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Characterization of tannase activity in cell-free extracts of
***Lactobacillus plantarum* CECT 748^T**

Héctor Rodríguez^a, Blanca de las Rivas^a, Carmen Gómez-Cordovés^b,
Rosario Muñoz^{a*}

^aDepartamento de Microbiología and ^bDepartamento de Tecnologías Sectoriales,
Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid

*Corresponding author. Tel.: +34-91-5622900; fax: +34-91-5644853

E-mail address: rmunoz@ifi.csic.es

25   **Abstract**

26

27   In foods, tannins are considered nutritionally undesirable. Spectrophotometric
28   methods have been used to detect tannin degradation by *L. plantarum* strains isolated
29   from food substrates. Enzymatic degradation of tannic acid by *L. plantarum* CECT 748^T
30   was examined in liquid cultures and in cell-free extracts by HPLC. Significant
31   reduction of tannic acid was not observed during incubation in the presence of *L.*
32   *plantarum* cells after 7 days incubation. However, tannic acid was effectively degraded
33   by cell-free extracts of *L. plantarum* during 16 h incubation. We have partially
34   characterized *L. plantarum* tannase activity by measuring its esterase activity on methyl
35   gallate. Tannase activity was optimal at pH 5.0 and 30 °C, and showed nearly 75% of
36   the maximal activity at 50°C. The biochemical characteristics showed by *L. plantarum*
37   tannase are considered favourable for tannin biodegradation in the food-processing
38   industry.

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43   *Keywords:* *Lactobacillus plantarum*, Tannase, Phenolic compounds, Tannic acid, Gallic
44   acid.

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49   **1. Introduction**

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51   Vegetable tannins are present in a variety of plants utilized as food and feed.
52   High tannin concentrations are found in nearly every part of the plant, such as bark,
53   wood, leaf, fruit, root and seed. Tannins also widely occur in common foodstuffs such
54   as banana, strawberry, raspberry, blackberry, grape, mango, cashew nut, hazelnut,
55   walnut and so on. Drinks like wines and tea also contain these phenolic compounds.
56   Tannins are considered nutritionally undesirable because they inhibit digestive enzymes
57   and affect the utilization of vitamins and minerals, they may be involved in cancer
58   formation, and hepatotoxicity. Therefore, it is not advisable to ingest large quantities of
59   tannins, since they may constitute a risk of adverse health effects (Chung et al., 1998).

60   Vegetable tannins can be classified into hydrolyzable and non-hydrolyzable or
61   condensed tannins, being tannic acid the most representative hydrolyzable tannins.
62   Tannic acid is one of the most abundant reserve materials of plants. Tannase (tannin
63   acyl hydrolase, EC. 3.1.1.20) catalyzes the hydrolysis of ester and depside linkages in
64   hydrolyzable tannins like tannic acid. The products of hydrolysis are glucose and gallic
65   acid. Gallic acid is used in the synthesis of propyl gallate, which is mainly used as
66   antioxidant in fats and oils, as well as in beverages. Tannase is also extensively used for
67   the preparation of instant tea, acorn wine, coffee-flavoured soft drinks, clarification of
68   beer and fruit juices, and detannification of foods (Aguilar and Gutiérrez-Sánchez,
69   2001; Aguilar et al., 2007; Lekha and Lonsane, 1997).

70   Bacteria, yeast and filamentous fungi are known tannase producers (Bhat et al,
71   1998). Normally, intestinal microbes have capability of degrading and detoxifying
72   many harmful and antinutritional factors into simpler and non-toxic constituents.
73   Therefore, some gastrointestinal microbiota has developed the ability to degrade tannins

to innocuous compounds. Osawa et al. (2000) isolated lactobacilli with tannase activity from human feces and fermented foods. This enzymatic property may have an ecological advantage for these *Lactobacillus* strains, as they are often associated with fermentation of plant materials. Therefore, lactobacilli may play an important role when tannins are present in food and intestine, because they may hydrolyse tannins and decrease adsorption on the cells. In addition, many beverages and teas that are routinely consumed in our society have been reported to contain various hydrolyzable tannins with marked pharmacological activities. The presence of lactobacilli with tannase activity in the human alimentary tract may thus have a significant effect on the medicinal properties of tannins (Osawa et al., 2000).

Since i) tannase is an enzyme extensively used in the food, feed, beverage, and brewing industries, ii) *L. plantarum* tannase activity has only been detected by spectrophotometric methods using methyl gallate as substrate (Osawa et al., 2000; Nishitani and Osawa, 2003; Nishitani et al. 2004; Vaquero et al., 2004), and iii) there is a constant search for new sources of tannase with more desirable properties for commercial application, the aim of this study is to confirm tannase activity in *L. plantarum* CECT 748^T and to characterize it biochemically.

2. Materials and methods

2.1. Strains and growth conditions

The type strain *L. plantarum* CECT 748^T (ATCC 14917, DSMZ 20174) isolated from pickled cabbage was purchased from the Spanish Type Culture Collection. This

99 strain was selected based on its high tannase activity previously reported (Nishitani and
100 Osawa, 2003; Nishitani et al., 2004; Vaquero et al., 2004). A modified basal medium
101 was used for the routine cultivation of this strain. The composition of the basal medium
102 described for *L. plantarum* was the following: glucose (2 g/l), trisodium citrate
103 dihydrate (0.5 g/l), D-,L-malic acid (5 g/l), casamino acids (Difco, Detroit, Mich) (1g/l),
104 yeast nitrogen base without amino acids (Difco) (6.7 g/l) and the pH adjusted to 5.5
105 (Rozès and Peres, 1998). This basal media was modified by the replacement of glucose
106 by galactose in order to avoid a possible glucose carbon catabolite repression
107 (Muscariello et al., 2001).

108 This modified basal medium was also used for *L. plantarum* growth in the
109 presence of 1mM tannic acid (Sigma, Germany). After 7 days incubation at 30 °C, an
110 aliquot of the medium was centrifuged in order to pellet the bacteria, and the
111 supernatant was subjected to HPLC analysis.

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113 *2.2. Analysis of tannase activity on cell-free extracts*

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115 *2.2.1. Preparation of cell-free extracts*

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117 *L. plantarum* CECT 748^T was grown in 400 ml of modified basal medium
118 (Rozès and Peres, 1998) at 30 °C until a late exponential growth phase. The cells were
119 harvested by centrifugation. The pellets were washed three times with 400 ml phosphate
120 buffer (50 mM, pH 6.5). The cell pellets were resuspended in 20 ml phosphate buffer in
121 preparation for cell rupture. This suspension was disintegrated twice by using the
122 French Press at 1100 psi pressure (Amicon French pressure cell, SLM Instruments). The
123 cell disruption steps were carried out on ice to ensure low temperature conditions

124 required for most enzymes. The disintegrated cell suspension was centrifuged at 12000
125 x g for 20 min at 4 °C. Supernatant from the centrifugation step was filtered aseptically
126 using sterile filters of 0.2 µm pore size (Sarstedt, Germany). Total soluble protein
127 concentration was determined using the Bio-Rad protein assay (Bio-Rad, Germany).

128

129 *2.2.2. Degradation of tannic acid by cell-free extracts*

130

131 The experiment was performed in 2-ml-Eppendorf tubes in a final volume of 1.1
132 ml containing 1 mM tannic acid final concentration. This involved the addition of 45 µl
133 of 25 mM stock solution of tannic acid (Sigma, Germany) to the cell-free extract. The
134 mixture was incubated in the dark at 37 °C for 16 h. The reaction products were
135 extracted twice with one third of the reaction volume of ethyl acetate (Lab-Scan,
136 Ireland). The solvent fractions were filtered through a 0.45 µm PVDF filter
137 (Teknokroma, Spain) and analysed by HPLC.

138

139 *2.2.3. HPLC analysis*

140

141 A Waters (Milford, MA) chromatograph equipped with a 600-MS system
142 controller, a 717 plus autosampler, and a 996 photodiode array detector were used. A
143 gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B
144 (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied to a reversed-phase Nova-
145 pack C₁₈ cartridge (25 cm x 4.0 mm i.d.; 4.6 µm particle size, cartridge at room
146 temperature) as follows: 0-55 min, 80% B linear, 1.0 ml/min; 55-57 min, 90% B linear,
147 1.2 ml/min; 57-70 min, 90% B isocratic, 1.2 ml/min; 70-80 min, 95% B linear, 1.2

148 ml/min; 80-90 min, 100% B linear, 1.2 ml/min; 90-100 min, washing (methanol), and
149 100-120 min, 1.0 ml/min reequilibration of the cartridge (Bartolomé et al., 2000).

150

151 2.3. Gallic acid analysis

152

153 The esterase activity of tannase was determined using a rhodanine assay specific
154 for gallic acid (Inoue and Hagerman, 1988). Rhodanine reacts only with gallic acid and
155 not with galloyl esters or other phenolics. Therefore, gallotannins are measured by
156 determining the quantity of gallic acid formed after hydrolysis of the tannins. Gallic
157 acid analysis in the reactions was determined using the following assay. Extract aliquots
158 of 100 µl were incubated with 100 µl of 25 mM methyl gallate in phosphate buffer (50
159 mM, pH 6.5) during 10 min at 37 °C. After this incubation, 150 µl of a methanolic
160 rhodanine solution (0.667% w/v rhodanine in 100% methanol) was added to the
161 mixture. After 5 min incubation at 30 °C, 100µl of 500 mM KOH was added and the
162 mixture was diluted to 900 µl with distilled water. After an additional incubation of 5-
163 10 min, the absorbance at 520 nm was measured on a Beckman-Coulter DU-70
164 spectrophotometer. A standard curve using gallic acid concentrations ranging from
165 0.125 to 1 mM was prepared.

166 One unit of tannase activity was defined as the amount of enzyme required to
167 release 1 µmol of gallic acid per minute under standard reaction conditions.

168

169 2.4. Enzyme characterization

170

171 The effect of different temperatures, substrate concentration, and additives on
172 tannase activity from *L. plantarum* cell-free extracts was studied. Tannase activity was

173 assayed by the rhodanine assay as described above. Unless otherwise stated, the
174 standard reaction assays contained methyl gallate (12.5 mM), and enzyme extract in 50
175 mM, pH 6.5, in a total volume of 200 μ l; the reactions, carried out at 37 °C were
176 terminated by the addition of 150 μ l of the methanolic rhodanine solution. All the
177 experiments were done in triplicate for each tube at each sampling condition, and the
178 mean values with the standard errors are reported.

179

180 *2.4.1. Optimum temperature and pH for tannase activity*

181

182 *L. plantarum* cell-free extracts were incubated during 10 min in phosphate buffer
183 (50 mM, pH 6.5) at various temperatures ranging from 20 to 70 °C, and the residual
184 enzyme activity was determined. To determiner the optimal pH of tannase, extracts
185 were incubated within different pH values (3-9) using acetate buffer for pH 3-5,
186 phosphate buffer for pH 6-7, Tris-HCl buffer for pH 8, and glycine-NaOH buffer for pH
187 9.

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189 *2.4.2. Temperature stability*

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191 The thermostability of tannase was determined by incubating the cell-free
192 extract in phosphate buffer (50 mM, pH 6.5) at several temperatures (25, 37, 45 and 55
193 °C) for different time intervals (from 5 min to 16 h). After the incubation periods,
194 reaction aliquots were withdrawn and assayed at standard assay conditions to determine
195 the residual tannase activities.

196

197 *2.4.3. Effect of substrate concentration*

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199   Methyl gallate solution of different concentrations (1.75 to 100 mM) was
200 prepared in phosphate buffer (50 mM, pH 6.5) and the effect of substrate concentration
201 on tannase activity was determined.

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203   2.4.4. *Effect of different additives in tannase activity*

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205   Different metal ions (like Mg^{2+} , K^+ , Ca^{2+} , Zn^{2+} , and Hg^{2+}) surfactans (Tween 80
206 and Triton X-100), denaturants (urea), chelators (EDTA), and inhibitors (DMSO, β -
207 mercaptoethanol), as well as a putative competitor substrate (ethyl gallate) were
208 dissolved in phosphate buffer (50 mM, pH 6.5) at a concentration of 0.1 M. Tannase
209 assay was performed using this buffer and the effect of these additives on tannase
210 activity was studied.

211

212   2.5. *Zymogram analysis*

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214   Aliquot of *L. plantarum* cell-free extract was precipitated with ammonium
215 sulphate to a 100% saturation in order to concentrate the proteins. The precipitated
216 proteins were dissolved and dialyzed against phosphate buffer (50 mM, pH 6.5) at 4 °C.
217 Duplicate aliquots of the concentrated proteins were subjected to PAGE in native
218 conditions (non-denaturing) or in presence of SDS. The zymogram analyses were
219 performed following a modified protocol based on a previous described method (Skene
220 and Brooker, 1995). Briefly, following electrophoresis, both gels were divided in half
221 and proteins in one half were localized by staining with Coomassie Blue R250. The
222 other half from the SDS gel was subjected to three washes at room temperature for 30

min per wash in a buffer containing 50 mM Tris-ClH, pH 8.0, 1 mM EDTA and 5 mM β -mercaptoethanol, in order to renature the proteins. After that, halves from SDS or native polyacrylamide gels were submerged in 25 mM gallic acid methyl ester solution, and incubated for 30 min at 37 °C. After incubation, both types of gel were submerged in 0.67% methanolic rhodanine solution for 5 min and then in 0.5 N KOH, in order to develop the purple-coloured rhodanine-gallate complex.

3. Results and discussion

3.1. Tannase activity in *L. plantarum*

Osawa et al. (2000) reported for the first time tannase activity in *L. plantarum* isolates. They used methyl gallate, a simple galloylester of methanol, as a substrate to be hydrolyzed by the bacterial tannase, and then, the gallic acid released from methyl gallate was oxidized to give a green to brown color, sufficient to be recognized visually or in a spectrometer (Nishitani and Osawa, 2003; Nishitani et al., 2004; Vaquero et al., 2004). Similarly, a spectrophotometric method was used by Lamia and Hamdi (2002) to demonstrate tannase activity in *L. plantarum* strains. So far, the action of *L. plantarum* tannase on complex hydrolyzable tannins have not been clearly demonstrated.

In order to demonstrate *L. plantarum* tannase activity on tannic acid, we used the following experimental approach. Since Lamia and Hamdi (2002) described that *L. plantarum* produced an extracellular tannase after 24 h growth on minimal medium of amino acids containing tannic acid, we grew *L. plantarum* CECT 748^T on a basal medium containing 1 mM tannic acid for a week. As control, we incubated the medium in the same conditions. From the *L. plantarum* culture, the cells were pelleted, and the

248 tannic acid in the supernatant was extracted and analyzed by HPLC. A commercial
249 tannic acid extracted from oak gall nuts from *Quercus infectoria* was used in the media.
250 The manufacturer indicated that the typical composition of this tannic acid is gallic acid,
251 and mono- to octagalloyl glucose products. Figure 1-A shows the chromatograms
252 obtained from the control (A, 1) and *L. plantarum* samples (A, 2). Both HPLC
253 chromatograms were similar, and only small variations were observed between them. If
254 *L. plantarum* produces an extracellular tannase able to degrade tannic acid, more
255 differences would be expected and a more simple chromatogram must be obtained. The
256 small differences observed could be due to a different stability of the tannic at different
257 pH, since the pH of the media containing *L. plantarum* become more acidic along
258 bacterial growth (data not shown). In spite of that Lamia and Hamdi (2002) reported the
259 presence of an extracellular tannase in *L. plantarum*, our results did not confirm their
260 results. However, both studies were done in different culture media and incubated under
261 different conditions. In addition, in *L. plantarum* resting cells, tannase activity was
262 reported on a simple compound, such as methyl gallate (Nishitani and Osawa, 2003;
263 Nishitani et al., 2004; Vaquero et al., 2004). It is possible that molecules smaller than
264 tannic acid, such as methyl gallate, would be able to enter inside the bacterial cell, be
265 enzymatically degraded, and followed by the release of its degradation products outside
266 the cell. However, complex compounds, like tannic acid, would be unable to pass into
267 the cell to be degraded.

268

269 In order to confirm tannic acid degradation by *L. plantarum*, cell-free extracts
270 containing the soluble proteins from *L. plantarum* were incubated in presence of tannic
271 acid. After 16 h incubation at 37 °C tannins were extracted and subjected to HPLC
272 analysis. Figure 1-B shows the chromatograms obtained. In this assay, a clear peak

273 reduction was observed in the sample incubated in presence of *L. plantarum* cell-free
274 extract (B, 2) as compared to the sample control (B, 1). It is interesting to note that both
275 control chromatograms (A, 1 and B, 1) showed differences among them. However, this
276 is not a surprising finding, since both controls were incubated in different media and
277 conditions, and Kumar et al. (1999) reported previously that a negligible amount of
278 tannic acid underwent autodegradation.

279 Tannic acid is a complex hydrolyzable tannin composed of polymers of gallic
280 acid esters with a sugar core. Tannic acid degradation into monomeric products by *L.*
281 *plantarum* is in accordance to the depolymerisation of phenolic compounds present in
282 olive mill wastewater observed by Kachouri and Hamdi (2004). They reported that
283 olive mill wastewater fermented with *L. plantarum* shows a depolymerisation of high
284 molecular weight of phenolic compounds and a reduction of low molecular weight
285 phenolics compounds. The application of *L. plantarum* favours the increase of all
286 phenolic compounds in olive oil, especially by depolymerisation, and inhibited the
287 polymerisation of phenolic compounds during storage being the responsible for the
288 darkening of the olive mill wastewater (Lamia and Hamdi, 2003).

289 The molar mass of tannin molecules affects tannin's characteristics directly. It
290 has been found that the higher the molar mass of tannin molecules is, the stronger the
291 anti-nutritional effects and the lower the biological activities are (Chung et al., 1998).
292 Small molecule tannins such as monomeric, dimeric and trimeric tannins are suggested
293 to have less anti-nutritional effects and can be more readily absorbed. Marker biological
294 and pharmacological activities such as anticarcinogenic activity, host-mediated
295 antitumor activity, antiviral activity, inhibition of lipid peroxidation have been shown
296 for medical herbs to contain small molecule tannins (Okuda et al., 1992).

297   Taking into account the tannic acid degradation observed by cell-free extract in
298 the described experiment, we unequivocally demonstrated for the first time that *L.*
299 *plantarum* is able to extensively degrade tannic acid.

300

301   3.2. Biochemical properties of *L. plantarum* tannase

302

303   Tannic acid is almost exclusively formed by poly-galloyl glucose derivatives
304 whose nature and complexity vary with the plant source. A certain proportion of the
305 galloyl groups are bound in the form of *m*-depsides. Therefore, tannic acid-
306 decomposing enzyme tannase contains two activities, an esterase and a depsidase with
307 specificities for methyl gallate and *m*-digallic acid ester linkages, respectively (Haslam
308 and Stangroom, 1966).

309   Since tannase catalyzes the hydrolysis of the galloyl ester linkage of the tannic
310 acid liberating gallic acid, the activity of tannase could be measured by estimating the
311 residual tannic acid or gallic acid formed due to enzyme action (Mueller-Harvey, 2001).
312 A method specific for the detection of gallic acid could be used for a reliable
313 quantification of tannase activity. Inoue and Hagerman (1988) described a rhodanine
314 assay for determining free gallic acid. Rhodanine reacts with the vicinal hydroxyl
315 groups of gallic acid to give a red complex with a maximum absorbance at 520 nm. The
316 red color formed only with free gallic acid and no with gallic acid esters, ellagic acid, or
317 other phenolics. Since the rhodanine assay using commercial tannic acid as substrate
318 give high absorbance values due to small amounts of free gallic acid present in the
319 preparation, we decided to use methyl gallate as substrate.

320   We used the rhodanine assay in cell-free extracts for a partial biochemical
321 characterization of *L. plantarum* tannase. Figure 2 showed some biochemical properties

322 of *L. plantarum* tannase. Tannase activity was optimal at pH 5, whereas at pH 6 the
323 enzyme retained only 40% of maximal activity (Figure 2A). However, it has been
324 reported that the optimum pH for *L. plantarum* tannase production is pH 6 (Lamia and
325 Hamdi, 2002). Rhodanine assay was performed at several incubation temperatures
326 ranging from 20 to 70 °C (Figure 2B). The optimum temperature for the enzyme activity
327 was found to be 30 °C, at which the enzyme activity was the highest (6.26 U/ml).
328 Similar optimal temperature was reported for fungal tannases (Aguilar and Gutierrez-
329 Sánchez, 2001); however, bacterial tannase from *Bacillus cereus* showed optimum
330 activity at 40 °C (Mondal et al., 2001). With further increase in temperature tannase
331 activity was found to decrease. There was considerably good activity even at 50 °C
332 (5.44 U/ml), this is an additional advantage since some of the processes assisted by
333 tannase are performed at increased temperatures. Prolonged incubation of cell-free
334 extracts at 22 or 37 °C did not showed detectable loss of activity under the experimental
335 conditions used (Figure 2C).

336 To check the effect of substrate concentration on tannase activity, the assay was
337 performed at several concentration of substrate, methyl gallate. Enzyme activity was
338 maximal at 6.25 mM methyl gallate (11.4 U/ml). Further increase in substrate
339 concentration was found to reduce tannase activity (data not shown). A lower substrate
340 level for the maximal activity of the enzyme was considered as a positive factor for
341 industrial applications.

342 Table 1 shows the effects of metal ions on the tannase activity. Most of enzymes
343 require the presence of metal ion activators to express their full catalytic activity. At low
344 concentrations, metal ions act as cofactors of many enzymes, thereby increasing the
345 catalytic activity of the enzyme whereas at high concentrations the catalytic activity
346 could be reduced. *L. plantarum* tannase was found to be partially inactivated by the

347 presence of Hg^{2+} and Mg^{2+} ions (Table 1). Tannase from *Aspergillus niger* was also
348 inhibited by ions like Mg^{2+} (Sabu et al., 2005). The inhibitory effect of heavy metal ions
349 is well documented in the literature (Valee and Ulmer, 1972). It is known that ions of
350 mercury react with protein thiol groups, converting them to mercaptides, and also react
351 with histidine and tryptophan residues. Moreover, by the addition of mercury, the
352 disulfite bond could also be hydrolytically degraded. Further, the decreased activity in
353 the presence of divalent cations could be due to non-specific binding or aggregation of
354 the enzyme.

355   Metal ions like K^+ , Ca^{2+} and Zn^{2+} did not affect *L. plantarum* tannase activity.
356 Sabu et al. (2005) reported that the activity of *A. niger* tannase was increased by the
357 addition of potassium and inhibited by Ca^{2+} and Zn^{2+} ions. *P. chrysogenum* tannase was
358 also inhibited by zinc ions and this inhibition could be strongly attenuated by adding
359 EDTA to the reaction (Lekha and Lonsane, 1997). The addition of some surfactants
360 (Tween 80), chelators (EDTA), inhibitors (DMSO), and denaturing agents (urea) does
361 not affect *L. plantarum* tannase activity at the concentration tested (Table 1). From these
362 results could be deduced that *L. plantarum* tannase activity is not affected by most of
363 the effector compounds tested. Competitive inhibition of tannase was observed by using
364 ethyl gallate, a ester derivative of gallic acid (Table 1). Since ethyl gallate was also
365 degraded by tannase (data not shown), this result might indicate that tannase shows
366 higher affinity for methyl gallate than for ethyl gallate, resulting in a lower gallic acid
367 formation.

368   Concentrated cell-free extracts were fractionated by denaturing SDS-PAGE, and
369 examined for enzyme activity by the zymogram procedure described in the Material and
370 methods section. In spite of that several renaturing procedures were checked, none of
371 them allowed the recovery of tannase activity in the gels. This activity inactivation

372 hinders the determination of the protein molecular size in PAGE gels. Reports reveal
373 that all tannases purified so far are multimeric with the molecular masses ranging from
374 186 to 300 KDa. The lack of tannase activity showed by washed SDS-PAGE gels
375 supports the idea of that *L. plantarum* tannase must be a multimeric protein. However,
376 in a non-denaturing PAGE gel, tannase activity was localized to a single band visible
377 from the concentrated cell-free extracts (Figure 3). The zymogram assay confirms that
378 *L. plantarum* cell-free extracts posses an enzyme able to hydrolyze the ester linkage of
379 methyl gallate, and therefore, of tannic acid.

380 In summary, tannase is an industrially important enzyme that is mainly used in
381 the food industry. As the range of applications of this enzyme is very wide there is
382 always a search for novel tannases with better characteristics, which may be suitable in
383 the diverse fields of applications. The present paper reports the characterization of a
384 tannase from *L. plantarum* CECT 748^T. It was found to give its optimum activity at
385 temperature around 30 °C. Furthermore, it was noted that the enzyme was active with
386 nearly 75% of the maximal activity at 50 °C. All these characteristics are considered
387 favourable for food industrial processing.

388 Tannase research will lead to obtain highly biological small molecule tannins
389 since biodegradation is one of the efficient ways to degrade large molecules tannins into
390 smaller molecule tannins with valuable bioactivities.

391

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393

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460 **Figure captions**

461

462 Figure 1. HPLC analysis of tannic acid. (A) Modified basal media containing 1 mM
 463 tannic acid was inoculated with *L. plantarum* CECT 748^T and incubated for 7 days at 30
 464 °C (2); a non-inoculated control medium was incubated in the same conditions (1). (B)
 465 *L. plantarum* CECT 748^T cell-free extract incubated for 16 h in presence of 1 mM
 466 tannic acid (2); phosphate buffer containing tannic acid was incubated in the same
 467 conditions and was used as control (1). Detection was performed at 280 nm.

469 Figure 2. Some biochemical properties of the *L. plantarum* CECT 748^T tannase. (A)
470 Optimum pH for activity. Tannase reactions were performed at pH ranging from 3 to 9.
471 (B) Optimum temperature for tannase activity. Cell-free extracts were incubated at
472 temperatures ranging from 20 to 70 °C. (C) Thermal stability of the tannase. Cell-free
473 extracts were incubated at 22 °C (●), 37 °C (■), 45 °C (▲), or 55 °C (X) in phosphate
474 buffer (50 mM, pH 6.5), at indicated times, aliquots were withdrawn, and analyzed as
475 described in the Material and method section. The experiments were done in triplicate.
476 The mean value and the standard error are showed.

477

478 Figure 3. Coomassie Blue (A) and tannase zymogram (B) of non-denaturing PAGE
479 analysis of concentrated cell-free extracts from *L. plantarum* CECT 748^T prepared as
480 described in the Material and methods section. The arrow indicate the location of the
481 tannase activity. A ruler indicate the distance migrated in a 8% polyacrylamide gel.

482

Table 1
Effect of different compounds on *L. plantarum* tannase activity

Additions (1 mM)	Residual activity (%)
None	100
MgCl ₂	84
KCl	100
CaCl ₂	100
ZnCl ₂	100
HgCl ₂	34
Tween 80	100
Triton X-100	68
Urea	100
EDTA	100
DMSO	100
β-mercaptoethanol	61
Ethyl gallate	80

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3