Entrapment of Penicillin G Acylase in sol-gel microparticles with magnetic properties

S.M.S.A. Bernardino[1], J.F.M. Gallegos, A. Santhagunam, D.P.C. de Barros, P. Fernandes[2] and L.P. Fonseca[3]

Institute for Biotechnology and Bioengineering, CEBQ, IST, Lisboa, Portugal
[1] susana1972@gmail.com
[2] pedro.fernandes@mail.ist.utl.pt
[3] pclfons@mail.ist.utl.pt

Introduction

Penicillin acylases (PAs) (penicillin amidohydrolase, EC 3.5.1.11) are a group of enzymes which are mainly involved in the industrial production of 6-aminopenicillanic acid (6-APA) (Fonseca et al; 1993), and 7-aminodesacetoxycephalosporanic acid (7-ADCA). These are key intermediates in the synthesis of semi-synthetic penicillins (SSP), and semi-synthetic cephalosporins (SSC), respectively (Arroyo et al; 2003). PAs belong to the class of the N-terminal nucleophile hydrolases, which have no catalytic triad but instead a N-terminal serine that is activated by a bridging water molecule (Duggleby et al., 1995).

The enzymatic synthesis of β-lactam antibiotics and their derivatives on a commercial basis is only feasible if immobilized biocatalysts are used, given the cost of enzyme production. The advantages of using immobilized penicillin acylases over the soluble form, e.g., simpler handling of the biocatalyst, facilitated separation of the biocatalyst from the product, prevention of product contamination, continuous use or re-use of the biocatalyst and enhanced stability and productivity, allow for significant cost saving in the production of SSP (Kraemer D and Ilhan F, 2001; Parmar et al., 2000; Schroën et al., 2001). However, immobilization brings along some drawbacks, such as loss of activity upon immobilization and diffusion limitations, which reduce the productivity of the immobilized enzyme system (Schroën et al., 2001,2002).

The various methods used for immobilization of penicillin acylase include adsorption, fiber entrapment, microencapsulation, covalent attachment, cross-linking and copolymerization. The two latter carrier-free immobilization approaches have been evaluated as alternatives in order to overcome the mass transfer limitations common to carrier-binding methods (Kallenberg et al. 2005). However, the mechanical resistance of the final biocatalyst is often rather poor.

Silica supports are widely used as enzyme carriers, since such materials are chemical and mechanically stable, non-toxic, hydrophilic and biological inert. Ionic or covalent attachment of the enzyme requires support activation, and loss of biocatalytic activity is often an unwanted outcome of such traditional immobilization processes.

A more recent approach for enzyme immobilization is based in the sol-gel process, which allows the room temperature synthesis of silica glasses, although suitably modified to exclude the typical harsh conditions that would cause enzyme denaturation.The concomitant material offers the same beneficial properties as traditional silica based matrices and allows a high biocatalyst load, since the enzyme is entrapped within the polymeric matrix as it forms (O'Neil et al., 2002).

A well-established sol-gel processing technique consists in hydrolyzing the adequate precursors in aqueous solutions to produce soluble hydroxylated monomers, followed by polymerization and phase separation to produce a hydrated metal or semi-metal oxide hydrogel. Removal of water from the wet gel, which is usually accompanied by changes in the structure of the pores and of the gel network, results in a porous xerogel. The most widely used precursors are alkyl-alkoxysilanes (Vidinha et al., 2006).

In this work, particular emphasis is given to advances in penicillin G acylase immobilization by entrapment in a silica matrix with magnetic properties. Mechanically stable xerogel carriers containing magnetite were produced from tetramethoxysilane (TMOS). The presence of magnetite enhances the recovery of the biocatalyst, in the form of macro and micro-carriers (1 – 10 μm), from the reaction media.

Material and methods

Penicillin G acylase from *Escherichia coli*, tetramethoxysilane (TMOS), 6-nitro–3 (phenylacetamide) benzoic acid (NIPAB), sodium dioctyl sulfosuccinate (AOT) and magnetite nanopower were all purchased from Sigma-Aldrich. Isooctane was supplied from Riedel de Haën. Penicillin G was obtained from Fersinca Gb. All other reagents used were either laboratory or analytical grade.

Enzyme assay

One unit of penicillin G acylase activity (IU) for the soluble and immobilized enzymes is defined as:
- the amount of enzyme required to produce 1 μmol of 6-APA per minute. Enzyme activity was determined in a small batch stirred reactor with automatic pH correction by the pH STAT method (Cardoso and Costa, 1986), using a 4% (w/v) penicillin solution in 0.02 mol dm^{-3} pH 8.0 phosphate buffer at 37ºC.
- the amount of enzyme required to produce 1 μmol of 3-amino-6-nitrobenzoic acid (NABA) at 30 ºC. The activity assay was based on the conversion of 6-nitro-3-(phenylacetamido)benzoic acid (NIPAB) to 3-amino-6-nitrobenzoic acid (NABA). The release of NABA in the assay mixtures was followed by recording the increase in absorbance at 405 nm and 30 ºC. The activity assay mixture contained 900 μL of 50 mmol dm^{-3} phosphate buffer pH 7.0 and 50 μL of 6 mmol dm^{-3} NIPAB in 50 mmol dm^{-3} phosphate buffer pH 7.0. The reaction was initiated by the addition of 50 μL of the enzyme solution (soluble or immobilized).

Sol-gels

The solution containing 100 μL TMOS (2.32 mol dm^3) and 40 μL HCl (1.37 mmol dm^3) was sonicated in a Transsonic T 460 sonicating water bath for 10-15 min until the hydrolysis reaction was complete. In a typical immobilization reaction 38 μL (26.6 mg/mL, 35 U/mg) of penicillin G acylase was suspended in 100 μL of 100 mmol dm^{-3} phosphate buffer, pH 7.5 and 14 μL of magnetite suspension 10% (w/v) in 100 mmol dm^{-3} phosphate buffer, pH 7.5, and then mixed with the sol solution. The reaction mixture was left at room temperature at least 22 hours. The solid xerogel was crushed in a mortar to a fine powder and washed with phosphate buffer to remove unreacted TMOS and methanol released during the formation of the sol-gel and suspended in 1 mL 100 mmol dm^{-3} phosphate buffer, pH 7.5.

Penicillin G acylase is inherently more sensitive to diffusion limitation than, for example, a lipase (Kallenberg et al., 2005). So, to obtain micro-particles (1-10 μm) and minimize difusional limitations 220 μL of the sol-gel solution with enzyme was immediately added to 1000 μL of 150 mmol dm^{-3} AOT/isoctane solution, before gelation. The resulting mixture was sonicated for 30 min, washed with 100 mmol dm^{-3} phosphate buffer, pH 7.5 and suspended in 1 mL of the same buffer.

Assay for protein concentration

The concentration of protein in the enzyme solution before attachment and in the supernatant and washings, after attachment, was determined through absorbance quantification at 280 nm. The amount of protein linked to the support was calculated by mass balance.

A*ctivity retention*
The percentage of activity retention was calculated as the ratio between the specific activities of the enzyme immobilized on the support and of the enzyme solution.

Results ans Discussion

Entrapment in a silica matrix showed to be effective and efficient with lipases, but when applied to penicillin G acylase, the activity recovery was a poor 10%, which the authors ascribed to diffusion limitation in the silica matrix (Kallenberg et al., 2005). In this investigation, the preliminar experiments led to high immobilization yields, 96.8% for macroparticles and 94.6% for microparticles. Activity retention ranged from roughly 23 to 64%, depending on the method used for activity quantification (Tables 1 and 2). The discrepancy in the activity determinations from the the two methods used most likely results from the different affinity of the two substrates towards the enzyme. Penicillin G used in pH STAT method is a natural substrate, whereas 6-nitro-3-(phenylacetamido)benzoic acid used in NIPAB method is an artificial substrate. Loss of activity due to enzyme leakage from the sol-gel particles to the buffer solution following immobilization was ruled out, since no enzyme activity was observed in the liquid phase. It is most likely due to the prolonged exposure of the enzyme to methanol, as well as to mass transport limitations.

Method	Initial activity (U)	Activity after immob. (U)	Activity retention (%)
pH STAT	27.74	17.66	63.66
NIPAB	10.66	2.73	25.61

Table 1: Immobilization of PGA in sol-gel (macroparticles).

Method	Initial activity (U)	Activity after immob. (U)	Activity retention (%)
pH STAT	20.90	9.47	45.32
NIPAB	9.35	2.20	23.52

Table 2: Immobilization of PGA in sol-gel in AOT/isoctane (microparticles).

The storage stability of the sol-gel preparation with macroparticles is quite high, since catalytic activity remained constant for at least 80 days (Figure 1). We didn't performed this study with microparticles yet.

The percent conversion of Penicillin G to 6- APA at 37ºC is given in Figure 2.

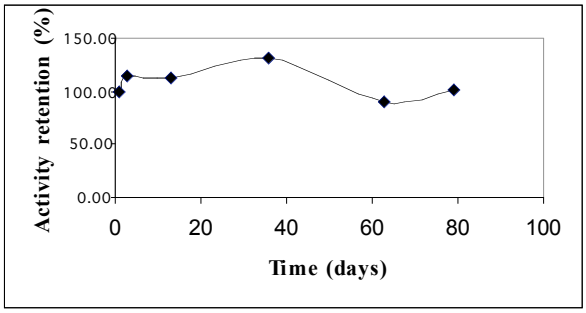

Figure 1: Time stability of immobilized PGA in sol-gel.

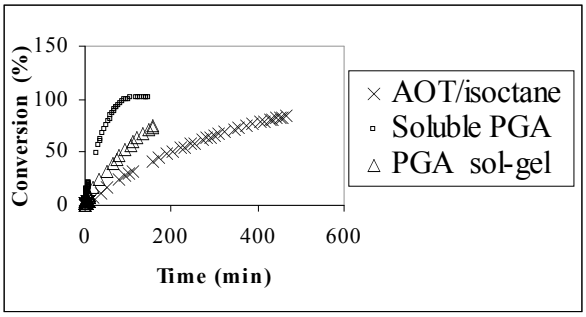

Figure 2: Hydrolysis of Penicillin G (pH STAT method) by immobilized PGA in sol-gel (12.51 U) and PGA in AOT/isoctane (7.89 U) and soluble PGA (14.60 U).

Some enzyme leakage from the sol-gels was observed during this trial, thus efforts are being currently carried out as to increase enzyme retention under operation conditions.

Conclusions

Successful immobilization of penicillin G acylase from *Escherichia coli* in sol-gel using an innovative approach has been demonstrated. The enzyme is catalytically active and can carry out hydrolytic reactions efficiently. Prevention of enzyme leakage under prolonged exposure to operational conditions is being looked at, in order to develop a biocatalyst that can be recycled many-fold/operate in continuous mode to yield an economically feasible bioconversion system.

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