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Characterization of a thermostable extracellular tannase produced under submerged fermentation by *Aspergillus ochraceus*

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Abstract

Background: Tannases are enzymes that may be used in different industrial sectors as, for example, food and pharmaceutical. They are obtained mainly from microorganisms, as filamentous fungi. However, the diversity of fungi stays poorly explored for tannase production. In this article, *Aspergillus ochraceus* is presented as a new source of tannase with interesting features for biotechnological applications. **Results:** Extracellular tannase production was induced when the fungus was cultured in Khanna medium with tannic acid as carbon source. The extracellular tannase was purified 9-fold with 2% recovery and a single band corresponding to 85 kDa was observed in SDS-PAGE. The native apparent molecular mass was estimated as 112 kDa. Optima of temperature and pH were 40ºC and 5.0, respectively. The enzyme was fully stable from 40ºC to 60ºC during 1 hr. The activity was enhanced by Mn^{2+} (33-39%) and NH_4^+ (15%). The purified tannase hydrolyzed tannic acid and methyl gallate with Km of 0.76 mM and 0.72 mM, respectively, and Vmax of 0.92 U/mg protein and 0.68 U/mg protein, respectively. The analysis of a partial sequence of the tannase encoding gene showed an open read frame of 567 bp and a sequence of 199 amino acids were predicted. TLC analysis revealed the presence of gallic acid as a tannic acid hydrolysis product. **Conclusion:** The extracellular tannase produced by *A. ochraceus* showed distinctive characteristics such as monomeric structure and activation by Mn^{2+}, suggesting a new kind of fungal tannases with biotechnological potential. Further, it was the first time that a partial gene sequence for *A. ochraceus* tannase was described.

Keywords: *Aspergillus ochraceus*, gallic acid, tannase, tannic acid, tannin acyl hydrolase

INTRODUCTION

Tannin acyl hydrolase (tannase, EC 3.1.1.20) catalyzes the breakdown of ester and depside bonds of hydrolysable tannins, as tannic acid, releasing glucose and gallic acid. Tannins are water-soluble molecules with different molecular weights (500-3000 Da) and high content of polyphenols, occurring in certain parts of plants, such as bark, wood, leaf, fruit, root and seed (Aguilar et al. 2007; Rodríguez et al. 2008). Tannins may be classified into three groups: condensed, complex and hydrolysable. This last class presents a sugar core linked by estherification to gallic acid (gallotannis) or to ellagic acid (ellagitannins) (Robledo et al. 2008).

Tannases have been used as clarifying agents in industrial processing of fruit juices and coffee-flavoured soft drinks, in the manufacture of instant teas, and in the production of gallic acid (Aguilar et

al. 2007). Gallic acid is in turn used to produce propyl gallate, mainly used as an antioxidant in fats, oils and beverages (Rodríguez et al. 2008). Tannases have further potential to treat wastewaters contaminated by polyphenolic compounds (Aguilar et al. 2007). Moreover, they have also been used for tannin removal from foodstuffs (Belmares et al. 2004) and animal feeds (Nuero and Reyes, 2002), since high concentrations of tannins linked to carbohydrates, proteins or minerals depress voluntary feed intake, digestive efficiency and animal productivity.

The production of tannin acyl hydrolase has been reported for bacteria (Kostinek et al. 2007), yeast (Zhong et al. 2004) and fungi, such as *Aspergillus versicolor* (Batra and Saxena, 2005), *Aspergillus niger* (Rana and Bhat, 2005; Mata-Gómez et al. 2009; Ventura et al. 2009), *Paecilomyces variotii* (Mahendran et al. 2006) and *Aspergillus ruber* (Kumar et al. 2007), among others. Nevertheless, searching for new sources of tannases with novel biotechnological properties is very attractive, since alternative producers may be found for future or new applications. In addition, the potential of fungal biodiversity for tannases production is yet poorly known. Previous studies in our laboratory showed that the filamentous fungus *A. ochraceus* was a good producer of enzymes with distinctive features (Guimarães et al. 2007). Hence, our aim was to study the production of a thermostable extracellular tannase by this filamentous fungus, characterize its biochemical properties and analyze the gene sequence for such enzyme, since this kind of information is scarce for filamentous fungi. Moreover, this is the first report on the biochemical properties of a fungal monomeric, manganese activated tannase.

MATERIALS AND METHODS

Microorganism and culture condition

Aspergillus ochraceus was isolated from Brazilian soil, identified by André Tosello Foundation (Campinas, São Paulo, Brasil), deposited at the Culture Collection of the Microbiology Laboratory of Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, University of São Paulo, and maintained on slants of PDA medium at 4ºC after growth for 1 week at 40ºC. Liquid cultures were performed in 125 mL Erlenmeyer flasks by the addition of 1 mL spore suspension (10^5 spores/mL) to 25 mL of Khanna medium (Khanna et al. 1995) containing different carbon sources (glucose, oatmeal, sugar cane bagasse, passion fruit peel, soybean flour, wheat bran, and crushed corn and corncob) at 1% (w/v) concentration. The initial pH was adjusted to 6.0, and the medium was previously autoclaved at 120ºC and 1.5 atm for 30 min. Alternatively, 2% (w/v) tannic acid (Sigma®, cat. number 403040), methyl gallate (Fluka®, cat. number 48690) or gallic acid (Mallinckrodt®, cat. number 3112-03), previously sterilized through 0.22 µm syringe filters (TPP®), were added as carbon sources to previously autoclaved Khanna medium. The mold was grown under orbital agitation (100 rpm), at 40ºC for 72 hrs.

Preparation of the enzymatic extract

Cultures were filtered through Whatman nº1 filter paper and the filtrate was named extracellular crude extract. Mycelia were rinsed with distilled water, blotted with filter paper, ground with acid cleaned sand in a mortar, at 4ºC, ressuspended in 100 mM sodium acetate buffer, pH 4.5, and centrifuged at 23000 *g* and 4ºC for 15 min). The supernatant was named intracellular crude extract. Both crude extracts were stored at 4ºC and used for further experiments.

Determination of tannase activity and protein

Tannase activity was quantified using 1% (w/v) methyl gallate as substrate in 100 mM sodium acetate buffer, pH 4.5. The reaction was carried out at 40ºC and the gallic acid produced was quantified using methanolic rhodanine as described by Sharma et al. (2000). One unit of enzymatic activity was defined as the amount of enzyme that produces 1 µmol of gallic acid per minute under the assay conditions. Proteins were quantified according to Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Enzyme purification

The extracellular crude extract obtained from cultures in medium containing tannic acid as carbon source was loaded into a DEAE-Cellulose chromatographic column (2 x 12 cm) equilibrated with 100

mM sodium acetate buffer, pH 5.0. The column was maintained at room temperature and fractions of 3 mL were collected at a flow rate of 1 mL/min. A single peak of tannase activity was eluted using a linear NaCl gradient (0-1 M), and active fractions were pooled, dialyzed against distilled water for 24 hrs at 4ºC, lyophilized and dissolved in 50 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl. The sample was then loaded onto a Sephacryl S-200 chromatographic column (2 x 80 cm) equilibrated with the same buffer. The column was maintained in a cold room at 4ºC and fractions of 1 mL were collected at a flow rate of 0.45 mL/min. Fractions with tannase activity were pooled, dialyzed against a large volume of distilled water for 24 hrs at 4ºC and lyophilized. SDS-PAGE analyses revealed the purity of the preparation of extracellular tannase.

Electrophoresis and molecular mass determination

Samples of purified extracellular tannase were submitted to denaturing polyacrylamide gel electrophoresis (SDS-PAGE) at 6% acrylamide slab gels. The font was adjusted to 120V and 40 mA, and after the run the gels were stained with silver nitrate (Blum et al. 1987). Alpha 2-macroglobulin (169 kDa), β-galactosidase (112.5 kDa), lactoferrin (92 kDa), pyruvate kinase (69 kDa), fumarase (59 kDa) and lactic dehydrogenase (26.5 kDa) were used as pre-stained molecular mass makers. Apparent native molecular mass was determined by gel-filtration in Sephacryl S-200, as described above. The molecular mass markers used were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa). All molecular mass makers were purchased from Sigma (SDS7B2).

Determination of kinetic parameters

The values of Km and Vmax were determined by Linewaver-Burk plots using methyl gallate and tannic acid as substrates in a concentration range from 0.25 mM to 10 mM. The catalytic efficiencies were estimated as Km/Vmax. Data fitting was carried out using the OriginPro 6.1 SRO software package (OriginLab Corp., Northampton, MA).

Analysis of hydrolysis products

The products of tannic acid hydrolysis were analyzed by thin layer chromatography (TLC) using benzene:ethyl ester:formic acid (18:15:1; v/v/v) as mobile phase. The spots were visualized by spraying with a 1% $FeCl_3$ solution. Gallic and tannic acids were used as standards.

DNA extraction, PCR and sequencing

The genomic DNA was extracted from mycelia according to the methodology from Weiland (1997), with slight modifications, and used for amplification of tannase partial sequence by PCR amplification with the primers (Sigma®) TTH1F (5'-CGACTACGAGAATTTCTACGTTGCTGG-3') and TTH2R (5'-GCAGCACAGTAGTAAGGCTCACCGATGATAGAG-3'). The reaction was performed with 100 ng genomic DNA and PCR conditions were 5 min at 95ºC, 35 cycles of: 95ºC for 1 min, 52ºC for 45 sec, 72ºC for 1 min and a final extension at 72ºC for 10 min. The PCR product was submitted to electrophoresis and the 650 bp fragment obtained was purified using Promega Wizard SV Gel Kit and PCR clean-up system. A 650 bp fragment was obtained and cloned into pCR 2.1-TOPO®(Invitrogen) following the manufacturer's instructions. The recombinant plasmid containing the PCR fragment was completely sequenced using the ABI Prism BigDye Terminator sequencing kit (Applied Biosystems) and primers T7 and M13R, essentially as described by Quiapim et al. (2009). The sequencing reaction products were analyzed on an ABI 3100 fluorescence automated sequencer (Applied Biosystems). The individual reads were evaluated and assembled by the Phred/Phrap/Consed package (Ewing and Green, 1998) generating a consensus sequence which was analyzed by the BLAST program at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The deduction of amino acid sequence from the nucleotide sequence obtained was performed using the Translate software in the ExPASy Bioinformatics Resource Portal (http://www.expasy.org).

Reproducibility

The experiments were performed in triplicate and the values obtained were expressed as media ± standard deviation (SD).

RESULTS AND DISCUSSION

Production of tannase activities

High levels of tannase were obtained when the fungus was grown for 72 hrs in Khanna medium using tannic acid as carbon source. Under this condition, the specific extracellular activity was twice higher than the intracellular activity (Table 1). The way of induction of tannase may be similar to cellulases induction, in which a basal level of extracellular enzyme is required to start the hydrolysis of the substrate to produce the true inducer. Basal levels of tannase production by *A. ochraceus* were observed when the fungus was grown in the presence of glucose only. Enzyme production in the presence of glucose was also observed for *A. japonicus* (Bradoo et al. 1997). Tannic acid cannot cross the cell membrane and, possibly, a low molecular weight intermediary compound is acting as the true inducer, but the real nature of the true tannase inducer deserves further investigation. On the other hand, the concentration of gallic acid in the initial culture medium with tannic acid as carbon source was 0.74 mM, and according to Bajpai and Patil (1997) methyl gallate and gallic acid may act as inducers of tannase production. Interestingly, the production of extracellular activity in the presence of oatmeal was higher, compared to that obtained with methyl gallate. Using methyl gallate, the content of gallic acid in the initial medium was 0.15 mM, and the use of gallic acid as single carbon source also promoted tannase production by *A. ochraceus* (0.17 U/mg of protein), although a reduction in the specific activity occurred, compared to that obtained in medium without carbon source. However, the specific activity obtained using gallic acid was quite similar to that observed for glucose, showing that there is no negative influence of gallic acid. This induction is probably related to the higher titers of enzyme produced constitutively if compared to the absence of carbon source, since the fungus was able to metabolize gallic acid. The intracellular activity had its maximal production using methyl gallate (0.61 U/mg protein), tannic acid (0.58 U/mg protein) and sugar cane bagasse (0.40 U/mg protein). Soybean flour and wheat bran were not able to stimulate the enzymatic production. When using these carbon sources, gallic acid content in the initial culture medium was undetectable.

Table 1. Influence of several carbon sources on tannase production by *A. ochraceus*. The microorganism was grown in Khanna medium with a desired carbon source for 72 hrs at 40ºC and 100 rpm.

Carbon source	Initial gallic acid content (mM)	Tannase activity (U/ mg of protein)	
		Extracellular	Intracellular
None	0	0.94 ± 0.02	0.17 ± 0.01
Tannic acid	0.74	1.1 ± 0.05	0.58 ± 0.02
Methyl gallate	0.15	0.16 ± 0.02	0.61 ± 0.01
Gallic acid	100.00	0.17 ± 0.04	0.30 ± 0.02
Glucose	0	0.12 ± 0.01	0.04 ± 0.01
Oatmeal	0.05	0.26 ± 0.03	0.09 ± 0.03
Sugar cane bagasse	0	0.56 ± 0.01	0.40 ± 0.04
Passion fruit peel	0	0	0.13 ± 0.01
Soybean flour	0	0	0.01 ± 0.01
Wheat bran	0	0	0
Crushed corn	0.05	0.33 ± 0.01	0.21 ± 0.02
Crushed corncob	0	0.14 ± 0.01	0

The best culture time (72 hrs) for extracellular tannase production in the presence of tannic acid as carbon source was confirmed (Figure 1). However, other tannin-rich sources have been used to induce tannase production, such as tar bush and creosote bush (Ventura et al. 2009). According to some authors, tannase is an inducible enzyme (Sharma et al. 2008), although some others have shown constitutive expression of this enzyme, independent of tannic acid presence, or even in the presence of unrelated substrates such as mono and disaccharides, among others (Bradoo et al. 1997). In spite of the intracellular and cell-wall bound predominance of tannases obtained under submerged fermentation, significant levels of extracellular enzyme have been mentioned for different fungal strains as *Verticillium* sp. (Kasieczka-Burnecka et al. 2007), *Paecilomyces variotii* (Mahendran et al. 2006) and *Emericella nidulans* (Gonçalves et al. 2011). It should be also considered that the term “intracellular” includes not only really intracellular enzymes, but also those contained in secretion vesicles (*in route*).

The ability to secrete tannase observed for *A. ochraceus* is an interesting characteristic for biotechnological applications, reducing the costs of enzyme extraction. Higher titles of extracellular tannase were observed when tannic acid was the carbon source, but the intracellular form was predominant when methyl gallate and gallic acid were used. However, the molecular and regulatory mechanisms of tannases secretion are still an open question and deserve a detailed investigation.

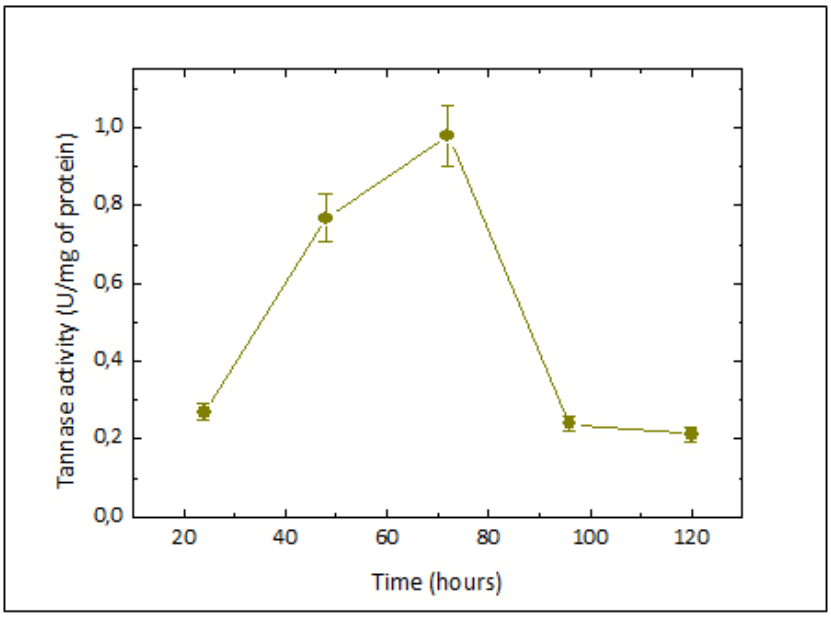

Fig. 1 Time-course for extracellular tannase production by the filamentous fungus *A. ochraceus* in submerged fermentation using tannic acid (2%, w/v) as carbon source.

Purification and molecular mass

Extracellular tannase eluted as single form from DEAE-Cellulose chromatography with 370 mM NaCl. When pooled and concentrated active fractions were applied onto a Sephacryl S-200, a single activity peak was also eluted. After these steps, the extracellular enzyme was purified 9.3-fold up to apparent electrophoretic homogeneity, with a recovery of 2.12% (Table 2).

Table 2. Purification of the extracellular tannase produced by *A. ochraceus*.

Step	Activity (Total U)	Protein (Total mg)	Specific Activity (U/mg)	Yield (%)	Purification factor
Crude extract	39.6	405	0.10	100	1
DEAE-Cellulose	6.34	10.34	0.61	16.01	6.10
Sephacryl S-200	0.84	0.90	0.93	2.12	9.33

The extracellular tannase from *A. ochraceus* is a monomer with native molecular mass of 112 kDa, determined by gel filtration, and 85 kDa, determined by SDS-PAGE (Figure 2). Most tannases are multimeric proteins, differing from the enzyme produced by *A. ochraceus*, which suggests a new group of fungal tannases. Monomeric enzymes have been mentioned for bacteria, as for instance *Lactobacillus plantarum* ATCC 14917 (Iwamoto et al. 2008). Moreover, the tannase produced by *Aspergillus niger* MTCC 2425 is constituted by two different polypeptides of 102 kDa and 83 kDa, determined by SDS-PAGE (Bhardwaj et al. 2003). Trimeric and tetrameric enzymes were also found (Barthomeuf et al. 1994; Mata-Gómez et al. 2009).

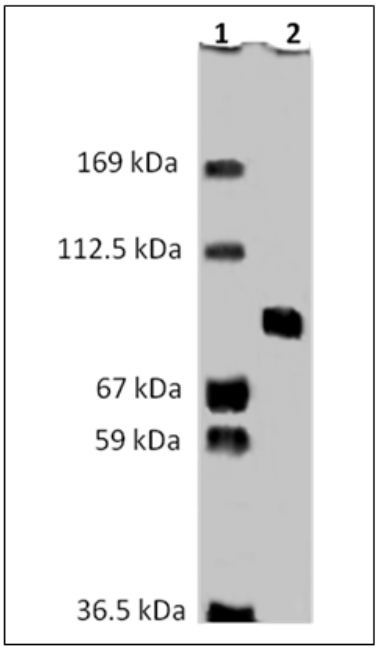

Fig. 2 SDS-PAGE of purified *A. ochraceus* tannase. Lane 1, molecular mass makers; lane 2, purified extracellular tannase. Protein bands were stained with silver. Details in **Material and Methods** section.

Temperature, pH and thermal stability

The maximum activity of the purified extracellular enzyme was observed in a range from 30ºC to 45ºC (Figure 3a) and at pH 5.5 (Figure 3b). This optimum temperature was higher than those reported for tannases from *Verticillium* sp. P9 (Kasieczka-Burnecka et al. 2007), *Aspergillus ruber* (Kumar et al. 2007), but similar to those of the enzymes from *Paecilomyces variotii* (Battestin and Macedo, 2007) and *Aspergillus foetidus* (Mukherjee and Banerjee, 2006). The optimum pH was similar to those reported for the enzyme from *Aspergillus awamori* MTCC9299 (Chhokar et al. 2010). Extracellular *A. ochraceus* enzyme was also very stable in a wide range of temperatures, from 40ºC to 70ºC, with a half-life (t_{50}) around 4 min at 80ºC (Figure 3c), higher than those reported for the enzymes from *Hyalopus* sp. (Mahapatra and Banerjee, 2009) and *Lactobacillus plantarum* CECT 748 (Rodríguez et al. 2008). The tannases produced by *Aspergillus niger* van Tieghem MTCC 2425 (Rana and Bhat, 2005) and *P. variotii* (Battestin and Macedo, 2007) were stable from 20ºC to 60ºC and 30ºC to 50ºC, respectively. Thermal stability is an important characteristic for many enzymes with biotechnological application, as for instance β-glucosidases (Yoon et al. 2008), esterases (Kim et al. 2008) and tannases (Mahapatra and Benerjee, 2009), among others. The thermal stability of *A. ochraceus* tannase is quite interesting, since some processes that use tannases need to be performed at elevated temperatures.

Influence of ions and EDTA

Extracellular tannase activity was stimulated by Mn^{2+} (33-39%) and NH_4^+ (15%) (Table 3). Most tannases are not affected or inhibited by Mn^{2+}, what makes *A. ochraceus* tannase interesting and different from the others. Magnesium ions significantly inhibited *A. ochraceus* tannase activity, but only at 10 mM concentration, different from the enzyme produced by *Fusarium subglutinans* (Hamdy, 2008). On the other hand, Mg^{2+} also inhibited activity from *A. niger* ATCC 16620 tannase (Sabu et al. 2005). All other compounds tested promoted inhibition, especially $CuSO_4$ and $HgCl_2$, that strongly decreased the activity by 82% to 97% at 1 mM concentration. Mata-Gómez et al. (2009) reported that Cu^{2+} and Zn^{2+} had a mild inhibitory effect on the enzyme produced by *Aspergillus niger* GH1. The tannases I and II from *Verticillium* sp. P9, among others, were also inhibited by Cu^{2+} and Hg^{2+} (Kasieczka-Burnecka et al. 2007). Mercury ions act on thiol groups and can also react with histidine and tryptophan residues,

while enzymatic activity reduction in the presence of divalent cations may be explained by nonspecific binding or aggregation of the protein, although various metal ions act as cofactors, increasing the activity of a great variety of enzymes. There was no significant influence of EDTA on tannase activity, similarly as observed for *A. niger* MTCC 2425 (Bhardwaj et al. 2003) and *L. plantarum* (Rodríguez et al. 2008) enzymes.

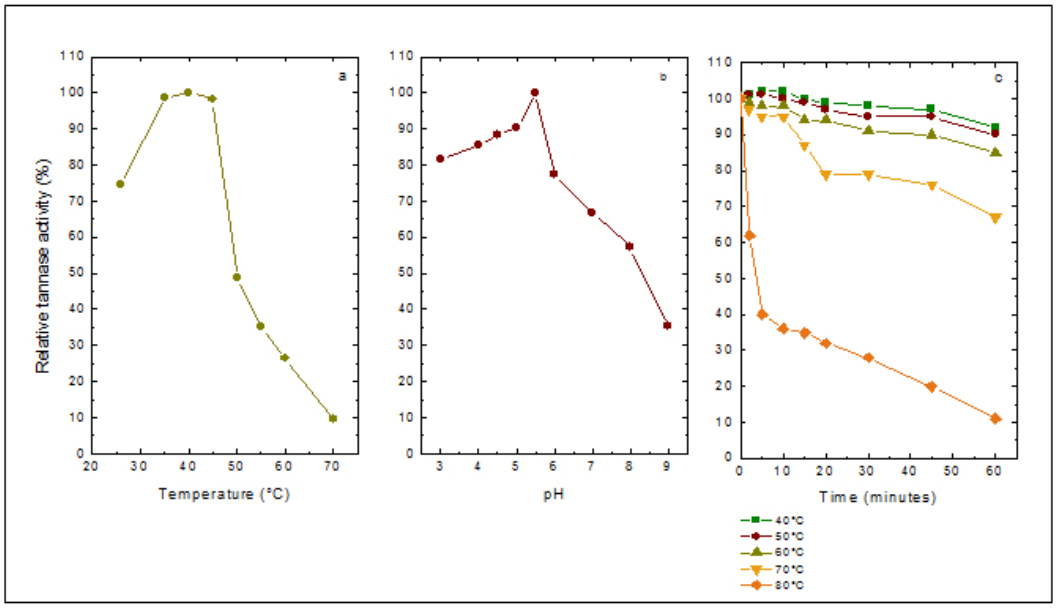

Fig. 3 Optima of temperature (a) and pH (b), and thermal stability (c) for extracellular tannase activity. Standard deviation varied from 1 to 3% for all points.

Table 3. Influence of different compounds on extracellular tannase activity from *A. ochraceus*.

Compounds	Relative Extracellular Tannase Activity (%)	
	1 mM	10 mM
None	100	100
$BaCl_2$	56 ± 2.99	4 ± 1.38
$CaCl_2$	76 ± 3.00	30 ± 2.14
$CuCl_2$	80 ± 4.93	82 ± 4.78
$CuSO_4$	18 ± 2.95	5 ± 1.96
EDTA	76 ± 3.96	95 ± 2.69
$HgCl_2$	3 ± 0.98	17 ± 2.01
$MgCl_2$	98 ± 3.96	38 ± 2.14
$MgSO_4$	87 ± 3.24	36 ± 1.45
$MnCl_2$	139 ± 2.94	133 ± 3.65
NaCl	79 ± 4.97	104 ± 3.35
NH_4Cl	115 ± 3.87	91 ± 2.39

Kinetic parameters and hydrolysis products analyses

Extracellular tannase from *A. ochraceus* hydrolyzed both tannic acid and methyl gallate, with Km values of 0.757 mM and 0.716 mM, respectively, and Vmax of 0.917 U/mg protein and 0.681 U/mg protein, respectively (Figure 4). The Km values were very close, showing similar affinities for both substrates. However, the enzyme showed higher catalytic efficiency (1.21 U/mg protein mM^{-1}) for

tannic acid hydrolysis, compared to that for methyl gallate (0.91 U/mg protein mM^{-1}). The affinities of *A. ochraceus* tannase for methyl gallate and tannic acid were higher than those observed for the enzymes from *Verticillium* sp. P9 (Km values of 1.44-10.0 mM) (Kasieczka-Burnecka et al. 2007), *Penicillium variable* (Km values of 14 mM for methyl gallate and 32 mM for tannic acid) (Sharma et al. 2008) and *Emericella nidulans* (Km values of 14.01 mM and 4.78 mM for tannic acid and methyl gallate, respectively) (Gonçalves et al. 2011).

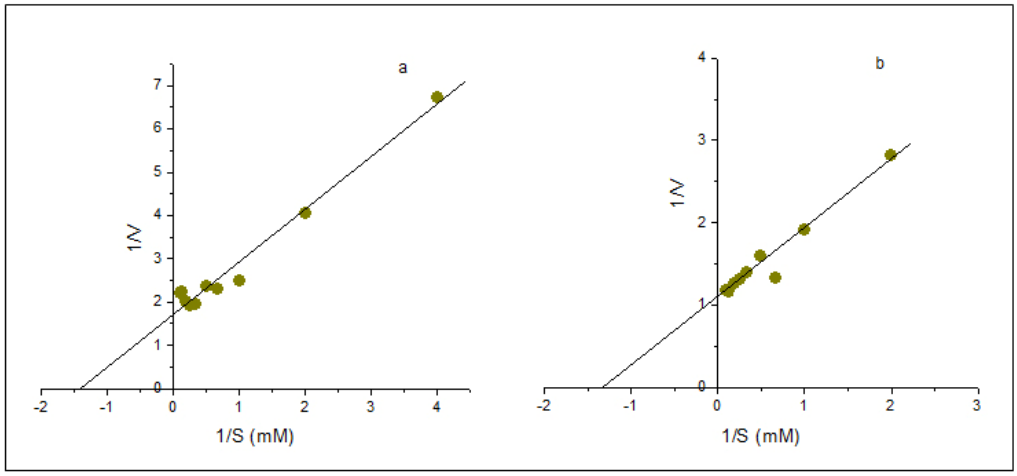

Fig. 4 Linewever-Burk plots for the hydrolysis of methyl gallate (a) and tannic acid (b) by the purified extracellular tannase. The reactions were conducted at 40ºC.

Gallic acid was the single product of tannic acid hydrolysis detected by TLC analysis, even in the presence of glucose (Figure 5). Gallic acid may be used as an antioxidant in foods and, additionally, is an important precursor for the synthesis of a variety of pharmaceutical drugs (Aguilar et al. 2007). On the other hand, gallic acid may be employed to synthesize ester gallates and pyrogalol, used as preservatives in food industries (Bajpai and Patil, 1997).

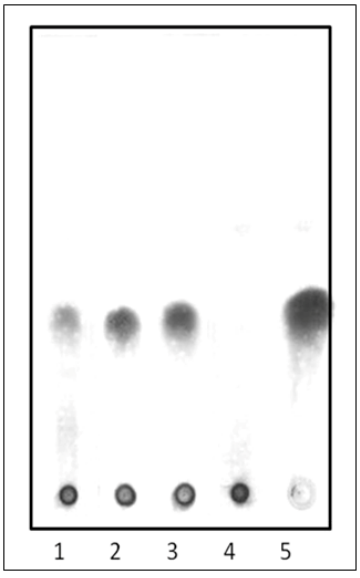

Fig. 5 TLC analysis of the products of tannic acid hydrolysis by the extracellular tannase. Time of reaction: 10 min (lane 1); 60 min (lane 2); 180 min (lane 3). Standards: tannic acid (lane 4) and gallic acid (lane 5). The reactions were conducted at 40ºC.

Molecular characterization

The partial sequence of the tannase encoding gene from *A. ochraceus* was determined and an open reading frame with 567 bp was obtained. The Translate (Expasy) predicted a sequence of 199 amino acids from the internal exons without introns. Hatamoto et al. (1996) reported that the encoding tannase gene isolated from *Aspergillus oryzae* genomic DNA is intronless. The tannase gene also has been characterized for bacteria (Sharma and John, 2011). The theoretical pI was 4.32, which is similar to that found (pI 4.31) for an acidophilic tannase from *Aspergillus awamori* BTMFW032 (Beena et al. 2010). The pI value is within the range (4.2-5.5) for fungal tannases observed by Banerjee et al. (2012) for 31 strains analyzed by in silico studies. The deduced amino acid sequence from the partial gene for *A. ochraceus* tannase is presented in Figure 6. The fragment obtained showed 87% identity with *Aspergillus niger* (DQ185610.2), and 80% with *Aspergillus flavus* NRLL3357 (XM_002385359.1) and *Aspergillus oryzae* (D63338.1) tannases. These similarities are relevant to recognize the high levels of conservation of fungal tannases. According to Banerjee et al. (2012), a motif of 29 amino acids (GCSTGGREALKQAQRWPHDYDGIIANNPA), that displays an important structure-function role, may be recognized as a signature for fungal tannases. Indeed, this motif (red highlighted) was observed in the deduced amino acid sequence for *A. ochraceus* tannase, although some amino acids of the motif (blue letters in Figure 6) are different from the regular sequence indicated above. Other motifs were also identified by Banerjee et al. (2012) by in silico studies, but these motifs were not identified in the deduced partial amino acid sequence from *A. ochraceus* tannase. Finally, there are few sequences of genes encoding fungal tannases described in the literature, and thus this first description of the partial sequence of the *A. ochraceus* tannase gene encoding is an important contribution to understand the genetic information for the production of such interesting and biotechnologically valuable enzymes.

Fig. 6 Nucleotide sequence of de amplified DNA fragment and deduced amino acid sequence for *A. ochraceus* tannase. The underlined regions represent the positions of the primers. The vector sequence was removed. In red is the motif that can be recognized as a signature for fungal tannases, with few different aminoacids in blue.

CONCLUDING REMARKS

The extracellular tannase produced by *A. ochraceus* showed distinctive features, such as monomeric structure and activation by Mn^{2+}, suggesting a new kind of fungal tannase. In addition, the enzyme showed good thermal stability, an interesting characteristic for its application in biotechnological

process, *e.g.* gallic acid production. Moreover, this is the first description of the partial gene sequence for *A. ochraceus* tannase, revealing a good identity with other fungal tannases.

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