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Synthesis of crosslinked lipase aggregates and their use in the synthesis of aspirin

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ABSTRACT

Biocatalytic synthesis of drugs is imperative to avoid the hazardous environmental effects of the pharmaceutical production processes. In view of this, biocatalytic synthesis of aspirin is discussed in the present work. A crude lipase extracted from *Geobacillus* sp. was crosslinked using *N*,*N*-methylenebisacrylamide (*N*,*N*-MBAAm) and two different initiators: ammonium persulphate (APS) and γ-irradiation. The crude lipase was crosslinked as such bypassing the separation procedures using a biocompatible crosslinker. The crosslinked lipase was characterized by various techniques and evaluated for its activity profile as a function of time, temperature, pH and substrate concentration and results were compared to its free form. The activity profile of the crosslinked lipase was observed to be only marginally lower than the free lipase. The crosslinked lipases were used as catalysts in the green synthesis of acetylsalicylic acid, aspirin, directly from the acetic acid. The reusability studies of the crosslinked lipases were carried out up to 10 cycles.

Keywords: Crude lipase; Crosslinking; Reusability; Activity profile

1. Introduction

Biocatalysts in the immobilized form are good candidates for developing green protocols in pharmaceutical chemistry. Applications of various enzymes in industrial biochemical transformations are restricted due to the operational and storage instability, time and solvent consuming separation processes and lack of reusability (Sheldon, 2007a). Immobilization of an enzyme can overcome many limitations of its free from and broaden its applications in a wide physical environment apart from increasing its reusability and recyclability (Cao et al., 2003; Khare and Nakajima, 2000). Enzyme immobilization by carrier or support bound techniques has been widely investigated (Chauhan et al., 2004; Oliveira et al., 2000). Among the different techniques reported for the immobilization of enzymes adsorption is a simple method, yet it has a limitation as the immobilized enzyme leaches out on reusability. Encapsulation of enzyme in matrix has some advantages, yet the diffusional limitations of both substrates to the enzyme active sites and products formed hinder potential of this technique (Erginer et al., 2000; Soares et al., 2004). The immobilization by the formation of covalent linkages between the functional groups of the support used and those of the enzyme results in a considerable stabilization of an enzyme (Letant et al., 2004; Miranda et al., 2006). However, the latter may involve the enzyme active sites in the covalent linkage resulting in the loss of enzyme activity (Villeneuve et al., 2000). Furthermore, this technique requires specially designed carriers or supports (Chauhan et al., 2007; Raj et al., 2011). The support-based immobilization techniques involve high cost in

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the form of support. The catalytic activity of enzyme is diluted as the non-catalytic mass, support, constitutes the major part of the total mass (Roessl et al., 2010). Hence, in the recent past there has been a great interest in developing support or carrier-free immobilized enzymes designated as crosslinked enzyme aggregates, CLEAs (Cao et al., 2000; Kim et al., 2007; Serrano et al., 2002; Wang et al., 2010). The carrier free technique, i.e. crosslinking of enzyme offers advantages including the exclusion of the expensive carrier. The later dilutes catalytic activity of enzyme due to the introduction of a large proportion (90–99% of the total) of the non-catalytic mass. The crosslinking of enzyme also results in the concentrated enzyme activity due to the negligible molecular weight of the crosslinking reagent as compared to the enzyme itself. The advantages such as reusability of the support-based immobilized enzymes are retained with an additional advantage of high catalytic activity and low production costs as the support is excluded (Schoevaart et al., 2004; Sheldon, 2007b; Sulek et al., 2011; Wilson et al., 2006). CLEAs are reported having high efficiency in performing various biotransformation (Rajendhran and Gunasekaran, 2007; Sheldon, 2007c; Sheldon et al., 2007).

In view of their ability to catalyze a wide range of reactions, applications of lipases have been exploited in many industrial processes (Rajendran et al., 2009; Yigitoglu and Temocin, 2010). The crosslinked lipase aggregates, CLLA, have been reported to be effective in resolving racemic mixture (Shah and Gupta, 2007), and as catalyst in the biodiesel production (Kumari et al., 2007). Crosslinked lipases are also stable in the ionic liquids of denaturing nature (Toral et al., 2007). It follows from the above discussion that the advantages of CLLAs are evident. However the protocol including the nature of the crosslinker still remains a challenge. Glutaraldehyde has been reported as crosslinker as its aldehyde groups react with the amino groups of enzymes to generate a CLEA (Park et al., 2001). However, it is of toxic nature and due to its small size it diffuses into the inner part of the enzyme (Sheldon et al., 2005). Hence, use of bulky crosslinking reagents as an alternate to glutaraldehyde needs to be investigated. We are interested in developing novel crosslinked enzymes by avoiding the use of glutaraldehyde because of lot of reported work on it and enough evidences for its toxic behaviour. *N*,*N*-MBAAm is comparatively less toxic and it polymerizes being tetrafunctional and does not remain of small size (Panajkar et al., 1997). Furthermore, it is a well reported crosslinker reported in many works for drug delivery, etc. (Bai et al., 2014).

In view of the above discussion, the present work is based on a simple protocol used to synthesize crosslinked lipase aggregate, CLLA, by following two different protocols. A biocompatible and tetrafunctional crosslinker, *N*,*N*-Methylenebisacrylamide (*N*,*N*-MBAAm), and ammonium persulphate (APS) or γ-radiation were used as the crosslinking initiators. Both the protocols are green as the crosslinking reaction was carried out by low energy intensive processes in water. Furthermore, the method involved crosslinking of crude lipase as such, hence, eliminate the separation and purification procedures of lipase from the crude lipase to pure form prior to immobilization by any techniques. The CLLA formed can be easily removed by simple filtration as reported in the present work. The activity of the CLLAs synthesized was investigated as a function of different parameters and compared with the pure free lipase. The CLLAs are recyclable and were used as catalysts to synthesize acetylsalicylic acid from salicylic acid and acetic acid.

2. Materials and methods

2.1. Chemicals

Ammonium persulphate, *N*,*N*-methylenebisacrylamide (Sisco Research Laboratory Pvt. Ltd., Bombay, India), Salicylic acid, Tris-(hydroxymethyl)-aminomethane (Tris buffer) (SD Fine Chem. Ltd., Mumbai, India), *p*-nitrophenyl palmitate (Lancaster England), acetic acid (Ranbaxy Chemicals Ltd., New Delhi, India), were used as received. Crude thermophilic lipase was obtained from the Department of Biotechnology, Himachal Pradesh University Shimla, India.

2.2. Synthesis of CLLAs

Crosslinking of lipase was achieved using *N*,*N*-MBAAm as crosslinker by two different methods. In the first method, 5% *N*,*N*-MBAAm and 1%APS was added to lipase solution and mixture was kept at 65 °C for 5 h to allow its crosslinking. In the second method, 5% *N*,*N*-MBAAm was added as crosslinker to lipase solution and the crosslinking was initiated by exposing the mixture to γ-radiation for 5 h at dose of 1.15 KGy. The solid crosslinked lipases obtained through both the methods were separated by simple filtration, washed with water and dried in air, and designated as CLLAs.

2.3. Characterization of CLLAs

CLLAs were characterized by Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM) and X-ray diffraction (XRD) to get evidence for crosslinking. FTIR spectrum was recorded on Perkin Elmer in transmittance mode in KBr. SEM images were recorded on SEM QUANTA 250 D9393. X-ray diffraction patterns of samples were recorded on PANalytic XRD with XPERT-PRO diffractometer system using a typical wavelength of 1.540,60 Å (Cu-Kα radiation). The diffraction angle 2θ was varied from 10° to 70°.

2.4. Activity assay

The activity of the free and CLLAs was determined spectrophotometrically by carrying out the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP). A solution of *p*-NPP (20 *m*M) was prepared in the HPLC grade isopropanol. The reaction mixture comprised of 80 μL of *p*-NPP stock solution, 10 μL free or 10 mg CLLA and Tris buffer (0.1 M, pH 8.5) to make final volume 3 mL. The reaction mixture was incubated at 45 °C. A control with a heat inactivated enzyme (by heating for 5 min in boiling water bath) was included with each assay. A calibration curve of the absorbance and known *p*-nitrophenol concentrations was plotted. Calculations were made by using commercial *p*-nitrophenol as standard. Enzyme activity was studied as a function of temperature (25–65 °C after 10 °C interval), pH (1.2, 4.0, 5.6, 7.0, 8.5 or 9.0) and concentration of substrate (5–30 mM each with 5 mM interval) after optimization of reaction time using the method discussed above. The reusability studies of the CLLAs were carried out for ten repeat cycles. All the experiments were carried out in duplicate and reported values are average of the two values.

2.5. Synthesis of acetylsalicylic acid using CLLAs

Salicylic acid and acetic acid were mixed together with 1:1.5 molar ratio. The mixture was shaken thoroughly in order to