# Hydrolysis of Tannic Acid Catalyzed by Immobilized-Stabilized Derivatives of Tannase from *Lactobacillus Plantarum*.

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**Abstract**

A recombinant tannase from *Lactobacillus plantarum*, overexpressed in *E. coli,* was purified in a single step by metal chelate affinity chromatography on lowly activated nickel supports. It was possible to obtain 0.9 grams of a pure enzyme by using only 20 mL of chromatographic support. The pure enzyme was immobilized and stabilized by multipoint covalent immobilization on highly activated glyoxyl agarose. Derivatives derivatives obtained by multipoint and multisubunit immobilization were 500- and 1000-fold more stable than both the soluble enzyme and the one point immobilized enzyme in experiments of thermal and cosolvent inactivation, respectively. In addition to that, up to 70 mg of pure enzyme were immobilized on 1 gram of wet support.

The hydrolysis of tannic acid was optimized by using the new immobilized tannase derivative. The optimal reaction conditions were 30% diglyme, at pH 5.0 and 4 $^{o}$C. Under these conditions, it was possible to obtain 47.5 mM of gallic acid from a 5 mM of tannic acid as substrate. The product was pure as proved by HPLC chromatography. On the other hand the immobilized biocatalyst preserved more than 95% of its initial activity after 1 month of incubation under the optimal reaction conditions.

**Keywords:** purification of recombinant enzymes with poly-His tags, enzymatic production of pure gallic acid.

## Introduction

Enzymes present important advantages in numerous areas of the Food Chemistry such as synthesis of prebiotics (1), modification of functional ingredients (2), synthesis of antioxidants (3), elimination of antinutrients, synthesis of bioactive peptides (4), design of enzymatic biosensors (5), etc. For the majority of the previously mentioned applications, utilization of immobilized enzymes is technologically advantageous (6, 7, 8). However, food technology must be so far economically viable, thus immobilized enzyme derivatives should be excellent in terms of activity, robustness and efficiency, etc. (9, 10).

The hydrolysis of tannic acid to obtain gallic acid and glucose is an important reaction in food chemistry (11). The substrate, tannic acid, is an abundant plant residue and it may be used to prepare different food preservatives as pyrogallol and propyl gallate (12, 13). In pharmaceutical chemistry gallic acid is also an important intermediate for the synthesis of the antibacterial drug trimethroprim (14). Practical implementation of this process requires the preparation of very active and stable immobilized derivatives of pure tannases able to hydrolyze different gallic-gallic and gallic-glucose bonds without suffering product inhibition (15-17).

Herein we will describe the preparation of a very active and stable derivative of a tannase from *Lactobacillus plantarum* (18). The enzyme was firstly purified to homogeneity by selective adsorption on small volumes of chelate activated agarose (19). The subsequent immobilization of the pure protein by covalent multipoint and multisubunit attachment on glyoxyl agarose stabilized the enzyme (20,21). Finally, the

optimal conditions for the production of pure and concentrated gallic acid were also investigated by using the best immobilized derivatives of tannase.

## Materials and methods

### Materials and bacterial strains

Crosslinked 6% agarose beads and cyanogen bromide activated Sepharose 4B (CNBr-agarose) were obtained from GE Healthcare (Uppsala, Sweden). Iminodiacetic acid disodium salt monohydrate (IDA) and nickel (II) sulphate 6-hydrate and methyl gallate were purchased from Fluka (Buchs, Switzerland). Tannic acid was obtained from Sigma (St. Louis, MO, USA). Epichlorohydrin and imidazole were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade. *L. plantarum* CECT 748$^T$ strain was purchased from the Spanish Culture Type Collection (CECT). *E. coli* JM109 (DE3) was used for tannase expression in pURI3 vector (*18*).

### Growth Conditions and Production of Recombinant Tannase

*L. plantarum* was grown in MRS medium at 30º C. *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 cloning and expression of the gene encoding the *L. plantarum* CECT 748$^T$ tannase, tanLp1 (named lp_2956 in the *L. plantarum* WCFS1 strain) was previously described (*18, 22* ). The growing of *E.coli* cells, their centrifugation and their disruption in order to obtain a of a crude protein extract were previously described (*18*). The crude tannase extract contained 8 mg of protein per mL.

**Purification of tannase by adsorption on lowly activated Ni-IDA- 6% agarose gels.**

Lowly activated Ni-IDA-agarose gels ( containing 10 µEqs of chelates per mL of 6% agarose) were prepared as previously described (*23*). The crude tannase extract was diluted 10-fold in 50mM sodium phosphate buffer containg 150mM NaCl and 20 mM of imidazole and adjusted at pH 7.0. 150 mM NaCl was added to the binding buffer to prevent unspecific ionic interactions between non-recombinant proteins and the support. 20 mM imidazol was used in order to minimize the adsorption of non-recombinant proteins on the lowly activated Ni-IDA-supports. 50 mL of the diluted crude tannase extract (0.8 mg/mL of protein concentration) were mixed with 1mL of lowly activated Ni-IDA-agarose support (*23*). The incubation was carried out at 25 ºC and under constant gentle magnetic stirring. After 1 h the enzyme was completely adsorbed on the chromatographic support. Then, the adsorbed enzyme was recovered by filtration and subsequently washed with 50 mL of 50 mM phosphate buffer pH 7.0 containing 50 mM imidazole and 150 mM NaCl in order to remove the traces of non-recombinant proteins adsorbed on the support. Finally, the desorption of tannase was performed by incubation of the chromatographic support for 30 min. with 50 mL of 50 mM phosphate buffer pH 7.0 containing 100 mM imidazole and 150 mM NaCl.

**Protein determination and enzymatic assays.**

Protein concentrations were determined by the Bradford´s method (*24*). Bovine serum albumin (BSA) was used as the standard. The esterase activity of tannase was determined using a rhodamine assay specific for gallic acid (*25*). A tannase activity unit was defined as the amount of enzyme needed to hydrolyze 1 µmol of methyl gallate per minute.

**SDS–PAGE analysis**

SDS–PAGE experiments were performed as described by Laemmli (*26*) in a SE 250-Mighty small II electrophoretic unit (Hoefer Co. San Francisco, USA) (www.hoeferinc.com) using gels of 12% polyacrylamide in a separation zone of 9 cm × 6 cm and a concentration zone of 5% polyacrylamide. Gels were stained with the Coomassie brilliant blue (R-250) method. Low-molecular mass marker kits from Pharmacia were used (*M*r = 14 000–94 000).

**Immobilization of Tannase on CNBr-activated support**

The immobilization on CNBr-activated support was carried out at pH 7, 4º C for 15 min in order to strongly reduce the possibilities of a multipoint covalent attachment. 5 g of CNBr-activated support was added to a solution of 50 mL of purified tannase preparation. After 15 min, around 30% of enzyme was immobilized on the support. The immobilization process was ended by incubating the support with 1 M

144 ethanolamine at pH 8 for 2 h. Finally, the immobilized preparation was washed with
145 abundant water.

146 **Immobilization of tannase on glyoxyl-agarose supports**

147 1g of glyoxyl-support was added to 40 mL of purified tannase solution in bicarbonate
148 buffer 100 mM pH 10.0. Immobilization was complete in less than 1 hour but the
149 immobilization suspension was incubated at pH 10 at different temperatures (25 ºC
150 and 4 ºC) during different incubation times (from 1hour up to 24 hour). Long
151 incubation times usually promote a more intense multipoint covalent immobilization
152 and a higher stabilizing effect (*27, 28*). A reference suspension, using reduced glyoxyl-
153 agarose, was used to discard unspecific adsorptions.

154 Finally, 10 mg sodium borohydride were added to the immobilization mixture and the
155 suspension was reduced at 25 ºC for 30 min under gentle magnetic stirring. Thereafter,
156 the immobilized derivatives were washed thoroughly with 50 mM phosphate buffer pH
157 7.0.

158 Four different TG derivatives were prepared under different conditions (see **Table 1**).

159

160 **Thermal stability of immobilized tannase.**

161 Different immobilized tannase derivatives were incubated in 5 mM sodium phosphate
162 buffer at pH 7 and 50 ºC (eg. 1 gram of derivative suspended in 10 mL of buffer).
163 Samples of the suspension (100 µl) were periodically withdrawn using a pipette with a

cut-tip and under vigorous stirring to have a homogenous biocatalyst suspension, and their residual activities were determined using gallic acid analysis.

**Inactivation of different immobilized enzyme derivatives in the presence of cosolvents.**

Enzyme derivatives were washed with an aqueous phase achieved after equilibrating the solutions of the desired water/cosolvents mixture at two pH (7 and 5), 25 °C, and 30% of propanol. Subsequently, the enzyme derivatives were resuspended in such solution and incubated at the temperature indicated. Samples were withdrawn periodically, and the residual activity was determined following the above assay. Experiments were carried out in triplicate, and standard error was never over 5%.

**Enzymatic hydrolysis of tannic acid**

Five hundred milligrams of immobilized preparation were added to 3mL of 1 mM tannic acid, in 25mM buffer at different conditions of pH (5 and 7) and temperature (4 ºC and 25 ºC), under continuous gentle stirring. The reaction was carried out in presence of 30% of dyglyme to avoid tannic acid decomposition. The conversion was analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450) using a Kromasil C18 (5 μm 250 mm×4.6 mm) column. Products were eluted at flow rate of 1.0 mL/min using methanol–10mM sodium acetate at pH 2.95

184 (25:75, v/v) and UV detection performed at 280 nm. Retention time of tannic acid was
185 4.07 min.

186 The time course of tannic acid hydrolysis was study by with 5 mM of substrate in 100
187 mM sodium acetate pH 5, 30% of dyglyme and 4 ºC using 1 g of TG -2 (50 mg pure
188 enzyme/g support) in 10 mL of solution. The conversion was analyzed by RP-HPLC.
189 Now, a tannase activity unit was defined as the amount of enzyme needed to
190 hydrolyze 1 µmol of tannic acid per minute.

191

192

193 **Results**

194 **Purification of recombinant tannase overexpressed in *E. coli*.**

195 As described previously (*17*), the recombinant tannase was overexpressed as analyzed
196 by SDS-PAGE (**Figure 1**, lane 2). It is also possible to observe a very selective adsorption
197 of the enzyme (approx. 90% purity) on poorly activated nickel chelate supports in the
198 presence of 20 mM imidazole (lane3). Contaminant proteins are only adsorbed in
199 traces and are easily desorbed with a first wash at 50 mM imidazol leaving the pure
200 tannase adsorbed to the support (lane 4). Pure tannase was eluted at 100 mM
201 imidazole (**Figure 1**, lane 5). Purification yield was 95% and purification factor was 15.
202 The specific activity of the pure enzyme for the hydrolysis of methyl gallate was 20
203 µmols hydrolyzed / min x mg of tannase. This value is 2 orders of magnitude higher

than the one obtained with commercial tannase from *Aspergillus ficuum* commercialized by Sigma Chem. Co.

Hence, in only one step it was possible to purify to homogeneity a His tagged recombinant protein from an *E. coli* crude extract even in the presence of nucleic acids. Moreover, the selective adsorption of the target enzyme facilitates the use of small volumes of chromatographic support and therefore simplifies and makes the purification more cost-efficient. In fact, up to 0.9 grams of enzyme could be purified by using only 20 mL of chromatographic support

**Preparation of Immobilized derivatives of tannase from *Lactobacillus plantarum*.**

We have prepared different immobilized derivatives of tannase namely: a.- tannase-CNBr-agarose (TCN) was prepared under very mild conditions in order to avoid the multi-point attachment of the enzyme on the support. Therefore, this derivative exhibits very similar activity and stability properties to those of the soluble enzyme (*20*). However, the full dispersion of immobilized enzyme molecules on the surface of the support allows its testing in reaction conditions where soluble enzyme would either aggregate or precipitated. , b.-TG-1 was prepared using highly activated agarose (HAS, 6% agarose gels contain 50 µEqs of glyoxyl per mL of supports) and the immobilization was carried out at 25 ºC, pH 10 for short periods of time (90 min) (unfavorable conditions for the multipoint covalent immobilization), c.- TG-2: tannase was immobilized on highly activated agarose (HAS, 6% agarose gels contain 50 µEqs of glyoxyl per mL of supports) and the immobilization was carried out at 25 ºC, pH 10 for

long time periods (24 h) enhancing multipoint attachment, d.- TG-3: tannase was immobilized by using glyoxyl-agarose with a lower number of reactive groups (MAS, 6% agarose gels contain 25 µEqs of glyoxyl per mL of supports) in order to prove the effect of the covalent multipoint attachment on the stability of the derivatives, e.- TG-4 was prepared with higly activated glyoxyl agarose but under unfavorable conditions for the multipoint covalent immobilization (pH 10, 24 h and 4 ºC). (*21*)

**Table 1** shows the immobilization yields and recovered activities for each immobilization strategy. A low enzyme concentration was used to avoid difussional problems. Immobilization on CNBr preserves 100% of its initial activity and the extent of activity retention in glyoxyl derivatives ranged from 78 to 85%. Having in mind that 70 mg of pure enzyme were immobilized per wet gram of 6% agarose, immobilized-stabilized tannase derivatives having an intrinsic activity of 1000 U/gr. could be prepared.

**Stability of the immobilized derivatives of tannase from *L.plantarum*.**

**Figure 2** showed the thermal inactivation of *L. plantarum* tannase derivatives. The glyoxyl derivatives were much more stable than the CNBr which was as stable as the soluble enzyme. Among the glyoxyl derivatives the one prepared on highly activated supports (HAS) at 25 ºC and for 24 h (TG2) reached the best stabilization factor (500 fold). The stabilization was higher when increasing the concentration of active groups, the temperature and the incubation time. These results may be an indication that the degree of stabilization is a direct consequence of a more intense multipoint covalent immobilization (*21*).

248 The most stable derivative (TG2) and the least stable (TCN) were also inactivated in the
249 presence of 2-propanol (**Figure 3**). As it was commented previously, aggregation
250 problems preclude the use of the soluble enzyme in this experiment that may main
251 either positive or negative artifacts. TG-2 was 1000 folds more stable than TGCN. The
252 stabilization against any inactivation agent is one of the advantages of the stabilization
253 by covalent multipoint immobilization: an increased rigidification of the enzyme
254 surface will promote the stabilization against several inactivating agents.

255

256 **Analysis by SDS-PAGE of subunits desorbed from the different covalently**
257 **immobilized derivatives**.

258 In order to study the multisubunit immobilization of tannase, boiling of the derivatives
259 in the presence of mercaptoethanol and SDS was carried out. This treatment causes
260 the desorption of all the subunits from a multimeric enzyme that were not covalently
261 attached to the support. **Figure 4** shows how TCN derivative desorbs at least one
262 subunit of the enzyme indicating that this tannase is a multimeric enzyme. Unlikely,
263 any subunit is desorbed from TG-2 derivative demonstrating that all the enzyme
264 subunits were covalently attached to the support.

265

266 **Reaction design of the hydrolysis of tannic acid.**

267 The hydrolysis of tannic acid was performed under 30% of different cosolvents in order
268 to avoid microbial contaminations (**Figure 5**). The best results were obtained using

diglyme or DMF (dimethylformamide). Diglyme was chosen as optimal cosolvent because it is a non toxic one and it is usually less harmful for enzyme stability (*27*).

We have also studied the reaction courses at different pHs (**Figure 6**). At pH 8 the reaction yields up to 50% of gallic acid before stopping. It seems that the gallic-gallic bonds are easier to hydrolyze than gallic-glucose ones. On the contrary at pH 5.0 the reaction course was linear up to very high hydrolysis percentages (eg., 95%)

The effect of the temperature was also studied . The reaction occurs approximately 4 folds slower at 4 ºC than at 25 ºC of the reaction product. However, at these conditions (pH 5.0, 30% dyglime and 4 ºC) the gallic acid purity was higher likely because there might be neither chemical nor microbial decomposition of the substrate or the product.

**Complete course of the hydrolysis reaction.**

Using a maximum load in the catalyst (70 mg of pure tannase per gram of catalyst) and a 1:10 ratio (weigh of catalyst: reaction volume) the complete course of tannic acid hydrolysis was followed under optimal reaction conditions (30% dyglime, pH 5.0 and 4 ºC) starting from 5mM of tannic acid (**Figure 7**). A fairly linear course of gallic acid production reaching a concentration of 47.5 mM (the commercial preparation contains approximately 10 molecules of gallic acid per molecule of tannic acid) was observed. When reaction was carried out under these conditions, a unique chromatographic peak was observed in HPLC indicating the total absence of byproducts (**Figure 8B**). On the contrary at pH 7.0 and 25 ºC several peaks , close to the one corresponding to gallic acid, appear after 5 h of reaction (**Figure 8A**). In those optimal conditions the

best tannase derivatives preserved more than 95% of its initial activity after 30 days of incubation (data not shown).

## DISCUSSION

### a.- Very Simple Immobilization Protocols.

The use of an over-expressed recombinant enzyme containing a poly-His tail hardly modifies the functional properties of industrial enzymes but strongly improves their purification. The combination of these enzymes with tailor-made lowly activated IMAC supports and the presence of moderated concentrations of imidazol during the adsorption of the crude protein extract allows the performance of a very selective adsorption of the target recombinant enzyme and small traces of other native proteins. In this way, 0.9 grams of recombinant enzyme could be fully purified by a single chromatographic step by using only 15 mL of chromatographic support in a batch reactor. However, if adsorption of the recombinant enzyme were not selective (eg., by using highly activated supports in the absence of imidazole) most of 90% of total proteins of the crude extract become adsorbed on IMAC supports (24 ). In this case we would need at least 300 mL of chromatographic support to get the adsorption of 15 grams of proteins from the crude extract and the subsequent purification of the 0.9 grams of target enzyme. The amount of chromatographic support needed to purify enzymes is not very relevant at laboratory scale but it becomes critical at industrial scale in order to get simpler and less expensive purification protocols.

312 On the other hand, the pure tannase from *Lactobacillus plantarum* exhibits a very high
313 catalytic activity (20 U/mg of enzyme). This activity is approximately 100 fold higher
314 than a fairly pure commercial tannase from *Aspergillus ficuum*.

315

316 **b.- Immobilization-Stabilization of Tannase.**

317 Tannases from other microbial sources have been already immobilized. In general
318 they were immobilized by using conventional techniques and enzyme stability has
319 been hardly improved (*16, 28*). For example, tannase from *Aspergillus niger* has been
320 immobilized by different techniques (encapsulation, covalent immobilization on
321 glutaraldehyde supports, etc.). In general, recoveries of activity after immobilization
322 were low (20%) and stabilization factors (compared to soluble enzyme) were only 2-3
323 fold. Moreover, reaction yields were not higher than 50%. (*28*).

324 A protocol for multipoint covalent attachment on glyoxyl agarose gels has
325 been developed in our laboratory and it has been already tested for many other
326 enzymes. In addition to the use of very highly activated supports, it has been
327 demonstrated that multipoint covalent attachment and the subsequent stabilization
328 are improved by using long incubations at pH 10.0 st 25 ºC (*29,30*) This method usually
329 promotes the highest stabilization factors achieved via immobilization techniques: eg.,
330 the most of derivatives of different enzymes were between 100 and 10.000 fold more
331 stable than the corresponding soluble enzymes or one-point immobilized derivatives
332 and they were stabilized against any distorting agent, heat, organic cosolvents, pH, etc,
333 (*21, 29, 30*). The application of this protocol to tannase has also given very promising

334 results: a very high stabilization plus the simultaneous immobilization of all enzyme
335 subunits. Again the enzyme was stabilized against temperature and against organic
336 cosolvents. In the first trial of cosolvent we have selected a distorting cosolvent (2-
337 propanol) in order to rapidly quantify the stabilization of the best immobilized
338 derivative regarding to the one-point immobilized one. However, other much milder
339 cosolvents were selected to prevent microbial contaminations during enzymatic
340 hydrolysis. In a previous paper we have reported that diglyme, ethanol and
341 dimethylformamide hardly exert harmful effects on most of immobilized enzyme
342 derivatives (*26*).

343 In addition to their good properties for immobilization-stabilization of enzymes, glyoxyl
344 agarose are very stable under immobilization conditions (eg., pH 10.0). In this way,
345 when using stable soluble enzymes, long immobilizations can be performed and the
346 support surface can be completely loaded with pure enzyme. In fact 50 mg of pure
347 tannase could be immobilized on 1 wet gram of 6% agarose gels and the resulting
348 derivatives exhibit a very high intrinsic activity: 1000 Units per gram of biocatalyst.
349 This activity was measured by following the hydrolysis of methyl gallate catalyzed by
350 fully loaded derivatives after breaking them (under a very strong magnetic stirring) in
351 order to get very small particle sizes and, in this way, minimize the difussional
352 limitations of the observed catalytic activity.

353

354 **c.- Enzymatic hydrolysis of tannic acid.**

On one hand, pH 5.0 was selected to get a quite linear and the almost quantitative hydrolysis of tannic acid (9.5 molecules of gallic acid were obtained from 1 molecule of commercial pure tannic acid). At other pH values or when using other tannases the reaction courses were much less linear and final yield were not quantitative. On the other hand, both gallic and tannic acids are very unstable against chemical and microbial degradation. The use of moderate concentrations of cosolvents (30% diglyme) and low temperatures prevent both degradations and then a chromatographically pure gallic acid was obtained. Under these mild reaction conditions, the stabilized derivatives of tannase from lactobacillus plantarum were extremely stable.

**d.- Practical remarks**

The multidisciplinary combination of good protocols from Microbiology, Molecular Biology, Enzyme purification using tailor-made chromatographic supports, Immobilization-Stabilization of enzymes by multisubunit and multipuntual immobilization, Process engineering, etc made possible the design of a relevant process in Food technology. We have been able to obtain a pure relevant product (gallic acid) using a vegetal byproduct as substrate (tannic acid) and very active, robust and economical immobilized enzyme preparations of a tannase from *Lactobacillus plantarum*. As far as we know, a set of a number of very interesting parameters for the biocatalyst and for the bioprocess has never been reported for the hydrolysis of tannic acid catalyzed by immobilized tannase:

1.- The soluble tannase enzyme had been is over-expressed in *E.coli* up to levels of 7-8% of enzyme versus total protein. In addition to that a poly-His tail had been added to the recombinant enzyme.

2.- the enzyme was fully purified through a single chromatographic step and the pure enzyme exhibited a high catalytic activity (20 Units /mg of protein)

3.- derivatives containing 50 mg of pure enzyme per gram of biocatalyst could be prepared and the intrinsic activity of these biocatalyst was 1000 Unites /wet gram of biocatalyst.

4.- The immobilized enzyme is stabilized 500-1000 fold regarding one-point covalent immobilized derivatives.

5.- at least a 95% of tannic acid is transformed into pure gallic acid

6.- the best enzyme derivative is extremely stable under optimal reaction conditions.

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## FIGURE LEGENDS

**Figure 1.- Analysis by SDS-PAGE (12%) of different samples of poly-His tagged tannase from *L. plantarum* : analysis of adsorbed and soluble proteins.** Adsorption of a crude extract from *E.coli* containing poly-His tagged tannase on lowly activated IDA-Ni agarose gels was carried out as described in Materials and methods. Lanes: (1) low molecular protein markers; (2) crude extract containing poly-His tagged tannase from *lactobacillus plantarum*; (3) proteins adsorbed on IDA-Ni-agarose ; (4) proteins adsorbed on IDA-Ni-agarose after washing with 50 mM of imidazole; (5) soluble tannase desorbed with 150 mM imidazole.

**Figure 2. Time-courses of thermal inactivation of different immobilized derivatives of tannase from *Lactobacillus plantarum*.** Inactivations were performed at pH 7 and 50 ◦C. Experiments carried out as described in Methods using lowly loaded enzyme preparations (derivatives with 1 mg protein/ g support). Circles: TG-2; triangle: TG-3 ; squares: TG-1; asterisk: TG-4 and rhombus: TCNBr.

**Figure 3.- Time-courses of Inactivation of different immobilized derivatives of tannase from *Lactobacillus plantarum* incubated with organic cosolvents.** Inactivations were performed at pH 7, 25 ◦C and 30% of propanol. Circles: TG-2 and squares: TCN.

**Figure 4.- Analysis by SDS-PAGE (12%) of different immobilized derivatives obtained from a crude extract of *E.coli* containing poly-His tagged tannase from *Lactobacillus plantarum*.** Immobilized experiments of tannase and preparation of the samples were carried out as described in Materials and methods. Lanes: (1) low molecular marker;

(2) crude extract from E.coli; (3) subunits of multimeric proteins covalently immobilized on CNBr-Sepharose support; (4) subunits of multimeric proteins covalently immobilized on highly activated glyoxyl support (TG-2).

**Figure 5.-Effect of solvent on the hydrolysis of tannic acid catalyzed by TG2.** . The hydrolysis reaction was performed at pH 7, 25 ºC in the presence of 30% of different cosolvents. Experiments carried out as described in methods using lowly loaded enzyme preparations. Circles: 30% Ethanol; squares: 30% diglyme; triangles: 30% DMF.

**Figure 6.- Effect of pH on the time-courses of hydrolysis of tannic acid catalyzed by TG2.** The hydrolysis was performed at 25 ºC in the presence of 30% of diglyme. Experiments were carried out as described in methods using lowly loaded enzyme preparations. Circles: sodium acetate 25 mM pH5; squares: sodium phosphate 25 mM pH 7; triangles: sodium phosphate 25 mM pH8.

**Figure 7.- Time-course of hydrolysis of 5 mM tannic acid catalyzed by TG2 in ammonium acetate 25mM pH 5, 4 ºC and 30% of dyglime**. Experiments were carried out as described in Methods by using highly loaded immobilized enzyme derivatives (70 mg of pure tannase / g catalyst).

**Figure 8.- UV-chromatograms of gallic acid obtained by enzymatic hydrolysis of tannic acid catalyzed by TG2.** Experiments were carried out as described in Methods. Concentration of tannic acid was 5 mM and reaction time was 5 h. Chromatogram A.- contaminated product obtained at pH 7.0, 25 ºC; Chromatogram B.- pure product obtained at pH 5.0, 4 ºC in the presence of 30% diglyme.

**Table 1.- Conditions of the immobilized derivatives of tannase from *Lactobacillus plantarum* on support glyoxyl-agarose.** Immobilizations were performed as described in the experimental section.

| Derivative | Activation grade | Time (h) | T (ºC) |
|---|---|---|---|
| TG-1 | HAS | 1.5 | 25 |
| TG-2 | HAS | 24 | 25 |
| TG-3 | MAS | 1.5 | 25 |
| TG-4 | HAS | 24 | 4 |

**Table 2.- Immobilization yield and recovered activity of the immobilized derivatives of tannase from *Lactobacillus plantarum*.** Immobilizations were performed as described in the experimental section.

| Derivative | Immobilization yield, % | Recovered activity (%) | Catalytic capability (mg) |
|---|---|---|---|
| TCN | 20 | 100 | 15 |
| TG-1 | >95 | 78 | 50 |
| TG-2 | >95 | 76 | 50 |
| TG-3 | >95 | 80 | 50 |
| TG-4 | >95 | 85 | 50 |

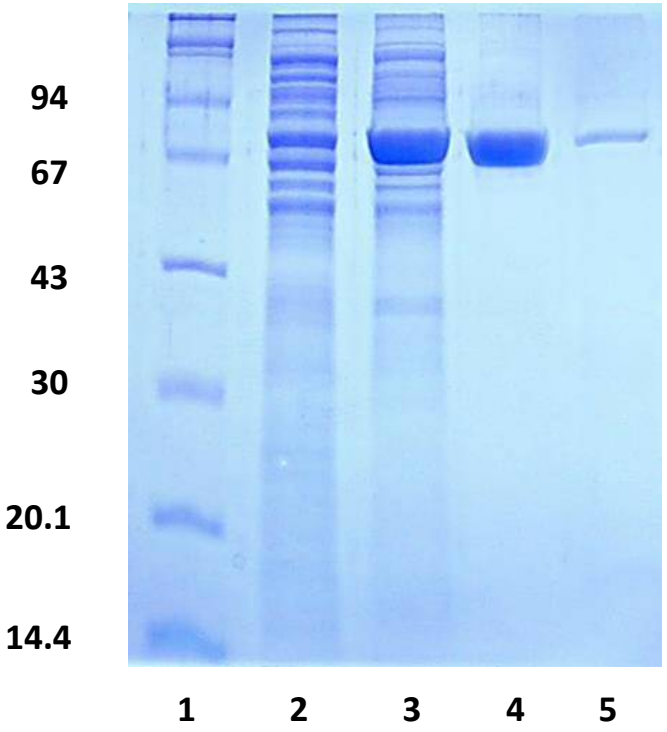
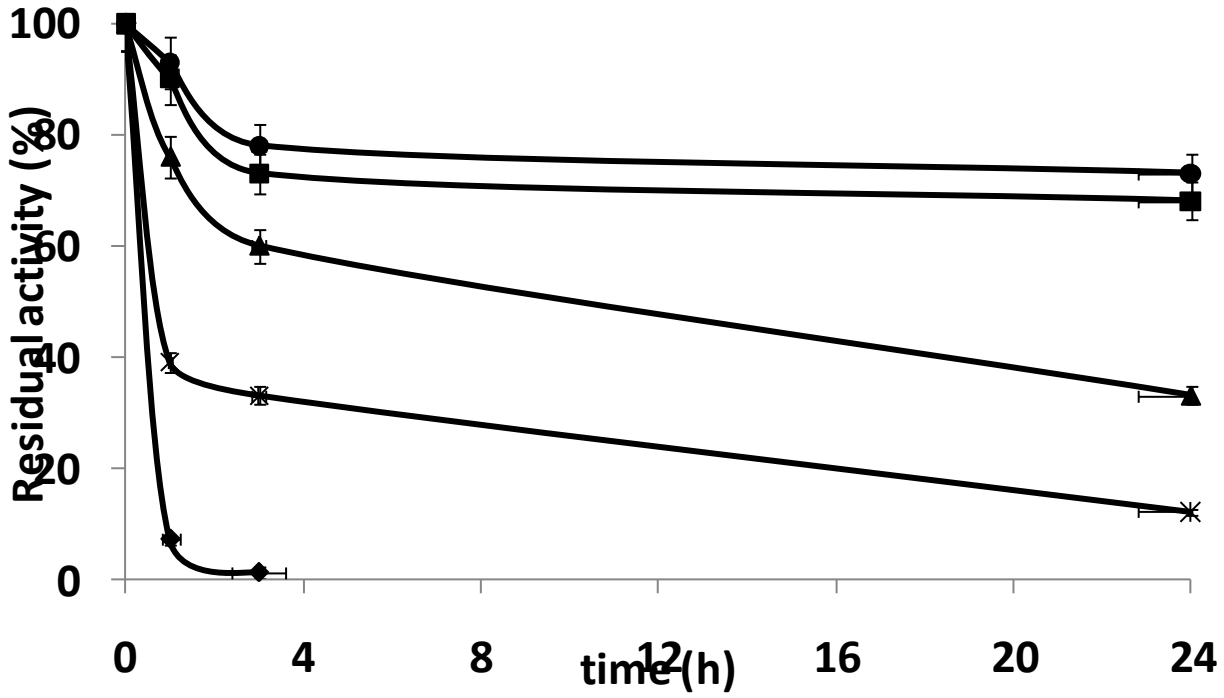



Figure 1

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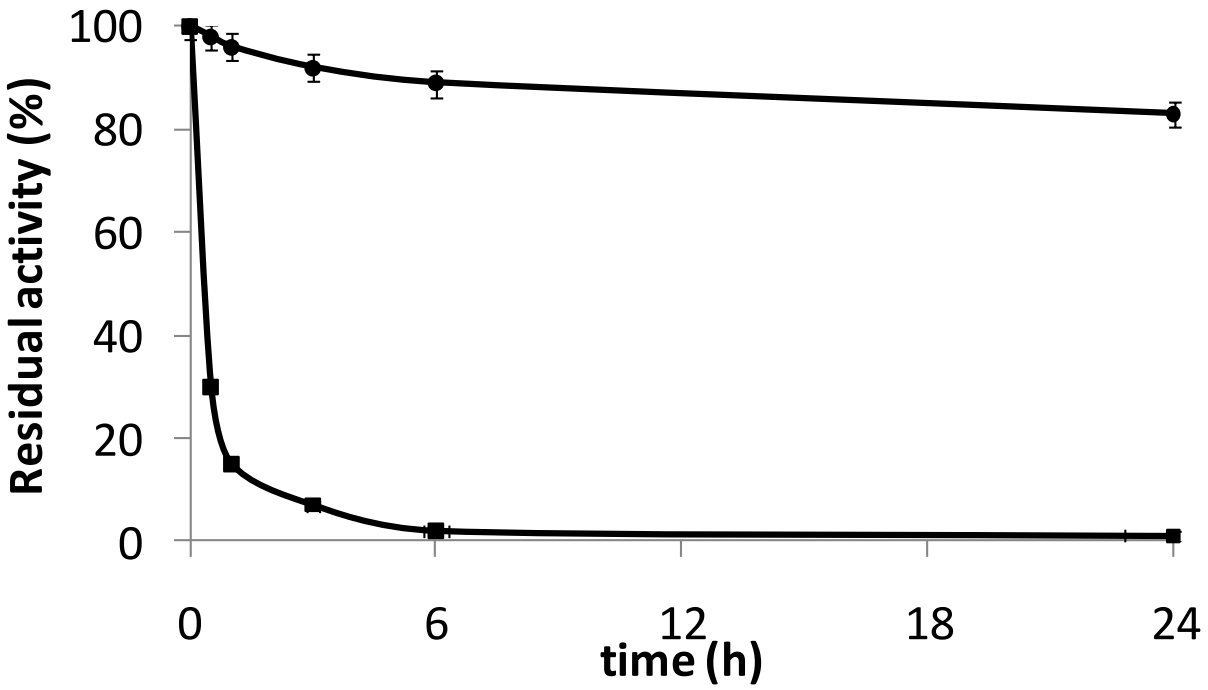


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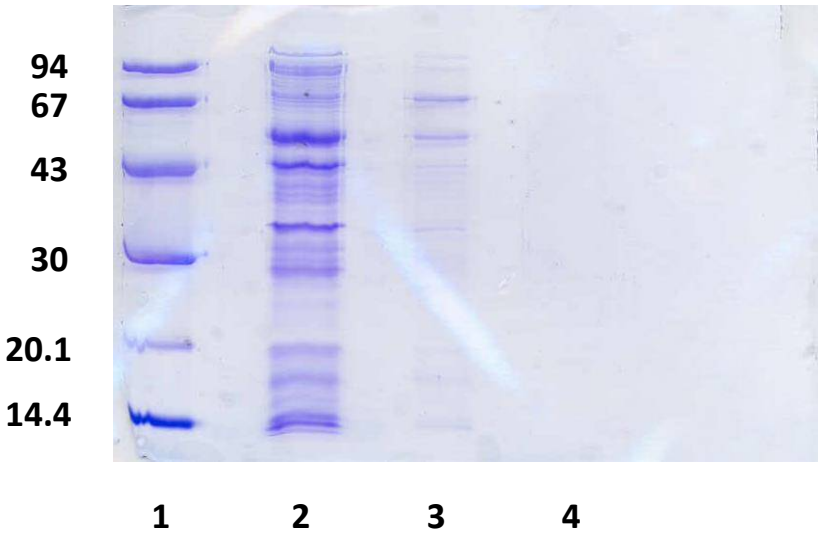
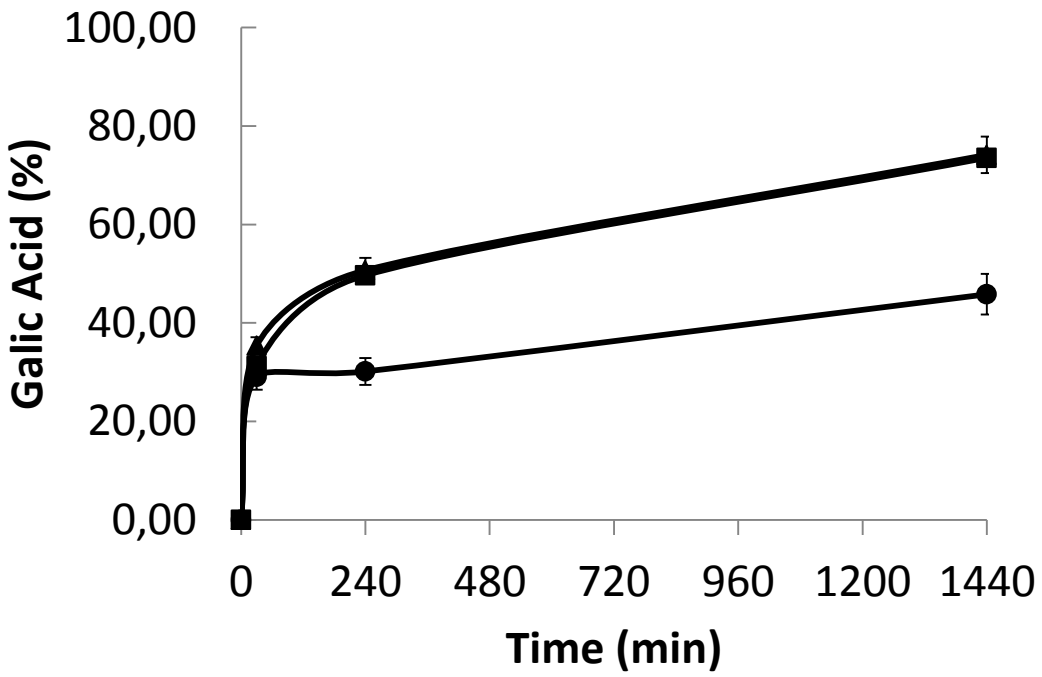



Figure 4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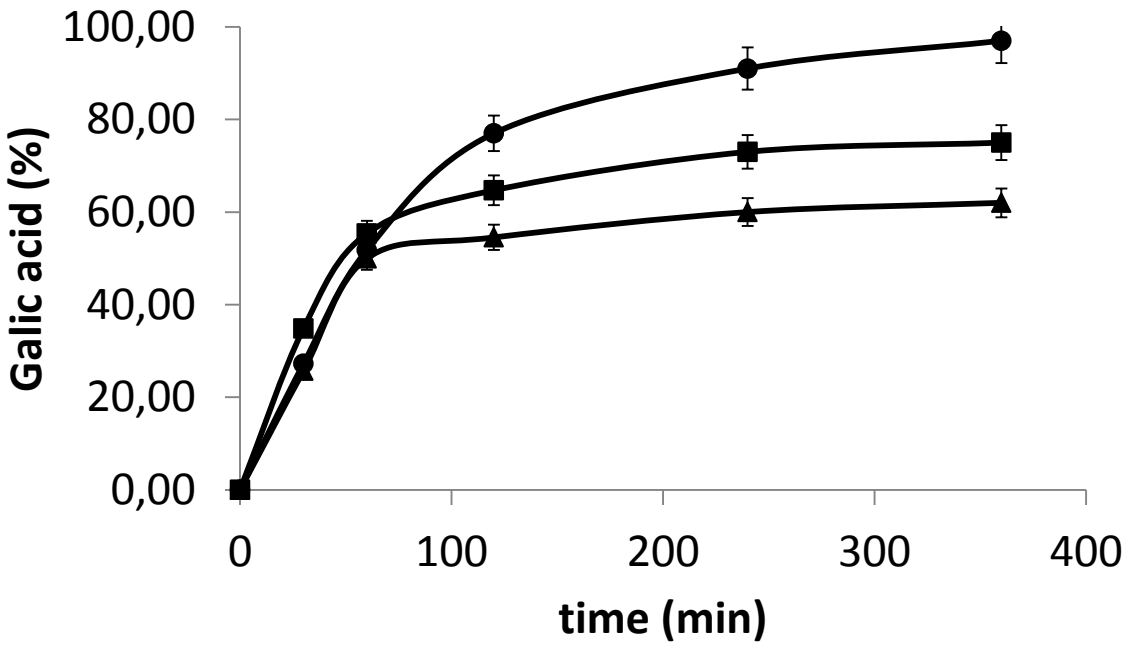
80,00
60,00
40,00
20,00
0,00
Galic Acid (%)
0
240
480
720
960
1200
1440
Time (min)

Figure 5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80,00
60,00
40,00
20,00
0,00
Galic acid (%)
0
100
200
300
400
time (min)

Figure 6

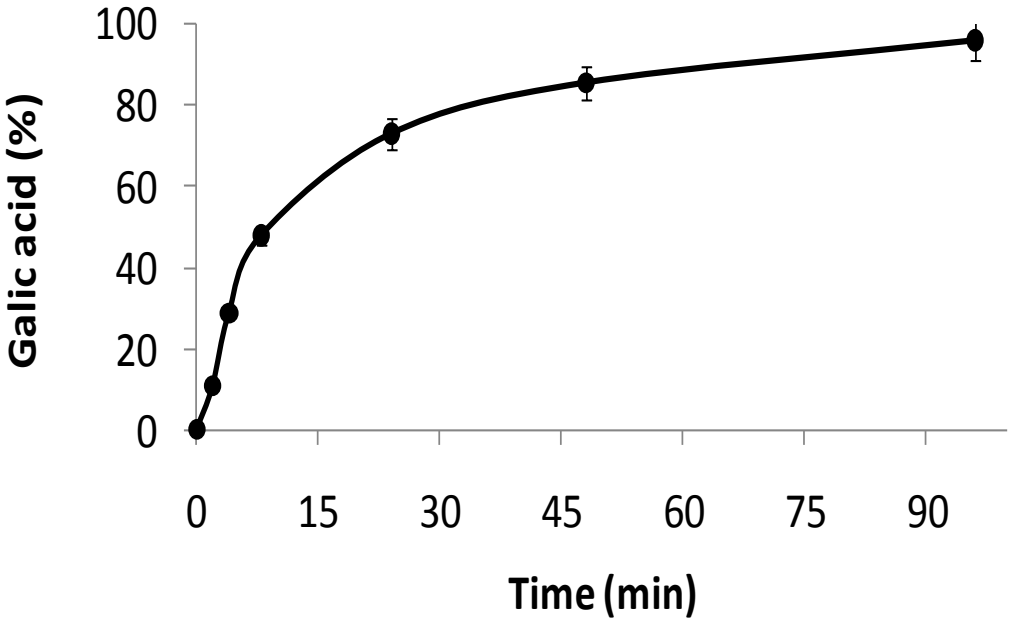
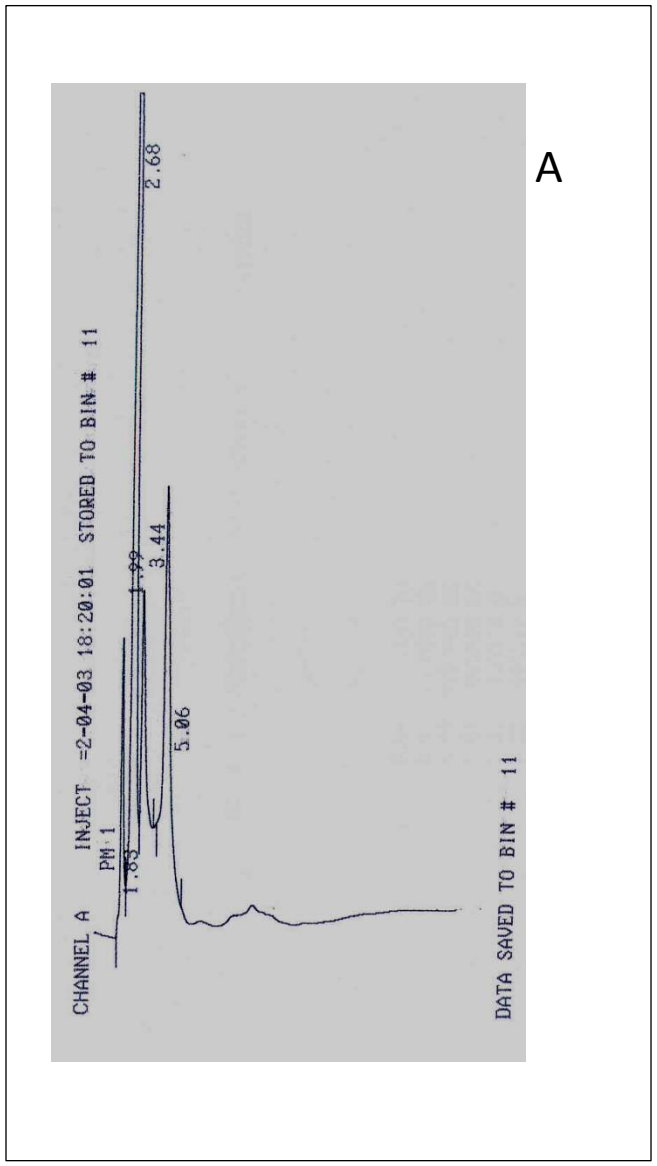



Figure 7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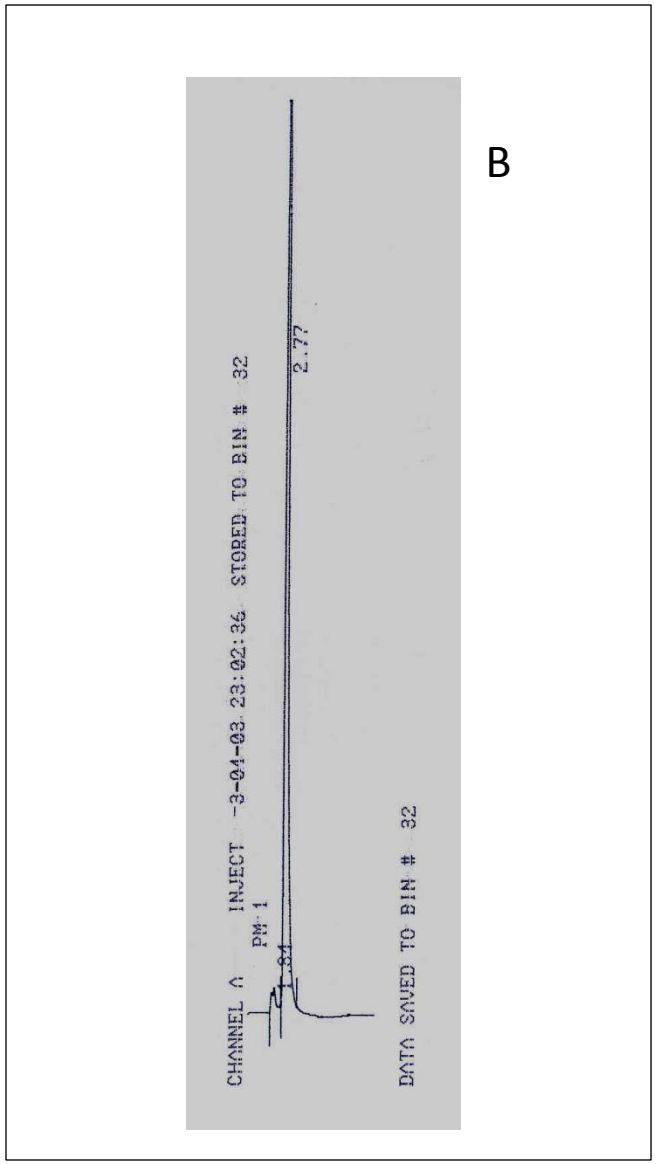
1.99
2.68
3.44
5.06
DATA SAVED TO BIN # 11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2.77
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Figure 8