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Molecular and biochemical characterization of extracellular tannin acyl hydrolase activity from a Mexican isolate of *Aspergillus niger*

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Microbial tannase, a hydrolysable tannin-degrading enzyme, is extensively used in manufacture of instant tea, beer, wine, and gallic acid. *Aspergillus niger* strain, obtained from a Mexican tannery wastewaters rich in gallic acid [Quebracho Phenolics-rich Tannery Wastewaters, (QPTW)], displayed a good growth and tannase activity in a minimal medium added with 1% (w/v) QPTW (Kr= 0.451 $mm.h^{-1}$). Using PCR and RACE 3´ and 5´methodologies, a complete cDNA of a tannase was cloned from this isolate. Nucleotide sequence of complete cDNA was of 4690 bp with a complete ORF of 1833 bp encoding 611 amino acids. Transcriptional induction was observed in mineral medium added with carbon sources as tannic acid alone (1 and 10 g/l), as well as mix of glucose (1 and 10 g/l) and tannic acid (1 g/l) in the media. However, neither glucose (1 and 10 g/l) and sucrose (1 and 10 g/l) nor (+)-catechin (1 and 10 g/l) as sole carbon sources displayed gene induction in *in vitro* assays. Thus, *A. niger*-GTO is a new strain with interesting characteristics for industrial tannase production purposes.

Keywords: Tannase, *Aspergillus niger* Guanajuato isolate, cDNA cloning

Introduction

Tannase (Tannin-acyl-hydrolase, E.C. 3.1.1.20) catalyzes hydrolysis of ester and depside bonds in gallotannins, as tannic acid, releasing glucose and gallic acid[1]. Gallotannins display high antimicrobial activity due to complexation with proteins and enzymes, but few fungal species develop resistance against gallotannin by production of tannase[2]. Family *Aspergilli* is widely used for production of food ingredients, pharmaceuticals and industrial enzymes, as well as for production of heterologous proteins[3-5]. In their natural habitat, *A. niger* strains secrete large amounts of a wide variety of enzymes needed to release nutrients from biopolymers. This high secretory capacity is exploited industrially in both solid and submerged fermentations[6,7]. In addition, *A. niger* has a long tradition of safe use in production of enzymes and organic acids, and these products have obtained GRAS status (generally regarded as safe)[8]. Species of *Aspergilli* are best producers of tannase[9,10], which is useful in manufacture of instant tea, acron wine, cofee-flavoured soft drinks, clarification of beer and fruit juices[11], food and beverage processing[12]. To increase production of tannase, identification of cognate ORF is to be expressed in homologous or heterologous systems[13]. So far, only tannase gene cloned and sequenced from *Asperlli* species is from *A. oryzae*[14]. Complete genome sequence of *A. niger* CBS 513.88, an early ancestor of current *A. niger* strains, has recently been reported[5]. A new strain of *A. niger*, isolated from Mexican tannery wastewaters, is highly-rich in gallic and protocatechuic acids[15] and expected to display enzymatic activities as tannase.

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This study characterized tannase activity, cloning a complete cDNA of a tannase gene (TG) and carried out studies on transcriptional regulation of TG from *A. niger* strain.

Experimental Section

Strain and Culture Medium

Quebracho phenolics-rich tannery wastewater (QPTW, 300 l) was collected from effluents of a tannery located in León, Guanajuato, México. Effluent was serially diluted in phosphate buffer (pH 7.0) and plated on solid minimal medium M9[16] [Na_2HPO_4-$7H_2O$ (12.8 g/l), KH_2PO_4 (3 g/l), NaCl (0.5 g/l), NH_4Cl (1 g/l), $CaCl_2$ (0.1 g/l), $MgSO_4$ (2 g/l)], supplemented with tannic acid (1 g/l; SIGMA, St. Louis, MO) as only carbon source. Petri dishes were incubated at 30°C during 7 days and colonies obtained were further submitted to identification.

Identification of *A. niger*-GTO

Identification of *A. niger* strains were carried out first by microbiological and microscopic methodologies[17]. For further identification of suspected *A. niger*, genomic DNA was extracted using protocol reported[18], and identified by amplifying and sequencing ITS1-5.8S-ITS2 regions of rRNA genes[19] and sequence comparison with Genbank on NCBI.

Evaluation of Fungal Radial Growth

Radial growth of *A. niger*-GTO and a strain of *A. oryzae* (CINVESTAV-Zacatenco, México), was determined using mineral medium M9 alone or supplemented either with tannic acid (1 g/l) or QPTW (1 g/l) as carbon source. A fungus mycelium disc (diam, 7 mm) was inoculated in center of Petri dishes with reported media [20,21]. Petri dishes were incubated at 30°C until mycelium growth covered all plate surface. Each 24 h radial mycelial growth was measured using a vernier (Industrias Vermar, Jalisco, México). Data were used to calculate velocity of radial growth as[22]

$$Kr\ (mm.h^{-1}) = (r_2 - r_1)\ (t_2 - t_1) - 1 \quad \ldots(1)$$

where r_1 and r_2 are mycelium radio at beginning (t_1) and end (t_2) of exponential growth of fungi. All experiments were carried out in triplicate and in three independent assays.

Determination of Tannase Activity

Tannase activity[23] of *A. niger*-GTO and *A. oryzae* was determined by using erlenmeyer flasks (250 ml) with sterile liquid medium M9 (50 ml) plus tannic acid (1 and 10 g/l) at an initial pH of 5.5, inoculated with 2.5 ml of a spore suspension (2 x 10^7 spores/ml) at 30°C on a rotary shaker (220 rpm); cultures were monitored every 2 h over a 20 h period. Biomass was separated by centrifugation at 13000 rpm during 10 min. Cell-free culture broth was assayed for tannase activity measuring absorbance at 260 nm. In addition, tannase activity was determined in mixture M9 media enmmended with tannic acid (10 g/l) plus glucose (1 and 10 g/l) for possible catabolite repression detection in the system. A tannase unit is amount of enzyme that catalyzes production of 1 mol of gallic acid per minute per ml of cell-free culture broth. All assays were carried out bytriplicate.

cDNA Cloning of a Tannase Gene (TG) from *A. niger*-GTO

Based on sequence of TG from *A. oryzae*[14], several primers were synthesized and pairwise evaluated in order to PCR amplify a segment of ORF of a TG from *A. niger*-GTO. Primer sequences were as follows: forward primers, Tan 1TF (5'-ATGCGCCAACACTCGCGCAG-3') and Tan 2TF (5'-CCTCCATCATCGGCCAGTCC-3´); and reverse primers Tan 1TR (5'-CGGAGCCATTCTGGGCGGGC-3´), Tan 2TR (5'-GCTGGTCCAGAGGGGACGAG-3') and Tan 3TR (5'-TTAGAAAACGGGCATCTTGA-3'). Sterile liquid M9 medium supplemented with tannic acid (1 g/l) was inoculated with 2 x 10^7 spores/ml and incubated during 48 h; then mycelium was collected by centrifugation and total RNA extraction was carried out using RNeasy kit (QIAGEN). Once obtained high quality total RNA, cDNA synthesis was carried out using SMART™ RACE cDNA amplification kit (ClonTech) according to manufacturer conditions. Amplicons obtained with different combinations of forward and reverse primers were sequenced and compared to Genbank, in order to determine homology to tannase from *A. oryzae*. Amplicons with high homology to TG of *A. oryzae* were obtained with primers Tan 1TF and Tan 2 TR and were used as substrate for 3´and 5´RACE methodology, using either forward (5´-GCTCTGCCAGTGGCCTTCCCG-3´) or reverse (5´- GTGCCATTGGAAGGAAGGGC-3´) primers synthesized based on nucleotide sequence of amplicon obtained from *A. niger*-GTO with homology to *A. oryzae* TG. Sequencing of TG was obtained by dideoxynucleotide chain termination method using an automatic sequencer (Applied Biosystems, Foster City, California).

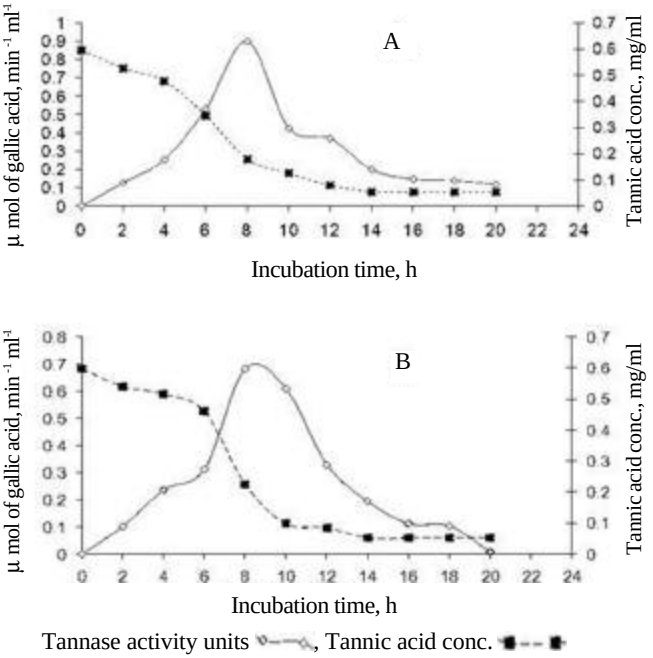

Fig. 1—Tannase activity and tannic acid degradation in: A) *A. oryzae* and B) *Aspergillus niger*-GTO

Southern, Northern and Dot Blot Hybridizations

Both Southern and Northern blot analysis were carried out by standard protocols[14]. Dot blot analysis was carried out as reported[24]. In all hybridizations, a fragment of 1833 bp encoding complete ORF of TG of *A. niger*-GTO was used as probe. For dot blot, probes were generated incorporating dUTP-11-fluorescein by a routine protocol (Gene Images CDP-Star Random prime labeling module; Amersham Pharmacia Biotech Inc. Piscataway, NJ, USA). Probe detection was made by conjugate alkaline antifluorescein-phosphatase and CDP-Star detection reactive (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). For Southern and Northern blot, probe was labeled using ^{32}P-dCTP by random priming. Inducer molecules glucose, catechin, tannic acid and sucrose were purchased to SIGMA-ALDRICH (St. Louis, MO, USA). Exposed X-ray film was scanned, and hybridization signals were quantified with a digital image system (1D Image Analysis Software Version 3.0.2, Kodak digital system, Rochester, New York).

Sequence Analysis

Sequences were compared based on data conducted with blastx algorithm[25] of National Center of Biotechnology Information (NCBI) and MegAlign tool of LASERGENE (DNASTAR software; Madison, WI, USA). Sequence of cDNA of TG from *A. niger*-GTO was deposited in Genbank database with accession number DQ185610.

Results and Discussion

Isolation of *A. niger*-GTO

During isolation of microorganisms from QPTW studies, two types of fungal colonies were detected. Majority of these colonies (98%), based on morphological and microscopic studies, were identified as *A. niger*. Rest (2%) of colonies were identified as *Talaromyces* sp. Molecular identification of *A. niger* was confirmed using ITS1-5.8S-ITS2 sequencing of this isolate. *A. niger* strain was named as *A. niger*-GTO.

Evaluation of Radial Growth of Fungi

Radial growth (Kr) on mineral medium (M9) plus QPTW of *A. niger*-GTO and *A. oryzae*, respectively, was found as: tannic acid, 0.506, 0.536 mm/h; and QPTW, 0.517, 0.451 mm/h. These results and reports on tannase activities in *A. niger* strains[12] suggested that *A. niger*-GTO strain could be a good source for tannase activity. In addition, Kr levels obtained for both *A. niger*-GTO and *A. oryzae* using either tannic acid or QPTW as carbon sources were similar, and that *A. oryzae* is reported[14] an efficient tannase producer.

Determination of Tannase Activity

Maximal tannase activity was detected at 8 h of culture, for *A. niger*-GTO (0.84 AU) and *A. oryzae* (0.68 AU) with a subsequent decline in enzyme activity, being more pronounced for *A. oryzae* (Fig. 1). Tannase activity increased to 1.82 AU when included glucose (10 g/l) plus tannic acid (10 g/l), indicating that catabolite repression was not present in the system at least in concentrations evaluated in this work (Fig. 2A). Decline in tannase activity of *A. niger*-GTO could be due to an enzyme inactivation as reports[26-28]. These results were similar to those reported for *A. niger* HA37, to degrade phenolics in wastewaters from olive mill[28]. Mineral medium (M9) is more complex than AT medium reported[28], could be likely that this aspect influenced differences in time and quantity of tannase activity showed by *A. niger*-GTO (this work) and *A. niger* HA37. In addition, it was shown a concomitant diminish in tannic acid level, once tannase activity increased from 2 h incubation for both *A. niger*-GTO and *A. oryzae* evaluated (Fig. 1).

Cloning a Complete cDNA of Tannase from *A. niger*-GTO

In order to provide a TG source for further studies on expression in homologous or heterologous systems, a complete tannase cDNA was isolated and sequenced from TG (Fig. 3). cDNA isolated corresponded to a 4673 bp sequence, with two putative polyadenilation signal and a

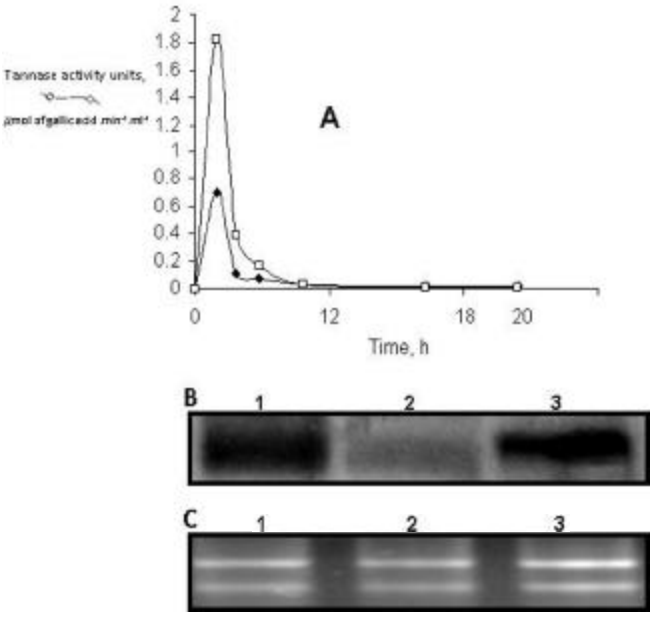

Fig. 2—Tannase activity in *A. niger*-GTO in: A) presence (□) and absence (■) of glucose 10g/l (panel A); B) Northern blot analysis for tannase gene expression [Tannic acid (10 g/l, lane 1), glucose (10 g/l, lane 2), and glucose plus tannic acid (10 g/l both, lane 3)]; and C) Total RNA loaded in each lane as control

Table 1—Homologies among tannase ORF of *Aspergillus niger* (GTO) and others aspergilli*

	% Identity	% Positives
A. niger CBS513.88(No.accesion)	86	90
A. oryzae(No.accesion)	75	84
A. terreus NIH2624(No.accesion)	76	84

* Each value is the % between *A. niger*-GTO and respective fungi in the first column

poly A tail. Complete ORF was of 1833 bp. The 5´ untranslated (UTR) region consisted of 1822 bp and a 3´ UTR of 1015 bp; both regions are substantially larger than the ones reported for a TG of *A. oryzae*[14]. UTR´s length in tannase cDNA of *A. niger*-GTO could be related to a highly regulated mechanism in comparison to *A. oryzae*. mRNA untranslated regions in fungi are involved in many post-transcriptional regulatory pathways that control mRNA localization, stability and translation efficiency[29-30]. Homology analysis of tannase ORF from *A. niger*-GTO and other *Aspergilli*, displayed an 86% identity with *A. niger* CBS513.88 (Table 1). Comparing sequence of a PCR amplified-genomic region using 5´and 3´primers flanking tannase ORF region of TG, displayed that there was no intron in TG. These results were similar to those reported for TG of *A. Oryzae*[14].

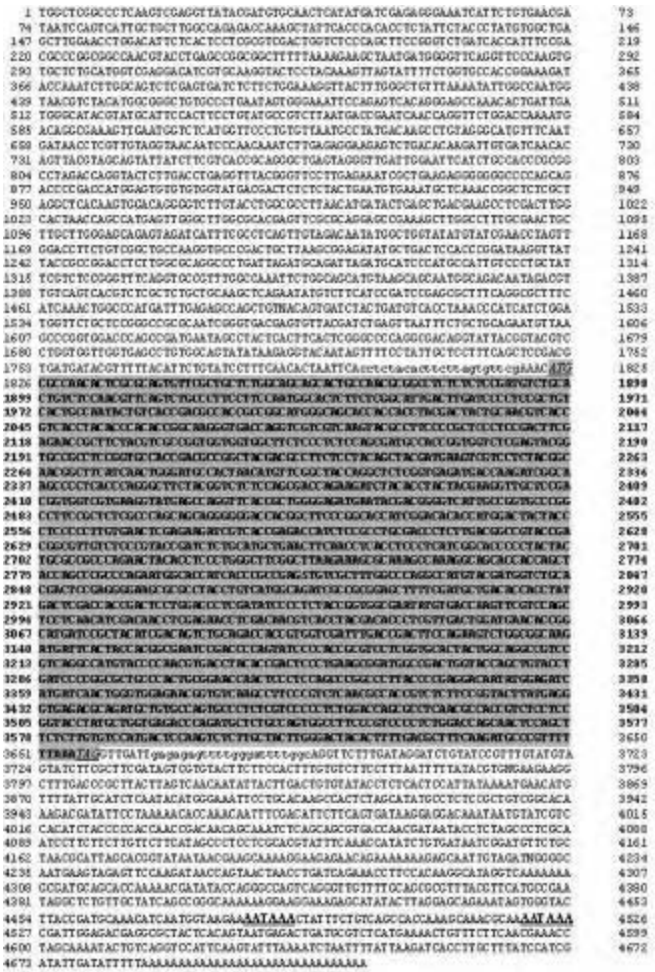

Fig. 3—Complete cDNA sequence isolated from *A. niger*-GTO. [Open reading frame is marked shaded and in bold letter. Start (ATG) and termination codons (TAG) are underlined and written in italics. Putative polyadenilation signal at 3´unstranslated region are indicated underlined and written in bold]

Tannase Gene Copy Numbers and Gene Expression Studies

In order to analyze number of copies of TG throughout *A. niger*-GTO genome, a Southern blot assay was carried out using genomic DNA of this strain. It detected (Fig. 4) only one signal using restriction digestions that based on sequence, and will not cleave within TG sequence. TG is present in one copy in *A. niger*-GTO genome, which could facilitate future approaches to improve expression of TG. This result is similar to that reported in *A. oryzae* and *A. niger* CBS 513.88[5,14]. On the other hand, mechanism of regulation of microbial TG induction is currently not deciphered although inducing effects of gallic acid fraction of tannic acid (or a derivative) have been suggested[30,31]. Moreover, catabolite repression by several carbon sources could be part of the mentioned mechanism

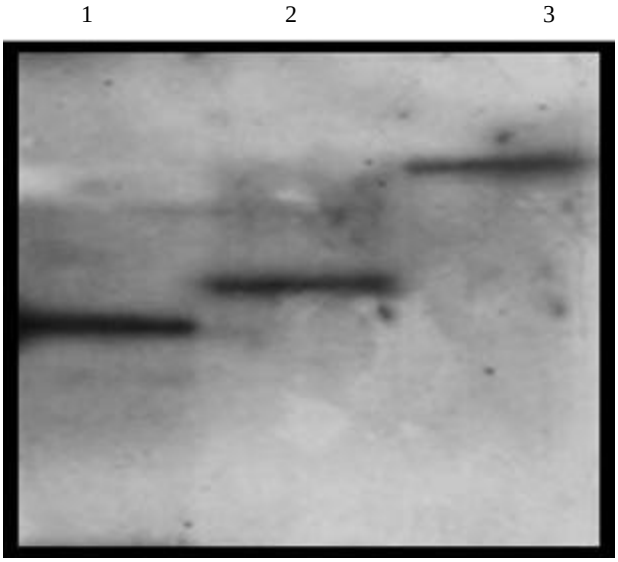

Fig. 4—Tannase gene copy numbers in *A. niger*-GTO (Lane 1, genomic DNA from *A. oryzae* digested with *Eco* RI; lanes 2 and 3, genomic DNA from *A. niger*-GTO digested with *Hin*d III and *Eco* RI enzymes, respectively. Complete ORF of tannase gene from *A. niger* radiactive ATP ^{32}P-labeled was used as a probe.)

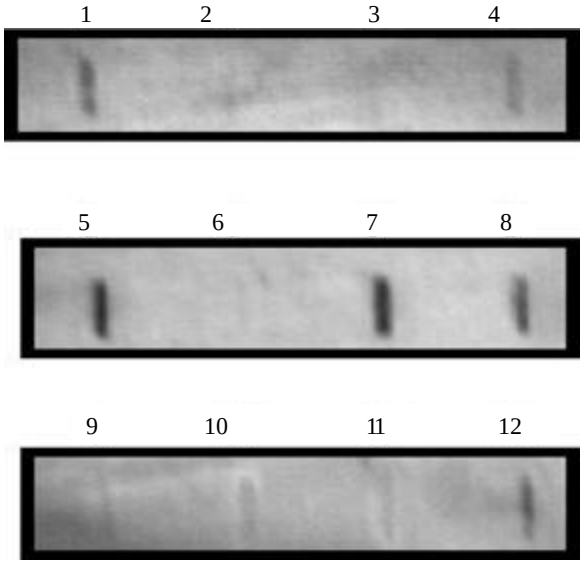

Fig. 5—Transcriptional studies of tannase gene expression of *A. niger*-GTO using different carbon sources. [Lane 1, tannic acid (1 g/l); lane 2, glucose (1 g/l); lane 3, glucose (10 g/l); lane 4, tannic acid + glucose (1 g/l both); lane 5, tannic acid (1g/l) + glucose (10 g/l); lane 6, sucrose (1 g/l), lane 7, tannic acid + sucrose (1 g/l both), lane 8, tannic acid (1g/l) + sucrose (10 g/l), lane 9, catechin 1g/l), lane 10, tannic acid + catechin (1g/l both), lane 11, tannic acid (1g/l) + catechin (10g/l), lane 12, tannase ORF used as a probe (positive control). Each lane was loaded with 10 mg of total RNA]

as reported[28]. Thus, in order to evaluate TG induction in *A. niger*-GTO, several transcriptional studies of TG were carried out using several carbon sources (Fig. 2B and 2C, and Fig. 5). Tannic acid (conc., 10 g/l) induced transcriptional TG expression in *A. niger*-GTO (Fig. 2B and 2C, lane 1). Moreover, when besides the latter carbon source, glucose 1 g/l was added, gene expression displayed no significant increase (Fig. 2B and 2C, lane 3). Tannase activity in these latter conditions (Fig. 2A) displayed a threefold increase (1.83 AU/ml) in comparison to tannic acid (10 g/l) alone. Thus, increase displayed in tannase activity was not associated with a concomitant increase in induction in TG, suggesting a post-transcriptional mechanism involved. This result agrees with Aguilar *et al*[27] who found in *A. niger* Aa-20, that in submerged fermentation tannase activity increased from 0.57 to 1.03 IU/ml, when initial glucose concentration increased from 6.25 to 25 g/l, but a strong catabolite repression of tannase synthesis was observed in SmF when an initial glucose concentration of 50 g/l was used. Thus, although different media were used in present work, it is likely that glucose concentrations used in present work were down the threshold required for catabolite repression of TG. For further TG induction studies, other several putative inducers as sucrose and catechin were also evaluated (Fig. 5). In two concentrations evaluated, neither glucose alone (Fig. 4), sucrose nor catechin were TG inducers (Fig. 5, lanes 2, 5 and 6 respectively). Catechin was a repressor for TG expression in *A. niger*-GTO (Fig. 5, lanes 6 and 7), the first report, in which it is demonstrated that TG repression using catechin.

Conclusions

Extracellular tannase from *A. niger*-GTO displayed molecular and biochemical features with potential applications in several industries. Also, authors claim that this is the first report on cDNA sequence for tannase gene in *A. niger*, which could be useful to future research in tannase activity improvement using molecular biology tools.

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