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Enhancement of transesterification-catalyzing capability of bio-imprinted tannase in organic solvents by cryogenic protection and immobilization

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ABSTRACT

Improvement of transesterification-catalyzing capability of bio-imprinted tannase is a crucial question of whether to be efficiently utilized in organic media. As for biotransformation of tannic acid to propyl gallate, bio-imprinting technique can dramatically enhance the transesterification-catalyzing capability of tannase. In this work, both cryogenic protection and immobilization were utilized to further improve its apparent catalytic capability in organic media. The results show that Triton-X-100, mannose, and magnesium ion all have a positive effect on cryogenic protection of the tannase. Particularly, combinational application of the three cryoprotectants increases its catalytic performance by 2.7-fold factor. Also, immobilization further elevates its catalytic capability by 2.1 folds. Noteworthily, the coupling application of immobilization and cryo-protection can cause the conversion rate of substrate of the bio-imprinted tannase to increase to a promising 70%. Consequently, it will be helpful to fully utilize tannase in organic phase.

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1. Introduction

A larger number of enzymes, in general, exhibit obviously lower activity in organic medium than in hydrous phase. Improvement of catalytic capability of biological enzyme in anhydrous media becomes a crucial question of whether the enzyme is efficiently utilized in industrial biotransformation. Just as tannase utilized in synthesis of propyl gallate (PG) from tannic acid (TA) in organic medium, it has not been applied commercially due to its lower biocatalytic capability [1,2].

Tannase is a tannin acyl hydrolase (EC 3.1.1.20), that cannot only hydrolyze the ester and depside linkages in hydrolysable tannins into glucose and gallic acid in aqueous medium [3] but also catalyze esterification between gallic acid and alcohol as well as the transesterification between TA and gallic acid esters in anhydrous medium [1,4]. Consequently, tannase has been widespreadly employed in food and pharmaceutical sectors [5,6].

Tannins are an abundant group of plant secondary metabolites, which are divided into condensed tannins and hydrolysable tannins [7]. TA is composed of a few monomers or dimmers of gallic acid [8] and belongs to hydrolysable tannins. Thus, it can be used as a cheap raw material for production of gallic acid esters, important one of which is PG. The gallic acid ester has an excellent antioxidative capacity and, in general, is used in the food industry (particularly in edible oils or lipid-based food) [9,10]. As a synthetic antioxidant, PG can improve the stability of biodiesel toward oxidation [11] and also has potential pharmaceutical properties such as an anti-tumor effect and an antinociceptive activity [12]. Accordingly, biotransformation of TA to PG owns great economic and ecological values.

Previously, few studies on tannase-catalyzed transesterification have been reported since tannase has an undesirable biocatalytic performance in the transesterification required for PG production on commercial scale [1,2]. For this reason, bio-imprinting technique was introduced to improve its biocatalytic performance. The technique, which was firstly defined by Mosbach [13], can induce a desirable conformation at the active site by pH tuning [14], substrate or its analogs imprinting [15], and interfacial activation [16,17] in an aqueous phase. The conformation with specific nano-sized cavities can lead to an efficient coupling between enzyme and substrate in organic medium. Whereby, our group has made combinational use of pH tuning and substrate imprinting to hyper-activate tannase. The modified enzyme holds 9.7-fold transesterification-catalyzing capability than the control [18]. Lyophilization, a required step in the protocol of bio-imprinting, aims to fix the favorable conformation of bio-imprinted enzyme. As is well-known, enzyme with three dimensional tertiary

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structure is so sensitive to temperature variation that mild freezing can result in the reduction of its biological activity. Hence, cryogenic protection of bio-imprinted enzyme is quite essential. But to our best knowledge, the relevant study has not been seen.

In this present work, combinational cryoprotective technique (including three cryoprotectants, such as Triton-X-100, mannose, and magnesium ion) was utilized to improve the biocatalytic performance of bio-imprinted tannase. Subsequently, an adsorptive immobilization was explored to further boost the apparent activity of the modified tannase. The immobilized and cryoprotective bio-imprinted tannase (IIt) was used to catalyze the transesterification from TA to PG. It is endeavored to enhance the catalytic performance of tannase in organic medium.

2. Materials and methods

2.1. Materials

Tannase produced by *Aspergillus oryzae* was purchased from Jinan Huazuan Trading Co., Ltd., China. Commercial TA ($CB_{76}HB_{52}OB_{46}$), citric acid monohydrate, Triton-X-100, mannose, magnesium sulfate, celite, n-propanol, and hexane were purchased from Sinopharm Chemical Reagent Co., Ltd. (SCRC), China, and all are of an analytical grade. PG (HPLC grade) was purchased from Sigma Co., USA. All other solvents and reagents were obtained commercially and were of analytical grade.

2.2. Bio-imprinting, cryogenic protection and immobilization

IIt was prepared based on our preliminary works [18]. This protocol is subjected to three steps as following:

Bio-imprinting (modification in aqueous medium): 50 mg TA was added to the tannase solution, which incorporated 16 IU (assayed by rhodanine-spectrophotometric method [19])tannase with 5 mL 90 mM pH 6.0 citrate buffer as solvent. The uniform enzyme–substrate mixture was maintained without agitation for 5 min at ambient temperature. The enzyme-substrate mixture was defined as a TA-pH treated tannase (TPt). (The lyophilized powder of TPt is considered as bio-imprinted tannase.)

Cryogenic protection (prior to lyophilization) and immobilization: 12.5 μL 200 mM magnesium ion solution, 0.625 mL 1 M mannose, 0.15 mL 10 mM Triton-X-100, and 0.5 g celite were decanted to TPt, in turn, and then the mixture was agitated for 5 min.

Lyophilization: The modified enzyme mixture was frozen at −20 °C overnight, followed by lyophilization using a freeze dryer (VirTis, SP Scientific USA). The lyophilized powders (i.e., IIt) were stored at 4 °C until use.

As for different requirements, the protocol was modified based on the above steps.

2.3. Transesterification synthesis of propyl gallate

An aliquot of IIt (containing 50 mg TA) was added into approximate 10 mL reaction medium, which is composed of 1 mL n-propanol, 9 mL hexane, and 0.1 mL distilled water. The reaction was performed at 40 °C and 200 rpm for 24 h. Conversion rate (CR) in the transesterification reaction refers to the mole percent of TA completely transformed to PG ($C_0 - C_t$) relative to the total dose of TA (a fixed concentration, C_0) before the equilibrium of reaction (in a fixed time, t). The reaction rate can be calculated as the following:

$$v = \frac{C_0 - C_t}{t} = \frac{(C_0 - C_t)C_0}{tC_0} = \frac{\mathrm{CR} \times C_0}{t}$$

If k is defined as C_0/t, $v = k\mathrm{CR}$, where v is the reaction rate of transesterification catalyzed by tannase, which, in general, denotes the transesterification-catalyzing capability of the enzyme. C_0 and C_t are the initial concentration of TA and the residual concentration of TA in a fixed time, t, respectively. In view of C_0 and t being constants, v is proportional to CR. Therefore, CR can be used to estimate the catalytic activity of the enzyme.

The activation factor (AF) refers to the CR of the modified tannase divided by one of the corresponding control, which intuitively denotes the effect of treatment on the catalytic activity of the enzyme. The control was prepared according to the different requirements. All experiments were performed in duplicate unless stated otherwise.

2.4. Assay of propyl gallate

Samples were assayed by HPLC (Waters 600, Waters, USA) with a Waters 996 photodiode array detector (PDAD) as well as a Phenomenex C18 column (250 × 4.60 mm, 4 μm). The mobile phase was composed of 50 mL of methanol, 50 mL of pure water, and 10 μL of acetic acid. The operating temperature was maintained at 35 °C. 20 μL of sample were injected and were detected at 274 nm with PG (HPLC grade) as the control at a flow rate of 1 mL/min.

2.5. Effect of sugar and metal ion on the catalytic capability of bio-imprinted tannase

Seven types of sugar (including sucrose, lactose, maltose, xylose, sorbitol, trehalose and mannose) at 100 mM, various concentrations of mannose (0–200 mM), six types of salts including different melt ions (i.e., manganese chloride, magnesium sulfate, zinc sulfate, calcium chloride, copper sulfate, and ferrous chloride) at 0.2 mM, and magnesium ions (0–4.0 mM) were added in IPt solution, respectively, and then the mixtures were frozen overnight and lyophilized for 24 h. All of these modified tannases were used to catalyze the transesterification for synthesis of PG, and the total amount of TA was unified as 50 mg in all reactions. The effects of the different treatments were estimated by comparing their CR values.

2.6. Effect of immobilization on the catalytic capability of bio-imprinted tannase

Various doses of celite (0–0.5 g) were added in IPt solution plus magnesium ions (0.5 mM), 125 mM mannose, and 0.3% Triton-X-100, respectively, and then these mixtures were frozen overnight and lyophilized for 24 h. All of these modified tannases were used to catalyze the transesterification for synthesis of PG, and the total amount of TA was unified as 50 mg in all reactions. The effects of the different treatments were estimated by comparing their CR values.

3. Results

3.1. Effect of sugar on the biocatalytic capability of bio-imprinted tannase

Generally, some sugars were utilized as cryoprotectants toward protein [20–23]. In this study, the effects of seven sugars on the biocatalytic activity of the tannase were analyzed. Fig. 2a shows that four sugars (i.e., lactose, xylose, sorbitol, and mannose) protect tannase activity better than the three others. Wherein, mannose is the best protector, by which the enzyme protected obtains the maximal CR, around 14%. The dependence of the protective effect on the concentration of mannose was inspected between 5 and 200 mM. As shown in Fig. 2b, the CR increases with mannose concentration rising up to 150 mM, at which the maximum CR is 16.3%, 1.6 times that of the control. Conversely, the CR decreases when the concentration increases further.

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