Carbohydrate Research 344 (2009) 1682–1686

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

The influence of N-glycosylation on biochemical properties of Amy1, an α-amylase from the yeast *Cryptococcus flavus*

Manoel Cardoso de Barros [a], Roberto do Nascimento Silva [b], Marcelo Henrique Soller Ramada [b], Alexsandro Sobreira Galdino [c], Lídia Maria Pepe de Moraes [c], Fernando Araripe Gonçalves Torres [c], Cirano José Ulhoa [b,*]

[a] *Laboratório de Bioquímica, Faculdade de Ciências Biológicas, Universidade de Rio Verde-Fesurv, Fazenda Fontes do Saber—s/n, Rio Verde, Goiás, GO, CEP 75901-970, Brazil*
[b] *Laboratório de Enzimologia, Universidade Federal de Goiás (ICB), Goiânia, GO, CEP 74001-970, Brazil*
[c] *Laboratório de Biologia Molecular, Universidade de Brasília, Brasília, DF, CEP 70910-900, Brazil*

ARTICLE INFO

Article history:
Received 26 February 2009
Received in revised form 29 May 2009
Accepted 1 June 2009
Available online 6 June 2009

Keywords:
Cryptococcus flavus
α-Amylase
N-glycosylation
Tunicamycin

ABSTRACT

The yeast *Cryptococcus flavus* secretes a glycosylated α-amylase (Amy1) when grown in a starch-containing medium. The effects of N-glycosylation on secretion, enzyme activity, and stability of this glycoprotein were studied. Addition of tunicamycin (TM) to the medium at a concentration higher than 0.5 μg mL^{-1} affected *C. flavus* growth. Amy1 activity increased by 55% in the intracellular fraction after *C. flavus* growth in the presence of 0.5 μg mL^{-1} TM. SDS–PAGE and gel activity detection showed that native enzyme and deglycosylated enzyme had apparent molecular mass of 68 and 64.5 kDa, respectively. The N-glycosylation process did not affect either optimum pH or optimum temperature. The K_M values of native and non-glycosylated α-amylases were 0.052 and 0.098 mg mL^{-1}, and V_{max} values were 0.038 and 0.047 mg min^{-1}, respectively. However, the non-glycosylated form was more sensitive to inactivation by both the proteolytic enzyme trypsin and high temperature. Furthermore, the activity of the non-glycosylated enzyme was affected by Hg^{2+} and Cu^{2+} suggesting that N-glycosylation is involved in the folding of Amy1.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Glycosylation is one of the major post-translation modifications that can affect a variety of enzyme functions including secretion, stability, and folding.[1] This process has been extensively investigated in yeast as well as in filamentous fungi.[2–4] N-Glycans are often attached to secreted proteins as the growing polypeptide enters the endoplasmic reticulum and modifications in the glycosylation pattern are attained during their transit through the inner membrane.[1] There are two main types of protein glycosylations: O-glycosylation at hydroxyl groups of serine and threonine residues and N-glycosylation at asparagine residues within the consensus sequence Asn-X-Ser/Thr.[1] The N-glycosylation seems to be restricted to the catalytic modules, and it is usually absent in other parts of the enzyme.[5]

Yeast belonging to the genus *Cryptococcus* can be found in different habitats including soil, air, organic material residues, flowers, and fruits.[6] The secretion of hydrolytic enzymes by yeast plays an important role in the uptake of various carbon sources, including starch, which is hydrolyzed by the action of different amylases. α-Amylases (EC 3.2.1.1) are *endo*-glycosyl hydrolases that catalyze the hydrolysis of internal α-1,4-glucosidic bonds in starch polymer, and they configure one of the enzymes that is most commonly exploited in the industry.[7] A *Cryptococcus flavus* isolate which showed a significant capacity for α-amylase production was isolated in a screen for amylolytic yeast from the Brazilian biodiversity.[6] The *C. flavus* α-amylase (Amy1) was biochemically characterized,[6] and its gene (*AMY1*) was recently cloned and heterologously expressed in *Saccharomyces cerevisiae*.[8] Amy1 has an apparent molecular mass of ~67 kDa, part of which is due to glycosylation.[8] Amy1 is known to be glycosylated, and three potential N-glycosylation sites have been predicted from its cDNA sequence.[6,8]

In order to gain insight into the role of glycosylation in the biochemical properties of Amy1, we have investigated the effects of tunicamycin (TM), a known glycosylation inhibitor. In this work we show that the inhibition of the glycosylation process exerts significant changes on the activity and secretion of Amy1.

2. Materials and methods

2.1. Organism and growth conditions

C. flavus (isolate I-11) was obtained from the yeast collection at the Molecular Biology Laboratory (Universidade de Brasilia, Brazil) and was maintained at 25 °C on YPD (1% yeast extract, 2% peptone,

* Corresponding author. Tel.: +55 (62) 3521 1473; fax: +55 (62) 3521 1190.
E-mail address: ulhoa@icb.ufg.br (C.J. Ulhoa).

0008-6215/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2009.06.006

and 2% glucose) or YM medium (0.67% YNB—yeast nitrogen base without amino acids and 2% glucose). For enzyme production, 1 mL of a pre-culture grown on YM medium was transferred into 500-mL Erlenmeyer flasks containing 100 mL of the yeast production medium YPM (0.67% YNB and 2% starch) in the absence or presence of tunicamycin (Sigma) at different concentrations. Cells were grown at 28 °C on a rotatory shaker at 200 rpm for 30 h. At different time intervals, 5-mL samples were collected and growth rate was determined by measuring cell density at $OD_{600\ nm}$. Samples were centrifuged at 2600*g* for 10 min and the supernatant and pellet were tested for amylase activity and total protein content.

2.2. Enzyme and protein analysis

α-Amylase activity was determined as described previously by monitoring starch hydrolysis according to Fuwa.[9] One unit of amylase activity was defined as the amount of enzyme necessary to hydrolyze 0.1 mg of starch per minute. Protein concentration was measured according to Bradford using serum albumin (Sigma) as a standard.[10] The intracellular crude protein extracts were prepared according to Rodríguez et al.[11] Briefly, after centrifugation, cells were washed with distilled water and centrifuged as before. The cell pellet was resuspended in 1 mL of extraction buffer: (20 mM Tris–HCl, 300 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, pH 7.8) with 0.1 mM of PMSF, and 2 μM of β-mercaptoethanol, and sonicated (at 16 μm) for 4 cycles of 5 min each. Cell debris was removed by centrifugation at 6600*g* for 15 min and the supernatant was used for protein analysis.

2.3. Enzyme purification

All purifications were carried out according to Wanderley et al.[6] with some modifications. Briefly, ~200 mL of crude extract, previously dialyzed and equilibrated with column buffer, was applied to a Q-Sepharose (Sigma) column (2.5 × 20 cm) equilibrated with 50 mM acetate buffer (pH 5.5). The column was washed with the same buffer and eluted with a linear gradient of 0–0.5 mM NaCl. Fractions of 3.0 mL were collected and monitored for protein ($A_{280\ nm}$) and amylase activity. Concentrated samples (1 mL) were loaded on a Sephacryl S-100 HR (GE Healthcare) column (1.7 × 100 cm) previously equilibrated with 50 mM acetate buffer (pH 5.5) containing 150 mM NaCl. The column was washed with the same buffer at a flow rate of 20 mL h^{-1}. Fractions containing Amy1 activity were pooled, dialyzed overnight at 4 °C against water, and stored at −20 °C until use.

2.4. Electrophoresis and detection of amylolytic activity on gel

Protein purity and apparent molecular mass determinations were performed through 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli.[12] Protein was silver-stained as described by Blum et al.[13] For amylolytic detection in gel, after non-denaturing PAGE, gels were washed with distilled water, and proteins were renatured by incubation with 50 mM sodium acetate (pH 5.5) for 60 min followed by incubation at 4 °C for 12 h in a solution containing 0.5% starch (in 50 mM sodium acetate buffer, pH 5.5). Finally, the gel was incubated at 37 °C for 2 h, and bands with amylase activity were detected after staining with iodine solution.

2.5. In vitro deglycosylation of Amy1

After purification, 7 ng of purified Amy1 was incubated with N-Glycosidase F (PNGase F) (New England Biolabs) according to manufacturer's instructions. Reaction products were analyzed by SDS–PAGE and gel activity analysis.

2.6. Enzyme characterization

The pH optimum for Amy1 in the presence or absence of TM was determined by measuring the relative activity after incubation for 20 min at 50 °C under conditions described by Wanderley et al.,[6] by varying the pH of the reaction mixtures using the following buffers (100 mM): sodium acetate (pH 3.0–5.5), sodium phosphate (pH 6.0–7.0), and Tris–HCl (pH 7.5–8.0). The temperature optimum for the enzyme was evaluated by measuring Amy1 activity at different temperatures (30–70 °C) in 100 mM sodium acetate (pH 5.5). The thermal stability was determined by incubating the enzyme solution at 50 °C for 15, 30, 45, and 60 min followed by enzymatic assay. The effect of trypsin on Amy1 structure was evaluated by measuring the amylase activity after incubation of the enzyme solution with trypsin (Sigma) according to Ulhoa et al.[14] Kinetic parameters (K_M and V_{max}) were determined under the best conditions for enzymatic assay, by measuring the initial rates of reaction with soluble starch at various concentrations (0–1.0 mg mL^{-1}). The constant values were calculated using the MICROCAL-ORIGIN 5.0 software and the Lineweaver–Burk regression model. For determination of metal ion effect on Amy1 activity, enzyme assays were performed after pre-incubation (50 °C for 10 min) of the enzyme with various metal ions at a final concentration of 4 mM. The activity assayed in the absence of metal ions was defined as the control.

3. Results and discussion

Tunicamycin (TM) is a drug that blocks the synthesis of dolichol pyrophosphate-*N*-acetylglucosamine, a key enzyme in glycoprotein biosynthesis.[15] This antibiotic has been used in a variety of N-linked glycosylation studies in fungi,[15–17] and it is reported that TM can affect many physiological processes in fungi, such as growth, protein secretion, and protein stability.[3] Unlike that of other organisms, such as *Trichoderma harzianum*,[4] *Cryptococcus albidus*,[17] and *Aspergillus oryzae*,[18] *C. flavus* growth is significantly affected by TM at concentrations higher than 1.0 μg mL^{-1} when compared with the control (Fig. 1).

In order to test the influence of TM on Amy1 activity and secretion, *C. flavus* was incubated with TM (0–0.5 μg mL^{-1}) in YPM, and then extracellular and intracellular Amy1 activities and total protein content were determined. The total amount of intracellular and extracellular proteins was not significantly affected after growth for 30 h in the presence of TM when compared with the controls (Table 1). However, the distribution of the Amy1 activity

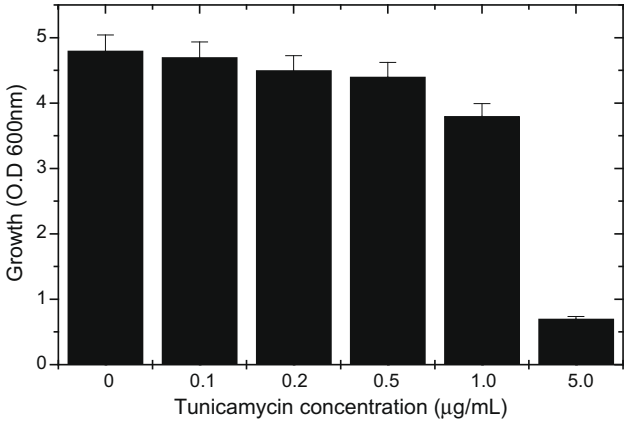

Figure 1. Effect of TM on *C. flavus* growth. Growth was measured after a 30-h incubation period in YPM containing different concentrations of TM (0–5 μg mL^{-1}). Tests were performed in triplicate, and results are presented as average.

Table 1
Distribution of amy1 activity and protein in intra- and extracellular portions in the presence and absence of TM

Tunicamycin (μg mL^{-1})	Amy1 activity (%)		Total protein (%)	
	Intra	Extra	Intra	Extra
0	8 ± 0.2	92 ± 6.0	52 ± 0.5	48 ± 0.2
0.1	21 ± 5.5	79 ± 2.8	44 ± 0.5	56 ± 0.5
0.2	32 ± 1.4	68 ± 2.5	49 ± 0.2	51 ± 0.1
0.5	63 ± 2.5	37 ± 1.4	49 ± 0.3	51 ± 0.8

in extracellular and intracellular fractions was affected (Table 1). Amy1 activity increased by 55% in the intracellular fraction after *C. flavus* growth in the presence of 0.5 μg mL^{-1} TM. The different patterns of Amy1 secretion were also confirmed by specific activity determination and by gel activity analysis (Fig. 2A and B). These data suggest that in the presence of TM (0.5 μg mL^{-1}), Amy1 remains mainly in the intracellular portion, suggesting the participation of N-glycosylation in the secretion Amy1 by *C. flavus*. However, the influence of glycosylation in the secretion of proteins other than Amy1 needs to be further investigated in more detail.

After standardization of TM concentration, a kinetic profile of Amy1 production was performed. The time course of Amy1 secretion and *C. flavus* growth during cultivation in YPM is shown in Figure 3. The optimum cellular growth of *C. flavus* was observed after 30 h incubation in the presence or absence of TM (Fig. 3A). However, growth in the presence of TM 0.5 μg mL^{-1} was about 10% less when compared with the control (without TM) confirming the effect of TM on *C. flavus* growth. Amy1 activity increased at all time points and reached 1.2 U mL^{-1} in the control and 1.0 U mL^{-1} in TM-treated cells after a 30-h incubation period (Fig. 3B). These results suggest that TM affects secretion and Amy1 structure as judged by the decrease in enzyme activity. Morsoli et al. reported a decrease in both secretion and xylanase activity in *C. albidus* when the medium was supplemented with TM (30 μg/mL), but no data about average deglycosylation were mentioned.[16] However, different results were observed in some filamentous fungi where both secretion and amylase activity were increased,[17] or there was no effect on these parameters.[14,18,19] These observations

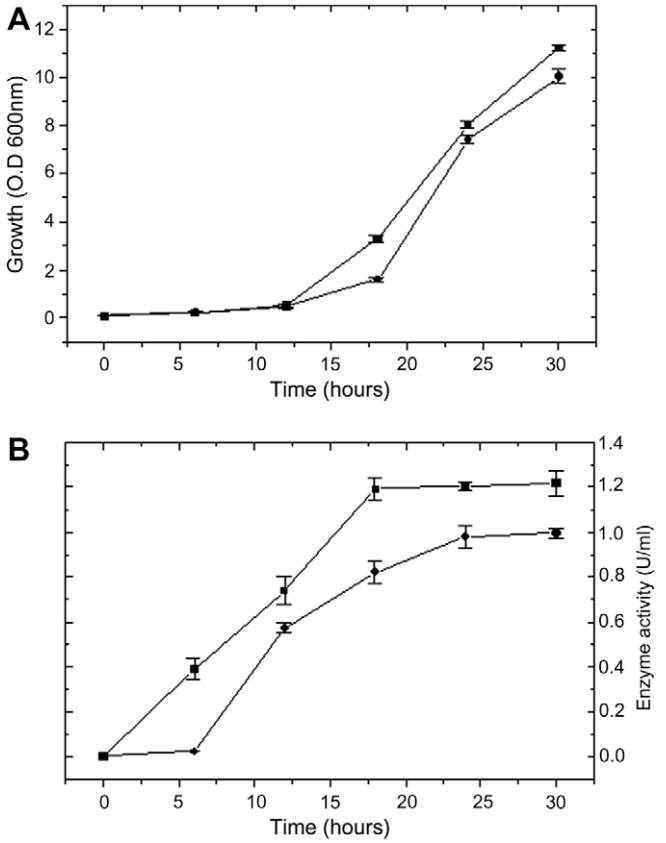

Figure 3. Time course of *C. flavus* growth (A) and Amy1 production (B) in YPM in the absence (■) or presence (●) of 0.5 μg mL^{-1} TM. Samples were measured in intervals of 6 h until 30 h of incubation at 28 °C and 200 rpm in YPM with TM (0–0.5 μg mL^{-1}).

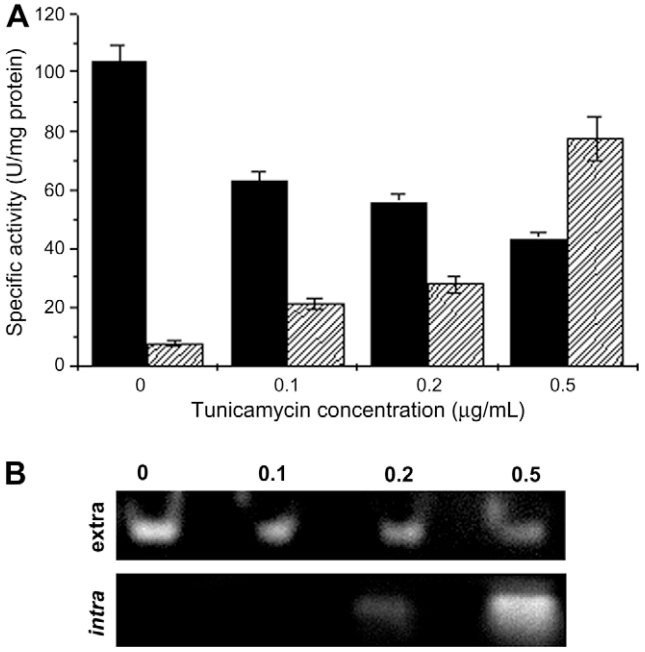

Figure 2. Effect of TM on Amy1 secretion. Specific activities of Amy1 (A) in intracellular (▨) and extracellular crude extracts (■). Samples were measured after 30 h of incubation at 28 °C and 200 rpm in YPM with TM (0–0.5 μg mL^{-1}). Samples were resolved in non-denaturing PAGE for amylolytic activity (B). 20 ng of total protein was loaded in each well.

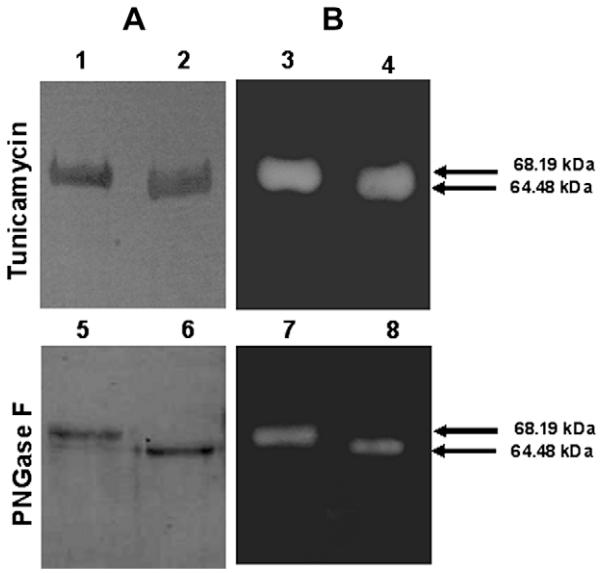

Figure 4. SDS–PAGE (A) and native PAGE (B) analyses of purified *C. flavus* Amy1. Lanes 1, 3, 5, and 7, native Amy1; Lanes 2 and 4, Amy1 produced in the presence of TM 0.5 μg mL^{-1}; and Lanes 6 and 8, Amy1 treated with PNGase F. The arrows indicate the molecular mass (kDa) of native and deglycosylated Amy1.

Table 2
Summary of the purification steps of Amy1 in the absence and presence of tunicamycin

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Supernatant	4.86 (4.94)	2105.08 (2110.66)	433.14 (427.17)	1 (1)	100 (100)
Q-Sepharose	2.05 (2.14)	986.41 (999.62)	479.77 (465.8)	1.10 (1.09)	42.3 (43.43)
Sephacryl	0.15	226.08	1458.62	3.04 (2.47)	7.53
S-100 HR	(0.21)	(243.32)	(1153.17)		(9.83)

The numbers in parentheses indicate results with TM.

suggest that N-glycosylation might have different effects on protein secretion in yeast and filamentous fungi.

We have purified Amy1 produced by *C. flavus*, after growth in the absence or presence of TM, using ion exchange chromatography on Q-Sepharose and gel filtration on Sephacryl S-100 HR. After purification, only one band with amylolytic activity was detected (Fig. 4). The native and non-glycosylated Amy1 were purified 3.48- and 2.71-fold with recovery of 10.7% and 11.5%, respectively (Table 2). The activities of native and non-glycosylated Amy1 were 1507.20 and 1158.67 U mg^{-1}, respectively (Table 2). SDS–PAGE analyses followed by gel activity detection showed that Amy1 secreted into the medium in the presence of TM shows a faster mobility when compared to the control (Fig. 4).

In order to confirm the efficacy of TM as an N-glycosylation inhibitor, in vitro deglycosylation with PNGase F was performed. Figure 4 shows that Amy1 produced in the presence of TM, and Amy1 treated with PNGase F had the same electrophoretic mobility. Amy1 produced in the presence of TM and the in vitro deglycosylated enzyme had an apparent molecular mass of 64.48 kDa, indicating that N-glycosylation is responsible for approximately 5.44% of protein mass (Fig. 4 and Table 3). Galdino et al. proposed the existence of three potential N-glycosylation sites in Amy1 located at ^{61}NGT, ^{190}NRT, and ^{269}NPS, which have not yet been experimentally confirmed.[8]

Biochemical properties of Amy1 produced by *C. flavus* in the absence or presence of TM are shown in Table 3. The optimal pH for the enzyme activity (5.5) was normal for enzymes and it is in good agreement with the data reported by Wanderley et al.[6] The optimal pH for yeast α-amylase activity is usually in the range of 4.0–6.0.[20] The optimum temperature was found to be 50 °C at pH 5.5 (Table 3), for both enzymes. Since N-glycosylation seems to have an influence on Amy1 structure, kinetic parameters were evaluated. The K_M of native Amy1 was substantially lower when compared to that of the non-glycosylated enzyme: 0.052 and 0.098 mg mL^{-1}, respectively (Table 3). The V_{max} of the non-glycosylated enzyme (0.047 mg min^{-1}) was higher than that of the native Amy1 (0.038 mg min^{-1}). These findings show that N-linked glycosylation of Amy1 plays an essential role in catalytic activity. Unlike those from *C. flavus*, the amylases from *A. niger,* and *Flavobacterium* sp. were not affected when N-linked sugars were enzymatically removed.[3,20]

The most common reported effect of deglycosylation on enzyme structure is on thermal stability.[3,14,20] The non-glycosylated form was more sensitive to inactivation by both the proteolytic enzyme trypsin and high temperature. The native and non-glycosylated forms of the enzyme, when incubated for 4 h in the presence of trypsin, retained 83.7% and 50.8% activities, respectively (Table 3). The native enzyme treated at 50 °C for 60 min retained 97.8% of its maximum activity, whereas the activity of the non-glycosylated form under the same conditions was lowered to about 94.5% (Table 3). The difference in the behavior 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.[2,19,20] These results suggest that the function of the N-linked carbohydrate may be to stabilize the structure of the α-amylase produced by *C. flavus* and so protect it from physical and proteolytic attack after secretion.

Table 3
Biochemical properties of Amy1 in the absence or presence of TM.

Parameter[a]	Control	Tunicamycin 0.5 μg mL^{-1}
Molecular mass (kDa)[b]	68.19	64.48
K_M (mg mL^{-1})	0.052 ± 0.019	0.098 ± 0.017
V_{max} (mg min^{-1})	0.038 ± 0.0071	0.047 ± 0.005
Optimum pH	5.5	5.5
Optimum temperature (°C)	50	50
Thermal stability pH 5.5/50 °C/60 min (%)	97.8	94.5
Thermal stability after trypsin[c] (%)	83.7	50.8

[a] All tests were performed in triplicate, and results are presented as average.
[b] Molecular mass was determined by SDS–PAGE.
[c] Amylase activity was determined after incubation of the enzyme solution with trypsin (300 mg mL^{-1}) at 50 °C for 4 h.

Table 4
Effect of ions on Amy1 activity in the absence or presence of TM

Compound (4 mM)	Control	Tunicamycin 0.5 μg mL^{-1}
Control	100	100
$Al_2(SO_4)_3$	99	80
$CaCl_2$	100	87
$CuCl_2$	61	19
$HgCl_2$	65	35
$MnCl_2$	97	85
$ZnSO_4$	97	87

[a] All tests were performed in triplicate, and results are presented as average.

It is known that some metal ions can promote or inhibit α-amylase activity.[7] Wanderley et al. reported that Amy1 activity is poorly affected by Mn^{2+}, Mg^{2+}, Zn^{2+}, and Ca^{2+} and inhibited in the presence of Hg^{2+}, Fe^{2+}, and Cu^{2+}.[1] Our data are in agreement and show that the non-glycosylated enzyme is more sensitive to Hg^{2+} and Cu^{2+} thus supporting the hypothesis of the influence of N-glycosylation on Amy1 structure (Table 4).

In amylases and many glycoside hydrolases the N-glycosylation seems to be restricted to the catalytic module.[5] The resolution of Amy1 structure is underway, and it will provide more information on N-linked oligosaccharides and catalysis mechanism of the enzyme.

Acknowledgments

This work was supported by a biotechnology research grant to C.J.U (CNPq and FUNAPE/UFG). M.C.B was supported by CAPES/MINTER.

References

1. Weerapana, E.; Imperiali, B. *Glycobiology* **2006**, *16*, 91R–101R.
2. Tull, D.; Gottschalk, T. E.; Svendsen, I.; Kramhoft, B.; Phillipson, B. A.; Bisgard-Frantzen, H.; Olsen, O.; Svensson, B. *Protein Expr. Purif.* **2001**, *21*, 13–23.
3. Jafari-Aghdama, J.; Khosro, K.; Bijan, R.; Mohsen, N. G. *Proteins Proteomics* **2005**, *1750*, 61–68.

4. Mukherjee, S.; Chowdhury, S.; Ghorai, S.; Pal, S.; Khowala, S. *Biotechnol. Lett.* **2006**, *21*, 1773–1778.
5. Gusakov, A. V.; Antonova, A. I.; Ustinova, B. B. *Carbohydr. Res.* **2008**, *343*, 48–55.
6. Wanderley, K. J.; Torres, F. A.; Moraes, L. M.; Ulhoa, C. J. *FEMS Microbiol. Lett.* **2004**, *231*, 165–169.
7. Sivaramakrishnan, S.; Gangadharan, D.; Nampoothiri, K. M.; Soccol, C. R.; Pandey, A. *Food Technol. Biotechnol.* **2006**, *44*, 173–184.
8. Galdino, A. S.; Ulhoa, C. J.; Moares, L. M. P.; Prates, M. V.; Bloch, C. B., Jr.; Torres, F. A. G. *FEMS Microbiol. Lett.* **2008**, *280*, 189–194.
9. Fuwa, H. J. *Biochemistry* **1954**, *41*, 583–603.
10. Bradford, M. M. A. *Anal. Biochem.* **1976**, *72*, 248–254.
11. Rodríguez, Á. P.; Leiro, R. F.; Trillo, M. C.; Cerdán, M. E.; Siso, M. I. G.; Becerra, M. *Microb. Cell Fact.* **2006**, *5*, 41–54.
12. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
13. Blum, H.; Beier, H.; Gross, H. *Eletrophoresis* **1987**, *8*, 93–99.
14. Ulhoa, C. J.; Sankievicz, D.; Limeira, P. S.; Peberdy, J. F. *Biochim. Biophys. Acta* **2001**, *1528*, 39–42.
15. Elbein, A. D. *Annu. Rev. Biochem.* **1987**, *56*, 497–534.
16. Morosoli, R.; Lécher, P.; Durand, S. *Arch. Biochem. Biophys.* **1988**, *265*, 183–189.
17. Eriksen, S. H.; Jensen, B.; Olsen, J. *Curr. Microbiol.* **1998**, *37*, 117–122.
18. Merivuori, H.; Sands, J. A.; Montenecourt, B. S. *Appl. Microbiol. Biotechnol.* **1985**, *23*, 60–66.
19. Kubicek, C. P.; Panda, P.; Schereferl-Kunar, G.; Messner, R. *Can. J. Microbiol.* **1987**, *33*, 698–703.
20. Takegawa, K.; Inami, M.; Yamamoto, K.; Kumagai, H.; Tochikura, T.; Mikami, B.; Morita, Y. *Biochim. Biophys. Acta* **1988**, *955*, 187–193.