Running title: *L. PLANTARUM* TANNASE

Production and physicochemical properties of recombinant *Lactobacillus plantarum* tannase

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Abstract

Tannase is an enzyme with important biotechnological applications in food industry. Previous studies have identified the tannase encoding gene in *Lactobacillus plantarum* and also have reported the description of the purification of recombinant *L. plantarum* tannase through a protocol involving several chromatographic steps. Here, we describe the high-yield production of pure recombinant tannase (17 mg/L) by a one-step affinity procedure. The purified recombinant tannase exhibits optimal activity at pH 7 and 40 ºC. Addition of Ca^{2+} to the reaction mixture greatly increased tannase activity. The enzymatic activity of tannase was assayed against 18 simple phenolic acid esters. Only esters derived from gallic acid and protocatechuic acid were hydrolyzed. In addition, tannase activity was also assayed against the tannins tannic acid, gallocatechin gallate and epigallocatechin gallate. Despite *L. plantarum* tannase representing a novel family of tannases which shows no significant similarity to tannases from fungal sources, both families of enzymes shared similar substrate specificity range. The physicochemical characteristics exhibited by *L. plantarum* recombinant tannase make it an adequate alternative to the currently used fungal tannases.

KEYWORDS: Tannin acyl hydrolase; tannase; hydrolyzable tannins; propyl gallate; gallic acid.

INTRODUCTION

The enzyme tannase (or tannin acyl hydrolase EC 3.1.1.20) catalyzes the hydrolysis of ester bonds in hydrolyzable tannins such as tannic acid, thereby releasing glucose and gallic acid (*1-3*). Currently, tannase plays an important role in the industries of drinks and foods, chemical-pharmaceutics, brewing and production of aminal feed. Thus, tannase is used in the elaboration of acorn liquor and also in the production of gallic acid, which is subsequently employed for the synthesis of propylgallate, a potent antioxidant. Also, tannase is used as clarifying agent in the manufacturing of instant tea, some wines, beers, juices or fruits and in coffee-flavored soft drinks (*1-4*).

It has long been known that several fungal species, such as *Aspergillus* spp., are capable of producing large amounts of tannase (*5*). However, the use of tannase on a large scale was limited by a variety of factors, including production costs and insufficient knowledge of the enzyme. The advent of recombinant DNA technology has revolutionized research in the field of enzymology. Hatamoto et al. (1996) cloned and sequenced the gene-encoding tannase from *Aspergillus oryzae* (*6*). Later, the *Aspergillus* tannase gene was heterologously expressed in *Saccharomyces cerevisiae*, although with a low yield of protein production (*3*). Conversely, large quantities of enzyme were obtained when produced in *Pichia pastoris* (*7*). Obviously, the high-yield production of fully active recombinant tannases is an attractive goal both for basic research and for industrial purposes.

In addition to fungal tannases, many bacterial species have been reported to produce tannase (*3*), and in fact several putative bacterial tannase sequences have been included in the databases. To our knowledge, the only bacterial tannases that have been analyzed both genetically and biochemically are those from *Staphylococcus lugdunensis* (*8*) and

Lactobacillus plantarum (*9*). As *S. lugdunensis* strains have been associated with human disease (*10-13*) and microorganisms used in the industrial production of food processing enzymes should be listed as GRAS (generally recognized as safe), so far, the *L. plantarum* protein is the only bacterial tannase alternative for its use in the food industry.

Since i) a low-yield protocol has been described for its production, and ii) the substrate specificity of the *L. plantarum* tannase remains unknown, the aims of this study were to design a protocol for a high-yield tannase production which could facilitate its biochemical characterization, as well as to make it highly attractive for their use in the industrial sector.

MATERIALS AND METHODS

Bacterial strains and materials. *L. plantarum* CECT 748^T strain was purchased from the Spanish Culture Type Collection (CECT). *E. coli* DH5α was used for all DNA manipulations. *E. coli* JM109 (DE3) was used for expression in pURI3 vector (*14*). *L. plantarum* strain was grown in MRS medium at 30 ºC without shaking. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 ºC and 200 rpm. When required, ampicillin was added to the medium at a concentration of 100 µg/mL.

The compounds assayed in this study were methyl gallate (Fluka 48690), ethyl gallate (Aldrich 48640), propyl gallate (Sigma P3130), lauryl gallate (Aldrich 48660), methyl benzoate (Fluka 18344), ethyl benzoate (Sigma E12907), methyl 4-hydroxybenzoate (Sigma H5501), ethyl 4-hydroxybenzoate (Aldrich 111988), propyl 4-hydroxybenzoate (Scharlau Hi200), butyl 4-hydroxybenzoate (Scharlau Hi180), methyl

vanillate (Aldrich 138126), methyl 2,4-dihydroxybenzoate (Aldrich M42505), methyl gentisate (Aldrich 426091), methyl salicylate (Sigma M6752), ethyl 3,4-dihydroxybenzoate (protocatechuic acid ethyl ester) (Aldrich E24859), ethyl 3,5-dihydroxybenzoate (Aldrich 541087), ferulic methyl ester (Extrasynthèse 6267), ferulic ethyl ester (Extrasynthèse 6275), tannic acid (Sigma T0125), ellagic acid (Sigma, E2250), chlorogenic acid (Aldrich C3878), quercetin (Sigma Q4951), catechin (Sigma C1251), epicatechin (Sigma E1753), gallocatechin (Fluka G6657), gallocatechin gallate (Sigma G6782), epigallocatechin (Fluka 08108), epigallocatechin gallate (Fluka 50299) and 4-nitrophenyl β-D-glucopyranoside (Sigma N7006).

DNA manipulations. Bacterial DNA was isolated from overnight cultures using a protocol previously described (*15*). DNA sequencing was carried out by using an Abi Prism 377 DNA™ sequencer (Applied Biosystems, Inc.). Sequence similarity searches were carried out using FASTA on EBI site (http://www.ebi.ac.uk). Signatures, pI/MW, etc. were analysed on EXPASY site (http//www.expasy.ch). Multiple alignments were done using CLUSTAL W on EBI site after retrieval of sequences from GenBank and Swiss-Prot.

Expression, and purification of recombinant tannase. The gene coding for the *L. plantarum* CECT 748^T tannase, *tanLp1* (named lp_2956 in the *L. plantarum* WCFS1 strain) was cloned and overexpressed following a strategy previously described (*14*). Briefly, the gene was PCR-amplified with Hot-start Turbo *Pfu* DNA polymerase by using the primers 513 (5´- *CATCATGGTGACGATGACGATAAG*atgagtaaccgattgatttttgatg) and 514 (5´- *AAGCTTAGTTAGCTATTATGCGTA*tcattggcacaagccatcaatccag) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the

tanLp1 gene sequence is written in lowercase letters). The corresponding 1.4-kb purified PCR product was inserted into pURI3 vector by using the restriction enzyme- and ligation-free cloning strategy described previously (*14*). Briefly, to clone any target protein into the pURI3 vector, PCR products of the gene of interest need to be generated with specific overhangs that are complementary to the integration site sequence of the vector. After the first PCR reaction, the fragment is added to the methylated recipient template plasmid. The elongated and modified strands are not methylated. *Dpn*I digestion is used to eliminate the methylated plasmid DNA. Expression vector pURI3 was constructed based on the commercial expression vector pT7-7 (USB) but expressing a protein containing the following leader sequence MGGSHHHHHHGDDDDKM consisting of a N-terminal methionine followed by three spacer amino acids, a six-histidine affinity tag, a spacer glycine residue, and the five-amino acid enterokinase recognition site (*14*). *E. coli* DH5α cells were transformed, recombinant plasmids were isolated and those containing the correct insert were identified by restriction-enzyme analysis, verified by DNA sequencing and then transformed into *E. coli* JM109(DE3) cells.

Cells carrying the recombinant plasmid, pURI3-TanLp1, were grown at 37 ºC in Luria-Bertani media containing ampicillin (100 µg/mL), and induced by adding 0.4 mM IPTG. After induction, the cells were grown at 22 ºC during 20 h and collected by centrifugation. Cells were resuspended in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl. Crude extracts were prepared by French Press lysis of cell suspensions (three cycles at 1100 p.s.i.). The insoluble fraction of the lysate was removed by centrifugation at 47 000 *g* for 30 min at 4 ºC.

The supernatant was filtered through a 0.45 µm filter and applied to a His-Trap™-FF crude chelating affinity column (Amersham Biosciences) equilibrated with 20 mM Tris-

HCl, pH 8.0, 100 mM NaCl containing 10 mM imidazole, to improve the interaction specificity in the affinity chromatograpy step. The bound enzyme was eluted by applying a stepwise gradient of imidazole concentration, from 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 10 mM imidazole to 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 500 mM imidazole. Fractions containing the eluted proteins were pooled and the protein was then dialyzed overnight at 4 ºC in a membrane (3500 cut-off) against 25 mM sodium phosphate buffer, pH 6.5. The purity of the enzymes was determined by 12% sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) in Tris-glycine buffer.

SDS-PAGE and determination of protein concentration. Samples were analyzed by 12 % SDS-PAGE under reducing conditions. Protein bands were visualized by Coomassie blue staining. The gels were calibrated using molecular weight markers. Protein concentration was measured according to the method of Bradford using a protein assay kit (Bio-Rad) with bovine serum albumin as standard.

Enzyme activity assay. Tannase activity was determined using a rhodanine assay specific for gallic acid (*16*). Rhodanine reacts only with gallic acid and not with galloyl esters or other phenolics. Gallic acid analysis in the reactions was determined using the following assay. Tannase enzyme (10 µg) in 700 µL of 50 mM phosphate buffer pH 6.5 was incubated with 40 µL of 25 mM methyl gallate (1 mM final concentration), during 5 min at 37 ºC. After this incubation, 150 µL of a methanolic rhodanine solution (0.667% w/v rhodanine in 100% methanol) was added to the mixture. After 5 min incubation at 30 ºC, 100 µL of 500 mM KOH was added. After an additional incubation of 5-10 min, the absorbance at 520 nm was measured on a spectrophotometer. A standard curve using gallic

acid concentrations ranging from 0.125 to 1 mM was prepared. One unit of tannase activity was defined as the amount of enzyme required to release 1 µmol of gallic acid per minute under standard reaction conditions.

To study the effect of substrate concentration, the standard activity assay was done by using solutions of methyl gallate at different concentrations (0.1 to 100 mM). To study the effect of pH and temperature, standard activity assays were done using the conditions described below.

Optimum temperature and pH-dependence. Activities of recombinant *L. plantarum* tannase were measured at 20, 30, 40, 50, 60, and 70 ºC to determine the optimal temperature for enzymatic activity. The optimum pH value for tannase activity was determined by studying its pH-dependence within the pH range between 3 and 10. Acetic acid-sodium acetate buffer was used for pH 3-5, citric acid-sodium citrate buffer for pH 6, sodium phosphate buffer for pH 7, Tris-HCl buffer for pH 8, glycine-NaOH buffer for pH 9, and sodium carbonate-bicarbonate for pH 10. A 100 mM concentration was used in all the buffers. The rhodanine assay was used for the optimal pH characterization of tannase. Since the rhodanine-gallic acid complex forms only in basic conditions, after the completion of the enzymatic degradation of methyl gallate, KOH was added to the reaction mixture to ensure that the same pH value (pH 11) was achieved in all samples assayed.

For temperature stability measurements, the recombinant tannase was incubated in 50 mM phosphate buffer pH 6.5 at 25, 30, 37, and 45 ºC for 15 min, 30 min, and 1, 2, 3, 4, and 20 h. After incubation, the residual activity was measured as above described.

Effect of additives on tannase activity. To test the effect of metals and ions on the activity of the recombinant tannase, the enzymatic activity was measured in the presence of different additives at a final concentration of 1mM. The additives analyzed were $MgCl_2$, KCl, $CaCl_2$, $HgCl_2$, $ZnCl_2$, Triton-X-100, Urea, Tween 80, EDTA, DMSO, and β-mercaptoethanol. The potential substrate ethyl gallate was also assayed.

HPLC analysis of *L. plantarum* tannase activity on several substrates. The activity of recombinant tannase against 28 potential substrates has been analyzed. The standard enzyme activity assay was modified by using 100 µg of tannase, 1 mM substrate, and 1mM $CaCl_2$ in the reaction mixture, and incubated at 37 ºC during 10 min. As controls, phosphate buffer containing the reagents but the enzyme were incubated in the same conditions. The reaction products were extracted twice with ethyl acetate (Lab-Scan, Ireland) and analyzed by HPLC-DAD.

A Thermo (Thermo Electron Corporation, Waltham, Massachussetts, USA) chromatograph equipped with a P400 SpectraSystem pump, and AS3000 autosampler, and a UV6000LP photodiode array detector were used. A gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied to a reversed-phase Nova-pack C_{18} (25 cm x 4.0 mm i.d.) 4.6µm particle size, cartridge at room temperature as follows: 0-55 min, 80% B linear, 1.1 mL/min; 55-57 min, 90% B linear, 1.2 mL/min; 57-70 min, 90% B isocratic, 1.2 mL/min; 70-80 min, 95% B linear, 1.2 mL/min; 80-90 min, 100% linear, 1.2 mL/min; 100-120 min, washing 1.0 mL/min, and reequilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm. Samples were injected in duplicate onto the cartridge after being filtered through a 0.45 µm PVDF filter. The identification of degradation compounds

was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers.

RESULTS AND DISCUSSION

Production and enzymatic activity of recombinant *L. plantarum* tannase.

Tannases are enzymes extensively used in the food industry. In previous studies we showed the existence of tannase activity in cell-free extracts from *L. plantarum* CECT 748^T (*17*). Later, it was reported the identification of a gene encoding a putative tannase (*9*), which was similar to the sequence of the *S. lugdunensis* tannase. The gene encoding the putative tannase from *L. plantarum* CECT748^T, *tanLp1*, has been cloned and hyperexpressed in *E. coli*; the recombinant tannase was further purified and biochemically characterized. The purification protocol comprised several chromatographic steps (Q-Sepharose Fast Flow, hydroxylapatite, and Mono-Q GL5/5 columns). With the aim of improving the purification of tannase from *L. plantarum* CECT48^T, we decided to use the expression vector pURI3 constructed in our laboratory (*14*). The pURI3 vector was created using the pT7-7 vector as template and contains an amino-terminal His-tag that allows convenient purification of the native protein directly from the crude cell extracts. In fact, we have previously employed this methodology to successfully overproduce other proteins from *L. plantarum* (*18, 19*).

The *tanLp1* gene from *L. plantarum* CECT 748^T (ATCC 14917^T) was PCR-amplified by using 513 and 514 primers and cloned into pURI3 vector. When the sequence of the recombinant plasmid pURI3-TanLp1 was analyzed, it could be observed that, in relation to the previously deposited *L. plantarum* ATCC 14917^T sequence, it contains two

nucleotide changes at positions 801 (C to T) and 962 (T to C). This last nucleotide change produces a conservative amino acid substitution at position 321 (Val-321 to Ala). The nucleotide changes were confirmed by the sequencing of the gene from chromosomal DNA. This was a surprising result, as both sequences proceed from the same strain. It is possible that the different evolution of both strain stocks could be responsible of these changes. In this sense, Iwamoto et al. (2008) described that the *tanLp1* sequence was 99.6% identical to that derived from the *L. plantarum* WCFS1 strain, lp_2956. These differences determine protein sequences differing in two amino acid residues (*9*). Therefore in relation to tannase from *L. plantarum* WCFS1, the sequence reported in this study, showed three amino acid substitutions, two non conservative changes Thr-154 to Ala, and Arg-406 to Gln, and the conservative Val-321 to Ala change.

To confirm that *L. plantarum* CECT 748^T *tanLp1* gene encodes a functional tannase, we expressed this gene in *E. coli* under the control of the T7 RNA polymerase-inducible ϕ10 promoter. Cell extracts were used to detect the presence of hyperproduced proteins by SDS-PAGE analysis. Control cells containing the pURI3 vector plasmid alone did not show overexpression, whereas expression of additional 50 kDa protein was apparent with cells harbouring pURI3-TanLp1 (**Figure 1**). As the protein was cloned containing an affinity hexa-His tag, recombinant *L. plantarum* tannase was purified on a His-Trap-FF chelating column and eluted with a stepwise gradient of imidazole. Highly purified recombinant tannase protein was obtained from pURI3-TanLp1 (**Figure 1**). The eluted protein was dialyzed to eliminate the imidazole and checked for its tannase activity on methyl gallate. The purification protocol previously described resulted in a production yield of 1.48 mg/L, with a specific activity of 84.3 U/mg (*9*). The one-step protocol herein

described, 17 mg of recombinant protein with specific activity of 2.35 x 10^6 U/mg were obtained per 1 L culture.

The biochemical characterization of tannase from *L. plantarum* has been previously reported from cell-free extracts (*17*) and also the pure recombinant enzyme (*9*). As some contradictory results were found among both studies, we decided to confirm the biochemical data on the pure recombinant *L. plantarum* CECT 748^T tannase by the colorimetric assay using methyl gallate as substrate. **Figure 2A** shows that recombinant tannase presented an optimal pH around 7, being also highly active at pH 6-8. This pH value is similar to that previously reported for the purified tannase (pH 8) (*9*), and clear differs from the value reported from cell-free extracts (pH 5). As recombinant tannases showed less than 20% activity at pH 5, the presence of a factor affecting tannase activity in the cell free extract at this pH must be considered. Although the dissimilar observed pH-dependences may result from the above described amino acid substitutions, the effect of experimental factors such as the different buffer systems or the standard assay used for the determination cannot be discarded.

The neutral optimum pH here reported for the recombinant *L. plantarum* tannase is in contrast to the pH dependence of fungal tannases which are acidic proteins with an optimum pH around 5.5 (*1*). **Figure 2B** shows the temperature dependence for recombinant tannase activity. The optimum temperature was found to be ~40 ºC, in agreement with previous results (*9*); similarly to cell-free extracts, tannase activity at 50 ºC was 75% of the maximal activity (*17*). Tannase from *Bacillus cereus* KBR9 also showed optimum activity at ~40 ºC (*20*). This temperature range represents an advantage since some processes assisted by tannases are performed at increased temperatures. **Figure 2C** shows that *L. plantarum* tannase activity was markedly decreased after incubation at a temperature of 37

ºC or higher. The pure recombinant enzyme appears to be more heat-labile that when present in cell-free extracts (*17*).

As a low substrate level for the maximal activity of the enzyme was considered a positive factor for industrial applications, the effect of substrate concentrations on tannase activity was studied. The assay was performed at substrate concentrations ranging from 0.1 to 100 mM. Enzyme activity was maximal a 0.7-0.9 mM methyl gallate, with further increases in substrate concentration resulting in reduced tannase activity (data not shown). This substrate concentration is lower that the previously reported from cell extracts (6.25 mM); however it should be considered that, in the previous work lower enzyme units were used in the assay.

Table 1 shows the effects of various additives (1 mM final concentration) on *L. plantarum* CECT 748^T recombinant tannase activity. Compared to the enzyme incubated in 50 mM phosphate pH 6.5, the enzymatic activity was increased by KCl and $CaCl_2$ (relative activity 126-181%), not significantly affected or partially inhibited by EDTA, $MgCl_2$, $ZnCl_2$, Triton-X-100, Tween-80, and DMSO (relative activity 80-107%), significantly inhibited by urea (relative activity 48%), and was greatly inhibited by $HgCl_2$ and β-mercaptoethanol (relative activity 15-22%). Whereas the great inhibitory effect of $HgCl_2$ agrees with the results derived from cells extracts (*17*), the positive effect of $CaCl_2$ was not identified. As cell extracts contained all the soluble cell components, the effect of a particular additive could be masked. Cell extracts could contain the Ca^{2+} ions needed for the optimal activity of *L. plantarum* tannase. As Ca^{2+} ions greatly increased tannase activity, the substrate specificity assays were done in the presence of 1 mM $CaCl_2$.

The addition of 1 mM ethyl gallate leads to a 52% relative activity suggesting that ethyl gallate acts as an operative competitive inhibitor of methyl gallate. Taking into

account that both compounds, methyl and ethyl gallate, are present at the same concentration on the reaction, this result must indicate that recombinant *L. plantarum* tannase showed similar affinity for both compounds.

Substrate specificity of recombinant *L. plantarum* tannase. When Osawa et al. (2000) reported for the first time tannase activity in *L. plantarum* strains by a colorimetric method, they used methyl gallate, a simple galloyl ester of methanol, as a substrate of tannase (*21*). When *tanLp1* was cloned from *L. plantarum* ATCC 14917^T only methyl gallate was assayed as substrate in the colorimetric assay. In order to analyze the substrate specificity of pure recombinant *L. plantarum* CECT 748^T tannase, 18 different simple phenolic acid esters were assayed. Among these compounds gallic esters (methyl gallate, ethyl gallate, propyl gallate, and lauryl gallate), benzoic esters (methyl benzoate, and ethyl benzoate), hydroxybenzoic esters (methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, and butyl 4-hydroxybenzoate), vanillic ester (methyl vanillate), dyhydroxybenzoic esters (methyl 2,4-dihydroxybenzoate, ethyl 3,4-dihydroxybenzoate or protocatechuic acid ethyl ester, and ethyl 3,5-dihydroxybenzoate), gentisic ester (methyl gentisate), salicylic ester (methyl salicylate), and ferulic esters (ferulic methyl ester, and ferulic ethyl ester) are found. Only all the esters derived form the gallic acid and the derived from the protocatechuic acid (ethyl 3,4-dihydroxybenzoate) were hydrolyzed (**Figure 3**). This result seems to indicate that other cinnamic acids without hydroxyl groups, and with substituents other than –H, or –OH at position 2 were not metabolized by recombinant tannase. It can be also concluded that there are not significant esteric hindrances regarding the aliphatic alcohol constituent of the ester bond, as ethyl as well as lauryl substituent could be effectively hydrolyzed by the *L. plantarum* tannase.

In vegetable foods and agricultural wastes, together with simple phenolic acid esters, complex tannins can be found, such as tannic acid or some flavonoids, which can be also potential substrates for the tannase enzyme (*22*). In this regard, it has been described that recombinant *E. coli* colonies producing *L. plantarum* tannase showed a clear zone on tannic acid-treated plates, which had an opaque surface due to the formation of a tannin-protein complex. This clear zone indicated apparent bacterial degradation of tannic acid. The effect of *L. plantarum* tannase on tannins was clearly demonstrated by incubating cell-free extracts on a tannic acid solution (*17, 23*). The tannic acid used is a gallotannin mainly composed of monomers to tetramers of galloyl glucose (*23*). *L. plantarum* extract degrades this gallotannin by depolymerization of high molecular weight tannins and a reduction of low molecular weight tannins, being gallic acid and pyrogallol the final metabolic products (*23*).

To corroborate these previous results, assays were performed to determine the activity of pure *L. plantarum* CECT 748^{T} recombinant tannase on tannic, ellagic, and chlorogenic acids, and on quercetin, catechin, epicatechin, gallocatechin, gallocatechin gallate, epigallocatechin, and epigallocatechin gallate. From these compounds, only tannic acid, gallocatechin gallate and epigallocatechin gallate were metabolized by tannase (**Figure 4**). Free gallic acid and pyrogallol were identified as the final products resulting from the degradation of hydrolyzable tannins by *L. plantarum* extracts (*18, 23*). As in our experimental conditions no gallate decarboxylase is present, the gallic acid thus formed cannot be decarboxylated becoming the only final product from tannic acid degradation. This is notable due to the antioxidant properties of gallic acid. In fact, among hydroxybenzoic acids, gallic acid is the most potent antioxidant, being 1.6 and 3.4-fold more active than protocatechuic and syringic acids, respectively (*24*). Therefore, the use of

L. plantarum tannase may provide an efficient means for obtaining molecules with valuable activities from the degradation of complex tannins present in food and agricultural wastes.

It has been reported that some fungal tannases exhibited also β-glucosidase activity (*25*). In order to know whether *L. plantarum* CECT 748^T tannase exhibits such an activity, the standard assay was performed by incubating recombinant tannase in the presence of 1mM 4-nitrophenyl β-D-glucopyranoside. No activity was detected against this substrate by using the colorimetric assay as well as by the HPLC analysis of the samples (data not shown).

Conversely, it has been reported that tannase enzymes cleave ester linkages in tannic acid (*26*), also acting on the ester linkages in methyl gallate and *m*-digallic acid, respectively. Tannases hydrolyze only those substrates that contain at least two phenolic OH groups in the acid component (**Figure 5**). The esterified COOH group must be on the oxidized benzene ring and must not be *ortho* to one of the OH groups. However, chlorogenic acid, originated from caffeic and quinic acid, is resistant to tannase activity. This is attributed to the presence of a double bond in the side-chain carrying the esterified COOH group (*1*). Despite that *L. plantarum* tannase represents a novel family of tannases showing no significant sequence similarity to fungal tannases, the reported substrate spectrum of fungal tannases and *L. plantarum* tannase seems to be very similar (*27*).

In conclusion we have described here an improved method for the high production of *L. plantarum* tannase. Although this protein is not similar to fungal tannases, the analysis of a broad range of potential substrates reveals that they shared common substrate specificity. Therefore the use of *L. plantarum* tannase is an adequate alternative to the fungal tannases currently used in the food industry.

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Table 1

Table 1. Effect of additives on recombinant *L. plantarum* CECT 748^T tannase activity

Additions (1 mM)	Relative activity (%)
Control	100
EDTA	107
KCl	126
$HgCl_2$	22
$CaCl_2$	181
$MgCl_2$	88
$ZnCl_2$	82
Triton X 100	80
DMSO	91
Tween 80	80
Urea	48
β-mercaptoethanol	15
Ethyl gallate	52

Figure 1

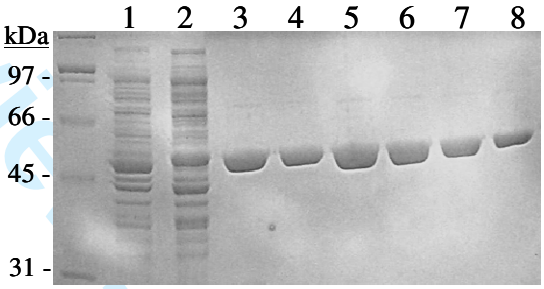

Figure 1. SDS-PAGE analysis of the expression and purification of the tannase protein from *L. plantarum* CECT 748^{T}. Analysis of soluble cell extracts of IPTG-induced *E. coli* JM109 (DE3) (pURI3) (lane 1) or *E. coli* JM109 (DE3) (pURI3-TanLp1) cultures (lane 2), or fractions eluted after His-Trap-FF crude chelating affinity column (lines 3 to 8). SDS-Polyacrylamide gel (12%) was stained with Coomassie blue. The positions of molecular mass markers (Bio-Rad) are indicated on the left.

Figure 2

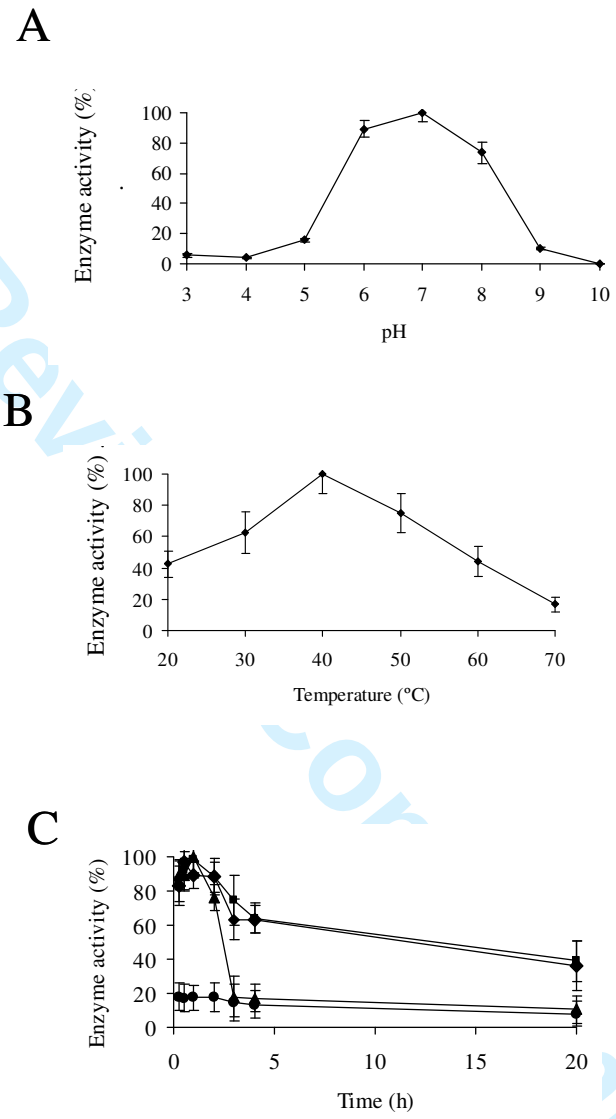

Figure 2. Biochemical properties of recombinant *L. plantarum* CECT 748^T tannase. (A) Relative activity of the tannase versus pH. Enzyme activity was assayed at 37 ºC. (B) Relative activity of tannase versus temperature. Enzyme activity was assayed at pH 6.5. (C) Residual activities of the recombinant *L. plantarum* tannase after preincubation at 25 ºC (♦), 30 ºC (■), 37 ºC (▲) or 45 ºC (●) in phosphate buffer pH 6.5 during 15 or 30 min, and 1, 2, 3, 4, and 20 h. In all the cases, the observed maximum activity was defined as 100%.

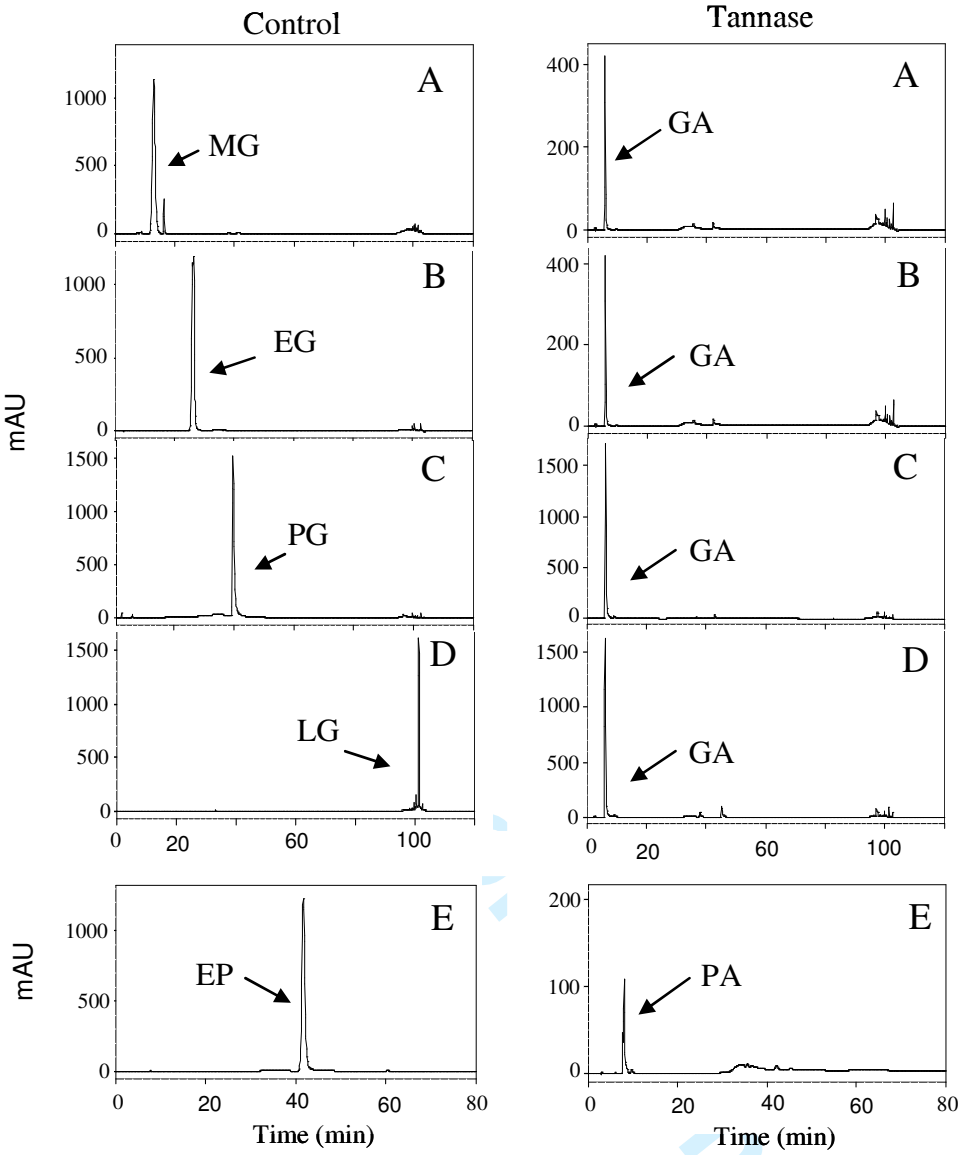

Figure 3. HPLC chromatograms of recombinant *L. plantarum* tannase on different gallic and protocatechuic esters. Tannase enzyme from *L. plantarum* CECT 748^T was incubated for 10 min in the presence of methyl gallate (A), ethyl gallate (B), propyl gallate (C), lauryl gallate (D) or protocatechuic acid ethyl ester (E). Chromatograms without protein (controls) are also showed. The chromatograms were recorded at 280 nm. MG, methyl gallate; EG, ethyl gallate; PG, propyl gallate; LG, lauryl gallate; EP, ethyl protocatechuate or protocatechuic acid ethyl ester; GA, gallic acid; PA, protocatechuic acid.

Figure 4

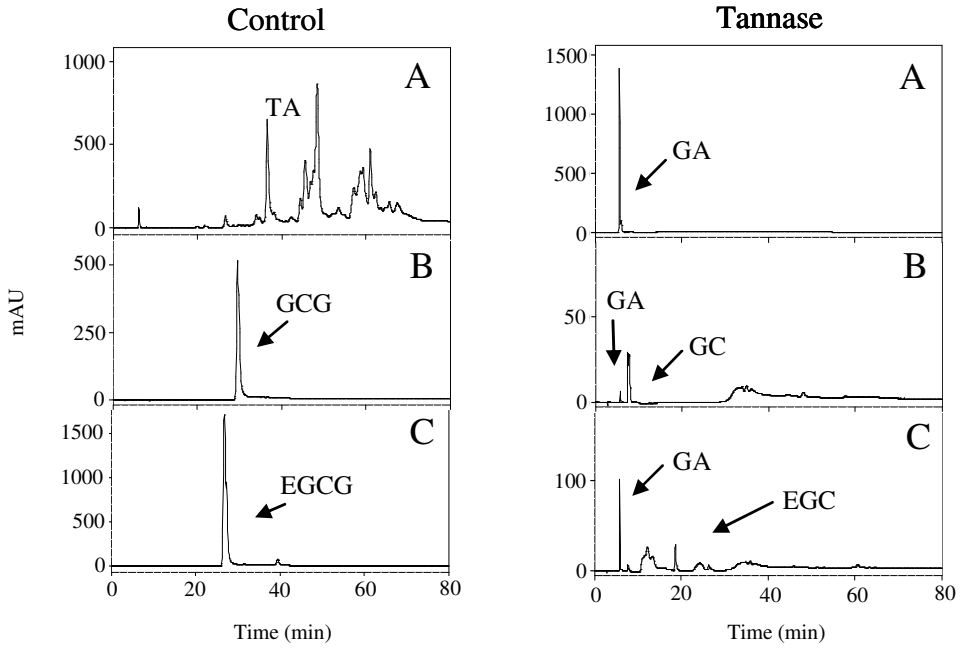

Figure 4. HPLC chromatograms of recombinant *L. plantarum* tannase on tannins. Tannase enzyme from *L. plantarum* CECT 748^T was incubated for 10 min in the presence of tannic acid (A), gallocatechin gallate (B) or epigallocatechin gallate (C). Chromatograms without protein (controls) are also showed. The chromatograms were recorded at 280 nm. TA, tannic acid; GCG, gallocatechin gallate; EGCG, epigallocatechin gallate; GA, gallic acid; GC, gallocatechin; EGC, epigallocatechin.

Figure 5

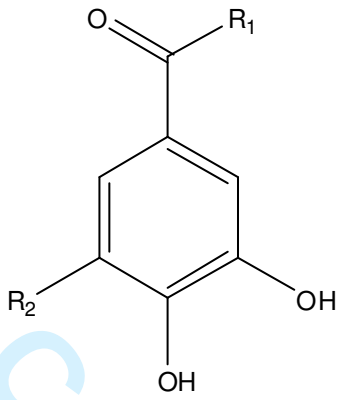

Figure 5. Schematic representation of the potential substrates of *L. plantarum* CECT 748^T tannase. The R_1 substituents could be $–OCH_3$, $-OCH_2-CH_3$, $-OCH_2-CH_2-CH_3$, $-OCH_2(CH_2)_{10}-CH_3$, glucose, gallic acid, gallocatechin, and epigallocatechin. The R_2 substituents could be –H or –OH.