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Purification and characterization of tannase and tannase gene from *Enterobacter* sp.

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ABSTRACT

Tannase of *Enterobacter* sp. was purified and characterized at molecular level. It was found to be 90 kDa in molecular weight. The purified enzyme showed maximum activity at 40 °C. The enzyme was also found to be active in acidic range of pH. The nucleotide and amino acid sequence of tannase exhibited resemblance with the other reported tannase sequences of bacteria, fungi and plants. Probably, this is the first report of tannase gene in *Enterobacter* sp. The investigation suggests that the purified enzyme can be useful to synthesize molecules of pharmaceutical interest. In addition to above, the enzyme tannase and the organism itself can also be employed to protect grazing animals and environment against the toxic effects caused by tannins in them.

1. Introduction

Tannins are defined as naturally occurring water soluble polyphenols of varying molecular weight. They represent the fourth most abundant group of plant's secondary metabolites after cellulose, hemicellulose and lignin [1] and second to lignin in plant phenolics [2]. These molecules are divided into two major groups; hydrolysable and condensed tannins. Hydrolyzable tannins are esters of gallic acid and ellagic acid with a sugar core. These are readily hydrolyzed by the action of either enzymes or acids into their respective monomeric units. Besides this, the condensed tannins also known as proanthocyanidins, are lacking in sugar core and are composed of flavanoids (flavan 3-ol or flavan 3,4-diol) units [3].

Generally tannins are quite resistant to microbial attack and thus are recalcitrant to biodegradation [4]. These compounds retard the rate of decomposition of soil organic matter and therefore slower down the process of soil formation [1]. Tannins are also responsible to reduce food digestibility in animals [3]. The above facts were suggested due to their ability to form complex with proteins, digestive enzymes including amylase, lipase, protease, β-galactosidase, cellulase and to a lesser extent with other macromolecules like cellulose and pectin by their phenolic hydroxyl groups [5]. In spite of the above harmful effects, few small molecules of tannins such as monomeric, dimeric and trimeric tannins had shown less inhibitory effect on proteins and enzymes. Moreover, these small molecules were also found to be associated with valuable pharmacological activities. This dual nature of tannins was recognized by Chung et al. [6] and thus they crowned tannins as double edged sword in biology. It was further supported by the fact that herbs containing smaller molecules of tannins possess anticarcinogenic activity, host-mediated antitumor activity, antiviral activity, etc. [7]. Therefore biodegradation seems one of the efficient methods to degrade larger molecule of tannins into small tannin molecules with valuable biological activities.

The degradation of tannins is initiated by tannase or tannin acyl hydrolase enzyme in living organisms. Many fungal species belonging to genera *Aspergillus* [8], *Paecilomyces* [9] and *Rhizopus* [10], etc. are very well characterized for tannase production. Besides fungi, bacterial species such as *Lactobacillus plantarum* [11] and *Bacillus licheniformis* [12] have been also identified as tannase producers. However, little is known about the genes encoding for bacterial tannases and their properties. In one study Noguchi et al. [13] investigated the association of tannase producing *Staphylococcus lugdunensis* with colon cancer and suggested that it tannase can be a good marker to identify *S. lugdunensis*. In this organism a gene named as tanA, encodes for a polypeptide of 613 amino acids of tannase activity. *L. plantarum* ATCC 14917^T was also isolated from fermented food products and characterized for various biochemical and molecular properties by Iwamoto et al. [14]. In this bacteria a regions of 1410 bp of bacterial genome code for a polypeptide consisting of 469 amino acids residues. It was also found 28.8% identical

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to tannase sequence of *S. lugdunensis*. But tannase of *L. plantarum* did not show any similarity in size, pH optima and kinetic constant to that of *Aspergillus oryzae*. So the present investigation was designed to characterize tannase at molecular level in *Enterobacter* sp. not only because of its importance in bioremediation, animal nutrition and pharmaceuticals but also because of inadequate information about tannase gene in literature till date.

2. Materials and methods

2.1. Chemicals

All chemicals unless specified otherwise were obtained from Sigma Chemical, USA and were of certified reagent grade. Rhodanine was purchased from Merck-Schuchardt, Germany. Sephadex G-100 was supplied by Pharmacia Fine Chemicals, Sweden and DEAE cellulose was obtained from GE healthcare, USA. Chemicals and reagents used in gene identification and cloning were purchased from Invitrogen, USA, and Bangalore Genei, India. Dialysis tubing of 10–12 kDa cut off and bacteriological medium were purchased from Hi-media, Mumbai, India.

2.2. Microorganism and growth conditions

A Gram negative bacterium which was identified as *Enterobacter* sp. on the basis of morphological, biochemical and 16S ribosomal RNA gene sequence features, isolated from normal soil to purify and characterize tannase and tannase gene. *Escherichia coli* DH5 α and pGEM® T Easy (Promega, Madison, USA) were used for cloning of tannase gene sequences. *Enterobacter* culture was maintained on a solid medium containing 0.05% of each KH_2PO_4, K_2HPO_4 and $MgSO_4$, 0.3% NH_4NO_3, 0.5% tannic acid and 2% agar. *E. coli* DH5 α cells were maintained on nutrient agar.

2.3. Enzyme assay

Tannase activity was determined spectrophotometrically by the method given by Sharma et al. [15]. In this method tannase reacts with methyl gallate and releases gallic acid which reacts subsequently with rhodanine and forms a complex. The formation of above complex (gallic acid and rhodanine complex) was recorded at 520 nm.

2.4. Purification

Purification was done by the methodology used by Zeida et al. [16]. The cells were grown overnight in nutrient broth medium of pH 7. 10% (v/v) overnight culture was used to inoculate a litre of defined medium of pH 6.0. (15 g tannic acid, 5 g sucrose, 2 g $(NH_4)_2HPO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4$, and 0.5 g yeast extract). The inoculated medium was incubated at 35 °C for 24 h with constant shaking (200 rpm). Cells were harvested by centrifugation (Sigma 2 16-PK) at 5000 × *g* for 10 min at room temperature. The pellet obtained was washed twice with 50 mM citrate buffer of pH 5.0 (containing 1 mM DTT and 50 mM $Na_2S_2O_3$) and suspended in minimum amount of the same buffer. The resting cells suspension was quantified for tannase enzyme activity and lysed by ultrasonication for 2 min in an ultrasonicator (Sonics Material Inc., USA) at 4 °C by giving pulse of 9 s on and 5 s off. The lysate was centrifuged at 4 °C for 15 min at 13,000 × *g*. The supernatant obtained was quantified for tannase activity. The protein concentration was also determined by Bradford method [17].

The lysate was further used as a source of crude enzyme for purification. The crude enzyme was fractionated with ammonium sulphate in increasing concentration. Three fractions of ammonium sulphate precipitation (0–30%, 30%, 60% and 60–100%) were obtained at 4 °C with constant gentle shaking. Each precipitated samples were suspended in 1 ml of 50 mM citrate buffer of pH 5.0 and dialyzed overnight against the buffer (50 mM citrate buffer of pH 5.0) containing 1 mM DTT and 50 mM $Na_2S_2O_3$ at 4 °C in dialysis tubing. The activity and protein content were determined as described above in all dialyzed samples.

Proteins precipitated at 30–60% concentration of ammonium sulphate subjected to dialysis and were loaded onto a pre equilibrated DEAE cellulose column (bed volume 20 ml) (Hi Prep™ 16/10 DEAE FF column). 50 mM citrate buffer of pH 5.0 containing 1 mM DTT and 50 mM $Na_2S_2O_3$ was used as pre equilibration buffer. Elution was done with increasing concentration of NaCl (0–0.5 M) in 50 mM citrate buffer of pH 5.0 containing 1 mM DTT and 50 mM $Na_2S_2O_3$ at room temperature with a flow rate of 5 ml/min. The eluted fractions were checked for tannase activity and active fractions were pooled. Pooled samples were concentrated by freeze-drying method and loaded onto G-100 Sephadex column (bed volume 60 ml). The protein was finally eluted with 50 mM citrate buffer of pH 5.0 containing 1 mM DTT, 50 mM $Na_2S_2O_3$ and 50 mM NaCl at a flow rate of 0.3 ml/min. The activity, protein content and purity were determined and the purified enzyme was characterized in terms of pH optima, temperature optima and K_m.

2.5. Effect of pH and temperature on enzyme activity

Effect of pH on purified enzyme was studied over a pH range of 4.0–6.5. The buffer systems used were 50 mM citrate buffer for pH 4.0–5.5 and 50 mM phosphate buffer for pH 6.0 and 6.5. To study the effect of temperature, the activity was determined at different temperatures (25–60 °C) under standard enzyme assay conditions.

2.6. Kinetic constant of Enetrobacter sp. tannase

Kinetic constant of tannase for most commonly used substrate methyl gallate (1–10 mM) was determined. The reactions were conducted under standard assay conditions. K_m value was calculated out using the Lineweaver–Burk transformation of Michaelis–Menten equation.

2.7. Isolation of genomic DNA

Genomic DNA was isolated from *Enterobacter* sp. by following the methodology of Laing et al. [18]. The purity and amount of DNA was calculated spectrophotometrically. Isolated genomic DNA was subjected to PCR along with various sets of degenerate primers.

2.8. PCR amplification, cloning and sequencing of tannase gene

The tannase gene sequences from five members of different classes (α, β and γ) of phylum proteobacteria (*Klebsiella* sp., *Pseudomonas* sp., *Ralstonia* sp., *Burkholderia* sp. and *Roseovarius* sp.) were aligned. Four sets of degenerate primers were designed from various locations of alignment file. Following pairs of primers were used to amplify tannase gene sequence in isolated organism; F1: 5′-Cgg HTg Cgg Ygg NYT gTg Cgg SA-3′; R1:5′-CCR TgC CAS Agg ATM AAK YTT gCC gCC-3′, F2: 5′-ggY TgC TCN AAY ggN ggN CgC VAR gCg-3′; R2: 5′-AAg ARS CgC gCR AAN gNC STS NgC-3′, F3: 5′-gAN TTC gAC ggC ATY NTC gCN ggC-3′; R3: 5′-ATC ATY TTg CCR CCN CgN T-3′, F4: 5′-CBg AYg ANT TCg ACg gYA TCN TCg NC-3′; R4: 5′-CCg CCN CgC CAR TgN BDC ATN CCN ggC-3′. The PCR mixture (25 μl) consisted of 2.5 μl of 10× buffer containing 10 mM $MgCl_2$, 0.4 μl of each dNTP (10 mM) 1 μl of each forward and reverse primer (10 μM), 2 μl of template (100 ng) and 0.25 μl of Taq DNA polymerase (5 U/ml). PCR was carried out in a thermal cycler [Gene Amp PCR system 9700 (Applied Biosystem, USA)] according to a programme encompassing initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 1 min, 72 °C for 2 min and final extension at 72 °C for 5 min. PCR products were electrophoresed on 1% agarose gel in 1× Tris–acetate–EDTA (0.04 M Tris–acetate, 0.001 M EDTA) buffer at 75 mAmp for 1.5 h. The gel was stained and desired fragment was excised, purified (Gen Elute™ Gel Extraction Kit of Sigma) and cloned into pGEM-T Easy cloning vector according to manufacturer instructions.

Transformed *E. coli* DH5α cells carrying the recombinant plasmid were selected by blue–white selection. The cloned product was sequenced by primer walking method using a BigDye terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, USA) in an ABI Prism 3130 *xl* Genetic Analyzer (Applied Biosystems) by following the manufacturer's instructions. Editing was done to remove the vector sequence and sequence was characterized with tannase gene sequences already available in GenBank using the BLAST programme (www.ncbi.nlm.nih.gov/blast/blast.cgi).

2.9. Characterization of tannase sequence

Tannase nucleotides and amino acids sequence of *Enterobacter* sp. was deposited in the GenBank database of NCBI. The deduced amino acid sequence was characterized in terms of percentage homology with the available tannase sequences through ClustalW2 (www.ebi.ac.uk).

3. Results and discussions

3.1. Purification and characterization

In the present investigation the enzyme tannase was purified from its native conditions. Osawa et al. [11] and Mondal and Pati [12] isolated bacterial species for tannase production but none of them characterized tannase at molecular level. Tannase was finally purified with a yield of 7.1% by using DEAE cellulose followed by Sephadex G-100 column. Bhardwaj et al. [8], Mahendran et al. [9] and Iwamoto et al. [14] also purified tannase from various sources and obtained the yield of process as 20%, 17% and 4.8%, respectively. In addition to it, the tannase of *Enterobacter* sp. was eluted as a single peak through DEAE cellulose and Sephadex G-100 column in the present investigation which is different from the observations made by Bhardwaj et al. [8] during the purification of tannase