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High pressure enhances activity and selectivity of *Candida rugosa* lipase immobilized onto silica nanoparticles in organic solvent

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ABSTRACT

High hydrostatic pressure has been increasingly utilized to improve functions of enzymes, and most of such studies are currently focused on free enzymes in aqueous solution or organic solvent. In this work, *Candida rugosa* lipase (CRL) was immobilized onto silica nanoparticles and its activity and enantioselectivity in organic solvent were evaluated at high pressures under different water activities. The application of high hydrostatic pressures (50–200 MPa) led to improved activities of immobilized CRL for transesterification of (*R*)-1-phenylpropan-2-ol with vinyl acetate by 4–6 folds. Additionally the immobilization of CRL resulted in a significant change of selectivities, shifting the enantiomeric excess from the (*R*)- towards (*S*)-1-phenylpropan-2-yl acetate product at atmospheric pressure. The application of high pressures led to either enantiomeric excess towards (*R*)-1-phenylpropan-2-yl or no enantiomeric selectivity, depending on the water activities in the organic solvent and the level of pressures. The interesting behaviour of immobilized CRL under high pressures offers new opportunities to modulate enzyme functions through combination of high pressures and enzyme immobilization.

1. Introduction

Enzymatic synthesis has been increasingly applied in fine chemical industry to produce enantiomeric pharmaceuticals (Stinson, 1998). Various chiral intermediates, including amino acids (Drauz, 1997), peptides (Gardossi et al., 1991; Sicard and Brennan, 2013), esters (Boland et al., 1991) and penicillin (Liu et al., 2011; Volpato et al., 2010) have been manufactured via enzymatic synthesis route. Sustainable application of enzymes for such productions require enzymes with high stability and selectivity under industrial processing conditions (Bornscheuer et al., 2012), and ideally, enzymes should be re-useable for a long period in order to keep the cost low (Mateo et al., 2007). Protein engineering and enzyme immobilization are two classical approaches to engineer enzymes with these desired features (Bornscheuer et al., 2012; Schmid et al., 2001). Alternatively, the performance of enzymes can also be enhanced through altering the reaction conditions such as

Abbreviations: a_w, water activity; CRL, *Candida rugosa* lipase; I-CRL, immobilized CRL; PP, 1-phenylpropan-2-ol; VA, vinyl acetate; SNP, silica nanoparticle; (*R*)-PPA, (*R*)-1-phenylpropan-2-yl acetate; (*S*)-PPA, (*S*)-1-phenylpropan-2-yl acetate; TEOS, tetraethylorthosilicate; PE, 1-phenylethanol.

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utilizing organic solvents as reaction medium (Klibanov, 2001), or employing high hydrostatic pressures (Eisenmenger and Reyes-De-Corcuera, 2009b).

Recently high hydrostatic pressure processes have shown potentials in enhancing performance of enzymes by increasing their stability and selectivity (Eisenmenger and Reyes-De-Corcuera, 2009b; Mozhaev et al., 1996b; Herbst et al., 2012, 2014). Although it is well known many proteins denature upon exposure to high hydrostatic pressures (Heremans, 1982; Mozhaev et al., 1996a), more than 25 enzymes have been reported with improved performances under extreme pressures (Eisenmenger and Reyes-De-Corcuera, 2009b). Despite these progresses, applications of bio-catalysis under high pressures are still limited, in part, due to poor knowledge of enzymes at extreme conditions (Berheide et al., 2010). Particularly, there is little understanding about how high pressures affect the function of enzymes in their immobilized form that is important for re-use of enzymes in industrial application (Eisenmenger and Reyes-De-Corcuera, 2009a).

Lipase catalysis in organic solvents is frequently used for enantiomeric products synthesis (Klibanov, 2001). Recently, high pressure has shown the ability to alter properties of free lipase in solvent including activity, stability and enantioselectivity (Eisenmenger and Reyes-De-Corcuera, 2009a,b; Mozhaev et al., 1996b; Herbst et al., 2012, 2014). The work by Herbst et al. (2012, 2014) has evaluated high pressure effects on *Candida rugosa* lipase (CRL), one of the most used lipase for industrial application (e.g. transesterification reactions). However, the effect of high pressure on lipase under its immobilized form has not yet been studied. Thus, in this work, we aim to investigate the effect of high hydrostatic pressure on immobilized CRL in organic solvent at different water activities. Solid silica nanoparticle was selected as carrier for CRL immobilization, based on the following considerations: (1) solid silica nanoparticles are stable under high pressures; (2) they have low mass transfer resistance due to their small size (Zaraki et al., 2015), and; (3) convenient surface functionalization is available for immobilization (Zhao et al., 2013a). Activity and enantioselectivity of immobilized CRL were evaluated and compared with free CRL under different pressures and water activities (a_w), using the established model reaction of the asymmetric transesterification of 1-phenylpropan-2-ol (PP) with vinyl acetate (VA) (Herbst et al., 2012, 2014).

2. Experimental

2.1. Materials

Lipase from *C. rugosa* in crude powder (ca. 2.5% enzyme by protein mass) was a gift from Amano Enzyme Inc. Nagoya, Japan. All chemicals and solvents were of at least analytical grade or higher. Chemicals for particle preparation and immobilization including tetraethyl orthosilicate (TEOS) and 3-glycidoxypropyltrimethoxysilane were purchased from Sigma-Aldrich Sydney, Australia. Chemicals for enzymatic reaction including vinyl acetate (VA), racemic 1-phenylethanol (PE), racemic 1-phenylpropan-2-ol (PP) and anhydrous hexane were obtained from Sigma-Aldrich Munich, Germany. Bradford assay reagent for protein assay was obtained from Sigma-Aldrich Munich, Germany.

2.2. Preparation of SNP and lipase immobilization

SNP with diameters around 250 nm were synthesized using the Stoeber method (Stoeber et al., 1968). The synthesized SNP were functionalized with epoxy groups using 3-glycidoxypropyltrimethoxysilane followed by immobilization of CRL using the method reported previously (Zhao et al., 2013b). Then, the particles were dried in fume hood before controlling their water activity. (Detailed information is given in the Supporting information).

2.3. CRL-catalyzed transesterification at ambient and high pressures

In a typical experiment, 18.75 mL hexane and 6.25 mL VA with certain water activity (a_w = 0.35 or 0.7, see control of water activity in the Supporting information) were mixed with PP at a final concentration of 0.41 M. After stirring the reaction media for 5 min at 300 rpm to dissolve PP, 150 mg crude CRL powder or immobilized CRL with the equivalent CRL mass was added. For the reactions at atmospheric pressure the lipase–substrate-mixture was transferred into sealed glass vials which were kept in an oven to control the reaction temperature. For reactions at high-pressures the lipase–substrate-mixture was transferred into a leak-proof high pressure vessel using the set-up reported previously (Herbst et al., 2014). Three pressure levels (50, 100 and 200 MPa) were applied for the experiments. The reaction solutions, both under atmospheric and high pressures, were continuously mixed with a stirrer bar at 300 rpm by a magnetic stirrer. The reaction was carried out for 48 h at 35 °C before samples were taken for analysis. All the experiments were conducted two times at least, with error bar which represent the standard deviation labelled on the data points of figures.

2.4. Analytical methods

The previous reported method was employed for the analysis of the (Herbst et al., 2014) product. Briefly, the product concentration of samples after the reaction were analyzed by gas chromatography GC-FID (Clarus 500, Perkin, Elmer Rodgau, Germany) utilizing a chiral Hydrodex-ß-PM column (Macherey & Nagel, Dueren, Germany; column length 50 m; column diameter 0.25 mm; condition: oven temperature from 80 °C (initial time 1 min) to 120 °C for 17 min with a heating rate of 20 °C/min, split ratio 1:20, He as carrier gas) to determine the specific activity and the enantiomeric excess. The enantiomeric excess ee_R [%] was calculated by:

$$ee_R = \frac{c_R - c_S}{c_R + c_S} \times 100$$

where c_R is the concentration of (*R*)-1-phenylpropan-2-yl acetate ((*R*)-PPA) and c_S is the concentration of (*S*)-1-phenylpropan-2-yl acetate ((*S*)-PPA). The enzymatic activity was calculated by the equation using previously reported method (Herbst et al., 2014) as below:

$$A = \frac{c_p \times V}{t \times m_E}$$

where c_p is the concentration of the product, V is the reaction volume, *t* is the reaction time, and m_E is mass of the enzyme.