Journal of Molecular Catalysis B: Enzymatic 63 (2010) 81–86

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Development of a high throughput screening tool for biotransformations utilising a thermophilic L-aminoacylase enzyme

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ARTICLE INFO

Article history:
Received 17 August 2009
Received in revised form
23 November 2009
Accepted 14 December 2009
Available online 24 December 2009

Keywords:
Thermophilic enzyme
L-Aminoacylase
High throughput screening
Biotransformations
Micro-reactor

ABSTRACT

Micro-reactors containing a monolith-immobilised thermophilic L-aminoacylase, from *Thermococcus litoralis*, have been developed for use in biotransformation reactions and a study has been carried out to investigate the stereospecificity and stability of the immobilised enzyme. The potential to use the developed micro-reactors as a tool for rapid screening of enzyme specificity was demonstrated, confirming that the L-aminoacylase showed a similar substrate specificity to that previously reported of the free enzyme. From this baseline, the technique was employed as a tool to evaluate potential unreported substrates with *N*-benzoyl- (L-threonine, L-leucine and L-arginine) and *N*-acetyl- (D,L-serine, D,L-leucine, L-tyrosine and L-lysine) protecting groups. The order of preferred substrates was found to be Phe > Thr > Leu > Arg for *N*-benzoyl substrates and Phe ≫ Ser > Leu > Met > Tyr > Trp for *N*-acetyl substrates.

It was found that by using the micro-reactor a significantly smaller quantity of enzyme and substrates was required. It was shown that the micro-reactors were still operational in the presence of selected organic solvents, such as ethanol, methanol, acetone, dimethylformamide (DMF) and dimethylsulfoxide (DMSO). The results indicated that a combination of a small amount of an appropriate solvent (5% DMSO) and a higher reaction temperature could be employed in biotransformations where substrate solubility was an issue.

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1. Introduction

Enzymes are continuing to be exploited for industrial purposes [1,2] and in particular, this area has received attention from pharmaceutical companies for the production of high value chemicals and new drug intermediates [3]. The use of enzyme catalysts allows high synthetic performance under mild conditions, minimising problems such as isomerisation, racemisation and epimerisation; which can reduce the quantity of side products that are generated during the process. In addition, enzyme reactions are stereoselective and therefore can produce optically pure products. The number of new pharmaceutical compounds on the market that are chiral is expected to increase to 70% by 2010 [4].

Enzymes are however often unstable under the necessary operating conditions of the commercial process and enzymatic processes can be more expensive to develop than those using traditional synthetic chemistry. Enzymes isolated from thermophilic organisms have evolved to operate optimally at high temperatures and are inherently more stable to elevated temperatures and organic solvents than their mesophilic counterparts. Although the thermophilic enzymes have optimal activity at elevated temperatures they are also active at lower temperatures which allows a broad operating window.

The L-aminoacylases are important enzymes for industrial applications since they can be used to resolve a racemic mixture of *N*-acyl amino acids to produce a range of L-amino acids and amino acid analogues. We have chosen to use a thermostable L-aminoacylase from the thermophilic archaeon *Thermococcus litoralis* which we have previously cloned, sequenced and overexpressed in *Escherichia coli* [5]. The purified enzyme has been characterised and found to have optimal activity at 85 °C in Tris–HCl buffer at pH 8.0 [5]. It has been shown to exhibit most specificity towards substrates containing *N*-benzoyl or *N*-chloroacetyl protected amino acids (Scheme 1). This is unlike the more common commercially available 'Amano' aminoacylase which has preferential selectivity for acetyl > chloroacetyl ≫ Boc > benzoyl. It is also has different substrate specificity to other thermophilic L-aminoacylases from *Pyrococcus furiosus* [6] and the related *Pyrococcus horikoshii* [7]. The *Thermococcus* L-aminoacylase is a homotetramer of 43 kDa monomers, and has an 82% sequence identity to the aminoacylase from *P. horikoshii* and 45% sequence identity to a carboxypeptidase from *Sulfolobus solfataricus*. Aminoacylase inhibitors, such as mono-*tert*-butyl malonate,

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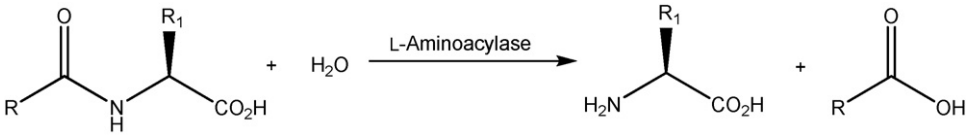
doi:10.1016/j.molcatb.2009.12.013



Scheme 1. Reaction catalysed by L-aminoacylase; R represents the *N*-protecting group [2,3].

have only a slight effect on the activity of the *Thermococcus* enzyme [5].

Previous work has been successful in immobilising the *T. litoralis* enzyme [8]. A column bioreactor containing the recombinant L-aminoacylase immobilised onto Sepharose beads was constructed with the substrate, *N*-acetyl-DL-Trp, continuously flowing at 60 °C for 10 days. Using a flow system has the added advantage that any problems with substrate and product inhibition are eliminated.

Microfluidic systems were first developed for analytical chemistry where they are commonly referred to as micro-total analytical systems (μTAS) [9]. More recently micro-reactors, having reaction channels between 200 and 1000 μm, have been used for fine chemical synthesis. It is now well established that chemical reactions conducted in such devices generate products in higher yield, purity and selectivity when compared to batch reactors [10,11]. The advantages of micro-reactors for enzymatic reactions have been reviewed by Miyazaki and Maeda [12].

The use of micro-reactors containing an immobilised enzyme offers an opportunity to minimise the quantities of enzyme, substrate and other reagents required, therefore reducing the costs when compared to a batch reaction. Several approaches have been taken towards immobilising enzymes in micro-reactors; the initial approach tended to be to immobilise the enzyme onto silica or polymer microparticles which were then packed within the micro-reactor [13]. A recent development by us has been to pack the reaction channel with an immobilised gamma lactamase catalyst and to produce an efficient micro-reactor [14]. The immobilisation of the enzyme onto the micro-reactor wall, can overcome problems associated with high back pressures, but the enzyme loading is lower [15]. The use of membranes within channels to immobilise enzymes has also been investigated, however this is difficult to achieve [16].

In comparison to these techniques, monoliths are well-ordered macroporous materials with low flow resistance, high reaction efficiency and good flow-through properties. They can be formed by *in situ* polymerisation making them ideal for use as components in micro-reaction systems. The application of monoliths in enzyme immobilisation in micro-reactors has proved to be successful for glucose oxidase [17] and a protease [18], where the monolith was prepared from a mixture of tetramethoxysilane and methyltrimethoxysilane.

The only reported use of an industrially important acylase enzyme within a micro-reaction system was for an analytical application in which the optical resolution of racemic amino acids was performed [19]. The work reported focused mainly on the extraction of the product L-amino acids from uncleaved D,L-substrates using an in-line aqueous extraction. The enzymatic reaction was carried out on acetyl-D,L-amino acids at 40 °C and using a flow rate of 0.5 μl min^{-1}. Although employing cross-linked polymerised aminoacylase (Amano) with a polylysine matrix on the surface of the micro-channels prevented high back pressure, a reduced surface to volume ratio results compared to immobilisation onto a monolith.

Recent developments involving monolith production over the past decade suggests that by careful selection of monomer and porogenic mixtures a higher degree of functionalisation and minimal back pressures can be achieved [20,21]. Based on this, the present study aimed to develop a micro-reaction system, where the thermophilic L-aminoacylase from *T. litoralis* was immobilised onto polymer monoliths formed inside micro-channels, which has allowed screening of the immobilised enzyme for activity towards a range of potential substrates with different protecting groups (benzoyl-, chloroacetyl-, acetyl-, Cbz- and Boc-) and a variety of amino acids (Phe, Met, Thr, Tyr, Trp, Leu, Ser and Arg), under a range of conditions.

2. Materials and methods

Chemicals were purchased from the sources indicated and were used as supplied; ethylene dimethacrylate, EDMA (98%, Sigma–Aldrich), glycidylmethacrylate, GMA (97%, Sigma–Aldrich), 2,2-dimethoxy-2-phenyl-acetophenone, 3-(trimethoxysilyl)propyl methacrylate (98%, Sigma–Aldrich), DMPA (99%, Sigma–Aldrich), tris(hydroxymethyl) aminomethane, Tris–buffer (Sigma–Aldrich), dodecanol (99%, Sigma–Aldrich), cyclohexanol (99%, Sigma–Aldrich), *N*-benzoyl-L-phenylalanine (Novabiochem), L-phenylalanine (99%, Sigma–Aldrich), *N*-acetyl-L-phenylalanine (98%, Sigma–Aldrich), *N*-chloroacetyl-L-phenylalanine (98%, Sigma–Aldrich), *N*-acetyl-L-tryptophan (98%, Sigma–Aldrich), *N*-benzoyl-L-leucine (98%, Sigma–Aldrich), *N*-benzoyl-L-threonine (98%, Sigma–Aldrich), *N*-acetyl-L-tyrosine (98%, Sigma–Aldrich), *N*-benzoyl-D,L-phenylalanine (98%, Sigma–Aldrich), *N*-*t*-Boc-L-phenylalanine (Sigma–Aldrich), *N*-CBZ-L-phenylalanine (99%, Sigma–Aldrich), *N*-benzoyl-L-arginine (99%, Alfa Aesar), *N*-acetyl-L-methionine (99%, Sigma–Aldrich), *N*-acetyl-L-serine (99%, Sigma–Aldrich), *N*-acetyl-D,L-leucine (98%, Sigma–Aldrich), *N*-acetyl-L-lysine (98%, Sigma–Aldrich). Ethanol and acetonitrile were HPLC grade and purchased from Fisher Scientific.

2.1. Preparation of monolithic micro-channels

The borosilicate glass micro-reactor used in this study was prepared 'in house' using standard fabrication techniques [22] and had channel dimensions of 100 μm depth, 300 μm width and 15 mm length. Poly(glycidylmethacrylate-*co*-ethylenedimethacrylate) monoliths were formed inside the micro-reactor channels using photoinitiation (Fig. 1) [23]. Fig. 1a shows where the monoliths were formed within the micro-channels and Fig. 1(b) is a schematic illustrating how the system was utilised for biotransformation reactions. The channel surfaces of the micro-reactor were pre-treated to enable covalent attachment of the monolith to the channel walls. This was achieved by firstly treating the channels with 0.2 M sodium hydroxide for 1 h, washing them with water and then filling them with 0.2 M hydrochloric acid for 1 h followed by a water and ethanol wash. The channels were subsequently filled with the silanising agent, 3-(trimethoxysilyl)propyl methacrylate (20% in ethanol, adjusted to pH 5 with acetic acid) and left for 1 h. After this the channels were dried with a stream of nitrogen.

To prepare the monolith, two portions of the monomer solution consisting of GMA and EDMA (3:1) (structures of both monomers are provided in Fig. 1) were mixed with one portion of the porogenic mixture containing 4:1 cyclohexanol:dodecanol, into which 1 wt% of the initiator (DMPA), with respect to total weight of monomers, was dissolved. The mixture was then sonicated to ensure thorough

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