Research Article

Catalytical Properties of Free and Immobilized Aspergillus niger Tannase

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A fungal tannase was produced, recovered, and immobilized by entrapment in calcium alginate beads. Catalytical properties of the immobilized enzyme were compared with those of the free one. Tannase was produced intracellularly by the xerophilic fungus Aspergillus niger GH1 in a submerged fermentation system. Enzyme was recovered by cell disruption and the crude extract was partially purified. The catalytical properties of free and immobilized tannase were evaluated using tannic acid and methyl gallate as substrates. $K_M$ and $V_{max}$ values for free enzyme were very similar for both substrates. But, after immobilization, $K_M$ and $V_{max}$ values increased drastically using tannic acid as substrate. These results indicated that immobilized tannase is a better biocatalyst than free enzyme for applications on liquid systems with high tannin content, such as bioremediation of tannery or olive-mill wastewater.

1. Introduction

Tannin acyl hydrolase (TAH) also known as tannase is an enzyme (EC 3.1.1.20) that catalyzes the hydrolysis of ester bonds present in gallotannins, complex tannins, and gallic acid esters [1, 2]. The major applications of tannase are in the elaboration of instantanous tea and acorn liquor, as well as in the production of gallic acid from tannin-rich agrowastes [3, 4]. Tannase is also utilized as clarifying agent in wine, beer, fruit juices, and coffee-flavored soft drinks [5, 6]. Moreover, it has been proposed the use of this enzyme for bioremediation of effluents from tanneries and to improve the nutritional properties of tannin-rich forage [7].

Despite the several important applications of tannase in food, feed, chemical and pharmaceutical industries, high scale use of this enzyme is severely restricted due to high production costs. Thus tannase is considered a specialty enzyme. Therefore, there is a continuous search for new sources of tannase [8–10], as well as improved methods for production, recovery, and application of the enzyme [11–13].

In order to overcome some limitations of free tannase, several attempts have been made to immobilize the enzyme on a suitable matrix [14–24]. Enzyme immobilization facilitates the efficient recovery and reuse of costly enzymes and enables their use in continuous, fixed-bed operation. Immobilized enzymes are more easy to handle and to separate from the product, thereby minimizing or eliminating protein contamination of the product. Additionally, immobilized enzymes are often more stable than the free ones, allowing the repeated reuse of the biocatalyst [25].

One of the most convenient methods for enzyme immobilization is the entrapment in Ca-alginate beads. The major advantage of this technique is the simplicity by which spherical beads can be obtained by dripping a polymer-cell suspension into a medium containing positively charged ions. The particles formed are transparent, mechanically stable, nontoxic, and cheap [26, 27].
We previously reported the production, and purification of a novel tannase from the xerophilic fungus Aspergillus niger GH1 [28]. This enzyme showed interesting properties such as good activity and stability at high temperature, low inhibition by metal ions and other additives and considerable stability at a wide range of pH. Therefore, in the present study, tannase from A. niger GH1 was immobilized in Ca alginate beads and the catalytic properties of immobilized tannase were compared with those of free enzyme.

2. Materials and Methods

2.1. Microorganism and Culture Conditions. Aspergillus niger GH1, utilized for tannase production, was obtained from the UAdeC-DIA culture collection. This strain was previously isolated from Mexican semidesert and characterized as tannase producer [29]. Fungal spores were stored at −40°C in a cryoprotectant medium composed of glycerol and skim milk.

Microorganism was propagated by transferring conserved spores to Erlenmeyer flasks with Czapek-tannic acid agar and incubating at 30°C for 4–6 days. After this, spores were harvested with Tween 80 (volume ratio of 0.01%) and counted in a modified Neubauer chamber before inoculation.

Tannase was produced by A. niger in submerged culture. The culture medium for tannase contained (g/L) KH₂PO₄: 2.19; (NH₄)₂SO₄: 4.38; MgSO₄·7H₂O: 0.44; CaCl₂·7H₂O: 0.044; MnCl₂·6H₂O: 0.009; NaMoO₄·2H₂O: 0.004; FeSO₄·7H₂O: 0.06. Salt solution was autoclaved at 12°C for 15 min and cooled at room temperature. Tannic acid was added to the salt solution to a final concentration of 12.5 g/L, then the pH was adjusted at 5.5 with 1 N NaOH, and then the culture media was sterilized again by filtration through 0.45 μm nylon membranes. Tannase production was carried out in 1 L Erlenmeyer flasks containing 250 mL of culture medium inoculated with 1 × 10⁷ A. niger spores/mL. Flasks were incubated for 24 h at 30°C with constant agitation at 250 rpm.

2.2. Tannase Recovery. Crude enzymatic extracts from SmF were obtained by filtering the biomass through Whatman no. 41 filter paper. Mycelial cells retained were washed with physiological solution, frozen with liquid nitrogen and milled in a mortar. The macerate was recovered from the acetate buffer (100 mM, pH = 5.5). Crude extract was put into a 10 kD MWCO cellulose membrane (Sigma, St. Louis, USA) and dialyzed against water. Dialyzed extract was concentrated (12.5-fold) with polyethylene glycol-6000 as described by Sharma and coworkers [30]. Concentrated extract was applied into a HiTrap G25 column, eluted with citrate buffer (100 mM, pH 5.0) and fractionated in an AKTA prime FPLC system (Amersham, Piscataway, USA).

2.3. Enzyme Immobilization. Tannase from A. niger was immobilized in Ca alginate beads. 4 mL of partially purified tannase (288 U/L) were mixed with 46 mL of 2.0% sodium alginate solution to get homogeneity. Then the mixture was added with constant agitation and a temperature of 4°C to 0.6 M CaCl₂ solution as droplets using a glass burette. The beads were kept in 0.1 M CaCl₂ at 4°C for about 2 h and then washed briefly with sterile water.

2.4. Kinetic Constants of Tannase. Values of Kₘ and Vₘₐₓ were calculated for free and immobilized tannase using methyl gallate and tannic acid as substrates in citrate buffer (50 mM, pH 5.5). Hydrolysis was carried out in a 250 mL glass jacketed bioreactor. 50 beads of immobilized tannase or the equivalent in free enzyme was added to 100 mL of substrate with constant agitation and controlled temperature at 30°C, and 1 mL samples were withdrawn at regular intervals. Reaction was stopped with 0.2 mL of 2 N HCl, and the reaction mixture was filtered through a 45 μm membrane and analyzed for gallic acid by an HPLC procedure [31]. Tannase activity rate was estimated as V (IU) following the Michaelis-Menten equation. Estimation of different parameters for the equation was obtained through the linearization method of Lineweaver-Burk, using the Solver utility of the program Excel (Microsoft, Redmond, USA).

2.5. Analytical Methods. Tannase activity was assayed using HPLC methodology, essentially as described by Beverini and Metche [31] with slight modifications. In brief, the enzyme (50 μL) was added to 1 mL of methyl gallate 3 mM. The reaction mixture was incubated at 30°C for 30 min. The reaction was stopped with 2M HCl. Each sample was filtered through a 45 μm nylon membrane prior to HPLC analysis. One unit of enzyme (IU) was defined as the amount of enzyme able to release 1 μmol gallic acid per minute of culture filtered under the standard assay conditions. Protein estimation was done as described by Bradford [32].

3. Results and Discussion

3.1. Tannase Production and Recovery. Tannase production was carried out by A. niger GH1 in submerged fermentation. Under the described conditions, tannase was expressed mainly intracellularly (data not shown). At the end of incubation, 1.16 g of biomass were obtained from 2 L of culture broth. After cell disruption it were recovered 200 mL of a crude extract with 15.68 IU of tannase per liter and a specific activity of 7.29 IU per mg of protein.

Tannase production was significantly lower than previously reported by our group [28]. This may be related with the culture system utilized. In that case, Mata-Gómez and coworkers [28] utilized a tannase produced by A. niger GH1 in a solid-state fermentation system. The enzyme was produced mainly extracellularly, and the volumetric activity reached about 400 IU/L. Lekha and Lonsane [33] reported that in submerged fermentation, tannase production by A. niger is intracellular during the first 48 h and the enzyme is subsequently excreted. In contrast, tannase production in solid-state fermentation is completely extracellular.

On the other hand Barthomeuf and coworkers found that, during the first hours of submerged fermentation, tannase remains strongly bound to the mycelium of Aspergillus
3.2. Enzyme Immobilization. Tannase was efficiently immobilized in Ca alginate beads. During immobilization, it was obtained 253 beads of 3.5 mm of diameter per mL of enzyme extract. Since no residual activity or protein was detected in CaCl₂ solution or in the wash water, it was assumed that the enzyme was completely trapped.

Recently, Schons and coworkers reported the immobilization of a Paecilomyces variotii tannase by entrapment into several polysaccharide matrixes [35]. Besides the fact that the best encapsulation efficiency (57%) was obtained with pectin, tannase immobilized in alginate beads (15% of encapsulation efficiency) was more efficient for hydrolysis of tannic acid.

3.3. Kinetic Constants of Tannase. Kinetic parameters of free and immobilized were evaluated at 30°C and a pH of 5.5 using tannic acid and methyl gallate as substrate. \( K_M \) and \( V_{max} \) were obtained with the linearization method of Lineweaver-Burk. The kinetic constants are summarized in Table 1, Lineweaver-Burk plots are showed in Figures 1 and 2.

| Microorganism      | Substrate     | Free tannase | Immobilized tannase |
|--------------------|---------------|--------------|---------------------|
|                    |               | \( K_M \) mM | \( V_{max} \) μmol/min | \( K_M \) mM | \( V_{max} \) μmol/min |
| \( Rhizopus oryzae \) | Sal seed      | 30.9*        | 4.4**               | 39.9*        | 4.0**               |
| \( Rhizopus oryzae \) | Myrobalan     | 26.3*        | 9.4**               | 29.0*        | 2.4**               |
| \( Rhizopus oryzae \) | Tea leaf      | 26.4*        | 0.47**              | 30.5*        | 0.46**              |
| \( Aspergillus oryzae \) | Tannic acid  | 7.35         | 80                  | 11.76        | 40                  |
| \( Aspergillus niger \) | Tannic acid  | 0.3          | 0.013               | 0.6          | 0.020               |
| \( Aspergillus niger \) | Tannic acid  | 1.1 \times 10^{-5} | 416           | 1.1 \times 10^{-5} | 131               |
| \( Aspergillus niger \) | Tannic acid  | 0.400        | 0.05                | 23.75        | 0.25                |
| \( Aspergillus niger \) | Methyl gallate | 0.433       | 0.033               | 0.529        | 0.003               |

These results are expressed as mg/mL. ** These results are expressed as U/mL/h.
macromolecules such as carbohydrates but, to maintain its binding capacity, tannins must have more than two acidic unit constituents esterified to the glucose core [4]. Thus, the interaction between alginate and tannic acid is much higher than that with methyl gallate, as indicated by these results.

On the other hand, the \( V_{\text{max}} \) value for intracellular tannase from \( A. \ niger \) is higher using tannic acid as substrate in both free and immobilized forms. In addition, immobilization resulted in an increase in \( V_{\text{max}} \) when tannic acid was used as substrate. Typically, immobilized enzymes have lower \( V_{\text{max}} \) values than their free counterparts [37], and this phenomenon is associated with conformational changes of the enzyme during immobilization [18]. Higher \( V_{\text{max}} \) values after immobilization have been reported for inulinase [38], invertase [39], and \( \beta \)-galactosidase [40]. However, this is an unusual behavior and the mechanistic basis for this positive feature requires further investigation [37].

The increase of the maximal velocity of reaction and the \( K_M \) value makes the immobilized tannase a better biocatalyst than free enzyme for applications on liquids with high tannin content, such as bioremediation of tannery or olive-mill wastewater. In addition, the immobilization technique allows the recovery and reutilization of the enzyme.

4. Conclusions

Tannase was efficiently immobilized in Ca alginate beads. The immobilized enzyme presented interesting catalytical properties, different from the free enzyme. These catalytical differences could be related with mass diffusion coefficients and conformational changes of the protein as well as the interaction of the substrate with the polymer matrix. These properties of the enzyme and the possibility of recovery and reutilization make the immobilized tannase from \( Aspergillus \)
niger GH1 an interesting biocatalyst with potential application in liquid systems with high tannin content, such as bioremediation of tannery or olive-mill wastewater.

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