Degradation of tannic acid and purification and characterization of tannase from *Enterococcus faecalis*

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**Abstract**

Tannins, present in various foods, feeds and forages, have anti-nutritional activity; however, presence of tannase in microorganisms inhabiting rumen and gastrointestinal tract of animals results in detoxification of these tannins. The present investigation was carried out to study the degradation profile of tannins by *Enterococcus faecalis* and to purify tannase. *E. faecalis* was observed to degrade tannic acid (1.0% in minimal media) to gallic acid, pyrogallol and resorcinol. Tannase from *E. faecalis* was purified up to 18.7 folds, with a recovery of 41.7%, using ammonium sulphate precipitation, followed by DEAE-cellulose and Sephadex G-150. The 45 kDa protein had an optimum activity at 40 °C and pH 6.0 at substrate concentration of 0.25 mM methyl gallate.

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1. Introduction

Tannins are the most common plant secondary metabolites that are poorly understood with respect to feeding habits, with both beneficial and adverse effects in ruminants (Getachew et al., 2000). Tannins (hydrolysable (HT) and condensed (CT)), being antimicrobial; resist microbial attack and remains as recalcitrant in the environment (Field and Lettinga, 1992). Though destructive in nature, the biological systems often have difficulty in removing toxic polyphenols to consistently low levels. However, some rumen microorganisms are shown to possess the ability to grow on tannins and degrade HTs, with a few evidences showing degradation of phenol ring of CTs (Bhat et al., 1998). These microorganisms have developed protective mechanisms such as secretion of binding polymers and synthesis of tannin resistant enzymes for their biodegradation (Goel et al., 2005a). A number of reviews on tannin biodegradation have appeared in the past, providing a general idea on the biodegradation of these polyphenols (William et al., 1986; Bhat et al., 1998). It is reported that tannase, the key enzyme, is involved in the hydrolysis of tannins (Aguilar and Gutierrez-Sanchez, 2001). The tannase catalyse the hydrolysis of ester and depside bonds presents in the hydrolysable tannins and gallic acid esters (Lekha and Lonsane, 1997). Among tannin degrading microbes, aerobic bacterial degraders and fungal species have been reviewed elsewhere (Bhat et al., 1998; Goel et al., 2005b). The fungi have a better activity in degrading hydrolyzable tannins however, a major problem in the utilization of fungal strains for industrial applications is that degradation by fungi is relatively slow (Beniwal et al., 2010). On the other end of the spectrum, bacterial tannase can degrade and hydrolyze natural tannins and tannic acid very efficiently (Lewis and Starkey, 1969; Deschamp et al., 1983). Therefore, the present investigation was undertaken to study the degradation profile of an anaerobic *Enterococcus faecalis* isolated from faeces of goat and to characterise tannase from this bacterium.

2. Materials and methods

2.1. Biodegradation of tannic acid

Tannic acid degrading *E. faecalis*, used in the present investigation was isolated from goat faeces (Goel et al., 2007). The overnight culture was centrifuged and cell pellet was collected and mixed (at the rate 10^6 cfu/ml) to the minimal media supplemented with 1% filter sterilized tannic acid (Nelson et al., 1995). The culture bottles were sealed under CO₂ and incubated at 37 °C for 72 h. The...
samples for analysis were removed aseptically from the bottles after every 12 h interval. The samples were kept at -20 °C till further analysis. The tannic acid degraded products were analysed by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). All the experiments were done in triplicates.

2.1.1. Thin Layer Chromatography (TLC)

Thin Layer Chromatography was done according to the protocol of Sharma et al. (1999). Briefly, slurry of silica (40 g in 80 ml of distilled water) was spread on glass plates (20 × 20 cm) to a thickness 0.5 mm. The plates were allowed to dry and were activated by placing in an oven at 110 °C for 1 h. The samples were centrifuged at 2000 × g and the supernatant was transferred in fresh tube. An aliquot of 10 μl was spotted on the TLC plate. A mixture of ethyl acetate: chloroform: water (50:50:1) was used as mobile phase and the detection was done using iodine vapours in a saturated chamber. Rf values were calculated and compared with the standards of gallic acid, pyrogallol and resorcinol.

2.1.2. RP-High Performance Liquid Chromatography

Tannin degraded products were estimated using RP-HPLC by the modified method of Makkar (2000). Briefly, the culture supernatant containing degraded tannic acid metabolites was filtered through Whatman filter paper. Equal volume of ethanol—ethyl acetate (95% EA + 5% ethanol) was added to the filtered broth. The contents were mixed, vortexed and stirred for 1 h. The contents were then transferred to separating funnel and partitioning was allowed for 1 h. The upper layer was collected and aliquots of 2 ml were made in duplicates. The solvents were removed under vacuum and the extracted samples were reconstituted by adding 2 ml HPLC grade methanol. The samples were filtered through Millipore membrane filter (0.22 μm) and the filtrate was used for gradient HPLC analysis using C18 spherisorb column (ODS 2, 4.6 × 250 mm, 5 μm particle size, 300 Å pore size) Waters HPLC system. The solvent system composed of degassed HPLC grade water (acidified with 0.025% phosphoric acid) and acetonitrile. Tannin degraded compounds were eluted by a gradient of water/acetonitrile from 100 to 0% in 30 min. The peaks were detected at two wavelengths of 250 nm and 280 nm.

2.2. Enzyme purification

E. faecalis was grown in minimal media containing 1% filter sterilised tannic acid (Sigma) for 24 h at 37 °C. The culture broth was centrifuged at 4000 × g for 20 min and the supernatant was used for purification of enzyme. The enzyme activity at each step was calculated as per Sharma et al. (1999). The enzyme activity and protein content (Lowry et al., 1951) at each step of purification was determined to calculate the specific activity.

2.2.1. Clarification and ammonium sulphate precipitation

The supernatant was treated with 1% activated charcoal to remove the colour and phenolic compounds (Mahendran et al., 2005) for 2 h with intermittent shaking. The filtrate was clarified by centrifugation at 4000 × g for 20 min at 4 °C. To 950 ml of clarified culture supernatant, ammonium sulphate was added to achieve 30–60% saturation and centrifuged at 16,000 × g for 20 min at 4 °C. The supernatant was subsequently adjusted to 80 and 100% saturation. The pellet in each case was dissolved in citrate buffer (0.05 M, pH 5.0). The enzyme preparation obtained after each ammonium sulphate fractionation step was dialysed using a 1000 MWCO (molecular weight cut-off) cellulose acetate membrane for about 18 h against citrate buffer (0.05 M, pH 5.0) with 3–4 intermittent changes of citrate buffer (0.05 M, pH 5.0).

2.2.2. DEAE-cellulose chromatography

A column was packed with DEAE-Cellulose to a bed size of 2.5 cm × 10 cm and was equilibrated with 0.05 M citrate buffer (pH 5.0). The elution was done in discontinuous gradient system with citrate buffer of increasing molarity (0.05, 0.1, 0.2, 0.3 M citrate buffer, pH 5.0). Five, 5 ml fractions for buffer of each molarity were collected and the fractions were monitored for the elution profile of protein and tannase activity as described above.

2.2.3. Gel filtration chromatography

Sephadex G-150 was soaked in 0.05 M citrate buffer (pH 5.0) overnight at room temperature and the fines were decanted off. The gel suspension, after deaerating for 10–15 min, was packed into a 55 cm × 2.5 cm glass column at the operating pressure head of 30 cm. The protein fractions were eluted from the column at a flow rate of 1 ml/min. Five millilitres fractions were collected after draining 80% of the void volume and analysed for tannase activity. The fractions showing tannase activity were pooled and concentrated using PEG-6000.

2.2.4. Characterization of tannase

The molecular weight of the tannase was determined by SDS-PAGE as per the protocol of Laemmli (1971). The molecular weight of the protein was determined by comparing with the protein marker standard (Low range marker from Biorad). The activity of partially purified tannase was studied at different temperatures (20, 30, 40, 50, 60 and 70 °C), pH (pH 2.0–9.0) and substrate concentration (methyl gallate at 0.0625, 0.125, 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0 and 5.0 mM). The enzyme activity was determined by the rhodanine method (Sharma et al., 1999).

3. Results and discussion

3.1. Degradation of tannic acid

Tannic acid degradation profile was observed using TLC and HPLC. The thin layer chromatogram showed two identification spots, namely gallic acid and pyrogallol when compared with standards. On TLC plate, tannic acid remained at the bottom line followed by gallic acid, pyrogallol and resorcinol with an Rf value of 0.26, 0.59 and 0.61, respectively. Gallic acid and pyrogallol were observed as major degraded product of tannic acid after 72 h of incubation (Fig. 1). However, HPLC results indicated that E. faecalis was able to degrade tannic acid to pyrogallol and further to resorcinol within 72 h of incubation. The retention time of 4.31 min, 4.57 min and 6.05 min was observed for gallic acid, pyrogallol and...
resorcinol, respectively. The peak area of the tannic acid at retention time (11.0 min) declined sharply with time of incubation with simultaneous increase in the peak area of pyrogallol.

The degradation profile of tannic acid by *E. faecalis* (Table 1) shows that initially at 12 h of incubation period, only gallic acid was formed indicating a 17% degradation of tannic acid. Further increase in incubation period up to 24 h resulted in maximum gallic acid formation and pyrogallol and traces of resorcinol. Pyrogallol was the major product after 48 h and 72 h of incubation. However, at 48 h of incubation period, all the metabolites i.e. gallic acid, pyrogallol and resorcinol were observed in significant amount resulting in 55% degradation of tannic acid and finally after 72 h of incubation, 92% of tannic acid was mineralized into pyrogallol and resorcinol as the major end products.

Several other ruminal microorganisms were reported to possess the ability to degrade phenolic monomers. The results are in agreement with reports from Tsai and Jones (1975) who showed that the isolated *Streptococcus* strains and *Coprooccus* strains from the bovine rumen were able to degrade 80% of phloroglucinol. Odenyo and Osuji (1998) have reported three strains of a tannin-tolerating bacterium (*Selenomonas* sp.) from the rumen microflora of sheep, goat and antelope that had either been fed or had browsed on tanniniferous forages. One of the strains (EAT2) of this ruminal bacterium could hydrolyse tannic acid to gallic acid and subsequently to pyrogallol, whereas the remaining two strains (ES3 and EG19) were able to hydrolyse tannic acid to gallic acid only. Zeida et al. (1998) also reported a combined activity of tannase and gallate decarboxylase activity in *Pantoea* agglomerans T71 which can be used for the biocconversion of tannic acid to pyrogallol. *Escherichia* oxidoreducens, a strictly anaerobe was reported to degrade gallate, phloroglucinol and pyrogallol to acetate and butyrate in the presence of hydrogen and formic acid (Krumholz and Bryant, 1986). Another anaerobe, *Syntrophococcus sucomutans* was reported to have ability to demethoxylate various phenolic monomers (Krumholz and Bryant, 1988). The degradation of tannic acid by our isolate indicates that the tannin-protein complexes, which were thought to be hydrolysed only under the acidic condition of the abomasum, may also be subject to microbial degradation in rumen.

### 3.2. Enzyme purification

The results pertaining stepwise purification of tannase are presented in Table 2.

#### 3.2.1. Clarification and ammonium sulphate precipitation

Culture filtrate treated with 1% activated carbon removed more than 80% of the colour according to the visual observation. The colour due to oxidation of gallic acid was removed by activated charcoal. The clarified filtrate was having an activity of 32.51 U/ml with a yield of 94.7%. Fractional precipitation with 50% ammonium sulphate removed some of the nonenzymatic proteins. Tannase was precipitated at 70% saturation. About 89.8% of the total tannase was recovered by 70% saturation with activity of 975.64 U/ml. Rajkumar and Nandy (1983) obtained a 69% of total recovery from ammonium sulphate at 100% saturation. Mahendran et al. (2005) obtained 78.7% of the total tannase at 70% saturation with ammonium sulphate. Chhokar et al. (2010a) reported maximum recovery of tannase at 80% ammonium sulphate fractionation.

#### 3.2.2. DEAE-cellulose chromatography

The 70% saturated fraction was further purified through DEAE-cellulose ion exchange chromatography. The elution profile yielded a single protein peak corresponding with the tannase activity. The tannase at this stage has total activity of 8980 U with a specific activity of 2986 U/mg. From DEAE-cellulose chromatography, the overall purification of 25.7 fold with a yield of 27.5% was obtained. Hamdy (2008) purified tannase from *Rusarium subglutinans* with 26.98 fold purification and 42.6% recovery by ammonium sulphate, ion exchange chromatography (DEAE-cellulose) and gel filtration (Sephadex G-150). An extracellular tannase from *A. heteromorphus* MTCC 8818 was also purified through ammonium sulphate precipitation and ion exchange chromatography leading to an

### Table 1

<table>
<thead>
<tr>
<th>Incubation Time (h)</th>
<th>Degraded products (mg/ml)</th>
<th>Tannic acid (TA) % TA degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gallic acid (GA)</td>
<td>Pyrogallol (PYR)</td>
</tr>
<tr>
<td>12</td>
<td>0.16 ± 0.013</td>
<td>0.09</td>
</tr>
<tr>
<td>24</td>
<td>0.28 ± 0.022</td>
<td>0.11 ± 0.011</td>
</tr>
<tr>
<td>36</td>
<td>0.20 ± 0.012</td>
<td>0.16 ± 0.023</td>
</tr>
<tr>
<td>48</td>
<td>0.11 ± 0.020</td>
<td>0.21 ± 0.034</td>
</tr>
<tr>
<td>60</td>
<td>0.08 ± 0.123</td>
<td>0.24 ± 0.077</td>
</tr>
<tr>
<td>72</td>
<td>0.00</td>
<td>0.35 ± 0.014</td>
</tr>
</tbody>
</table>

Values presented are mean ± SD.

<table>
<thead>
<tr>
<th>Time (h)</th>
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<tr>
<td>12</td>
<td>0.012</td>
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<tr>
<td>24</td>
<td>0.20</td>
<td>0.07</td>
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<td>36</td>
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<td>48</td>
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<td>60</td>
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<tr>
<td>72</td>
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</table>

### Table 2

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Tannase activity (U/ml)</th>
<th>Total Activity (U)</th>
<th>Protein (mg/ml)</th>
<th>Total Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>1000</td>
<td>32.58</td>
<td>32,580</td>
<td>0.29</td>
<td>290</td>
<td>112.3</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Clarification</td>
<td>950</td>
<td>32.51</td>
<td>30,884</td>
<td>0.29</td>
<td>275.5</td>
<td>112.1</td>
<td>1</td>
<td>94.7</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>30</td>
<td>975.64</td>
<td>29,268</td>
<td>0.89</td>
<td>26.7</td>
<td>1096</td>
<td>9.7</td>
<td>89.8</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>10</td>
<td>898</td>
<td>8980</td>
<td>0.31</td>
<td>3.1</td>
<td>2896</td>
<td>25.7</td>
<td>27.5</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>15</td>
<td>906.81</td>
<td>13,602</td>
<td>0.43</td>
<td>6.45</td>
<td>2109</td>
<td>18.7</td>
<td>41.7</td>
</tr>
</tbody>
</table>

Total activity = Activity (U) × Total volume (ml).

Total protein = Protein (mg) × Total volume (ml).

Specific activity = Total activity (U)/Total protein (mg).

Purification fold = Specific activity of the sample/Initial specific activity.

Yield = [Total activity of the sample/Initial total activity] × 100.
overall purification of 39.74 fold with a yield of 19.29% \cite{Chhokar et al., 2010b}.

### 3.2.3. Sephadex G-150 chromatography

The elution profile of the enzyme from gel filtration resulted in four protein peaks of which two were prominent. One of the prominent peak between fractions from 14 to 28 coincides with the peak of tannase activity, thus those fractions were pooled and concentrated. The tannase activity was not observed in other peaks. The tannase at this stage has total activity of 13,602 U with a specific activity of 2109 U/mg. At gel filtration the enzyme was purified up to 18.7 folds with a yield of 41.7\% \cite{Rajkumar and Nandy, 1983} obtained 18.5\% recovery from the Sephadex G-200 column eluted with 0.02 M acetate buffer. \cite{Sharma et al., 1999} also obtained four peaks of the protein content with a single peak of tannase activity where the purification was obtained upto 29 folds with 2.7\% recovery of the total tannase. \cite{Mahendran et al., 2005} obtained three protein peaks when tannase was eluted from Sephadex G-200, yielding about 17.6\% of total tannase.

### 3.3. Molecular weight (Mr) determination

The purified protein resulted in a single band of 45 kDa (Fig. 2). The tannases with a molecular weight of 59 kDa from \textit{Selenomonas ruminantium} \cite{Skene and Brooker, 1995} and 45 kDa from \textit{Paecilomyces variotii} \cite{Mahendran et al., 2005} have already been reported. However, \cite{Yamada et al., 1968} and \cite{Hatamoto et al., 1996} have reported that the multimeric tannases from \textit{Aspergillus flavus} and \textit{Aspergillus oryzae}, respectively, with molecular weight of 186 to over 300 kDa. \cite{Bhardwaj et al., 2003} reported that the total tannase activity was made up of nearly equal activity of esterase and depsidase with a molecular weight 102 and 83 kDa.

### 3.4. Properties of tannase

#### 3.4.1. Effect of temperature

The temperature for the optimum activity of tannase from \textit{E. faecalis} was 40°C (Fig. 3). With the further increase in temperature, the tannase activity was found to decline sharply with no activity at 80°C as increase in temperature increases the rate of denaturation of the enzyme, with the loss of secondary and tertiary structures \cite{Sabu et al., 2005}. Tannase from other sources like \textit{A. oryzae}, \textit{Penicillium chrysogenum}, \textit{Aspergillus niger} have been reported to have their optimal activity at 30–40°C \cite{Iibuchi et al., 1968; Rajkumar and Nandy, 1983; Lekha and Lonsane, 1997; Sabu et al., 2005}. A lower temperature optima (30°C) has been reported for tannase from \textit{A. niger} Van Tieghem MTCC 2425 \cite{Sharma et al., 1999} and \textit{Aspergillus awamori} \cite{Chhokar et al., 2010a}.

#### 3.4.2. Effect of pH

The tannase from \textit{E. faecalis} possess an activity between 5.0 and 7.0 with an optimum activity at pH of 6.0 (Fig. 4). The same pH range was reported for other tannase obtained from \textit{A. niger} \cite{Ramirez-Coronel et al., 2003; Srivastava and Kar, 2009}, \textit{Cryphonectria} \cite{Farias et al., 1994} and \textit{P. variotii} \cite{Mahendran et al., 2005; Battestin and Macedo, 2007}. However, tannase from \textit{A. niger} Van Tieghem MTCC 2425 as reported by \cite{Sharma et al., 1999} resulted in two different peaks of tannase activity at a pH of 6.0 and a secondary peak at 4.5 which were reported to be due to different depsidase and esterase activities \cite{Barthomeuf et al., 1994; Lekha and Lonsane, 1997}.

#### 3.4.3. Effect of substrate concentration

The analysis of the graph of substrate concentration against tannase activity yielded \textit{Km} values of 0.43 μmol. This implies that tannase of \textit{E. faecalis} has higher affinity for methyl gallate. The \textit{Km} values for tannases from \textit{A. flavus}, \textit{S. ruminantium}, \textit{Cryphonectria}, \textit{A. niger} Van Tieghem using methyl gallate as substrate have been found to be 0.86 mM \cite{Yamada et al., 1968}, 1.6 mM \cite{Skene and Brooker, 1995}, 7.49 mM \cite{Farias et al., 1994} and 0.2 mM \cite{Sharma et al., 1999}, respectively.

From the present investigation, it can be concluded that the potential tannin degrading microorganisms reside in the gut of small ruminants. Being grazers, goats are found to be more adapted to tannins in feeds as they harbour tannin degrading microflora \textit{in situ}. Hence, a considerable potential exists for the exploitation of such potential isolates using \textit{in vivo} studies in order to use them for trans-inoculation into other animal species to improve the nutritional status and productivity of the livestock. The results presented in this work shows that \textit{E. faecalis} tannase has interesting and attractive properties for industrial use for production of pyrogallol.
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