RESEARCH LETTER

Cloning, molecular characterization and heterologous expression of *AMY1*, an α-amylase gene from *Cryptococcus flavus*

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Received 11 October 2007; accepted 14 December 2007.
First published online February 2008.

DOI:10.1111/j.1574-6968.2007.01059.x

Editor: Diethard Mattanovich

Keywords
Cryptococcus flavus; α-amylase; heterologous expression; *Saccharomyces cerevisiae*.

Abstract

A *Cryptococcus flavus* gene (*AMY1*) encoding an extracellular α-amylase has been cloned. The nucleotide sequence of the cDNA revealed an ORF of 1896 bp encoding for a 631 amino acid polypeptide with high sequence identity with a homologous protein isolated from *Cryptococcus* sp. S-2. The presence of four conserved signature regions, (I) ^{144}DVVVNH^{149}, (II) ^{235}GLRIDSLQQ^{243}, (III) ^{263}GEVFN^{267}, (IV) ^{327}FLENQD^{332}, placed the enzyme in the GH13 α-amylase family. Furthermore, sequence comparison suggests that the *C. flavus* α-amylase has a C-terminal starch-binding domain characteristic of the CBM20 family. *AMY1* was successfully expressed in *Saccharomyces cerevisiae*. The time course of amylase secretion in *S. cerevisiae* resulted in a maximal extracellular amylolytic activity (3.93 U mL^{-1}) at 60 h of incubation. The recombinant protein had an apparent molecular mass similar to the native enzyme (*c.* 67 kDa), part of which was due to N-glycosylation.

Introduction

α-Amylases (EC 3.2.1.1) are endo-glycosyl hydrolases that randomly cleave the α-1,4-glycosidic bonds present in starch. This reaction generates linear and branched oligosaccharides of various lengths. The α-amylase family consists of several enzymes that share common characteristics, such as a parallel $(\alpha/\beta)_8$ barrel structure and the catalytic mechanism (Van der Maarel *et al.*, 2002). They also contain four highly conserved regions comprising the catalytic centre, the substrate and Ca^{+2}-binding sites (Janeček, 1997; MacGregor *et al.*, 2001). Only *c.* 10% of the known amylases are capable of binding and degrading raw starch and these enzymes possess a distinct sequence-structural module called a starch-binding domain (SBD) (Machovič & Janeček, 2006).

α-Amylases are among the most important industrial enzymes, with many applications in starch processing, brewing, alcohol production, textile and other industries (Gupta *et al.*, 2003). Industrial processes for hydrolysis of starch to glucose rely on inorganic or enzymatic catalysis. The use of enzymes is preferred as it offers a number of advantages, including improved yields and cost savings (Satyanarayana *et al.*, 2004). Enzyme hydrolysis also allows greater control over the specificity of the reaction and the stability of the generated products. The enzymes used in the industrial conversion of starch are estimated to account for 10–15% of the total world enzyme market (Satyanarayana *et al.*, 2004).

Yeast species such as *Schwanniomyces occidentalis*, *Lipomyces kononenkoae* and *Streptomyces fibuligera* are known to produce highly active extracellular α-amylases and glucoamylases (Steyn & Pretorius, 1990). During the course of screening for amylolytic yeasts in the Brazilian biodiversity, a species classified as *Cryptococcus flavus* was isolated and its secreted amylase was biochemically characterized (Wanderley *et al.*, 2004). The purified α-amylase (hereafter called Amy1) exhibited important properties for biotechnological applications such as a low K_m (0.0056 mg mL^{-1}) for soluble starch, high stability at pH 5.5 and optimal temperature at 50 °C. These features prompted the authors to explore the use of Amy1 in biotechnological processes such as starch conversion for the production of ethanol.

Although *C. flavus* is able to metabolize starch, it cannot produce high yields of ethanol and may be pathogenic to humans. On the other hand, the baker's yeast *Saccharomyces cerevisiae* is widely used for the industrial production of ethanol and has the Generally Regarded as Safe (GRAS) status. However, *S. cerevisiae* cannot degrade starch to fermentable sugars unless an external source of amylase is provided or the yeast is genetically modified to express these enzymes (Steyn & Pretorius, 1990).

The present study describes the isolation of an α-amylase gene (*AMY1*) from the yeast *C. flavus*. This gene was successfully expressed in *S. cerevisiae*, showing that this system may be used in starch-conversion processes using Amy1 as a source of α-amylase activity.

Materials and methods

Strains and plasmids

The *C. flavus* strain used in this work was maintained at the Molecular Biology Laboratory (Universidade de Brasília, Brazil). *Escherichia coli* strain DH5α and plasmid pGEM-T® (Promega) were used for general DNA manipulations. Plasmid YEp351PGK (Moraes *et al.*, 1995) was used to express *AMY1* in *S. cerevisiae* CENPK2 (*MaTa/α, ura3-52/ura3-52, leu2-3,112, trp1-289/trp1-289, his3-1/his3-1*).

Culture conditions

Cryptococcus flavus was cultured at 25 °C in YPD (1% yeast extract, 2% peptone and 2% glucose) or synthetic starch medium (SSM: 0.67% YNB, 2% starch). For heterologous expression in *S. cerevisiae*, synthetic dextrose (SD) minimal medium containing 0.67% yeast nitrogen base (YNB), 2% glucose, 50 mM acetate buffer pH 5.5 and the appropriate amino acid supplements was used. SDA medium (0.67% YNB, 1% starch, 2% glucose, 50 mM acetate buffer, pH 5.5, 2% agar and appropriate amino acid supplements) was used for plate detection of amylolytic activity. Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.3) containing 100 μg mL^{-1} ampicillin was used for selection and growth of bacterial transformants.

Protein sequencing and molecular biology techniques

After growing *C. flavus* on SSM media for 60 h at 25 °C, α-amylase was purified from culture supernatants as described previously (Wanderley *et al.*, 2004). The internal fragments were produced by proteolytic digestion using immobilized bovine trypsin (Pierce) in a solution of ammonium bicarbonate, pH 7.8. The mixture of amylase/immobilized trypsin was incubated at 37 °C for 4 h in an orbital shaker. The supernatant was collected after centrifugation and directly spotted onto a matrix-assisted laser desorption ionization (MALDI) plate with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid. Peptide sequences of the native secreted enzyme were identified by monoisotopic mass analyses and sequenced using MS/MS data produced by past source decay (PSD) and collision induced dissociation (CID) fragmentation experiments obtained on a MALDI TOF-TOF Ultraflex® II spectrometer (Bruker Daltonics, Billerica). All DNA manipulations were carried out as described in Sambrook & Russel (2001). Restriction enzymes were obtained from New England Biolabs and Promega and used as instructed by the suppliers. All plasmid DNA was prepared with the QIAprep® Spin Miniprep Kit (Qiagen).

Cloning of *AMY1*

Based on the sequence of amy-CS2, the *Cryptococcus* sp. S-2 α-amylase gene described by Iefuji *et al.* (1996), primers CF5 and CF3 were designed (Table 1), which were used in a PCR reaction containing 20 ng of *C. flavus* genomic DNA, 10 pmol of each primer, dNTPs at 2.5 mM and 1 U of *Taq* DNA polymerase (Cenbiot, Brazil) in a final reaction volume of 50 μL. The system was submitted to 30 amplification cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1.5 min and a final elongation cycle (5 min for 72 °C). A *c.* 0.9-kb amplicon was cloned into the pGEM-T® vector and sequenced. Because the cloned fragment showed high sequence identity to amy-CS2, primers CFAMY5 and CFAMY3 were designed (Table 1) to clone the entire genomic coding region (*c.* 2.0 kb) by PCR using *Tgo* DNA polymerase (Roche Molecular Biochemicals) and the following conditions: 35 amplification cycles of 94 °C for 30 s, 55 °C for 60 s, 72 °C for 2 min, followed by a final elongation cycle (72 °C for 7 min). A reverse transcriptase (RT)-PCR procedure was used to clone the *c.* 1.9-kb *AMY1* cDNA. Briefly, total RNA from *C. flavus* cells grown in SSM was isolated using the TRizol® method (Invitrogen) and cDNA was synthesized using the SuperScript® III First Strand Synthesis System for RT-PCR (Invitrogen) following the supplier's recommendations. PCR was carried out with *Tgo* DNA polymerase using primers CFAMY5 and CFAMY3 as described above. The nucleotide sequence for *AMY1* was deposited at GenBank under accession number EU014874.

Table 1. Primers used in this work

Primer	Sequence (5′ → 3′)	RS*
CF5	GCTTACCACGGTTACTGG	–
CF3	CCACAGTGCCTCTCGGT	–
CFAMY5	AGATCTACCATGGCTCCTGTCCGCTCCCTAG	Bgl II
CFAMY3	AGATCTCTAGGAGGACCACGTAAACTC	Bgl II

*RS, restriction site (underlined).

DNA sequencing, protein and gene analysis *in silico*

DNA sequencing was performed using the MegaBACE® Dye Terminator procedure (GE Healthcare) and reactions were analysed in a MegaBACE® 1000 automatic DNA sequencer (GE Healthcare). The web interface at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) was used to conduct the search for sequence similarity via BLAST tools (Altschul *et al.*, 1990). Putative N-glycosylation sites were predicted using the NetNglyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). The Compute Pi/Mw tool (http://www.expasy.org/tools/pi_tool.html) was used for protein molecular mass calculations. Sequence alignments were performed using CLUSTAL W (Thompson *et al.*, 1994).

Heterologous expression and amylase production

In order to express *AMY1* in *S. cerevisiae,* the cDNA was amplified by RT-PCR using primers CFAMY5 and CFAMY3, which introduce Bgl II sites at amplicon ends (Table 1). The cloned cDNA was digested with Bgl II, and the *c.* 1.9-kb fragment was isolated and cloned into the yeast expression vector YEp351 PGK linearized with the same enzyme. The resulting vector was named YEpAMY1. *Saccharomyces cerevisiae* CENPK2 was transformed as reported previously (Chen *et al.*, 1992). Transformants expressing α-amylase were selected by the production of hydrolysis haloes after 72 h of incubation at 30 °C in SSM and stained with iodine vapour as described by Moraes *et al.* (1995). A colony of *S. cerevisiae* harbouring YEpAMY1 was precultured in SD medium and 2 mL of this culture was transferred to a 1-L conical flask containing 200 mL of the same medium. Cells were incubated at 30 °C on a rotatory shaker at 200 r.p.m., and cell growth was monitored at 600 nm. At different time intervals, 5 mL samples were collected and centrifuged at 5000 ***g*** for 10 min and the supernatant was used for further analysis. α-Amylase activity was determined by monitoring starch hydrolysis as described by Moraes *et al.* (1995). One unit of amylase activity was defined as the amount of enzyme necessary to hydrolyse 0.1 mg of starch per minute. Protein deglycosylation was performed with PNGase F (New England Biolabs) according to the manufacturer's instructions.

Electrophoresis and enzymatic activities in gel

The apparent molecular mass of the recombinant enzyme was determined in a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Proteins were visualized after silver staining as described by Blum *et al.* (1987). Molecular mass markers were as follows: β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), REase Bsp98l (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) (Fermentas Life Sciences). Activity gels were prepared as described by Wanderley *et al.* (2004). Briefly, after samples were resolved on a 12% SDS-PAGE, the gel was washed with distilled water, incubated with 50 mM sodium acetate (pH 5.5) for 60 min and then incubated at 4 °C for 12 h in a solution containing 0.5% starch (in 50 mM sodium acetate, pH 5.5). After this incubation period, the gel was further incubated at 37 °C for 2 h and bands displaying amylase activity were detected after staining with iodine solution (1% I_2 in 0.5 M KI).

Results and discussion

Isolation of the *AMY1* gene

A secreted α-amylase from *C. flavus* was purified from culture supernatants and sequence analysis of two tryptic fragments revealed 100% identity to positions 21–38 and 420–433 of AMY-CS2, the α-amylase isolated from *Cryptococcus* sp. S-2 (Iefuji *et al.*, 1996). Because the two proteins shared high sequence identity, primers were designed based on the sequence from amy-CS2 and an *c.* 2.0-kb amplicon corresponding to the genomic version of *AMY1* was obtained. *AMY1* contains two introns at positions 867–912 and 1024–1060. These introns begin with GT(G/A)AGT and end with AG, which are general intron signatures. The first intron in *AMY1* is of a size equal to the first intron present in amy-CS2 (46 bp) while the second intron (37 bp) is 16 bp smaller. The *AMY1* cDNA was cloned by RT-PCR using mRNA isolated from cells grown in the presence of starch. The cDNA sequence showed the highest identities with the *Cryptococcus* sp. S-2 and *Aspergillus niger* α-amylase genes: 92% and 89%, respectively. The deduced protein, Amy1, has 631 amino acid residues and its sequence exhibits homology to α-amylases from *Cryptococcus* sp. S-2 (97%), *Aspergillus terreus* (46%), *Aspergillus fumigatus* (45%), *Aspergillus clavatus* (44%) and *Aspergillus kawachii* (44%). Sequence comparison with other amylases places Amy1 in the GH13 family because it has the four conserved signature regions (I, II, III, IV) present at positions 144–149, 235–243, 263–267 and 327–332 and the highly conserved catalytic residues Asp-239, Glu-264 and Asp-332 present in regions II, III and IV, respectively (Fig. 1). Furthermore, the five amino acid residues His-149, Asp-239, Glu-264, Gln-331 and Asp-332 in Amy1 correspond to residues His-122, Asp-206, Glu-230, His-296 and Asp-297 in Taka-amylase (Janeček, 1997). Asp-206, Glu-230 and Asp-297 were found to play a role in catalysis while His-122, His-210 and His-296 have been shown to be involved in substrate recognition in the active site in mammalian pancreatic α-amylases (Ishikawa *et al.*,

Source	Region I	Region II	Region III	Region IV
Bacteria				
Bacli	129 DVVINH	256 GFRLDAVKH	289 AEYWQ	352 FVDNHD
Bacsu	138 DAVINH	213 GFRFDAAKH	248 GEILQ	305 WVESHD
Ecoli	101 DVVVNH	231 GFRIDAVKH	264 AEYWS	327 LVANHD
Archaea				
Pyrfu	132 DVVINH	219 GWRFDYVKG	246 GEYWD	309 FVANHD
Pyrsp	133 DIVINH	220 AWRFDYVKG	247 GEYWD	310 FVANHD
Thchy	129 DIVINH	216 AWRFDYVKG	244 GEYWD	306 FVANHD
Eucarya				
Aspor	138 DVVANH	223 GLRIDTVKH	250 GEVLD	313 FVENHD
Crysp	144 DVVVNH	235 GLRIDSLQQ	263 GEVFN	327 FLENQD
Cryfl	144 DVVVNH	235 GLRIDSLQQ	263 GEVFN	327 FLENQD
BarHIG	101 DIVINH	200 GWRFDFAKG	226 AEIWT	308 FVDNHD
Consensus	DvViNH	* gfRfD vK	* gEyw	* fvdnhD

Fig. 1. Multiple sequence alignment of the conserved signature regions (I, II, III and IV) of α-amylases from several organisms. The figure compares amino acids of Bacli (*Bacillus liqueniformis* strain ATCC 27811, accession no. P06278), Bacsu (*Bacillus subtilis*, accession no. P00691), Ecoli (*Escherichia coli* strain JA11, accession no. P26612), Pyrfu (*Pyrococcus furiosus* strain DSM 3638, accession no. U96622), Pyrsp (*Pyrococcus* sp strain KOD1, accession no. D83793), Thchy (*Thermococcus hydrothermalis* strain AL662, accession no. AF068255), Aspor (*Aspergillus oryzae*, accession no. P10529), Crysp (*Cryptococcus* sp, accession no. D83540), Cryfl (this study, *Cryptococcus flavus*, accession no. EU014874) and BarHIG [*Hordeum vulgare* (barley), accession no. P04063]. The three proposed catalytic residues are marked by *asterisks*. A residue is written in the consensus sequence (*consensus*) if it is present in more than half of the α-amylases.

1992, 1993) and in the transition state stabilization, but not directly in catalysis of barley α-amylase (Søgaard *et al.*, 1993b). Interestingly, regions II and IV in Amy1 contain uncharged Gln (Gln-241 and Gln-331) in place of the highly conserved His residues (His-210 and His-296 in Taka-amylase A, respectively). A similar substitution was also observed in region II of the *Lipomyces kononenkoae* α-amylase (Kang *et al.*, 2004). Janeček *et al.* (1999) observed that a glycine replacing the histidine at the end of region II was a general feature of archaeal as well as plant α-amylases. It has been proposed that in *Thermococcus profundus*, these residues may be involved in the catalytic mechanism (Lee *et al.*, 1996). The histidine residue of the *Bacillus stearothermophilus* α-amylase that is equivalent to His-210 in Taka-amylase may control the specificity and thermal stability of the enzyme (Vihinen *et al.*, 1990). A similar function has been reported for the same residue in the enzyme from *Bacillus subtilis* (Takase, 1994). In CGTases, histidine residues are probably responsible for both cycling and amylolytic activities (Mattsson *et al.*, 1995). However, activity as well as substrate specificity of these enzymes could be modified by mutation of nonessential amino acid residues adjacent to or near the catalytic residues (Takase, 1992; Inohara-Ochiai *et al.*, 1997).

Iefuji *et al.* (1996) proposed that the C-terminal region of AMY-CS2 is implicated in raw starch binding. The SBD is usually a distinct sequence-structural module that improves the efficiency of an amylolytic enzyme, allowing it to bind and digest raw and granular starch (Svensson *et al.*, 1982; Goto *et al.*, 1994; Chen *et al.*, 1995; Lo *et al.*, 2002). Most carbohydrate-binding modules (CBMs) have been classified into seven CBM families: CBM20, CBM21, CBM25, CBM26, CBM34, CBM41 and CBM45 (Coutinho & Henrissat, 1999; Machovič & Janeček, 2006). The CBM20 family is the best-studied one; in this family, all real SBD modules are positioned C-terminally with regard to the catalytic domain (Machovič *et al.*, 2005). Because of the high sequence identity between Amy1 and AMY-CS2, these amylases should be placed in the CBM20 family.

Heterologous expression of Amy1 in *S. cerevisiae*

The *AMY1* cDNA was cloned into YEp351PGK, which allows constitutive expression of foreign genes under the control of the yeast *PGK1* promoter. The resulting plasmid, YEpAMY1, was used to transform the *S. cerevisiae* CENPK2 strain and successful α-amylase expression was observed after the formation of distinct hydrolysis haloes around colonies (Fig. 2a–d). No starch hydrolysis halo was observed in either untransformed cells or cells transformed with YEp351PGK (Fig. 2e and f). The time course of growth and secreted amylase production by a selected *S. cerevisiae* transformant during batch culture is shown in Fig. 3. The extracellular amylolytic activity increased during growth and reached a maximal value ($3.93\,U\,mL^{-1}$) at 60 h of incubation (Fig. 3a). Cell growth was equivalent to that of the clone transformed with the vector alone (data not shown), indicating that the amylase production did not impair cell growth, and resulted in constitutive and cumulative production of α-amylase. Amylolytic activity was

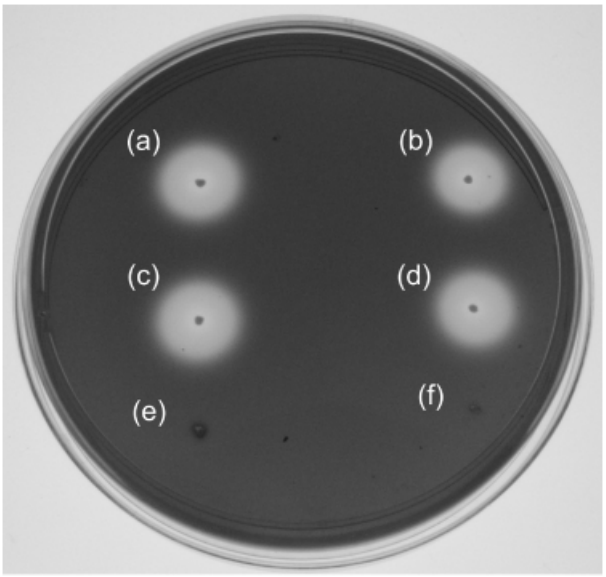

Fig. 2. Amylolytic activity of yeast transformants in plate assays. *Saccharomyces cerevisiae* CENPK2 was transformed with the indicated plasmids and plated in SDA medium. After 72 h of growth at 30 °C, the plate was stained with iodine vapour. *Saccharomyces cerevisiae* CENPK2 colonies transformed with YEpAMY1 (a, b, c and d); YEp351 PGK (e) and untransformed cells (f).

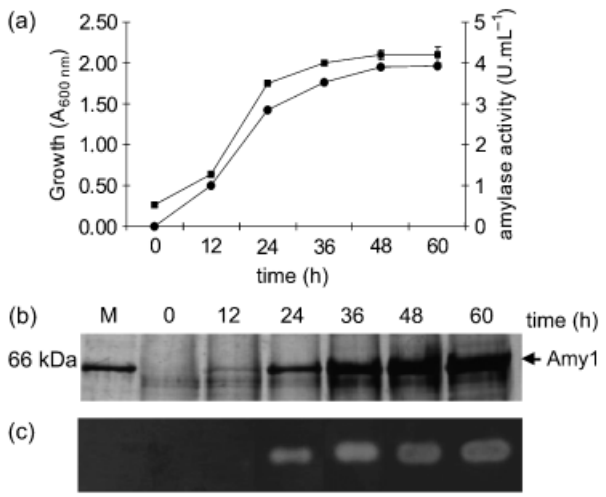

Fig. 3. Kinetics of Amy1 production by recombinant *Saccharomyces cerevisiae* CENPK2 grown in liquid SD medium. (a) Growth curve (■) and amylase activity (●) of a clone expressing *AMY1*. SDS-PAGE (b) and zymogram (c) analysis of supernatants from different time courses. M, molecular mass marker.

correlated to a single band with an apparent molecular mass of *c*. 67 kDa, which is similar to that from the native enzyme (Fig. 3b and c). After deglycosylation with PNGase F, which removes the glycan portion of N-glycoproteins, both native and recombinant enzymes showed a molecular mass of

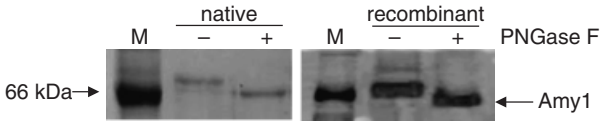

Fig. 4. N-Glycosylation state of Amy1. Twelve per cent SDS-PAGE analysis of native and recombinant Amy1 with or without treatment with PNGase F. M, molecular mass marker.

c. 66 kDa, which is in good agreement with the predicted size of the mature protein (65 865 Da) (Fig. 4a and b). Three potential N-glycosylation sites were identified in Amy1 (^{61}NGT, ^{190}NRT and ^{269}NPS) but more experimental data are needed to confirm the precise location of these sites. Although Amy1 has 97% sequence identity with AMY-CS2, the latter could only be efficiently secreted in yeast when its C-terminal was deleted (Iefuji *et al*., 1996). The only striking differences in the C-terminal region of both enzymes are three substitutions at positions 606 (Pro → Thr), 608 (Asn → Ser) and 623 (Ala → Thr). It is unclear whether these substitutions alone are enough to promote efficient secretion of Amy1 in *S. cerevisiae*.

This work represents the first report of the cloning and secretion of a *C. flavus* α-amylase gene in *S. cerevisiae*. The successful expression of Amy1 in yeast shows that this enzyme may be used in biotechnological processes. In fact, work is under way to add a glucoamylase gene to system, which should enable the resulting strain to ferment starch to ethanol directly.

Acknowledgements

This project was supported by FAP/DF, CNPq and CAPES (Brazil). The authors are indebted to Hugo Costa Paes for manuscript revision.

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