cAMP phosphodiesterase by caffeine and IBMX, activation of Gs proteins by cholera toxin and AlF$_4^-$, inhibition of Gi proteins by pertussis toxin or addition of the membrane permeable cAMP analogue. A *T. harzianum* 42 kDa endochitinase was also shown to be repressed by the modulators suggesting that the chitinolytic enzymes may be regulated by common signalling pathways.

The cAMP signaling cascades seem to be conserved among fungi (3). In these organisms, cAMP activates a cAMP-dependent protein kinase (PKA) that phosphorylates enzymes involved in intermediary metabolism (particularly carbohydrate metabolism) and transcription factors which are key regulators of stress-responsive gene expression. For instance in *Sacharomyces cervisiae* glucose and other fermentable sugars are sensed extracellularly by G-protein coupled receptors and raise cAMP levels (through a signalling pathway composed of a G protein and adenylate cyclase) activating PKA. Elevated PKA activity results in induction of glycolytic enzymes and cell proliferation indicating that cAMP pathway signals nutrient availability to the cell cycle machinery (3). It was previously reported that glucose and other easily metabolized carbon sources repressed NAGase production by *T. harzianum* in chitin-containing medium (15). Although it is not clear whether the repression involved cAMP this might be true considering that glucose is able to elicit a rapid increase in cAMP in *Trichoderma reesei* (12).

Aditional work using other modulators such as PKA inhibitors will be necessary to confirm that a signal transduction pathway involving G proteins and cAMP is responsible for the production of chitinolytic enzymes by *T. harzianum*. On the other hand, a more complete proteomic approach, including two-dimensional electrophoresis and mass spectrometric protein identification, will be necessary to obtain a more complete view of *T. harzianum* intracellular and secreted proteins that have their production affected by cAMP and G protein modulators.

RESUMO

Envolvimento de proteínas G e cAMP na produção de enzimas quitinolíticas por *Trichoderma harzianum*

O efeito de cAMP e de moduladores de proteínas G sobre a produção de N-acetilglicosaminidase (NAGase) foi investigado durante o crescimento de *Trichoderma harzianum* em meio contendo quitina. Cafeína (5 mM), dBcAMP (1mM) e IBMX (2 mM) provocaram diminuições na atividade extracelular de NAGase em 80%, 77% e 37%, respectivamente. Por outro lado, a presença de AlCl$_3$/KF nas concentrações de 100 µM/10 mM e 200 µM/ 20 mM causou decréscimo na atividade em 85% e 95%, respectivamente. A toxina do cólera (10 µ/mL) e a toxina pertussis (20 µ/mL) também afetaram a atividade de NAGase, causando um decréscimo de aproximadamente 75%. Análises eletroforéticas mostraram que todos os tratamentos citados causaram diminuição no sinal de bandas correspondendo a polipeptídios de 73 kDa, 68 kDa e 45 kDa, enquanto uma banda de 50 kDa foi intensificada apenas com tratamento com as toxinas do cólera e pertussis. Análises de sequenciamento N-terminal permitiram a identificação das proteínas de 73 kDa e 68 kDa como sendo NAGase de *T. harzianum* em duas formas diferentemente processadas enquanto a banda de 45 kDa correspondeu a uma endoquitinase de *T. harzianum*. A proteína de 50 kDa mostrou similaridade de sequência com uma celobiohidrolase de *Coriolus vesicolor*. Os resultados sugerem que uma via de sinalização composta por proteínas G, adenilato ciclase e cAMP possa estar envolvida na produção de quitinases *T. harzianum*.

Palavras-chave: *Trichoderma*, N-acetilglicosaminidase, quitinase, cAMP, proteína G.

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