Journal of Biotechnology 156 (2011) 30–38

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Enhancement of activity of cross-linked enzyme aggregates by a sugar-assisted precipitation strategy: Technical development and molecular mechanism

Mengfan Wang, Wei Qi*, Chenxi Jia, Yufei Ren, Rongxin Su, Zhimin He

State Key Laboratory of Chemical Engineering, Chemical Engineering Research Center, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, PR China

ARTICLE INFO

Article history:
Received 27 May 2011
Received in revised form 21 July 2011
Accepted 3 August 2011
Available online 10 August 2011

Keywords:
Cross-linked enzyme aggregates
Sugar
Penicillin G acylase
Stabilization
Immobilization

ABSTRACT

The precipitation of enzyme causes the major activity loss in the conventional protocol for CLEAs preparation. Herein, a sugar-assisted strategy was developed to minimize the activity loss in the step of enzyme precipitation by adding sugar as the stabilizer, which contributed to improve the activity yield of resulting CLEAs. Penicillin G acylase (PGA) was employed as a model enzyme. The effects of glucose, sucrose and trehalose on the activity yields of CLEAs were investigated. The highest activity was obtained in the case of adding trehalose. Confocal laser scanning microscopy and Fourier transform infrared spectroscopy showed that the polar microenvironment and the secondary structure of native enzyme were preserved to some extent when PGA was prepared as sugar-assisted CLEAs, resulting in PGA's higher activity than sugar-free CLEAs. Scanning electron microscope revealed the different inner morphologies, and the kinetic studies showed the higher affinity and resist-inhibition capacity of sugar-assisted CLEAs. Furthermore, stability experiments demonstrated that CLEAs prepared in sugar-assisted strategy remained higher thermal stability when it was incubated at high temperature.

1. Introduction

Using enzymes as biocatalysts in chemical reactions has numerous advantages including selectivity, specificity, mild reaction conditions, stereoselectivity with pure enantiomers as products, and it is an environmental-friendly process (Fazary et al., 2009). However, the enzymes are sensitive and fragile to temperature, system pH and reaction medium. Therefore, the stabilization of enzymes has attracted more and more attention. Design of new enzyme immobilization techniques for the preparation of industrial biocatalysts has led to new opportunities in biocatalysis and biotransformation (Cao et al., 2003; Kim et al., 2006).

Recently, cross-linked enzyme aggregates (CLEAs) were developed as an efficient approach to obtain immobilized enzymes without the use of any pre-existing carriers (Aytar and Bakir, 2008; Cao et al., 2000; Gupta et al., 2009; Sheldon et al., 2005). The final preparation of CLEAs is pure protein with a high concentration of enzyme per unit. CLEAs are stable over a wide pH and temperature ranges, tolerant to organic medium, reusable, and cost effective. Due to the strong stability and high unit activity, it has been used in many biocatalysis processes such as hydrocyanation of a wide range of aldehydes (van Langen et al., 2004), transesterification of ferulic acid into 1-butyl ferulate (Vafiadi et al., 2008), and aerobic oxidation of linear aliphatic alcohols (Matijosyte et al., 2010). Besides, continuous stirred membrane reactor (CSMR) (Malandra et al., 2009) and perfusion basket reactor (BR) (Cabana et al., 2009) were also developed for the application of CLEAs as biocatalyst.

Many researches have focused on the further improvement of CLEAs' activity and stability. Shah et al. (2006) used bovine serum albumin as a proteic feeder in order to obtain high activity when the enzyme concentration is low or the enzyme activity is vulnerable under high concentration of cross-linking agent. López-Serrano et al. (2002) added surfactants to lipase so as to keep a more active conformation when it was prepared as CLEAs. Our group (Wang et al., 2011) prepared porous CLEAs through adding starch as a pore-making agent, which facilitates CLEAs in cases that the substrates of enzyme are macromolecules. Besides, the combination of CLEAs technology with other immobilization methods was developed. For example, our group (Wang et al., 2010) immobilized cross-linked papain aggregates on the commercial macroporous silica gel through one-step cross-linking strategy which exhibited higher thermal and storage stability, as well as good durability after repeated use. Sangeetha and Emilia Abraham (2008) entrapped CLEAs in the hydrogel composite beads which were resistant to low pH conditions in the stomach and thus was found to be useful for the oral drug delivery.

The conventional protocol for preparation of CLEAs consists of two steps: precipitation and cross-linking. First, the soluble enzymes are precipitated to form enzyme aggregates by water miscible organic solvents, salts or non-ionic polymers. Second, the formed aggregates are cross-linked by the bifunctional agent to produce final CLEAs. However, the water-miscible organic solvents

* Corresponding author. Tel.: +86 22 2740 7799; fax: +86 22 2740 7599.
E-mail address: qiwei@tju.edu.cn (W. Qi).

0168-1656/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2011.08.002

usually induced the denaturation of enzyme (Bellezza et al., 2009; Cardoso et al., 2009; Öztürk et al., 2010), which leads to the activity loss of enzyme and finally lower the activity yield of resulting CLEAs. Several mechanisms have been proposed to explain the influence of organic solvents on enzyme activity (Serdakowski and Dordick, 2008), such as disruption of enzyme structure (Michels et al., 1997), decrease of conformational dynamics (Soares et al., 2003), alteration of thermodynamics of the reaction medium (Kim et al., 2000) and strip of crucial water layer from the enzyme surface (Gorman and Dordick, 1992). Therefore, this study aims to develop a practical strategy to protect enzyme from denaturation during precipitation and thus minimize the activity loss for CLEAs preparation.

Sugar is well known as the stabilizer of various proteins in natural dehydrated environments and during the lyophilization process because sugar can preserve the crucial water layer at the protein surface and protect the protein secondary structure (Sun and Davidson, 1998; Sun et al., 1998). In this work, glucose, sucrose or trehalose was first introduced in the precipitation step to prepare the sugar-assisted CLEAs. The commercial penicillin G acylase (PGA) was employed as a model enzyme to test this technique. The activities, morphologies, fluorescent images, protein secondary structures, kinetic parameters and thermal stabilities of sugar-assisted CLEAs and the sugar-free CLEAs (prepared by conventional protocol) have been systematically studied in order to demonstrate and elaborate the function of sugar. It is indicated that sugar protected the enzyme protein from denaturation through the "water-substitute" function which could minimize the activity loss of final CLEAs. The obtained results can provide a practical and effective method to improve CLEAs performance for biocatalysis and biotransformation processes.

2. Material and methods

2.1. Reagents

Free PGA (156 U/mg) was obtained from Shijiazhuang Pharmaceutical Group Co., Ltd. (Hebei, China). Glucose, sucrose, trehalose, glutaraldehyde and other reagents were of analytical grade and obtained from common commercial sources without further purification.

2.2. Preparation of sugar-assisted CLEAs

A 2.04 mg (320 U) of the free PGA was first dissolved in 300 μL phosphate buffer (0.2 mol/L, pH 8.0) to form 6.8 mg/mL PGA solution. Sugar-assisted CLEAs were prepared by slowly adding 500 μL organic precipitant (methanol, ethanol or acetone) to 300 μL PGA solution containing a certain amount of sugar (glucose, sucrose or trehalose). After incubation at 4 °C for 30 min, 6 μL glutaraldehyde (50% w/v) was added and vortexed, and then cross-linking for 2 h at 4 °C. The mixture was centrifuged at 10,000 rpm for 2 min. The supernatant was decanted and the insoluble residue was washed for 3 times with distilled water. Finally, the CLEAs sample was stored in 0.2 mol/L phosphate buffer (pH 8.0) at 4 °C.

2.3. Bradford assay for protein quantitation

The protein quantitation of PGA was determined by Bradford (1976) method using bovine serum albumin as standard. For each sample, 10 μL of PGA solution was diluted in 1 mL phosphate buffer (pH 8.0; 0.2 mol/L) and mixed with 4 mL Bradford reagent. After incubation at room temperature for 30 min, the protein content was measured from the absorbance at 595 nm. A calibration using standard bovine serum albumin solution was carried out for calculating the protein content in PGA solution.

For the determination of the free PGA residue after precipitation, the insoluble enzyme aggregates were centrifuged and the supernatant was collected. Then, the protein content in supernatant was determined by Bradford method.

2.4. Activity assay

The activities of free PGA and CLEAs were measured by the p-dimethyl-aminobenzaldehyde (PDAB) method (Shewale et al., 1987). 1 mg of enzyme samples was added to 50 ml 0.2 mol/L phosphate buffer (pH 8.0), containing 4% (w/v) penicillin G at 37 °C. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 μmol 6-APA (6-aminopenicillanic acid) per minute. For the activity assay of enzyme aggregates, the precipitates were redissolved into clear solution with phosphate buffer (0.2 mol/L, pH 8.0) and detected by PDAB method.

The activity yield was defined as:

$$\text{Activity yield (\%)} = \frac{\text{The total activity detected (U)}}{\text{The total activity of the initial free PGA preparation (U)}} \times 100 \quad (1)$$

2.5. Morphology studies

The structure of lyophilized CLEAs samples were detected by emission scanning electron microscope (SEM). All the samples were sputter-coated with platinum using E1045 Pt-coater (Hitachi High-technologies CO., Japan), and then imaged by an S-4800 field emission scanning electron microscope (Hitachi High-technologies CO., Japan) at the acceleration voltage of 5 kV.

2.6. Confocal laser scanning microscopy

The fluorescent morphologies of CLEAs were obtained by Olympus Fluoview FV-1000 confocal laser scanning microscopy (CLSM). The excitation was set at 488 nm with the same intensity and the emission at 525 nm wavelength was collected. The fluorescent intensities of CLSM images were examined using Image J software (National Institutes of Health, Bethesda, MD) and represented as a color-coded height map (Brunecky et al., 2009).

2.7. Fourier transform infrared spectroscopy

For Fourier transform infrared (FTIR) measurements, the infrared spectra of enzyme samples (free PGA, sugar-assisted and sugar-free CLEAs) were recorded by Nicolet Nexus 870 FT-IR spectrometer from 1400 to 400 cm^{-1} with samples powder dispersed in the pressed KBr discs. The spectral data were corrected for the background spectrum, displayed in the absorbance mode, and analyzed using OMNIC 8.0 (Thermo Nicolet). Peak frequencies in amide I region (1600–1700 cm^{-1}) were smoothed with a 13-point Savitzky–Golay to remove noise and identified using the secondary derivative. Then, a multipeak fitting program with Gaussian function in Origin 8.0 was used to quantify the multicomponent peak areas in protein amide I bands. The relative amounts of β-sheet (1613–1640 cm^{-1}, 1682–1689 cm^{-1}), random coil (1640–1645 cm^{-1}), α-helix (1645–1662 cm^{-1}) and β-turn (1662–1682 cm^{-1}) based on the modeled peak area were calculated according to the report generated by the software (Goormaghtigh et al., 1990; Yu et al., 2004).

2.8. Determination of kinetic parameters

The kinetic parameters of sugar-assisted and sugar-free CLEAs for catalyzing the hydrolysis reaction of penicillin G were determined by measuring the initial reaction rates with varying