Microbiology (2003), **149**, 2941–2946 DOI 10.1099/mic.0.26346-0

A novel tannase from *Aspergillus niger* with β-glucosidase activity

M. Ascención Ramírez-Coronel,[1,2] Gustavo Viniegra-González,[1] Alan Darvill[3] and Christopher Augur[2]†

[1]Departamento de Biotecnología, Universidad Autónoma Metropolitana, Av. Michoacán y la Purísima s/n, Colonia Vicentina, Iztapalapa, México DF, CP 09340, Mexico

[2]IRD-México, Cicerón 609, Los Morales, CP 11530, México DF, Mexico

[3]Complex Carbohydrate Research Center, The University of Georgia, 220 Riverbend Road, Athens, GA 30602-4712, USA

Correspondence
Christopher Augur
caugur@esil.univ-mrs.fr

Received 12 March 2003
Revised 25 June 2003
Accepted 7 July 2003

An extracellular tannase was produced from solid-state cultures of *Aspergillus niger*. The enzyme was purified to homogeneity from the cell-free culture broth by preparative isoelectric focusing and by FPLC using anion-exchange and gel-filtration chromatography. SDS-PAGE analysis as well as gel localization studies of purified tannase indicated the presence of two enzyme forms, with molecular masses of 90 kDa and 180 kDa. The tannase had an isoelectric point of 3·8, a temperature optimum of 60–70 °C and a pH optimum of 6·0. The substrate specificity of the tannase was determined by HPLC analysis of tannin substrates and products. The enzyme was able to remove gallic acid from both condensed and hydrolysable tannins. Internal sequences were obtained from each of the gel-purified and trypsin-digested tannase forms. The peptide sequences obtained from both forms were identical to sequences within a β-glucosidase from *Aspergillus kawachii*. The purified tannase was tested for β-glucosidase activity and was shown to hydrolyse cellobiose efficiently. However, no β-glucosidase activity was detected when the enzyme was assayed in the presence of tannic acid.

INTRODUCTION

Tannase (tannin acylhydrolase, EC 3.1.1.20) is an enzyme produced in the presence of tannic acid by various filamentous fungi, principally *Aspergillus* and *Penicillium*. Other tannase producers include bacteria (Deschamps *et al.*, 1980; Kumar *et al.*, 1999; Mondal & Pati 2000) and yeast (Aoki *et al.*, 1976). Tannase production in plants has also been reported in pedunculate oak (Niehaus & Gross, 1997). Tannase hydrolyses ester bonds of tannins such as tannic acid to produce gallic acid and glucose. The enzyme is utilized in a number of industrial applications, including the manufacture of instant tea (Agbo & Spradlin, 1995; Barmentlo *et al.*, 1993; Boadi & Neufeld, 2001), wine (Yamada & Tanaka, 1972) and gallic acid. Tannase has been produced essentially by submerged fermentation (SmF). Tannases reported in the literature vary greatly in molecular mass, ranging from 186 to 300 kDa (Adachi *et al.*, 1968; Aoki *et al.*, 1976; Barthomeuf *et al.*, 1994; Farias *et al.*, 1994). In general, tannases produced by SmF have an isoelectric point around 4·0 and an optimum temperature between 30 and 40 °C. Their optimum pH is between 5·0 and 7·0.

Unlike SmF, studies on tannase produced by solid-state fermentation (SSF) are recent. The enzyme produced in SSF is extracellular, facilitating its recovery. Lekha & Lonsane (1994) reported the production of an extracellular thermostable tannase, with higher titres in SSF as compared to SmF. During SSF, most of the supports are also substrates. Sugarcane pith bagasse has been used as solid support, absorbing the liquid medium used in the SmF process for extracellular tannase production (Lekha & Lonsane, 1994). Pearl barley has also been used as solid support (Seth & Chand, 2000). Polyurethane foam (PUF) has been utilized as an inert support for enzyme production, with higher enzyme titres obtained in SSF (Díaz-Godínez *et al.*, 2001; Romero-Gómez *et al.*, 2000).

The original purpose of this work was to study the tolerance limit of fungal strains to tannic acid in order to produce useful strains for industrial purposes. A strain of *Aspergillus niger* was among the few organisms able to grow on 20 % tannic acid (García-Peña *et al.*, 1999). The enzymes involved in tannic acid degradation by this strain were therefore investigated. The enzyme was partially sequenced.

†Present address: Laboratoire de Microbiologie, IRD, Université de Provence CESB/ESIL, Case 925, 163, Avenue de Luminy, F-13288 Marseille Cedex 9, France.

Abbreviations: HPAEC-PAD, high-performance anion-exchange chromatography-pulsed amperometric detection; PUF, polyurethane foam; SmF, submerged fermentation; SSF, solid-state fermentation.

Sequences obtained were identical to the predicted protein sequence of a β-D-glucosidase from *Aspergillus kawachii* (Iwashita *et al*, 1999). The purified tannase was able to cleave the β,1–4 linkage of a disaccharide, cellobiose, resulting in a novel function for the tannase from *A. niger*.

METHODS

Reference compounds. Tannic acid, gallic acid and standard tannins [(−)-catechin gallate, (−)-epicatechin gallate, gallocatechin gallate, (−)-epigallocatechin gallate and gallic acid methyl ester] were purchased from Sigma.

Micro-organism and culture medium. *Aspergillus niger* (IRD-UAM collection strain no. Aa20) used in the present study was selected for its ability to produce tannase. Spores were stored at −20 °C in protect-cryo blocks (bead storage system, Technical Service Consultants, Heywood, UK).

The fermentation medium for tannase production contained (g l^{-1}): KH_2PO_4, 5; NH_4NO_3, 10; $MgSO_4.7H_2O$, 1; $CaCl_2.6H_2O$, 0·1; $MnCl_2.6H_2O$, 0·02; $NaMoO_4.2H_2O$, 0·01; $FeSO_4.7H_2O$, 0·125; glucose, 2·5. The pH was adjusted to 5·5 with 100 mM NaOH. This mineral medium was autoclaved at 121 °C for 15 min. A tannic acid (Sigma) solution was adjusted to pH 5·5, filter-sterilized and added to a final concentration of 0·1 % to the fermentation medium.

Cultures. Inocula were prepared by transferring the spores to potato dextrose agar and incubating for 4 days at 30 °C. Spores were then scraped into a sterile 0·02 % Tween 80 solution and counted in a Neubauer chamber. Liquid medium to be added to each SSF support was inoculated with the spore suspension at 5×10^7 spores per g carbon source.

Tannase production in solid-state fermentation (SSF). Polyurethane foam (PUF) was used as solid support to absorb the inoculated liquid medium. The density of the PUF was 17 kg m^{-3} (EXPUFLEX, Mexico). The foam was cut into 0·5 cm cubes, and washed twice with cold water followed by a wash with water at 60 °C. The PUF was then dried at 60 °C in an incubator before autoclaving at 121 °C for 15 min. Erlenmeyer flasks containing 10 g PUF were impregnated with 250 ml inoculated medium, prepared as described above. Flasks were incubated at 30 °C for 120 h before harvesting.

Production and concentration of enzyme extract. Extract (about 2 l) containing extracellular tannase was obtained by compressing PUF in a Buchner funnel. The PUF was washed with 100 mM citrate/phosphate buffer pH 7·0. The extract was then centrifuged (15 min, 7000 r.p.m., Beckman JA14 rotor at 4 °C) and concentrated to 100 ml by ultrafiltration on an Amicon membrane with a 50 kDa molecular mass cut-off (Millipore). The extract was dialysed against water prior to preparative isoelectric focusing.

Tannase activity assay. Tannase activity was assayed using the HPLC methodology essentially as described by Beverini & Metche (1990). In brief, an enzyme extract (50 µl) was added to 1 ml 0·3 mM tannic acid in 100 mM sodium acetate pH 5·5. The reaction mixture was incubated at 30 °C for 30 min. Freezing the samples at −80 °C stopped the reactions. Each sample was filtered through a 0·22 µm membrane prior to HPLC analysis.

HPLC analysis of tannase substrates and products. Chromatographic analyses were performed on a Thermo Separation Products HPLC (Software PC1000) with a quaternary HPLC pump, an auto sampler with a 20 µl injection loop and a detector at 280 nm. Compounds were separated on a Phenomenex prodigy 5µ, ODS 3, 100Å, column (250 × 4·6 mm) at room temperature, with a flow rate of 1·5 ml min^{-1}, by a gradient elution system. The eluents were methanol for pump A, and formic acid and water (1 : 99, v/v) for pump B. Compounds were separated by a linear gradient from 10 % to 100 % B in 5 min. Gallic acid was identified by comparison of its retention time with that of the pure product (Sigma). Different tannins were tested as tannase substrates for tannase activity: (−)-catechin gallate, (−)-epicatechin gallate, (−) epigallo catechin gallate, (−)-gallocatechin gallate, tannic acid and gallic acid methyl ester. Each substrate was diluted in 100 mM acetate buffer pH 5·5 to a final concentration of 1 mg ml^{-1}. The hydrolysis of the different substrates was followed by HPLC. The activity (units) of the enzyme was expressed in µmol gallic acid released in 1 min at 30 °C. All experiments were done in duplicate.

Protein determination. Protein concentration was determined by the Bio-Rad assay system according to the manufacturer's procedures. BSA was used as a standard.

Purification steps

Preparative isoelectric focusing. Preparative isoelectric focusing was performed using a Rotofor cylindrical focusing chamber (Bio-Rad) with a capacity of approximately 50 ml, enabling half of the sample to be processed at a time. The chamber is divided into 20 discrete compartments by a membrane core. Ampholytes, pH 3–10 (Biolyte 3–10, Bio-Rad) were added to the enzyme solution (about 45 ml) to 1·3 % (v/v). This solution was loaded into the Rotofor chamber and maintained at 4 °C for 20 min before being electrofocused at 12 W constant power for 3 h with a model 3000Xi power supply (Bio-Rad). The contents of the focusing chamber were collected into twenty 2·5 ml fractions. Each fraction was analysed for pH and tannase activity. The run was repeated with the other half of the sample. Tannase-containing fractions from both runs were pooled, and dialysed against 50 mM acetate buffer pH 5·5 prior to anion-exchange chromatography. The proteins in each of the fractions were monitored by SDS-PAGE.

Anion-exchange chromatography. The Rotofor-purified tannase sample (13 ml) was filtered and applied at 1 ml min^{-1} to a 7 × 52 mm MonoQ (Bio-Rad) column equilibrated in 50 mM sodium acetate pH 5·5 (buffer A). The column was washed with 50 mM sodium acetate pH 5·5, until the A_{280} was equal to that of the buffer. The column was eluted at 1 ml min^{-1} with 50 mM sodium acetate pH 5·5 containing 1 M NaCl (buffer B), in a stepwise manner starting with 10 % B for 10 ml, followed by a linear gradient from 10 to 60 % B for 25 ml, ending with 100 % B for 7 min. Each fraction was analysed for pH and tannase activity. Tannase-containing fractions were pooled and concentrated to 500 µl in a Centricon 30 (Millipore) prior to gel-filtration chromatography. The proteins in each of the fractions were monitored by SDS-PAGE.

Gel filtration. The concentrated sample (500 µl) was applied to a Sephadex G-100 (1·6 × 35 cm) gel-filtration column pre-equilibrated with 50 mM sodium acetate pH 5·5 and eluted with the same buffer at 0·7 ml min^{-1}. Fractions were analysed for tannase activity and protein content. The column was pre-calibrated with gel-filtration molecular mass standards in order to estimate the molecular mass of the tannase.

Molecular mass of tannase in SDS-PAGE. SDS-PAGE was conducted using a 10 % (w/v) polyacrylamide gel based on the protocol of Laemmli (1970). Protein bands were detected by either Coomassie blue staining or silver staining. Molecular mass markers were purchased from Bio-Rad.

Gel localization of tannase activity. The sample with tannase activity was mixed with SDS sample buffer without *β*-mercaptoethanol and heated at 40 °C for 5 min. Samples were loaded and run at 70 V per gel, for 2·5 h at 4 °C, in a Mini Protean II electrophoresis cell (Bio-Rad). The location of tannase activity within the gel was determined essentially as described by Aoki *et al.* (1979). The gel was washed for 1 h in 100 ml 2·5 % (v/v) Triton X-100 followed by two 45 min washes with 100 ml 10 mM acetate buffer pH 5·5 with constant shaking. This was followed by incubation of the gel containing tannic acid 0·5 % (w/v) in 0·1 M acetate buffer pH 5·5 at 30 °C, with constant shaking. The tannic acid solution was discarded and replaced with 100 ml 0·5 % (w/v) quinine hydrochloride solution in 0·05 M acetate buffer pH 5·5 at room temperature. Tannase activity appeared as a clear band on a white background.

Tannase characterization

Optimum temperature. Samples were prepared by adding 50 µl purified tannase to 1 ml 0·3 mM tannic acid solution in 100 mM sodium acetate pH 5. Samples were incubated at 30, 40, 50, 60, 70, 80 and 90 °C for 30 min. For analysis, 20 µl of reaction mix was applied to the HPLC chromatography column and eluted as described. Experiments were done in duplicate.

Optimum pH. The tannic acid substrate solution (0·3 mM final concentration) was dissolved in 0·1 M citrate/phosphate buffer solution. Tannase activity was assayed at pH 3·5, 4·0, 4·5, 5·0, 5·5, 6·0, 6·5 and 7·0. Assays were performed at 30 °C. Enzyme activity was assayed as described above. Experiments were done in duplicate.

HPAEC-PAD analysis. Cellobiose (Sigma) and its degradation product, glucose, were analysed by anion-exchange chromatography with a Dionex AL50 HPLC and a Dionex CarboPAC PA1 column (4 × 250 mm) coupled to a Dionex pulsed amperometric detector. For profiles of the effect of the purified tannase on cellobiose, samples were prepared for HPLC by adding 20 µl purified tannase to 200 µl 1·5 mM solution of cellobiose in 100 mM sodium acetate pH 5·5. The reaction mixture was incubated at 30 °C for 1 h. Freezing the samples at −80 °C stopped the reactions. For analysis, 5 µl of sample was applied to the chromatography column and eluted in 90 mM NaOH and 100 mM sodium acetate over 15 min.

Comparison of tannase and *β*-D-glucosidase activities. Enzyme assays were carried out as follows. Purified enzyme (20 µl) was added to 200 µl 100 mM sodium acetate pH 5·5, containing either 50 mM tannic acid or 50 mM cellobiose or an equimolar concentration of both added together. Each reaction mixture was incubated at 30 °C for 1 h. Freezing the samples at −80 °C stopped the reactions. Tannase activity was assayed as described above. The *β*-D-glucosidase activity of the pure enzyme was assayed by glucose determination using the GOD-PAP Trinder enzymatic kit from SPINREACT, Spain. All the experiments were carried out in duplicate.

Protein sequencing. The gel-filtration-purified tannase protein was subjected to SDS-PAGE and the Coomassie-blue-stained bands corresponding to the 90 kDa and 180 kDa forms were excised, washed twice with 0·5 ml 50 % acetonitrile in HPLC-grade water, and sent for sequencing. Prior to sequencing, samples were reduced, *S*-carboxyamidomethylated and tryptic-digested. Sequencing of the tryptic fragments was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (µLC/MS/MS) on a Finnigan LCQ quadrupole ion-trap mass spectrometer as described by Chittum *et al.* (1998).

RESULTS

Purification of tannase

A. niger secreted tannase into the culture medium when grown in SSF on PUF. The extracellular extract was harvested 120 h after inoculation, as tannase activity was known to be optimum at that stage (Ramirez-Coronel, 2000). The tannase was partially purified from low-molecular-mass proteins after ultrafiltration (50 kDa cut-off). Tannase was further purified by a combination of preparative isoelectric focusing, and Mono-Q and Sephadex G-100 chromatography. The purification steps were monitored by SDS-PAGE (data not shown). It was not possible to construct a purification table, as in the initial stages residual tannic acid interfered with protein assays (Kilkowski & Gross, 1999). On preparative isoelectric focusing, fractions 2–7 (data not shown) containing tannase activity were pooled. The isoelectric point of the enzyme was between pH 3·7 and pH 3·9. The analysis of fractions obtained after Mono-Q chromatography showed a single broad peak of tannase activity. Tannase-containing fractions were pooled, dialysed, concentrated and subjected to gel-filtration chromatography on Sephadex G-100 (data not shown). Resulting fractions were collected and analysed for tannase activity and protein content. Fractions from Sephadex G-100 were collected and subjected to SDS-PAGE. Fractions yielding pure tannase were collected. These fractions were combined (Fig. 1, lane 2) and run on an SDS-PAGE gel. The first half of the gel was silver stained, revealing two polypeptides, with molecular masses of 90 kDa and 180 kDa, as shown in Fig. 1, lane 2. The second half of the polyacrylamide gel, also containing the combined fraction (from Sephadex G-100), was analysed for specific detection of tannase activity within the gel. A band

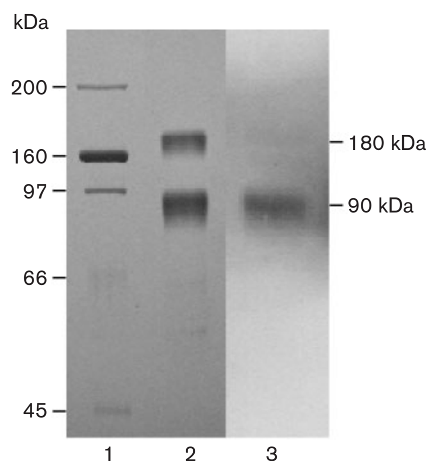

Fig. 1. Analysis of Sephadex G100-purified tannase by SDS-PAGE (lanes 1 and 2) and by tannase activity staining (lane 3). Lane 1, molecular mass markers (silver stained); lane 2, purified tannase after Sephadex G-100 chromatography (silver stained); lane 3, localization of tannase activity.

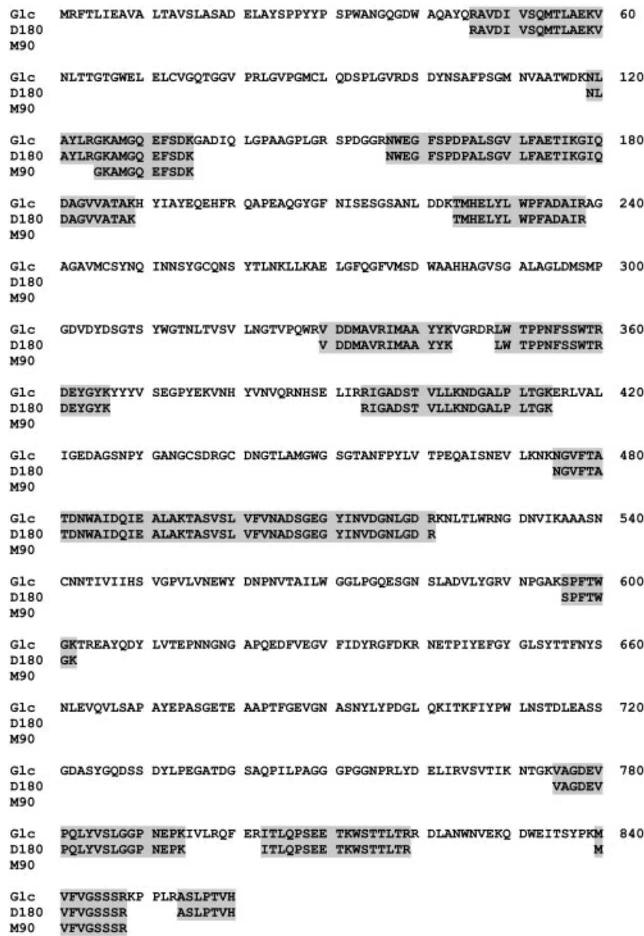

Fig. 2. Alignment of the amino acid sequences obtained from 90 kDa and 180 kDa forms of the *A. niger* tannase with the deduced amino acid sequence of *A. kawachii* β-D-glucosidase (Glc), (accession no. BAA19913, gi|2077896|). Thirteen sequences were obtained from the 180 kDa form (D180) and two sequences from the 90 kDa form (M90). Identical amino acids are shaded.

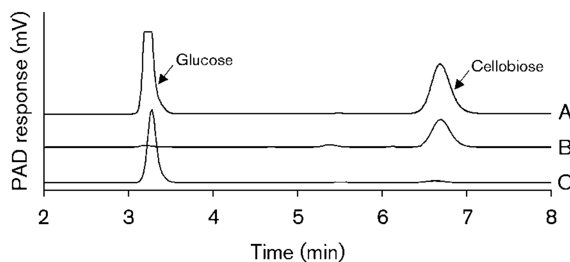

Fig. 3. Hydrolysis of cellobiose by purified tannase. Cellobiose and glucose were separated using HPAEC on a Dionex Carbopac PA-1 column (4 × 250 mm) with PAD detection. A, glucose and cellobiose standards; B, cellobiose before tannase treatment; C, product generated by tannase digestion of cellobiose.

with tannase activity (Fig. 1, lane 3) coincided with the 90 kDa silver-stained band. After prolonged incubation of the activity gel (over 12 h), a second tannase activity band was visible, coinciding with the 180 kDa silver-stained polypeptide (data not shown). It was therefore considered that the tannase from *A. niger* had been purified to homogeneity.

Protein sequencing

To characterize the two tannase polypeptides further, a gel was prepared and stained with Coomassie blue. The two Coomassie-stained bands were excised, trypsin-digested and sequenced. Thirteen different peptide sequences were obtained for the 180 kDa protein and two sequences for the 90 kDa protein. Both sequences from the 90 kDa protein were present within the sequences obtained from the 180 kDa protein, indicating sequence similarity between the two tannase forms. A search of the SWISS-PROT/GenBank/PIR databases indicated that each of the *A. niger* tannase peptides was present within the amino acid sequence of a 91 kDa β-glucosidase from *Aspergillus kawachii* reported by Iwashita *et al.* (1999) (Fig. 2).

Tannase and β-glucosidase activity

In light of the above results, the purified tannase was tested for β-glucosidase activity. The enzyme was able to hydrolyse cellobiose effectively into glucose (Fig. 3). However, no β-glucosidase activity was detected when the reaction was carried out in the presence of tannic acid (Table 1).

The enzyme was tested for tannase activity, under standard assay conditions, against a wide array of putative substrates (Table 2). The enzyme is non-specific, with the ability to remove gallic acid from a variety of substrates.

The optimum temperature and pH for tannase activity were determined. Under our assay conditions the tannase showed activity at all the temperatures tested (30–90 °C), although activity was markedly reduced above 70 °C. Activity was highest at 60–70 °C. The tannase was active over a broad pH range (3·5–7) with an optimum at pH 6·0.

Table 1. Substrate specificity of the purified enzyme from *A. niger*

The results are means ± standard deviation ($n=2$). ND, Not determined.

Substrate	Enzyme activity (U ml^{-1})	
	Tannase	Glucosidase
Tannic acid 50 mM	0·228 ± 0·004	ND
Cellobiose 50 mM	ND	0·253 ± 0·02
Tannic acid 50 mM + cellobiose 50 mM	0·215 ± 0·007	0

Table 2. Specific activity of purified tannase on different tannins

The results are means ± standard deviation ($n=2$).

Substrate (1 mg ml^{-1})	Tannase activity [μmol min^{-1} (mg protein)$^{-1}$]
Gallocatechin gallate	1·13 ± 0·01
Gallic acid methyl ester	0·87 ± 0·07
Tannic acid	0·84 ± 0·02
Epigallocatechin gallate	0·64 ± 0·02
Epicatechin gallate	0·43 ± 0·02
Catechin gallate	0·18 ± 0·009

DISCUSSION

Enzymes involved in fungal degradative pathways of aromatic compounds are of considerable interest for bioremediation and biodegradation of organic waste products. They can also be used in industrial applications; for example, tannase has found extensive use in the manufacture of instant tea (Agbo & Spradlin, 1995). We have described the purification and characterization of a novel 90 kDa protein from *A. niger*. The extracellular tannase was purified through a three-step procedure involving preparative isoelectric focusing, anion-exchange chromatography and gel filtration chromatography.

The optimum pH of the *A niger* tannase characterized in this study, pH 6·0, was similar to those reported by other workers (Barthomeuf *et al.*, 1994; Farias *et al.*, 1994; Suseela & Nandy, 1983). Farias *et al.* (1994), working on a *Cryphonectria* tannase, detected activity between pH 2·5 and 8·5, and high and stable activity was observed between pH 4 and pH 7. We determined a temperature optimum for the *A. niger* tannase activity of 70 °C. This value was considerably higher than those of tannases produced in liquid-state fermentation (Barthomeuf *et al.*, 1994; Suseela & Nandy, 1983). Tannase production by SSF resulted in a thermostable extracellular enzyme. This confirmed earlier studies by Lekha & Lonsane (1994), who compared tannase production by *A. niger* grown in solid and liquid media. They found that enzyme production with SSF was 2·5 times higher compared to liquid fermentation. The enzyme they obtained was completely extracellular in SSF compared to partially intracellular in liquid fermentation. The tannase from SSF exhibited increased thermostability.

All tannases purified so far are multimeric, with molecular masses of the monomeric forms varying from 186 kDa to over 300 kDa (Adachi *et al.*, 1968; Hatamoto *et al.*, 1996). The purified tannase from the present study is therefore the smallest known active tannase to date, with a molecular mass of 90 kDa. The enzyme is also present in a less active form of 180 kDa, suggesting the presence of a homodimer with two identical subunits.

Prediction of function is based on sequence similarity with proteins of known function. To date, the only gene sequence of a functional tannase (not derived solely from homology studies) is that from *Aspergillus oryzae* (Hatamoto *et al.*, 1996). The amino acid sequences resulting from tryptic digestion of both the 90 kDa and 180 kDa tannase from *A. niger* (Fig. 2) show no sequence homology to that of *A. oryzae*. Surprisingly, however, the same peptide sequences showed 100 % homology to a 91 kDa β-glucosidase from *A. kawachii*. In addition, the purified tannase was able to hydrolyse cellobiose, a β-glucosidase substrate. Bifunctional glucosidases have been reported (Sang *et al.*, 1995), exhibiting both endo and exo activities towards specific oligosaccharide substrates. However, tannases have hitherto only been shown to exhibit esterase activity. Such bifunctionality may be a way for micro-organisms such as *Aspergillus* to survive in extreme environments or to efficiently degrade cellulose and tannins, abundant in decaying plant material. It is unknown whether the two functions (esterase for the tannase and hydrolase for the β-glucosidase) are carried out by one or two domains within the protein structure. However, as stated by Hegyi & Gernstein (1999), there is a low chance that a single-domain protein, highly homologous to a known enzyme, has a different function.

There are similarities between the tannase from *A. niger* and the β-glucosidase from *A. kawachii*. Both enzymes were partially intracellular when produced by liquid fermentation. On the other hand, under solid culture conditions, both enzymes were secreted into the medium (Iwashita *et al.*, 1998, 1999; Ramirez-Coronel, 2000; Aguilar *et al.*, 2001). Both enzymes were also thermostable and unspecific. The *A. niger* tannase was able to remove gallic acid from all the hydrolysable and condensed tannins tested (Table 2).

Concluding remarks

A thermostable tannase, active at 70 °C, has been purified to homogeneity and characterized. The protein was present in both a monomeric and a dimeric form. The enzyme is non-specific and was able to degrade all tested tannins that contained a gallic acid residue within their structure. More importantly, sequence identity with a β-glucosidase was observed. The tannase exhibited cellobiase activity. However, no β-glucosidase activity was detected when the enzyme was assayed in the presence of tannic acid. This is believed to be the first report of a bifunctional tannase.

ACKNOWLEDGEMENTS

The present work was performed as part of cooperative agreement between the National Council of Science and Technology (CONACyT, Mexico) and the Institut de Recherche pour le Développement (IRD-France) within a specific programme undertaken at the Universidad Autónoma Metropolitana (México). Part of this research was funded by the European Community through an INCO DC grant (BIOPULCA #IC*CT970185) and from the US Department of Energy, grant DE-FG 05-93ER20097. A. Ramírez-Coronel had a pre-doctoral fellowship (#124739) from CONACyT, Mexico.

REFERENCES

Adachi, O., Watanabe, M. & Yamada, H. (1968). Studies on fungal tannase. Part II. Physicochemical properties of tannase of *Aspergillus flavus*. *Agric Biol Chem* **32**, 1079–1085.

Agbo, F. & Spradlin, J. E. (1995). *Enzymatic clarification of tea extracts*. US Patent no. 5 445 836.

Aguilar, C. N., Augur, C., Favela-Torres, E. & Viniegra-González, G. (2001). Induction and repression patterns of fungal tannase in solid-state and submerged cultures. *Process Biochem Int* **36**, 565–570.

Aoki, K., Shinke, R. & Nishira, H. (1976). Chemical composition and molecular weight of yeast tannase. *Agric Biol Chem* **40**, 297–302.

Aoki, K., Tanaka, T., Shinke, R. & Nishira, H. (1979). Detection of tannase in polyacrylamide gels. *J Chromatogr* **170**, 446–448.

Barmentlo, B., Bel, W. J., Hoogstad, B., Pendlington, S. & Slater, N. K. (1993). *Process of preparing a tea product*. US Patent no. 5 258 188.

Barthomeuf, C., Regerat, F. & Pourrat, H. (1994). Production, purification and characterization of a tannase from *Aspergillus niger* LCF 8. *J Ferment Bioeng* **77**, 320–323.

Beverini, M. & Metche, M. (1990). Identification, purification and physicochemical properties of tannase of *Aspergillus oryzae*. *Sci Aliment* **10**, 807–816.

Boadi, D. K. & Neufeld, R. J. (2001). Encapsulation of tannase for the hydrolysis of tea tannins. *Enzyme Microb Technol* **28**, 590–595.

Chittum, H. S., Lane, W. S., Carlson, B. A., Roller, P. P., Lung, F. D. T., Byeong, J. L. & Hatfield, D. L. (1998). Rabbit *β*-globin is extended beyond its UGA stop codon by multiple suppression and translational reading gaps. *Biochemistry* **37**, 10866–10870.

Deschamps, A. M., Mahodeau, G., Conti, M. & Lebeault, J. M. (1980). Bacteria degrading tannic acid and related compounds. *J Ferment Technol* **58**, 93–97.

Díaz-Godínez, G., Soriano-Santos, J., Augur, C. & Viniegra-González, G. (2001). Exopectinases produced by *Aspergillus niger* in solid-state fermentation and submerged fermentation: a comparative study. *J Ind Microbiol Biotechnol* **26**, 271–275.

Farias, G. M., Gorbea, C., Elkins, J. R. & Griffin, G. J. (1994). Purification, characterization and substrate relationships of the tannase from *Chryphonectria parasitica*. *Physiol Mol Plant Pathol* **44**, 51–63.

Garcia-Peña, I., Favela-Torres, E. & Augur, C. (1999). Purificación parcial de tanasa producida por *Aspergillus niger* en fermentación en medio sólido. In *Topicos in Biotecnología, Avances en Purificación y Aplicación de Enzimas en Biotecnología*, pp. 247–261. Edited by A. Prado-Barragan, S. Huerta-Ochoa, G. Rodriguez-Serrano & G. Saucedo Castañeda. Mexico City: UAM-I.

Hatamoto, O., Watarai, T., Kikuchi, M., Misusawa, K. & Sekine, H. (1996). Cloning and sequencing of the gene encoding tannase and structural study of the tannase subunit from *Aspergillus oryzae*. *Gene* **175**, 215–221.

Hegyi, H. & Gernstein, M. (1999). The relationship between protein structure and function: a comprehensive survey with application to the yeast genome. *J Mol Biol* **288**, 147–164.

Iwashita, K., Todoroki, K., Kimura, H., Shimoi, H. & Ito, K. (1998). Purification and characterization of extracellular and cell wall bound *β*-glucosidases from *Aspergillus kawachii*. *Biosci Biotechnol Biochem* **62**, 1938–1946.

Iwashita, K., Nagahara, T., Kimura, H., Takano, M., Shimoi, H. & Ito, K. (1999). The *bglA* gene of *Aspergillus kawachii* encodes both extracellular and cell wall-bound beta-glucosidases. *Appl Environ Microbiol* **65**, 5546–5553.

Kilkowski, W. J. & Gross, G. G. (1999). Color reaction of hydrolyzable tannins with Bradford reagent Coomassie brilliant blue. *Phytochemistry* **51**, 363–366.

Kumar, R. A., Gunasekaran, P. & Lakshmanan, M. (1999). Biodegradation of tannic acid by *Citrobacter freundii* isolated from a tannery effluent. *J Basic Microbiol* **39**, 161–168.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lekha, P. K. & Lonsane, B. K. (1994). Comparative titers, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid-state, liquid surface and submerged fermentations. *Process Biochem* **29**, 497–503.

Mondal, K. C. & Pati, B. R. (2000). Studies on the extracellular tannase from newly isolated *Bacillus licheniformis* KBR 6. *J Basic Microbiol* **40**, 223–232.

Niehaus, J. U. & Gross, G. G. (1997). A gallotannin degrading esterase from leaves of pedunculate oak. *Phytochemistry* **45**, 1555–1560.

Ramírez-Coronel, M. A. (2000). *Purificación y caracterización de una tanasa de A. niger AA20 producida por fermentacion en medio sólido*. Master's thesis, Universidad Autónoma Metropólitana Iztapalapa, Mexico.

Romero-Gómez, S., Augur, C. & Viniegra-González, G. (2000). Invertase production by *Aspergillus niger* in submerged and solid state fermentation. *Biotechnol Lett* **22**, 1255–1258.

Sang, J. H., Yong, J. Y. & Hyen Sam, K. (1995). Characterization of a bifunctional cellulase and its structural gene. The *cel* gene of *Bacillus* sp. D04 has exo and endoglucanase activity. *J Biol Chem* **270**, 26012–26019.

Seth, M. & Chand, S. (2000). Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori*, optimization of process parameters. *Process Biochem* **36**, 39–44.

Suseela, R. G. & Nandy, S. C. (1983). Isolation, purification and some properties of *Penicillium Chrysogenum* tannase. *Appl Environ Microbiol* **46**, 525–527.

Yamada, D. & Tanaka, T. (1972). *Wine making using tannase in fermentation process*. Patent. Ger. Offen. 2.224 100 (cl. 12g). Japanese Patent Application no. 71 33, 151.