Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Molecular Catalysis B: Enzymatic 39 (2006) 105–111
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Kinetically controlled synthesis of ampicillin and cephalexin in highly condensed systems in the absence of a liquid aqueous phase

Alessandra Basso, Patrizia Spizzo, Micaela Toniutti, Cynthia Ebert, Paolo Linda, Lucia Gardossi *

Laboratory of Applied and Computational Biocatalysis, Dipartimento di Scienze Farmaceutiche, Università degli Studi, Piazzale Europa 1, 34127 Trieste, Italy

Available online 28 February 2006

Abstract

Advantages of performing penicillin G amidase catalysed synthesis of ampicillin and cephalexin by enzymatic acyl transfer to the β-lactam antibiotic nuclei in a highly condensed system using mainly undissolved substrates, with no apparent aqueous liquid phase, were demonstrated. It was shown that synthesis can be performed in the absence of a liquid phase formed by water or an organic co-solvent. This highly condensed system is formed by a liquid phase given by one of the reactant, the phenylglycine methyl ester (PGM), that remains liquid in these operative conditions and the partially dissolved β-lactam nucleus. Operating in such highly condensed system, the water that causes the hydrolysis of PGM is limited to the water hydrating the support on which the enzyme is covalently immobilised. In this way the reaction system is maintained at a controlled degree of hydration.

In the present work the reaction system was modulated by eliminating the solvent (aqueous or aqueous/organic), reducing the amount of water to the minimum for the biocatalytic activity and using PGM as solvent and reagent at the same time. The synthesis was conducted with equimolar amounts of PGM and the β-lactam nucleus, with a reduced hydrolysis of the activated acyl donor. We have also studied a simple and efficient method for the workup of the reaction where the unreacted reagents can be recovered after selective filtration and precipitation.

Keywords: Penicillin G amidase; β-Lactam antibiotic; Synthesis; Cephalexin; Ampicillin

1. Introduction

β-Lactam antibiotics, and in particular cephalexin and ampicillin, are among the most widely used in therapy as they are suitable for a wide spectrum of bacterial infections and have a good level of activity and tolerability. For example, nowadays cephalexin has, at worldwide level, a consumption that reaches 3000 t per year.

Currently the biocatalytic production of semisynthetic β-lactam antibiotics exploiting penicillin G amidase catalysed synthesis has become a practical alternative to the conventional chemical synthesis methods [1]. Biocatalysed synthesis has the unquestionable advantage of reducing the condensation reaction to a single step that can be carried out in aqueous environment and at temperature ranging from 0 to 20 °C, conditions that represent an improvement compared to the chemical processes where, as an example, the Dane anhydride formation proceeds at −60 °C.

Specificity of penicillin G amidase for the acyl donor is not only limited to phenylacetic acid but includes also D-phenylglycine and D-hydroxyphenylglycine, so that the enzyme can be exploited for the biocatalytic synthesis of β-lactams antibiotics as ampicillin, amoxicillin, cephalexin, cephadroxil and cephachlor.

An efficient synthesis of β-lactam antibiotics with D-phenylglycine, or its derivatives, as side chain, can be accomplished only by using a kinetically controlled approach via acyl group transfer from an activated side chain donor. D-Phenylglycine can be used as ester, usually methyl (PGM) or ethyl (PGE), or as amide. In fact, the enzymatic synthesis cannot be performed using directly the D-phenylglycine as acyl donor, due to the totally unfavoured thermodynamic equilibrium of the reaction [2] and to the presence of a positive charge of the zwitterionic D-phenylglycine that prevents the binding in the active site of the enzyme [3].

* Corresponding author. Tel.: +39 0405583110; fax: +39 04052572.
E-mail address: gardossi@units.it (L. Gardossi).

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.01.029

In the kinetic approach water acts as competing nucleophile so that the acyl donor is partially hydrolysed during the enzymatic reaction and at the end of the reaction the overall hydrolysis exceeds the antibiotic synthesis. Of course, the product, whose accumulation shows a clear-cut maximum, can be recovered at its maximum concentration but the competing hydrolysis of the acyl donor represents one of the major drawbacks in the industrial application of enzymatic antibiotic synthesis.

Many approaches have been reported to improve the efficiency of the synthetic reaction that go from pH and temperature optimisation [4–7], use of co-solvents [8–10], regulation of the thermodynamic water activity (a_w) of the system [9], modification of the catalytic properties of the enzyme through mutagenesis [11,12] or immobilisation [13,14] and also control of reagents concentration [15,16].

This latter aspect has been extensively studied demonstrating that highly concentrated or supersaturated solutions of activated acyl donors and β-lactam nuclei have a beneficial impact on both the synthetic efficiency (a lower acyl donor/nucleus ratio can be used) and on the productivity (improving the final yield of antibiotic) [17–20].

Here we present an innovative highly condensed system for the enzymatic synthesis of ampicillin and cephalexin that is formed by substrates partially dissolved in the liquid reactant. Neither liquid aqueous phase nor organic solvents were used for improving the dispersion of the reagents. The absence of an apparent aqueous phase in the reaction mixture reduces the incidence of the hydrolytic reaction. A simple method for the recovery of the reagents – D-phenylglycine and the β-lactam nucleus – and of the final product of the reaction by means of selective precipitations with organic solvents and pH shift is also described [21].

Dissolved enzymes are commonly used in academic studies, but in industrial biocatalytic processes, for economic reasons, immobilised enzymatic preparations are usually employed [22]. In the enzymatic synthesis of β-lactam antibiotics, the use of immobilised biocatalysts introduces mass transfer limitations, which have to be considered in process development. Therefore, in this study a commercial preparation of immobilised penicillin G amidase (PGA-450 from Roche) was used.

2. Experimental

2.1. Materials

PGA-450 was a generous gift of Roche. It consists of penicillin G amidase from *Escherichia coli* covalently immobilised on a polymer the chemical nature of which is not disclosed by the producer. PGA-450 has a water content of 62.3% and it was partially dehydrated before use with the aid of Celite R-640 (Fluka), according to reported procedures [23], to reach a final water content of 27% (specific activity of 453 U/g_{dry}). Batches were prepared on a gram scale and there was no decrease in activity over at least four months. As required, enzymatic samples were withdrawn and the volatile organic solvent used for the storage was removed from the enzymatic sample at room temperature and atmospheric pressure without causing any detrimental effect to the catalyst.

All reagents were purchased from Sigma–Aldrich and were used without any further purification.

The dipeptide phenylglycine–phenylglycine methyl ester (PG–PGM) that was used as HPLC standard was kindly supplied by Dr. Luuk M. van Langen (Delft University of Technology, The Netherlands).

2.2. Preparation of PGM free base

D-Phenylglycine methyl ester hydrochloride PGM·HCl (20 mmol) was suspended in 20 ml of dry dichloromethane together with 30 mmol of $Na_2CO_3 \cdot 10H_2O$. After 15 min of magnetic stirring the organic solution was filtered and dried over anhydrous sodium sulphate. Finally, the solvent was removed under reduced pressure to obtain the free D-phenylglycine methyl ester as a liquid with a yield of 70%. Products were characterised by ^{1}H NMR and compared to reported data [24].

2.3. Attempted synthesis of dipeptide PG–PGM in organic solvent

One hundred milligrams of dehydrated PGA-450 (33 U) was added to 2 mmol of D-phenylglycine methyl ester (PGM) in 1 ml of dry toluene and the reaction was incubated at 30 °C in an orbital shaker.

The reaction was monitored both by RP-HPLC and by thin layer chromatography (TLC). No product corresponding to PG–PGM was observed after one-week reaction.

2.4. Synthesis of ampicillin and cephalexin

6-Aminopenicillanic acid (6-APA) or 7-aminodesacetoxycephalosporanic acid (7-ADCA) was suspended in the liquid PGM at room temperature. The reactions were carried out in a 20 ml glass test tube having a cap with a Teflon septum and they were started by adding PGA-450 and by mixing the reaction components using a spatula. The reaction system was maintained at 4 °C in a thermostatted bath without stirring. The composition of the various synthetic mixtures at the maximum conversion (ampicillin, AMP; cephalexin, CEX; phenylglycine, PG; dipeptide, PG–PGM) was quantified by RP-HPLC as indicated in Tables 1 and 2.

2.5. Workup of highly condensed systems and recovery of products

At the maximum of conversion the reaction was stopped by suspending the reaction mixture in 100 ml of dichloromethane and by removing the enzyme using a porosity 0 glass filtration system (step A, see Scheme 2). The PGM dissolved in dichloromethane (DCM) was then separated from the undissolved β-lactam nucleus and precipitated products by a second selective filtration using a porosity 4 glass filtration system (step B, see Scheme 2). The solid residue was repeat-