Bioresource Technology 102 (2011) 469–474

Contents lists available at ScienceDirect

Bioresource Technology

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

# Stable and efficient immobilization technique of aldolase under consecutive microwave irradiation at low temperature

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ARTICLE INFO

*Article history:*
Received 19 June 2010
Received in revised form 16 August 2010
Accepted 17 August 2010
Available online 24 August 2010

*Keywords:*
Consecutive microwave irradiation
Covalent immobilization
Mesocellular siliceous foams
Aldolase
2-Deoxy-D-ribose-5-phosphate

ABSTRACT

To establish a stable and efficient immobilization technique under microwave irradiation, a focused microwave reaction system was used, where the temperature was set appropriately in the microwave system and cooling module to produce consecutive microwave irradiation. 2-Deoxy-D-ribose-5-phosphate aldolase (DERA) was rapidly and efficiently immobilized in mesocellular siliceous foams (MCFs) under microwave irradiation. When the output power in the microwave system was set to 30 W, after 3 min, 88.4% of the enzyme protein was coupled to the wall of the support pores and the specific activity of the immobilized enzyme was 2.24 U $mg^{-1}$, 149.2% higher than that of the free enzyme and 157.0% higher than that of the non-microwave-assisted immobilized enzyme. In catalysis, microwave-assisted immobilized DERA tolerated a wider range of both pH and temperature than other DERA preparations. The thermal and storage stabilities were also significantly improved. This focused; microwave-assisted immobilization technique has proven to be simple, stable and highly efficient. This technique could also be applied to other enzyme immobilizations.

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## 1. Introduction

In enzyme biocatalysis, aldolase has attracted the interest of organic chemists because of its ability to catalyze the formation of C–C bonds under mild conditions with stereospecific control and thus direct the synthesis of a single product (Bolt et al., 2008; Gillingham et al., 2010; Walters and Toone, 2007). Stereo-controlled C–C bond-forming reactions catalyzed by aldolase are also highly valuable tools in the synthesis of chiral molecules, especially in the pharmaceutical industry (Jennewein et al., 2006). 2-Deoxy-D-ribose-5-phosphate aldolase (DERA), the only member of the family of acetaldehyde-dependent aldolases, is the only known aldolase that catalyzes the aldol reaction between two aldehydes, glyceraldehyde 3-phosphate and acetaldehyde (Dean et al., 2007). It is capable of sequentially adding two acetaldehyde molecules to the acceptor aldehyde (Sakuraba et al., 2007). The common use of DERA in the synthesis of the key fragment of atorvastatin has attracted attention to this aldolase in recent years (Dean et al., 2007).

Although a DERA-catalyzed reaction offers the potential to greatly simplify the process of synthesizing the chiral side chain of statins, several issues limit its practicability for large-scale production. In the catalyzing process the catalyst load is highly consumed, using more than 200 mg of DERA per gram of isolated product (RACl), or 20% (w/w). The use of such a high concentration of enzyme would make the process prohibitively expensive, in addition to making the isolation of the product from the reaction mixture difficult (Greenberg et al., 2004). Enzyme immobilization can offer the advantages, for example repetitive uses, the possibility to stop the reaction easily and the availability which cannot be contaminated by the enzymes (Cabana et al., 2009; Zhang et al., 2010). Moreover, novel immobilization method and technology, however, could supply the efficiently immobilized enzyme with a high enzyme load and activity (Wang et al., 2008a; Zhao et al., 2010). This would be a suitable approach for obtaining a high catalyst concentration without the laborious product isolation difficulty. Moreover, the ability to stabilize and reuse an enzyme catalyst through immobilization has also proven to be one of the key steps in rendering an enzymatic process economically viable (Cao and Schmid, 2005; Tzialla et al., 2010).

Until now, there have been few reports on the immobilization of 2-deoxy-D-ribose-5-phosphate aldolase (DERA). The immobilization of other aldolases has also not been reported frequently in previous biocatalyst immobilization research (Suau et al., 2009). Although the immobilized preparations exhibited an improved optimum reaction temperature and enhanced stability, they often retained no more than 60% of their original activity when a non-physical adsorption method was used in the immobilization (Ardao et al., 2006; Seelan et al., 2006). In the present work,

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doi:10.1016/j.biortech.2010.08.048

consecutive microwave irradiation technology was applied in the covalent immobilization of 2-deoxy-D-ribose-5-phosphate aldolase (DERA) at low temperature. A CEM auto-focused microwave synthesis system, Model Discover, was used and was expected to give a consecutive microwave irradiation that would perform a repeatable, efficient immobilization when compared to a multimode microwave system. Based on the cleavage activity of DERA using DRP as substrate, the immobilization of DERA in the present work was evaluated.

## 2. Methods

### 2.1. Materials

*E. coli* BL21 (DE3) (pET303-DERA027) was a gift from Dr. Qiuyan Wang. Tetraethylorthosilicate (Shanghai, Sinopharm) and 3-glicydoxypropyltriethoxysilane (GPTS) (Jingzhou Jianghan Fine Chemical Co., Ltd.) were obtained commercially and were used as such. Poly (ethylene glycol)-block-poly (propyleneglcol)-block-poly (ethyleneglycol) (P123) of the highest commercially-available grade was obtained from Sigma–Aldrich. 2-Deoxy-D-ribose-5-phosphate (DRP), triose-phosphate isomerase (TPI), and glycerol-3-phosphate dehydrogenase (GDH) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were provided by Sinopharm Chemical Reagent (Shanghai, Sinopharm) and were analytical grade. Deionized water with a resistance greater than 18 MQ was obtained from a Millipore-Q Plus water purifier.

### 2.2. Expression and purification of 2-deoxy-d-ribose-5-phosphate aldolase

The recombinant strain *E. coli* BL21 (DE3) (pET303-DERA027) was used for the 2-deoxyribose-5-phosphate aldolase expression. The strains were cultivated in LB medium containing 100 mg/L of ampicillin at 37 °C until the OD600 reached 0.6. Expression was induced by adding IPTG at a final concentration of 0.5 mM, and cultivation was continued for an additional 5 h at 37 °C. Cells were harvested by centrifugation at 15,000*g* for 20 min and the pelleted cell paste was resuspended in 100 mM sodium phosphate (pH 7.5) and 200 mM sodium chloride. Cell disruption was performed with 300 cycles of ultrasonication (Sonicator Labsonic 200B, BRAUNS.A.) for 30 min in an ice water bath. After centrifugation, the supernatant was incubated with Ni–NTA agarose (Qiagen, Hilden, Germany) for 1 h at 4 °C, and then the mixture was loaded onto a chromatography column. The column was washed with a buffer containing 100 mM sodium phosphate (pH 7.5), 200 mM sodium chloride, and 10 mM imidazole. The C-terminal His-tagged DERA was eluted from the column with the same buffer containing 500 mM imidazole. Then, the eluted enzyme protein was ultrafiltered to desalt the imidazole and concentrated with polyethylene glycol (PEG) 20,000. The purity of the DERA after the metal affinity chromatography was analyzed by SDS–PAGE, and the enzyme protein concentration was determined by the Bradford method.

### 2.3. Covalent immobilization of DERA under continuous microwave irradiation

The preparation and functionalization of the mesocellular siliceous foam (MCF) supports were performed as previously described (Wang et al., 2008b, 2009). Briefly, 20 mg of functionalized MCFs were incubated in 3 ml of 0.1 M p-benzoquinone at room temperature for 1 h, then washed twice with an alcohol solution (20%, V/V) and distilled water. The carrier was then suspended in 3 ml of 0.1 M phosphate buffer, pH 7.0, containing DERA. The container with this mixture was put in the microwave reactor coupled with a cooling module and irradiated (Discover CoolMate, CEM, USA) for a certain period. The consecutive irradiation was induced by controlling the temperature difference between the microwave reaction system and the cooling module. The immobilized enzyme was separated and washed using a phosphate buffer (pH 7.0, 0.01 M) until no protein could be detected in the supernatant. The amount of enzyme remaining in the supernatant was measured using a Bradford assay.

To verify the covalent immobilization of DERA under consecutive microwave irradiation, the immobilized enzyme was hydrolyzed using 6 mol/L HCl at 110 °C for 24 h. The hydrolyzed sample was then washed with DI water for 4 times and dried at 80 °C for 12 h, followed with FT-IR spectra characterization.

### 2.4. Enzyme activity assay

The free and immobilized DERA assay was carried out by two steps method as the previous report (Horvath et al., 1989). At first, the mixture containing DRP (0.3 mM) was incubated at 50 °C for 5–10 min. When DERA preparation was added into the mixture, the cleavage of DRP was initiated and lasted for 5 min at 50 °C. Then the mixture containing substrate and enzyme preparations was cooled at once in the ice-water for 10 min and then the liquid solution was filtered by using a centrifugal filter device (Microcon YM-10; Millipore). Secondly, the content of glyceraldehyde-3-phosphate was measured by following the oxidation of NADH of 60 min at 25 °C in a coupled assay that converts glyceraldehyde-3-phosphate, one of the cleavage products of DRP, to glycerol 3-phosphate by TPI and GDH. TPI convert dihydroxyacetone phosphate to glyceraldehyde 3-phosphate and GDH convert glyceraldehyde 3-phosphate to glycerophosphate coupled with oxidation of NADH. The reaction mixture contained 100 mM sodium acetate buffer (pH 6.5), 0.1 mM NADH, 11 U triose-phosphate isomerase, 4 U glycerol-3-phosphate dehydrogenase. The decrease in the absorbance at 340 nm was monitored and the oxidation of NADH was measured with the coupled reaction for determining the content of glyceraldehyde-3-phosphate, which was used to determine the DRP cleavage activity.

The decrease in the NADH level was monitored at 340 nm using a UV–Visible spectrophotometer (Shimadzu Co., Kyoto, Japan). The activity was defined as the amount of DERA required to catalyze the cleavage of 1 μmol of DRP per minute. The coupled yield, relative activity after coupling were defined as follows,

$$\text{Coupled yield } (\%) = \frac{(A-B)}{A} \times 100 \quad (1)$$

$$\text{Relative activity } (\%) = \frac{C}{(A-B)} \times 100 \quad (2)$$

In above equations, *A* represented the total activity of enzyme added in the initial immobilization solution; *B* was the activity of the same amount of enzyme in supernatant after the immobilization procedure; and *C* was the activity of the immobilized DERA. The amount of enzyme protein in supernatant after the immobilization procedure is determined using the Bradford assay method. Each activity assay was repeated at least three times, with each replicate performed at a different time.

### 2.5. Determination of the $k_m$ and $V_{max}$ for DERA preparations to hydrolyze the DRP substrate

The kinetic constant $k_m$ and the $V_{max}$ values of free and immobilized DERA preparations were determined by measuring the initial rates of the reaction with DRP as the substrate in phosphate buffer solution (0.01 M, pH 6.5) at 50 °C. Five concentrations of DRP, ranging from 0.05 to 0.3 mM, were used to determine the

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