Process Biochemistry 45 (2010) 593–597

Contents lists available at ScienceDirect

Process Biochemistry

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

Development of a monolith based immobilized lipase micro-reactor for biocatalytic reactions in a biphasic mobile system

Ping He, Gillian Greenway, Stephen J. Haswell *

Department of Chemistry, University of Hull, Hull HU6 7RX, UK

ARTICLE INFO

Article history:
Received 1 October 2009
Received in revised form 6 December 2009
Accepted 10 December 2009

Keywords:
Micro-reactor
Immobilized lipase
Monolith
Biphasic system
Hydrolysis reaction
Enzyme kinetics

ABSTRACT

This paper reports a simple method for producing macroporous silica-monoliths with controllable porosity that can be used for the immobilization of lipases to generate an active and stable micro-reactor for biocatalysis. A range of commercially available lipases has been examined using the hydrolysis reactions of 4-nitrophenyl butyrate in water–decane media. The kinetic studies performed have identified that a similar value for k_{cat} is obtained for the immobilized *Candida antarctica* lipase A (0.13 min^{-1}) and the free lipase in solution (0.12 min^{-1}) whilst the immobilized apparent Michaelis constant K_m (3.1 mM) is 12 times lower than the free lipase in solution (38 mM). A 96% conversion was obtained for the immobilized *C. antarctica* lipase A compared to only 23% conversion for the free lipase. The significant higher conversions obtained with the immobilized lipases were mainly attributed to the formation of a favourable biphasic system in the continuous flowing micro-reactor system, where a significant increase in the interfacial activation occurred. The immobilized *C. antarctica* lipase A on the monolith also exhibited improved stability, showing 64% conversion at 80 °C and 70% conversion after continuous running for 480 h, compared to 40 and 20% conversions under the same temperature and reaction time for the free lipase.

1. Introduction

Due to the existence of the interfacial activation lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) represent versatile group of enzymes with various biocatalytic activities which include triacylglycerols hydrolysis, esterification, trans-esterification, alcoholysis and acidolysis to yield a wide range of useful biological and chemical derivatives [1–5]. Furthermore, lipases are also enantioselective catalysts and can be used for the resolution of chiral compounds and the synthesis of high-value pharmaceutical intermediates [6–8]. However, the high cost of lipase makes enzymatically driven processes in the soluble homogenous form economically unattractive. A practical solution to this problem is to use an immobilized lipase that can significantly reduce the cost and increase the ratio of product to lipase used [9,10]. The use of an appropriate immobilization method may promote enzyme-support multipoint or multi-subunit interactions and enhance enzyme stabilization. Such a method of immobilization will also enhance other lipase properties including activity [11,12] whilst facilitating the separation of products from the enzyme, and optimization of lipase/substrate contact time by using continuous flow systems [2,10–13]. Traditional methods for lipase immobilization are largely based on lipase adsorption onto hydrophobic polymers such as polypropylene, alkyl-agarose, polyacrylate and polystyrene, lipase stability can, however, still be significantly affected by the polymer surfaces and activity is typically limited by surface coverage [14–16]. Recently, hydrophobic sol–gel supports, obtained by using alkyl-modified silane ($RSi(OCH_3)_3$), have been reported for the entrapment of lipases [17,18]. Since lipases are interface-active enzymes with lipophilic domains, lipophilic interactions between hydrophobic parts of gel and lipase favour the stabilization of the open form of the lipase which offers in turn enhanced activity [11,12]. However, if the substrate is large or hydrophilic, the near presence of the hydrophobic support surface may generate some steric hindrances, reducing the activity of the lipase. More recently chemical covalent bonding and cross-linking as well as physical encapsulation processes have been reported for the enzyme immobilization but these methods can still cause structural deformation of the enzyme or introduce steric hindrance to the catalytic sites, leading to reduction in enzyme activity [19–23].

The use of micro-reactors in conjugation with enzymatic processes is now attracting increasing attention due to their ability to spatially control localized concentration of reactants, intermediates and products within the flow region of such systems

* Corresponding author. Tel.: +44 01482 465469; fax: +44 01482 466410.
E-mail address: s.j.haswell@hull.ac.uk (S.J. Haswell).

1359-5113/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2009.12.008

offering a significant reduction in both reagent consumption and operating costs [24]. In addition, chemical and biological processing at the microscale offers a number of reaction and practical benefits that are unattainable when using a macroscale configurations [25–27]. However, packed-bed micro-reactors often generate increased backpressure that gives rise to leaking or blocking problems [28,29]. Recently, silica-based monoliths have been used as an attractive alternative to packed columns for the analysis of proteins, peptides and nucleic acids as they offer low diffusion resistance during mass transfer, controllable porosity and low back pressure compared to packed column reactors [30–35]. The preparation of these monoliths by sol–gel chemistry however usually requires the addition of other additives such as polyethylene glycol (PEG) and dextrin to stabilise the structure [21,22] and fabrication can be time consuming (ca. 3–15 days) [21,22].

In this paper we report a simple method for generating a stable monolith for the immobilization of lipase with in a micro-reactor device. The macroporous silica-monoliths with controllable porosity were prepared within a capillary from two precursors tetramethoxysilane (TMOS) and methyltrimethoxysilane (MTMOS) using a sol–gel method, followed by lipase immobilization *via* multipoint interactions between lipase and the surface of silica-monoliths to generate immobilized lipase micro-reactors based on a range of commercially available lipases. Through multipoint immobilization the lipases can be immobilized in an active conformation that significantly enhances thermal and reactive stability. In addition, the use of mobile water–decane biphasic system for the hydrolysis reaction of 4-nitrophenyl butyrate can significantly increase the lipase interfacial activation. Kinetic parameters, i.e. apparent Michaelis constant K_m and turnover number k_{cat} for the free and immobilized lipase were determined. An evaluation of the stability for the free and immobilized lipase at elevated temperatures and over extended run times was also carried out.

2. Experimental

2.1. Materials

Six lipases (EC 3.1.1.3) including *Aspergillus niger* lipase (ANL), *Burkholderia* sp. Lipase (BCL), *Candida antarctica* lipase A (CAL), *C. antarctica* lipase B (CBL), *Penicillium camembert* lipase (PCL) and *C. antarctica* lipase A cross-linked enzyme aggregate (CLEA–CAL) were purchased from Sigma–Aldrich (UK) and used without further purification. Decane (≥99%), 4-nitrophenyl butyrate (4-NPB, ≥98%) and 4-nitrophenol (4-NP, ≥98%) were also obtained from Sigma–Aldrich. Polyethyleneimine (PEI, molecular weight 10,000), KCl (99%), HCl (37%), tetramethoxysilane (TMOS, 99%) and methyltrimethoxysilane (MTMOS, 99%) were purchased from Fluka. Glass capillary (0.6 ± 0.05 mm ID and 25 mm length) was obtained from Brand GMBH (Germany). Milli-Q water (18 MΩ cm) was used to prepare all aqueous solutions.

2.2. Preparation of silica-monoliths within a capillary

Silica-monoliths were prepared from two precursors TMOS and MTMOS by using a sol–gel method [36]. In a typical preparation, 18 μl of TMOS and 69 μl of MTMOS (at 1:4 molar ratio) were added to dilute HCl solution (8 μl of 1 mM HCl and 13.2 μl water) and allowed to hydrolyse for 15 min under sonication at room temperature to form a homogeneous sol of silicic acid. A 12.6 μl aliquot of sol was then mixed with 9.4 μl of water and 22 μl of 0.2 M (pH 7) Tris–HCl buffer, and the resultant liquid mixture was immediately loaded into a capillary (4 cm length) and allowed to polymerize for 15 h at room temperature, followed by vacuum drying for 1 h to form a silica-monolith with void volume of 10 ± 1 μl.

2.3. Lipase immobilization

An aqueous solution (lipase in 0.05 M pH 7 Tris–HCl buffer, final lipase concentration 0.1–1 mg/ml) was pumped through a monolith micro-reactor at flow rate of 10 μl/min for 10 min, then the monolith micro-reactor was kept in a fridge (4 °C) for 1 h, followed by vacuum drying for 30 min to give a monolith immobilized lipase micro-reactor (note: after an initial evaluation of a range of lipase substrates only monoliths based on immobilized *C. antarctica* lipase A (CAL–monolith) were used in this paper). Before measurement was taken, the micro-reactor was washed with 0.05 M neutral Tris–HCl buffer (flow rate 10 μl/min) for 5 min to remove unentrapped lipase and washing liquid was collected.

2.4. Assay of the free solution and immobilized lipase activity

4-NPB, a well-known substrate for lipase hydrolysis activity, was used as the substrate in this work to evaluate the performance of free solution and immobilized lipase in the media consisted of 0.05 M pH 7 Tris–HCl buffer and decane solvent. The solubility of the reactant 4-NPB, the product 4-NP and lipases was checked in both water and decane solvent, it was found that the reactant 4-NPB was dissolved in the organic phase (decane) whilst the product 4-NP and lipase (for solution based reactions only) were present in water phase.

The assay for free lipase activity was carried out at the desired temperature (usually room temperature 25 °C) under agitation (with a magnetic stirrer at 100 rmp) in 1 ml final volume, containing 0.4 ml of organic phase (1–15 mM 4-NPB in decane) and 0.6 ml of aqueous phase (free lipase in 0.05 M pH 7 Tris–HCl buffer). After a given time (1, 2.5, 5, 10 and 15 min), aqueous phase samples (10–100 μl) were removed by a pipette and mixed with a 0.1 M neutral Tris–HCl buffer solution to quench the reaction (final volume 1 ml in a disposable plastic UV–vis cuvette). The production of 4-NP was determined using an UV–vis spectrophotometer (Chemspec M508) by reading absorbance at 400 nm and using a calibration curve of 4-NP concentration versus absorbance. The conversion was calculated using Eq. (1):

$$\text{Conversion} = \frac{C_p}{C_0} \times 100 \quad (1)$$

where C_0 was initial concentration of 4-NPB and C_p concentration of 4-NP in the samples collected at a given time. All data reported are average values from at least triplicate measurements and with standard error less than 6%.

A schematic of the apparatus used for the evaluation of the immobilized lipase activity is shown in Fig. 1 and consists of two syringe pumps for controlling flow rate of the aqueous and organic solutions, a capillary mixer, and a monolith immobilized lipase micro-reactor. An aqueous solution (0.05 M neutral Tris–HCl buffer) without lipase present and an organic solution (1–15 mM 4-NPB in decane) were separately pumped through the capillary mixer and monolith micro-reactor with the product being collected from the outlet of the micro-reactor under steady-state condition. The organic phase was then removed by a pipette and the aqueous phase was treated and analysed using the same methodology as described above for the assay of the free lipase activity.

2.5. Determination of lipase loading in the immobilized monolith micro-reactor

The quantity of the immobilized lipase was determined by calculating the difference between the amount of lipase in the initial loading solution and that in the residual solution collected after the entrapping process, which was quantified using a calibration curve of lipase activity versus lipase concentration within a linear range. The quantity of immobilized lipase reported is an average value from at least triplicate measurements with a standard error less than 5%.

3. Results and discussion

3.1. Initial solution study into the catalytic activity of different lipases and effect of water content

The activity of the different lipases and the effect of water content were first investigated in order to aid the selection of lipase and determine the optimum water content to use in the immobilization methodology. Six commercially available lipases including CAL, BCL, ANL, PCL, CBL and CLEA–CAL were examined in

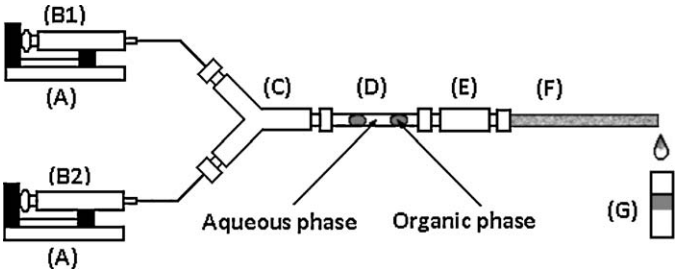

Fig. 1. Schematic diagram of the reaction system used to assay immobilized lipase activity, consisting of (A) syringe pump, (B1) syringe (volume 1 ml) for organic solution and (B2) syringe (volume 1 ml) for buffer solution, (C) small PEEK Y-shape connector (pore size 0.5 mm diameter), (D) the capillary mixer (0.6 mm ID and 3 cm length), (E) 2-way PEEK connector (pore size 0.5 mm diameter), (F) the monolith micro-reactor (0.6 mm ID and 4 cm length), (G) sample collection vial. Within the capillary mixer, water was the continuous phase and the organic solution the segmented phase. The length of the organic plugs was ca. 1.5 mm.