Original article

Identification, isolation, cloning and sequencing of tannase gene from *Aspergillus niger*

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Abstract
Introduction and objective: Tannin acyl hydrolase (tannase E.C. 3.1.1.20), commonly referred to as tannase, hydrolyses the 'ester' bond (galloyl ester of an alcohol moiety) and the 'depside' bond (galloyl ester of gallic acid) in substrates such as tannic acid, methylgallate and *m*-digallic acid. This is an inducible enzyme produced by various filamentous fungi. The aim of present research was to isolate, clone and sequence partial gene of tannase from *Aspergillus niger.*
Materials and methods: *Aspergillus niger* was grown in a selective medium and then genomic DNA was directly extracted from fungal mycelium. Using designed primers, PCR carried out and a 950bp expected band obtained that was ligated into cloning vector and then cloned into *Escherichia coli* Top-10F′. In order to screen transformed cells, the blue/white bacterial colony selection was carried out and expected the inserted DNA was extracted from putative transformants cells.
Results: Extracted DNA was sequenced using ABI automatic system and a 908bp intronlees motif was obtained using BlastX analaysing software that showed 36% identity with tannase precursor of *A. oryzae* and 29% with *Debaryomyces hansenii* tannase gene in amino acid level.
Conclusion: In conclusion, the identification, isolation, cloning and sequencing of a 908bp partial sequence of tannase gene from *A. niger* which is considered as an important bioreactor and industrial fungus were reported.

Keywords: Cloning; Tannase; Gene sequence; *Aspergillus niger*

Introduction

Microorganisms need to produce a combination of enzymes, primarily carbohydrases and 'esterases' (able to remove side chain substituents) that act synergistically, in order to increase their ability to use different substrates [1,2]. Between these enzymes, microbial esterases are a broad group of enzymes able to hydrolyse the ester bond of a variety of naturally occurring esters [3].

Tannin acyl hydrolase (tannase E.C. 3.1.1.20), commonly referred to as tannases, hydrolyses the 'ester' bond (galloyl ester of an alcohol moiety) and the 'depside' bond (galloyl ester of gallic acid) in substrates such as tannic acid, methylgallate and *m*-digallic acid [1,4,5]. In some filamentous fungi like *A. oryzae*, the presence of two separate isoenzymes, tannase I and tannase II, with esterase and depsidase activity, respectively, have been described [6]. These are inducible enzymes produced mainly by *Aspergillus*, *Penicillium* and *Rhizopus* species [4,7-9] but have also been described in yeast [4], bacteria [10], and plants such as *Pedunculate oak* [11].

Gallic acid is the product of acidic or enzymatic hydrolysis of tannic acid and is taken into account as an important substrate for the synthesis of propyl gallate in the food industry and trimethoprim in the pharmaceutical industry. Pyrogallol, the product of tannase and gallic acid decarboxylase, has widespread industrial applications; it is used as a developer in photography, for staining leather, fur, and hair, as a precursor for dyes, and for determining oxygen concentrations in gas analyses [1,6,9]. Gallic acid decarboxylases catalyze the second step in the degradation of the polyphenol tannic acid, the decarboxylation of gallic acid to pyrogallol [1,4]. Tannase also has potential applications in the clarification of beer and fruit juices, manufacture of coffee flavoured soft drinks, improvement in the flavour of grape wine and as an analytical probe for determining the structures of naturally occurring gallic acid esters [3].

Aspergillus niger has been used as an important bioreactor to prepare different type of industrial enzymes and materials. *A. niger* also releases large amounts of citric acid into the environment, causing it to become acidic. To adapt to this acidic environment, some of the enzymes of *A. niger* such as tannase, amylase, protease, cellulase, and hemicellulase, are more acid stable than the same enzymes secreted by other bacterial and fungal species. Moreover optimum temperature for the *A. niger* tannase activity (70°C) was considerably higher than those of tannases produced in other experiment [4,7]. The purified tannase so far from different sources have been shown to have a molecular mass of 90kDa to over 300 [12].

Identification and sequencing of tannase gene in *A. niger* has not been preformed till this study and the aim of present study was to isolate, clone and sequence partial gene of tannase from *A. niger*.

Materials and methods

Wild type *A. niger* PTCC 5012 (similar to *Csp* A1 derivative from ATCC 9029) was obtained from Iranian Centre of Industrial and Medical Fungi and Bacteria Collection. *Escherichia coli* Top-10F´ competent cells were obtained from Invitrogen and were used for all cloning procedures. Potato dextrose agar (Oxoid, UK) was used for routinely growing fungus strain and Modified Vogel's Medium was prepared by filter sterilizing (0.2µm) a 50x stock of Vogel's salts solution. For agar plates, 1.5% (w/v) agarose (Lucas Meyer, USA) was

included with the sugar to solidify the medium.

Luria-Burtani Medium (LB) was prepared by autoclaving a solution containing standard agents. When required, 0.1% (v/v) of a filter sterilised solution of 50mg ml⁻¹ ampicillin was added after autoclaving the medium. For the production of *A. niger* spores, fungus were grown at 25°C for five days in Vogel's modified agar (Lucas Meyer, USA). To prepare spores, 10ml of sterile 0.1% (v/v) Tween 80, was spread on the surface of the mycelium and spores were collected by gently scraping the surface of the colonies with a flame-sterilised glass spreader and filtered through Whatman lens tissue to remove mycelial fragments and then and stored at 4°C.

To prepare plasmid, 2ml or 4ml of LB broth (containing 1-2µl of a filter sterilised 50mg ml⁻¹ ampicillin stock solution), was inoculated with *E. coli* Top-10F' from a colony and incubated overnight at 37ºC (250rpm). Genomic DNA was extracted from approximately 1.4g of mycelium (wet weight) from 18h liquid cultures of *A. niger* using the method described by Griffin *et al.* [13]. To rapidly screen the recombinant transformants, plasmid DNA was prepared from cultures using the boiling method and stored at -20ºC until required. Digestion of DNA was carried out using restriction endonucleases (Boegringer Mannheim) and the appropriate 1x buffer, usually in a volume of 10µl. For genomic DNA, the digestion was performed overnight at 37°C. To obtain plasmid DNA, digests were usually performed at 37°C for 2h.

Usually, PCR products were gel purified and cloned into *E. coli* Top-10F´ (Stratagene) using standard cloning procedures. PCR products were cloned using the pGEM®-T easy vector system (Promega, USA). Ligation reactions were typically carried out by incubating 1µl of 10x reaction buffer, 1µl vector (50ngµl⁻¹),

100 ngµl⁻¹ purified PCR product and sterile ddH₂O (to make a final volume of 10µl) and incubated at 4°C overnight.

Blue/white colony selection
The insertion of DNA into α peptide, part of the β-galactosidase gene encoded by pGEM®-T easy vector inactivates the gene. Therefore, transformed colonies containing the PCR product plasmid can not produce the active enzyme. This effect can degrade the colorimetric substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and release the blue coloured product. After growing transformed colonies blue-white colony selection was carried out on ampicillin agar plates containing 0.0265mg ml⁻¹ X-gal and 0.3mg ml⁻¹ Isopropyl β-D-thiogalactopyranoside (IPTG).

To confirm the presence of expected DNA insertion, the plasmid was digested by *Eco*R1 restriction enzyme. Digested plasmid was run on 1% (w/v) agarose gel which was stained using ethidium bromide bath. All DNA products that required sequencing were cloned using the pGEM®-T easy vector and sent either to MWG or Lark Technology for sequencing.

Forward and reverse degenerate primers (TTCTGCTCTGGATCGCAATCTG and ACTAGTGATTGATGGGGGAGAGG) for tannase gene were designed from highly conserved regions of amino acids from an alignment of the translated sequences of tannase gene from several sources. The process of PCR degenerate was performed as follows: 3min 94ºC x1, 1min 94ºC, 1min 48ºC, 3mins 72ºC x 29, 1min 94ºC, 1min 48ºC, 7mins 72ºC x1. PCR with above conditions produced a clear product of ca. 950bp with *A. niger* genomic DNA which was the size of product predicted (Fig. 1).

Bioinformatics tools
A range of web-based tools were used for analysis of DNA and protein sequences.

Novel DNA sequences were submitted for analysis through the BLASTX. BLAST was accessed through the NCBI (http://www.ncbi.nlm.nih.gov/) or through the Bioinformatics Centre, University of Kyoto (http://blast.genome.ad.jp/) to search for sequence similarity. DNA-to-protein translation of DNA sequences was performed using the EXPASY translate tool (http://au.expasy.org/tools/dna.html) using the standard genetic code. To compare reverse and forward strand sequences, one strand was reverse complimented prior to alignment using the reverse and compliment program at Lipper Centre for Computational Genetics, Harvard (http://arep.med.harvard.edu/labgc-/adnan/projects/Utilities/revcomp.html).

Results

Sequence analysis

Three clones containing product inserts of ca. 950bp were sent externally to sequence after plasmid DNA isolation. After removal of 5' and 3' vector sequence and primers, sequences were subjected to multiple alignments. All three clones were identical except for the clone which was on the opposite strand to the others. This was confirmed by multiple alignments of the nucleotide sequences after reverse complementing the third clone. When the sequence of the 950bp PCR product (Fig. 2) was analysed by BLASTX, the translated sequence showed a significant homology to fungal and other eukaryotic *tannase* genes. The partial *tannase* sequence was named *antan1* and shared a 36% identity with tannase precursor *A. oryzae* and 29% with *Debaryomyces hansenii* tannase at the amino acid level.
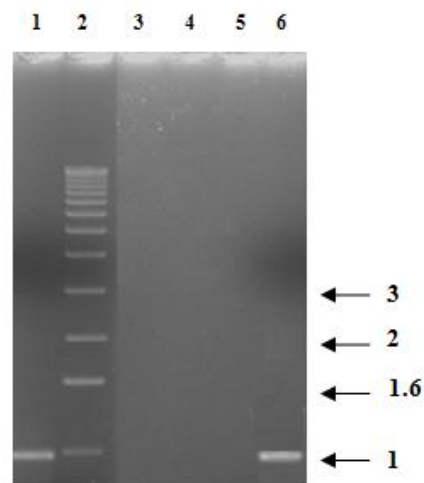

Fig. 1: Agarose gel electrophoresis of PCR products

PCR was performed and products were visualised by agarose gel electrophoresis. Lanes 3, 4 and 5 show the results of the PCR in the absence of genomic DNA or forward primer (lane 4) or reverse (lane 5). Lanes 1 and 6 show the PCR products obtained with both primers. Lane 2 shows the molecular weight marker, with band sizes (Kb) indicated at the right

Southern analysis

The 950bp insert from plasmid pGEM®-T Easy vector was cut from the plasmid with *Eco* RI and labelled with digoxigenin and was used to probe *A. niger* DNA restricted with *Apa* I, *Asp* 718, *Hind* III, *Sac* I, *Sma* I, *Xba* I and *Xho* I. Southern analysis showed that the probe hybridised to a single band with each digest except *Sac* I (Fig. 3). The *Apa* I digest produced a band of 1.8Kb and a second band of 3.1Kb, the *Asp* 718 digest a 4.7Kb band, the *Hind* III digest a 6.5Kb band, the *Sac* I digest a band of 10.5Kb, the *Sma* I digest a 4.7Kb band, the *Xba* I digest a 1.4Kb band and the *Xho* I digest a 4.1Kb band.

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1    TTCTGCTCTG GATCGCAATC TGTTGAGCAA GTTTACGGCA CCGTGGTGTC ATTTCGAGGT
61   GTTTAAAACA GGCAGCTTTA TCCTATCGCA CAGGGGGCAT GCAGCAGGCG GGACGGTGTT
121  CGTTTTGTCG GGCCTACATC CAGGTTAAAC GCCGAGGCCG ACCCGGCTTC TCGCAAAGAA
181  CCCCCTAAGT AGGGAAATGC GGGAAAGCTT TCTCCACATG AAAAGCCCCG TTGCACCTGG
241  ATCGTCAGGC CACATTTTAA AACTATATGC CTACGTAACA AGCACTTTCA GATGTAGCAA
301  CGCGTCTCAA CAATTATTTT CGATAATTGG GGAGACATGA AACAGGTAAA CTTGGGCCCG
361  AGATGCCGAT GCAAATGCCA AAGTATGTAC TATATAAAGT ACCAGTTTTC CGCCTCTGAT
421  AAAGAGCGTT GAAGTCAGGT GGCTATACGA ATCGCGGTGA TGCTACTAGT ACAGCCTGGC
481  TTAATCTGGT AAAGTCAGTC CATGCCCTGC TTGGTATTAG CATAGCTTGC ATCATTACTT
541  CGGTCACTTC TCATCATTAT ATTCCACACA TCCTTGTTCT TTCATCTGAG ATGGGGTGTC
601  TGCCACTAGT CATCATCCTT GGGGCGACAC TGGTTCACGC CGTCACCACT TCCACCACTC
661  TCGATGATGT CTGCACCCCT GCATACGTTC GATCTAAGTT GCCCGTCGAT AATGTTTATC
721  CTGGGATCAC TATTAGCCAC ACCAACCTCT CCGCTAACCC TGTCTACAAC GTCTCTACTA
781  GCGGCTCTGC GGTATACCCA GCCACCACGA TCGACTATTG CAACGTAACC TTGGCCTACT
841  CCCACAATGG CCGGGATGAC ACCGTCCAGA TGCGCTTCTG ATTATCCTCT CCCCCATCAA
901  TCACTAGT
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NCBI gene bank accession No.: AY727901

LLWIAIC-ASLRHRGVISRCLKQAALSYRTGGMQQAGRCSFCRAYIQVKRRGRPGFSQRT
P-VGKCGKAFST-KAPLHLDRQATF-NYMPT-QALSDVATRLNNYFR-LGRHETGKLGPE
MPMQMPKYVLYKVPVFRL--**RALKSGGYTNRGDATSTAWLNLVKSVHALLGISIACIITS
VTSHHYIPHILVLSSEMGCLPLVIILGATLVHAVTTSTTLDDVCTPAYVRSKLPVDNVYP
GITISHTNLSANPVYNVSTSGSAVYPATTIDYCNVTLAYSHNGRDDTVQMRF**-LSSPPSI
TS

Fig. 2: Sequence of a partial tannase gene from *A. niger* (*antan1*) and its translation using Expasy bioinformatic software. A valid part of translated amino acid is indicated in **Bold**. Results of nucleotide sequence analysis have been highlighted as described in discussion

Northern analysis

Cultures of *A. niger* were grown for 72h in modified Vogel's and after RNA extraction, the quality of total *A. niger* RNA was examined by non-denaturing gel electrophoresis. Two bands representing the 18S and 28S rRNA were clearly visible. After membrane blotting, the membrane was probed with the same digoxigenin labeled probe as used for Southern analysis. Northern analysis revealed a single transcript of ca. 3.8Kb hybridised to the *antan1* probe at stringency condition (a) and the other at non-stringency condition (b) (Fig. 4)
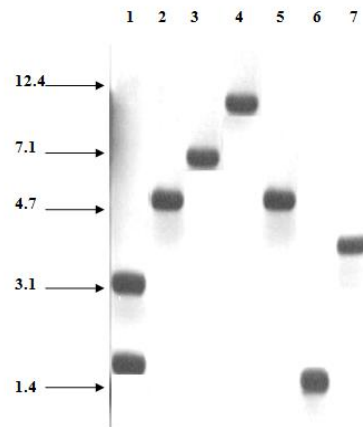

Fig. 3: Southern analysis of *A. niger* genomic DNA;

A. niger genomic DNA was extracted, restriction digested and subjected to agarose gel elctrophoresis. Following blotting, the membrane was hybridised with probe. Lane 1 shows the molecular weight marker, with band sizes (Kb) indicated to the left. Lanes 1-7 shows *A. niger* genomic DNA restricted with *Apa* I, *Asp* 718, *Hind* III, *Sac* I, *Sma* I, *Xba* I *and Xho* I respectively.
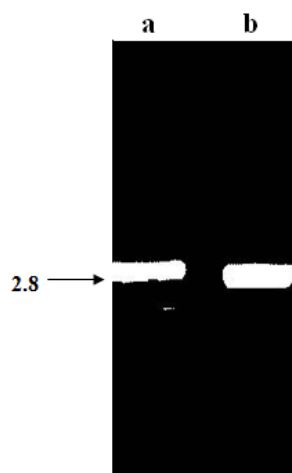
Fig. 4: Northern analysis of *A. niger* total RNA using the *antan1* probe

Total RNA was extracted from crushed mycelia and subjected to non-denaturing gel electrophoresis (a). Following membrane blotting, the membrane was cut into two and one half hybridised with the probe of tannase at stringency condition (a) and the other at non-stringency condition (b).

Discussion

Although during last few decades some tannase enzyme have been isolated, purificated and characterizated from *Aspergillus* species (*A. oryzae, A. kawachii, A. avuleatus, A. niger, A. awamori*) and described [4,7,8]; so far, the only genomic sequence of a functional tannase gene has been described from *A. oryzae* [12]. Both tannase from *A. niger* and the ß-glucosidase from *A. kawachii* are partially intracellular when produced by liquid fermentation, extracellular under solid culture conditions [8] and also thermostable and unspecific. Such bifunctionality may be a survival way for micro-organisms such as *Lactobacillus plantarum* and *A. niger* during natural selection period to survive in extreme environments or to efficiently degrade cellulose and tannins, abundant in decaying plant material [8,12,14-17].

It is unknown whether the two functions (esterase for the tannase and hydrolase for the ß-glucosidase) are carried out by one or two domains within the protein structure [18]. However there is a low chance that a single-domain protein, highly homologous to a known enzyme, has a different function [3,14]. In the present study, we cloned the partial sequence of gene the encoding tannase in *A. niger* and analyzed the sequence.

Nucleotide sequence analysis

The obtained sequence was a 908bp without any intron that encodes a 302 amino acid protein. From this 302 amino acid sequences only a 152 amino acid motif look like a valid motif that has been highlighted (Bold) at Figure 2. The amino acid sequence exhibited high levels of homology with the amino acid sequences of two tannase genes at NCBI.

To obtain partial sequence, we were neither able to recognize intron(s), TATAA-like, CCAAT (CAAT box), poly (A) sequences and nor predict starting or stopping codons and the CT rich sequence before starting codon. Therefore we were not able to hypothesize the C- and N terminal of peptide. Five possible CREA binding sites (5'-[G/C][C/T]GG[G/A]-3') [19] were present at positions 198, 592, 629, 721, and 851bp (under lined sequences) in the nucleotide sequence of motif suggesting significant carbon catabolite repression of tannase gene and deacylase activity can be affected at different carbon sources.

This sequence encoding is a binding site for the carbon catabolitite repression transcripn factor CREA [19]. Also no binding site was found for the transcriptional regulator PacC that regulate gene expression in response to external pH [20] (GCCARG) suggesting tannase gene is not PH regulated. Consensus internal intron sequence, TACTCAC did not found between GTATGT sequence and CAG sequence at 5' and 3' end of the above motif despite the presence of these

sequences (Bold highlighted) in motif so it look like intronless motif as tannase full sequence from *A. oryzae* that has been cloned by Hatamoto *et al.* [12].

Also sequence encoding a binding site for the carbon catabolite repression transcription factor CREA [19] (SYGGRG) was not present in the amino acid level of motif suggesting no or very poor carbon catabolite repression of tannase gene and deacylase activity was found not to be significantly affected at different carbon sources. Also no binding site was found for the transcriptional regulator PacC [20] that regulate gene expression in response to external pH (GCCARG) suggesting tannase gene is not pH regulated [21].

Comparison of amino acid sequences

We searched the SwissProt database for sequences similar to the deduced amino acid sequence of obtained sequence. Significant levels of similarity were found with Tannase precursor (EC 3.1.1.20) [Contains: Tannase 33 kDa Subunit and Tannase 30kDa subunit] from *A. oryzae* with 37% identities (Medline and PubMed accession No. 97074675; & 8917102 respectively) and below characters: EMBL; D63338; BAA09656.1; PIR; JC5087; JC5087; InterPro; IPR011118; Tannase; Pfam; PF07519; Tannase; 1. and Similar sequence to Sp. P78581 *A. oryzae* from *Debaryomyces hansenii* chromosome A of strain CBS767 with 29% identity and below character: EMBL; CR382133; CAG84949.1; InterPro; IPR011118; Tannase; Pfam; PF07519; Tannase; 1.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been submitted to NCBI GenBank under accession number AY727901. We emphasize that additional experiments are needed to clone and sequencing the full length of tannase gene from *A. niger* and then more fully understand the factors that regulate the activity and control the destination of enzyme and it's probable role in medical field [22]. Also additional studies of tannase mRNA transcription in solid and broth cultures are needed to understand the effective factors for expression of enzyme.

Conclusion

In conclusion, the ease of *A. niger* culture, the high level of enzyme production on different media, the high activity of tannase from this mould, and its very high tolerances to acidic condition and high temperature (70°C) are particularly favourable for the application of this enzyme in the biotechnological industry.

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