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Hydrolysis of Tannic Acid Catalyzed by Immobilized−Stabilized Derivatives of Tannase from *Lactobacillus plantarum*

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A recombinant tannase from *Lactobacillus plantarum*, overexpressed in *Escherichia coli*, was purified in a single step by metal chelate affinity chromatography on poorly activated nickel supports. It was possible to obtain 0.9 g 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 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, up to 70 mg of pure enzyme was immobilized on 1 g 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 °C. Under these conditions, it was possible to obtain 47.5 mM gallic acid from 5 mM tannic acid as substrate. The product was pure as proved by HPLC. On the other hand, the immobilized biocatalyst preserved >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 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*), and design of enzymatic biosensors (*5*). For the majority of the previously mentioned applications, utilization of immobilized enzymes is technologically advantageous (*6*−*8*). However, food technology must be 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 product inhibition (*15*−*17*).

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Herein we describe the preparation of a very active and stable derivative of a tannase from *Lactobacillus plantarum* (*18*). The enzyme was first purified to homogeneity by selective adsorption on small volumes of chelate-activated agarose (*19*). Subsequent immobilization of the pure protein by covalent multipoint and multisubunit attachment on glyoxyl agarose stabilized the enzyme (*20*, *21*). Finally, 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. Cross-linked 6% agarose beads and cyanogen bromide activated Sepharose 4B (CNBr-agarose) were obtained from GE Healthcare (Uppsala, Sweden). Iminodiacetic acid disodium salt monohydrate (IDA), nickel(II) sulfate 6-hydrate, and methyl gallate were purchased from Fluka (Buchs, Switzerland). Tannic acid was obtained from Sigma (St. Louis, MO). 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). *Escherichia 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 30 °C. *E. coli* strains were cultured in Luria−Bertani (LB) medium at 37 °C 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

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Table 1. Conditions of the Immobilized Derivatives of Tannase from *Lactobacillus plantarum* on Glyoxyl−Agarose Support[a]

derivative	activation grade	time (h)	T (°C)
TG-1	HAS	1.5	25
TG-2	HAS	24	25
TG-3	MAS	1.5	25
TG-4	HAS	24	4

[a] Immobilizations were performed as described under Materials and Methods.

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 to obtain a crude protein extract were previously described (*18*). The crude tannase extract contained 8 mg of protein/mL.

Purification of Tannase by Adsorption on Poorly Activated Ni-IDA- 6% Agarose Gels. Mildly activated Ni-IDA-agarose gels (containing 10 μequiv of chelates/mL of 6% agarose) were prepared as previously described (*23*). The crude tannase extract was diluted 10-fold in 50 mM sodium phosphate buffer containg 150 mM NaCl and 20 mM imidazole and adjusted at pH 7.0. NaCl (150 mM) was added to the binding buffer to prevent unspecific ionic interactions between nonrecombinant proteins and the support. Imidazole (20 mM) was used to minimize the adsorption of nonrecombinant proteins on the poorly activated Ni-IDA supports. Fifty milliliters of the diluted crude tannase extract (0.8 mg/mL of protein concentration) was mixed with 1 mL of poorly activated Ni-IDA-agarose support (*23*). Incubation was carried out at 25 °C 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 to remove the traces of nonrecombinant 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 according to 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/min.

SDS-PAGE Analysis. SDS-PAGE experiments were performed as described by Laemmli (*26*) in an SE 250-Mighty small II electrophoretic unit (Hoefer Co., San Francisco, CA) (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 = 14000−94000).

Immobilization of Tannase on CNBr-Activated Support. The immobilization on CNBr-activated support was carried out at pH 7 and 4 °C for 15 min to strongly reduce the possibilities of a multipoint covalent attachment. Five grams 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 ethanolamine at pH 8 for 2 h. Finally, the immobilized preparation was washed with abundant water.

Immobilization of Tannase on Glyoxyl−Agarose Supports. One gram of glyoxyl support was added to 40 mL of purified tannase solution in bicarbonate buffer, 100 mM, pH 10.0. Immobilization was complete in <1 h, but the immobilization suspension was incubated at pH 10 at different temperatures (25 and 4 °C) during different incubation times (from 1 to 24 h). Long incubation times usually promote a more intense multipoint covalent immobilization and a higher stabilizing effect (*27*, *28*). A reference suspension, using reduced glyoxyl−agarose, was used to discard unspecific adsorptions.

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

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

Thermal Stability of Immobilized Tannase. Different immobilized tannase derivatives were incubated in 5 mM sodium phosphate buffer at pH 7 and 50 °C (e.g., 1 g of derivative suspended in 10 mL of buffer). Samples of the suspension (100 μL) were periodically withdrawn using a pipet with a cut-tip and under vigorous stirring to have a homogeneous 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 equilibration of the solutions of the desired water/cosolvents mixture at two pH values (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 the standard error was never > 5%.

Enzymatic Hydrolysis of Tannic Acid. Five hundred milligrams of immobilized preparation was added to 3 mL of 1 mM tannic acid, in 25 mM buffer at different conditions of pH (5 and 7) and temperature (4 and 25 °C), under continuous gentle stirring. The reaction was carried out in the presence of 30% of diglyme 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 a flow rate of 1.0 mL/min using methanol/10 mM sodium acetate at pH 2.95 (25:75, v/v) and UV detection performed at 280 nm. The retention time of tannic acid was 4.07 min.

The time course of tannic acid hydrolysis was studied with 5 mM substrate in 100 mM sodium acetate, pH 5, 30% of diglyme and 4 °C using 1 g of TG-2 (50 mg of pure enzyme/g of support) in 10 mL of solution. The conversion was analyzed by RP-HPLC. Now, a tannase activity unit was defined as the amount of enzyme needed to hydrolyze 1 μmol of tannic acid/min.

RESULTS

Purification of Recombinant Tannase Overexpressed in *E. coli*. As previously described (*17*), the recombinant tannase was overexpressed as analyzed by SDS-PAGE (**Figure 1**, lane 2). It is also possible to observe a very selective adsorption of the enzyme (approximately 90% purity) on poorly activated nickel chelate supports in the presence of 20 mM imidazole (lane 3). Contaminant proteins are only adsorbed in traces and are easily desorbed with a first wash at 50 mM imidazole, leaving the pure tannase adsorbed to the support (lane 4). Pure tannase was eluted at 100 mM imidazole (**Figure 1**, lane 5). Purification yield was 95%, and purification factor was 15. The specific activity of the pure enzyme for the hydrolysis of methyl gallate was 20 μmol of hydrolyzed/min·mg of tannase. This value is 2 orders of magnitude higher than the one obtained with commercial tannase from *Aspergillus ficuum* commercialized by Sigma Chemical 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 g of enzyme could be purified by using only 20 mL of chromatographic support.

Preparation of Immobilized Derivatives of Tannase from *L. plantarum*. We have prepared different immobilized derivatives of tannase (**Table 1**), namely, tannase−CNBr-agarose (TCNBr) was prepared under very mild conditions to avoid the multipoint attachment of the enzyme on the support. Therefore, this derivative exhibits activity and stability properties very similar to those of the soluble enzyme (*20*). However, the full dispersion of

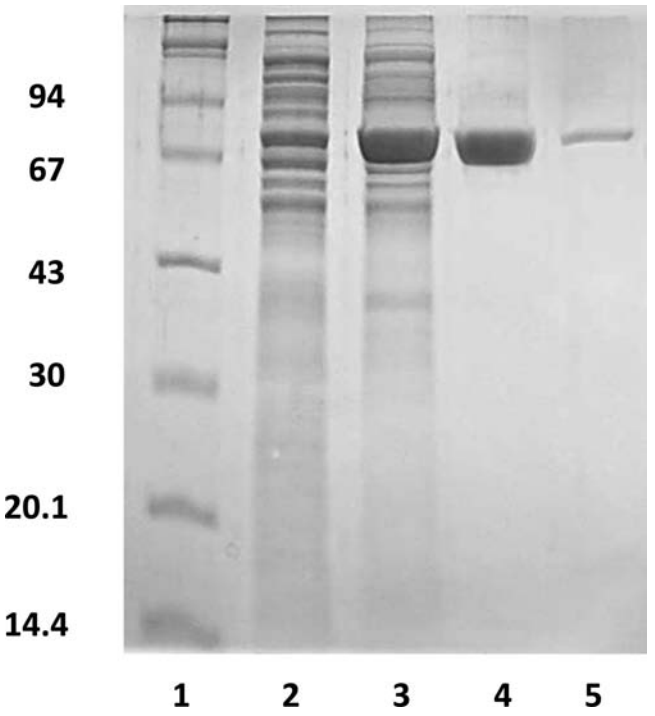

Figure 1. Analysis by SDS-PAGE (12%) of different samples of poly-His-tagged tannase from *L. plantarum*: analysis of adsorbed and soluble proteins. Adsorption of a crude extract from *E. coli* containing poly-His-tagged tannase on poorly activated IDA−Ni agarose gels was carried out as described under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

immobilized enzyme molecules on the surface of the support allows its testing in reaction conditions where soluble enzyme would either aggregate or precipitate. TG-1 was prepared using highly activated agarose (HAS, 6% agarose gels contain 50 μequiv of glyoxyl/mL of supports), and the immobilization was carried out at 25 °C, pH 10, for short periods of time (90 min) (unfavorable conditions for the multipoint covalent immobilization). For TG-2, tannase was immobilized on highly activated agarose (HAS, 6% agarose gels contain 50 μequiv of glyoxyl/mL of supports), and the immobilization was carried out at 25 °C, pH 10, for long time periods (24 h), enhancing multipoint attachment. For TG-3, tannase was immobilized by using glyoxyl−agarose with a lower number of reactive groups (MAS, 6% agarose gels contain 25 μequiv of glyoxyl/mL of supports) to prove the effect of the covalent multipoint attachment on the stability of the derivatives. TG-4 was prepared with highly activated glyoxyl−agarose but under unfavorable conditions for the multipoint covalent immobilization (pH 10, 24 h, and 4 °C) (*21*).

Table 2 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%. Because 70 mg of pure enzyme was immobilized per wet gram of 6% agarose, immobilized−stabilized tannase derivatives having an intrinsic activity of 1000 U/g could be prepared.

Stability of the Immobilized Derivatives of Tannase from *L. plantarum*. **Figure 2** shows 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.

Table 2. Immobilization Yield and Recovered Activity of the Immobilized Derivatives of Tannase from *L. plantarum*[a]

derivative	immobilization yield (%)	recovered activity (%)	catalytic capability (mg)
TCNBr	20	100	15
TG-1	>95	78	50
TG-2	>95	76	50
TG-3	>95	80	50
TG-4	>95	85	50

[a] Immobilizations were performed as described under Materials and Methods.

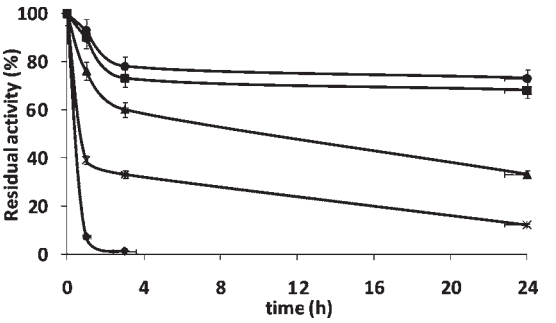

Figure 2. Time courses of thermal inactivation of different immobilized derivatives of tannase from *L. plantarum*: (●) TG-2; (▲) TG-3; (■) TG-1; (∗) TG-4; (◆) TCNBr. Inactivations were performed at pH 7 and 50 °C. Experiments carried out as described under Materials and Methods using poorly loaded enzyme preparations (derivatives with 1 mg of protein/g of support).

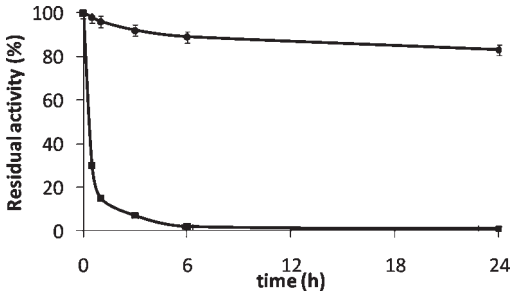

Figure 3. Time courses of inactivation of different immobilized derivatives of tannase from *L. plantarum* incubated with organic cosolvents: (●) TG-2; (■) TCNBr. Inactivations were performed at pH 7, 25 °C, and 30% of propanol.

Among the glyoxyl derivatives the one prepared on highly activated supports (HAS) at 25 °C and for 24 h (TG-2) reached the best stabilization factor (500-fold). The stabilization was higher when the concentration of active groups, the temperature, and the incubation time were increased. These results may be an indication that the degree of stabilization is a direct consequence of a more intense multipoint covalent immobilization (*21*).

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

Analysis by SDS-PAGE of Subunits Desorbed from the Different Covalently Immobilized Derivatives. To study the multisubunit

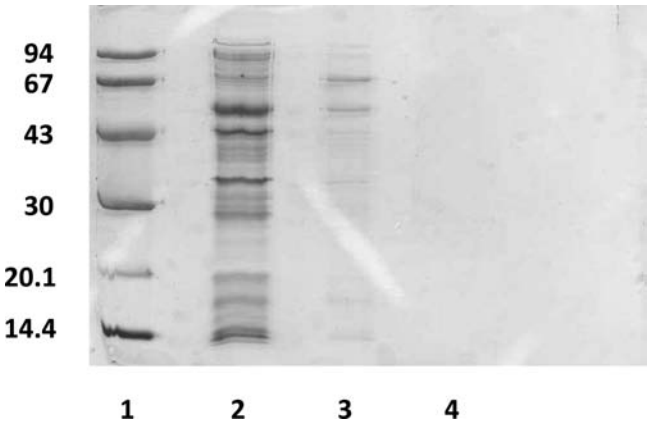

Figure 4. Analysis by SDS-PAGE (12%) of different immobilized derivatives obtained from a crude extract of *E. coli* containing poly-His-tagged tannase from *L. plantarum*. Lanes: (1) low molecular weight marker; (2) crude extract from *E. coli*; (3) subunits of multimeric proteins covalently immobilized on CNBr-Sepharose support; (4) subunits of multimeric proteins covalently immobilized on highly activated glyoxyl support (TG-2). Immobilized experiments of tannase and preparation of the samples were carried out as described under Materials and Methods.

immobilization of tannase, boiling of the derivatives in the presence of mercaptoethanol and SDS was carried out. This treatment causes the desorption of all the subunits from a multimeric enzyme that were not covalently attached to the support. **Figure 4** shows how the TCNBr derivative desorbs at least one subunit of the enzyme, indicating that this tannase is a multimeric enzyme. It is unlikely that any subunit is desorbed from the TG-2 derivative, demonstrating that all of the enzyme subunits were covalently attached to the support.

Reaction Design of the Hydrolysis of Tannic Acid. The hydrolysis of tannic acid was performed under 30% of different cosolvents to avoid microbial contaminations (**Figure 5**). The best results were obtained using diglyme or dimethylformamide (DMF). Diglyme was chosen as optimal cosolvent because it is nontoxic and is usually less harmful to enzyme stability (*27*).

We have also studied the reaction courses at different pH values (**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 (e.g., 95%).

The effect of the temperature was also studied . The reaction occurs approximately 4-fold more slowly at 4 °C than at 25 °C of the reaction product. However, at these conditions (pH 5.0, 30% dyiglyme. and 4 °C) 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/g 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% diglyme, pH 5.0, and 4 °C) starting from 5 mM 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/molecule of tannic acid) was observed. When the 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 °C several peaks, close to the one corresponding to gallic acid, appear after 5 h of reaction (**Figure 8A**). In those optimal conditions the

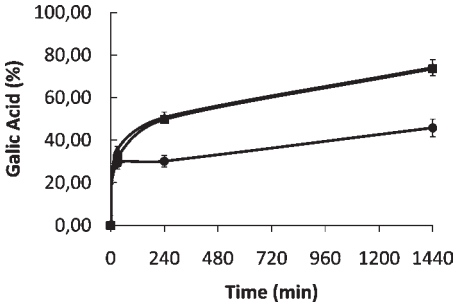

Figure 5. Effect of solvent on the hydrolysis of tannic acid catalyzed by TG-2. The hydrolysis reaction was performed at pH 7 and 25 °C in the presence of 30% of different cosolvents: (●) 30% ethanol; (■) 30% diglyme; (▲) 30% DMF. Experiments were carried out as described under Materials and Methods using poorly loaded enzyme preparations.

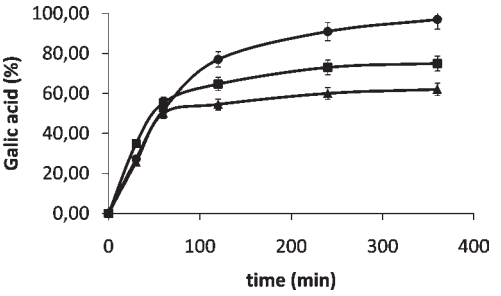

Figure 6. Effect of pH on the time courses of hydrolysis of tannic acid catalyzed by TG-2. The hydrolysis was performed at 25 °C in the presence of 30% of diglyme: (●) sodium acetate, 25 mM, pH 5; (■) sodium phosphate, 25 mM, pH 7; (▲) sodium phosphate, 25 mM, pH 8. Experiments were carried out as described under Materials and Methods using poorly loaded enzyme preparations.

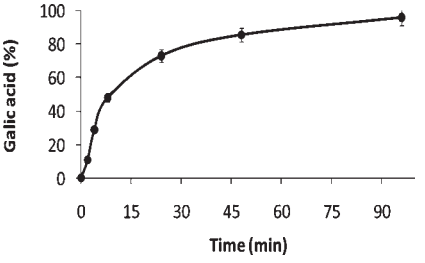

Figure 7. Time course of hydrolysis of 5 mM tannic acid catalyzed by TG-2 in ammonium acetate, 25 mM, pH 5, 4 °C, and 30% of diglyme. Experiments were carried out as described under Materials and Methods by using highly loaded immobilized enzyme derivatives (70 mg of pure tannase/g of catalyst).

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

DISCUSSION

Very Simple Immobilization Protocols. The use of an overexpressed 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 poorly activated IMAC supports and the presence of moderate concentrations of imidazole 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 g of

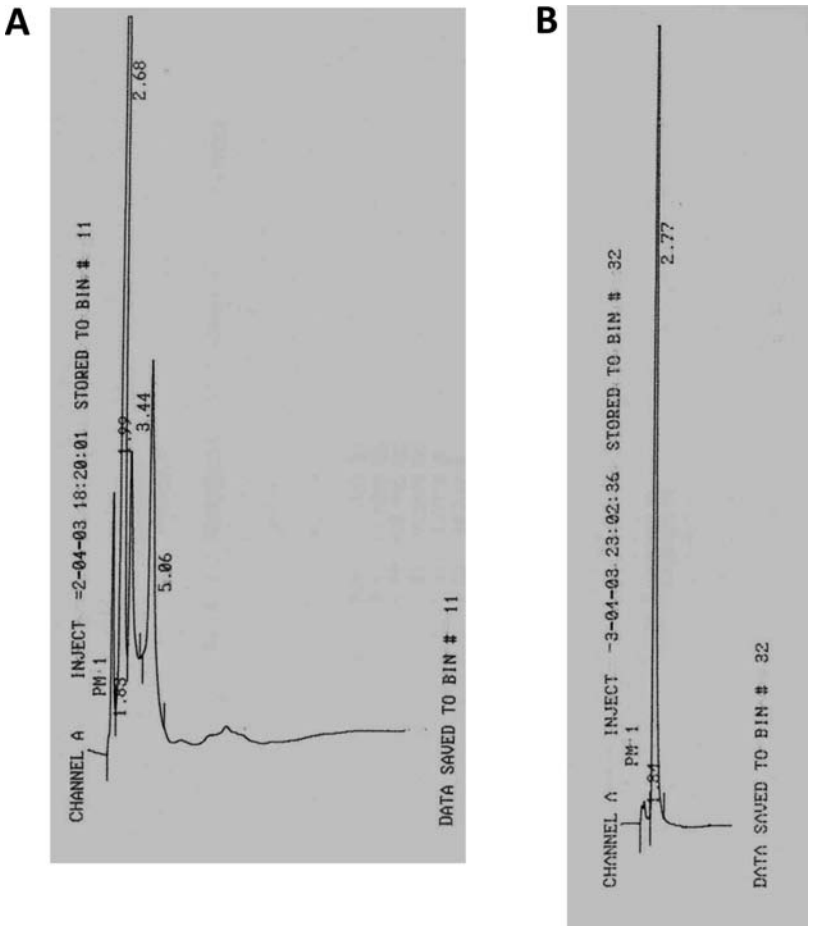

Figure 8. UV chromatograms of gallic acid obtained by enzymatic hydrolysis of tannic acid catalyzed by TG-2. Experiments were carried out as described under Materials and Methods: (**A**) contaminated product obtained at pH 7.0, 25 °C; (**B**) pure product obtained at pH 5.0, 4 °C, in the presence of 30% diglyme. Concentration of tannic acid was 5 mM, and reaction time was 5 h.

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 (e.g., by using highly activated supports in the absence of imidazole), 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 g of proteins from the crude extract and the subsequent purification of the 0.9 g 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 to achieve simpler and less expensive purification protocols.

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

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

A protocol for multipoint covalent attachment on glyoxyl agarose gels has been developed in our laboratory, and it has been already tested for many other enzymes. In addition to the use of very highly activated supports, it has been demonstrated that multipoint covalent attachment and subsequent stabilization are improved by using long incubations at pH 10.0 and 25 °C (*29*, *30*) This method usually promotes the highest stabilization factors achieved via immobilization techniques: for example, most of the derivatives of different enzymes were between 100- and 10000-fold more stable than the corresponding soluble enzymes or one-point-immobilized derivatives, and they were stabilized against any distorting agent, heat, organic cosolvents, pH, etc. (*21*, *29*, *30*). The application of this protocol to tannase has also given very promising results: a very high stabilization plus the simultaneous immobilization of all enzyme subunits. Again, the enzyme was stabilized against temperature and against organic cosolvents. In the first trial of cosolvent we have selected a distorting cosolvent (2-propanol) to rapidly quantify the stabilization of the best immobilized derivative with regard to the one-point-immobilized one. However, other much milder cosolvents were selected to prevent microbial contaminations during enzymatic hydrolysis. In a previous paper we have reported that diglyme, ethanol, and DMF hardly exert harmful effects on most of immobilized enzyme derivatives (*26*).

In addition to its good properties for immobilization−stabilization of enzymes, glyoxyl agarose is very stable under immobilization conditions (e.g., pH 10.0). In this way, when using stable soluble enzymes, long immobilizations can be performed and the support surface can be completely loaded with pure enzyme. In fact, 50 mg of pure tannase could be immobilized on 1 g of wet 6%

agarose gels, and the resulting derivatives exhibit a very high intrinsic activity: 1000 units/g of biocatalyst. This activity was measured by following the hydrolysis of methyl gallate catalyzed by fully loaded derivatives after breaking them (under a very strong magnetic stirring) to get very small particle sizes and, in this way, minimize the difussional limitations of the observed catalytic activity.

Enzymatic Hydrolysis of Tannic Acid. On the one hand, pH 5.0 was selected to get a quite linear and 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 other tannases were used, the reaction courses were much less linear and the final yields 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 prevents both degradations, and then a chromatographically pure gallic acid was obtained. Under these mild reaction conditions, the stabilized derivatives of tannase from *L. plantarum* were extremely stable.

Practical Remarks. The multidisciplinary combination of good protocols from microbiology, molecular biology, and 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 *L. 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 is overexpressed in *E. coli* up to levels of 7−8% of enzyme versus total protein. In addition, a poly-His tail has 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/g of biocatalyst could be prepared, and the intrinsic activity of these biocatalysts was 1000 units/wet g of biocatalyst.

(4) The immobilized enzyme is stabilized 500−1000-fold with regard to one-point covalent immobilized derivatives.

(5) At least 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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