Journal of Molecular Catalysis B: Enzymatic 104 (2014) 29–34

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Efficient resveratrol production by immobilized β-glucosidase on cross-linked chitosan microsphere modified by L-lysine

Jie Zhang[a], Dan Wang[a,*], Jiachuan Pan[b], Jing Wang[a], Hongbo Zhao[a], Qiang Li[c], Xiaohua Zhou[a,*]

[a] College of Chemistry and Chemical Engineering, Chongqing University, Chongqing 400044, China
[b] Department of Biomedical and Chemical Engineering, Syracuse University, Syracuse, NY 13244, USA
[c] National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China

ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form 16 February 2014
Accepted 8 March 2014
Available online 19 March 2014

Keywords:
Resveratrol
β-glucosidase
Immobilization
Cross-linked chitosan microsphere
L-Lysine

ABSTRACT

Resveratrol and its glycosidic derivative polydatin are the major stilbenes in *Polygonum cuspidatum*, a most widely used traditional herbal medicine. To produce resveratrol *via* enzymatic hydrolysis of polydatin, β-glucosidase was immobilized on cross-linked chitosan microspheres modified by L-lysine. The condition of enzyme immobilization was optimized, and the characterization of the immobilized enzyme was carried out. K_m value of immobilized β-glucosidase was calculated as 47.7 ± 0.7 mM, and V_{max} value was 34.6 ± 0.5 U/mg. Under optimized condition, which was 45 °C, pH 6.0, with the initial polydatin concentration of 2.0%, and the flow rate of 1.0 mL/min, the overall hydrolysis yield could reach 85.8 ± 0.6%, and the activity of the immobilized enzyme remained 92.2 ± 0.9% after continuous hydrolysis for 8 h. Enzymatic hydrolysis of polydatin extracted from *P. cuspidatum* is a promising method to increase the supply of resveratrol in pharmaceutical markets worldwide, which also showed a new application of immobilized β-glucosidase in biotechnological industry.

1. Introduction

Resveratrol, 3,5,4′-trihydroxy-*trans*-stilbene, is a natural phenol, and a phytoalexin naturally produced by several plants when under attack by pathogens such as bacteria or fungi [1,2]. It has been widely considered beneficial to human health for its physiological properties, including anti-oxidation, anticancer, and anti-inflammatory, especially the property to reduce neuronal injury both *in vitro* and *in vivo* [3,4].

Resveratrol was firstly extracted from skin of red grapes, and now it could be produced by chemical synthesis or biotechnological synthesis adopting metabolic engineered microorganisms [5,6]. *Polygonum cuspidatum* Sieb. et Zucc., a traditional, popular Chinese medicinal herb, is widely distributed in southern China and Japan. The root of *P. cuspidatum* has been used in treatment of inflammation, infection, jaundice, skin burns and hyperlipemia diseases, in China and Japan. Resveratrol and its glycosidic derivative polydatin are the major stilbenes in *P. cuspidatum*, which can be used in the treatment of atherosclerosis, cough, asthma, hypertension, cancer, *etc.* [1,3]. Considering the popularity of resveratrol in pharmaceutical markets worldwide, it is cost-effective to transform polydatin to resveratrol to produce the latter. This purpose could be well achieved by the mild and effective enzymatic reaction catalyzed by β-glucosidase [7].

β-Glucosidases (EC 3.2.1.21) are biologically important enzymes and catalyze the transfer of glycosyl group between oxygen nucleophiles [8]. They are, therefore, accountable for the hydrolysis of β-glycosidic linkages in amino-, alkyl-, or aryl-β-D-glucosidase, cyanogenic glycosides, and di- and short chain oligo-saccharides. In this study, β-glucosidase could catalyze the hydrolysis of terminal non-reducing β-D-glucose residues in polydatin with the release of β-D-glucose.

The immobilization of β-glucosidase in a solid carrier offers the prospect of cost saving and widens the flexibility of process design, by enabling continuous operation and simplifying downstream processing. Till now, several natural and synthetic materials have been explored as potential supports for β-glucosidase immobilization, such as alginate gel beads, polyacrylamide-magnetite beads, chitosan, chitosan–clay composite, and new ionic liquid sol–gel matrices [9–13]. Among these materials, chitosan is a cheap,

* Corresponding authors at: Chongqing University, College of Chemistry and Chemical Engineering, Chongqing 400044, China. Tel.: +86 23 65111179; fax: +86 23 65111179.
E-mail addresses: danwang088@gmail.com (D. Wang), zhou65306590@tom.com (X. Zhou).

http://dx.doi.org/10.1016/j.molcatb.2014.03.003
1381-1177/

non-toxic, and non-harmful support to the enzymes. It possesses many advantages such as excellent hydrophilicity, high porosity, and large adhesion area. Chitosan has hydroxyl (–OH) and amino ($-NH_2$) groups, which easily links with enzymes and, can be cross-linked with glutaraldehyde to prevent dissolution in acidic solution (pH < 2) [14,15]. The cross-linked chitosan microspheres are more applicable in biochemical engineering due to their greater mechanical strength. However, there are still operational defects; for example, its reactive primary amino groups are directly bonded with pyranoid rings, which would cause large steric hindrance. And the cross-linking reaction would consume a large portion of amino groups, which reduced the enzyme load and subsequently decreased the volumetric productivities. These problems could be overcome by a novel synthesized resin prepared in our lab, which is a cross-linked chitosan resin modified by L-lysine, named LMCCR [16]. A six-carbon L-lysine residue acted as flexible spacer arm to decrease the steric hindrance and the epsilon-NH_2 of L-lysine residues would replace the less reactive amino groups of cross-linked chitosan during the cross linking reaction.

In this study, to produce resveratrol by hydrolysis of polydatin, β-glucosidase was immobilized on LMCCR to facilitate the enzymatic reaction. The characteristics of the immobilized enzyme were explored and the whole transformation process was studied in detail.

2. Materials and methods

2.1. Materials

The enzyme β-glucosidase extracted from almonds was purchased from Sigma with an activity of 3.4 U/mg protein. And β-glucosidase from almonds has high efficiency of hydrolyzing resveratrol glucoside than the same enzyme from *Aspergillus niger* and *Saccharomyces cerevisiae yeast* [17,18]. L-lysine was purchased from Merck. Chitosan oligosaccharide (M_w < 5000 Da), with the deacetylation degree of 90.3%, was purchased from Yuhuan Biochemical Co. in Zhejiang, China. Polydatin crude product in the form of powder with a purity of 80% was purchase from Yangling Dongke Madisen Medical Pharmaceutical Co. in Xian, China. The standard product of resveratrol and polydatin were supplied by China National Institutes for Food and Drug Control. All other chemicals were of analytical grade.

2.2. Experimental methods

2.2.1. Preparation of LMCCR

LMCCR was prepared following the methods described in detail in the previous paper [16]. The simplified synthesis process of LMCCR contains two steps. In the first step, chitosan solution was poured into a dispersion medium, paraffin oil and Tween-80. The dispersion medium was vigorously stirred by a mechanical stirrer. Thirty minutes later, glutaraldehyde solution and $CaCO_3$ powder were continuously added into the dispersion medium. After that, the product was filtered by vacuum and washed continuously with petroleum ether and ethanol. Then the solid product was poured into HCl solution and the cross-linked chitosan resin (CCR) was obtained. In the second step, CCR was suspended in L-lysine solution, and then DCC was added to induce the acylation reaction. When the reaction finished, the product was then filtered by vacuum and washed with 95% ethanol. Finally, the solid product was dialyzed with deionized water and filtered again to obtain the final product (LMCCR).

2.2.2. Immobilization of enzyme and activity determination

The LMCCR beads, 15 g, were suspended in 100 mL buffer composed of disodium hydrogen phosphate (0.2 M) and citric acid (0.1 M)) for 60 min at 25 °C. Then they were mixed with 20 mL of 80 U/L β-glucosidase in a shaker for 1 h at 4 °C. Aqueous glutaraldehyde solution of 1.0% (v/v) was added dropwise and the suspension was then incubated for several hours. After the reaction mixture was filtered by vacuum and washed with 0.2 mol/L phosphate buffer (PB, pH 6.8) to remove excessive glutaraldehyde, the immobilized beads were stored at 4 °C. The immobilization efficiency was defined as percentage of total enzyme protein content immobilized on the support and calculated according to Eq. (1).

$$\text{Immobilization efficiency} = \frac{\text{preliminary enzyme content} - \text{enzyme content in the filtrate}}{\text{preliminary enzyme content}} \times 100\% \quad (1)$$

The activity of β-glucosidase in the solution was determined by adding 0.1 mL sample to 0.9 mL of 0.1 M acetate buffer (pH 3.5), which contained 5 mM of *p*-nitrophenyl β-D-glucopyranoside (Sigma Co., N7006) substrate [19]. The mixture was incubated with stirring at 25 °C for 1 min and stopped by adding 2 mL of 1 M Na_2CO_3. The absorbance of the product *p*-nitrophenol at 400 nm was measured using an UV/visible spectrophotometer (Jasco V-530), and the activity was calculated based on a molar extinction coefficient of 18,300 $dm^3/(mol\,cm)$. One activity unit (U) of β-glucosidase is defined as the amount of this enzyme required for hydrolyzing 1 μmol of substrate per minute. The activity of β-glucosidase immobilized on the LMCCR was similarly measured, except that 0.1 mL of the solution was replaced by 0.1 mL deionized water and a given amount of LMCCR. The activities of the free β-glucosidase and the immobilized β-glucosidase were given as U/mg protein and U/mg immobilized β-glucosidase, respectively. The relative activity was calculated using Eq. (2).

$$\text{Relative activity} = \frac{\text{activity}}{\text{maximum activity}} \times 100\% \quad (2)$$

And the enzyme activity recovery was defined as follows:

$$\text{Enzyme activity recovery} = \frac{U_{act}}{U_{total} - U_{remain}} \times 100\% \quad (3)$$

where U_{act} is the activity of immobilized enzyme, U_{total} is the activity of total enzyme used, and U_{remain} is the activity of β-glucosidase in filtration.

2.2.3. Resveratrol production by immobilized enzyme

15.0 g wet immobilized β-glucosidase was packed in a 40 cm × Φ1 cm column with thermal insulation jacket (Shanghai Jinhua Co., Ltd., China), which was then connected to constant temperature water bath and peristaltic pump YZ1515X (Baoding Longer Precision Pump Co., Ltd., China) to construct the immobilized enzyme reactor, as it was shown in Fig. 1.

After sample loading using peristaltic pump, the effluent was taken intermittently to determine the glucose concentration in the hydrolysate and calculate the hydrolysis rate of the polydatin. The concentration of the initial polydatin solution and the sample flow rate were changed to examine their effects on the hydrolysis rate of the process.

2.2.4. Analytical methods

The concentration of polydatin and resveratrol were measured by high-performance liquid chromatography (Agilent, USA) equipped with an Agilent model DAD UV–VIS detector and a discovery C_{18} column (250 mm × 4.6 mm ID, 5 μm, Supelco, USA).