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

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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].

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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-

Table 1
Composition of the highly condensed systems at the beginning of the reaction and at the maximum AMP conversion

Entry	Reagents (mmol)				Products (mmol) (conversion (%))[a]			Composition
	PGA-450 (U)[b]	PGM	6-APA	PGM/6-APA	AMP	PG	PG–PGM	AMP_{max}/PG
1	261	2.126	0.458	4.6	0.055 (12)	0.128 (6)	0.021 (1)	0.43
2	52	2.126	0.458	4.6	0.101 (22)	0.106 (5)	–[c]	0.95
3	261	1.060	0.458	2.3	0.073 (16)	0.848 (80)	–[c]	0.09
4	52	1.060	0.458	2.3	0.064 (14)	0.127 (12)	0.011 (1)	0.50
5	261	2.126	1.143	1.9	0.069 (6)	0.064 (3)	–[c]	1.08
6	106	1.575	0.801	2.0	0.104 (13)	0.110 (7)	0.016 (1)	0.94
7	52	2.126	1.143	1.9	0.171 (15)	0.128 (6)	0.021 (1)	1.34
8	52	1.101	1.143	1.0	0.366 (32)	0.371 (35)	0.053 (5)	0.99

[a] Conversion of AMP is calculated as millimoles of ampicillin over the initial millimoles of 6-APA, conversions of PG and PG–PGM are calculated over the initial millimoles of PGM.
[b] PGA-450 (initial water content of 62.3%) was dehydrated to a final water content of 27% (specific activity of 453 U/g_{dry}).
[c] No formation of dipeptide was detected.

edly washed with DCM. The organic phases were concentrated at reduced pressure obtaining a yellow oily residue that was analysed by RP-HPLC and characterised by ^1H NMR. After the washing step with DCM, the solid residue was suspended in 20 ml of ultrapure water, subsequently acidified to pH 1.5 with concentrated hydrochloric acid to obtain a precipitate of the β-lactam nucleus that was recovered by filtration at reduced pressure. The remaining solution (slightly yellow) was adjusted to pH 5.0 (corresponding to the isoelectric point of the β-lactam antibiotics) with a 28% NH_4OH solution. Acetonitrile (∼40 ml) was added to the solution until formation of a white precipitate, composed by AMP (or CEX) and PG.

2.6. Analysis by RP-HPLC

Analyses with RP-HPLC were carried out by dissolving small portions of the heterogeneous reaction mixture in 2.5 ml of ultrapure water acidified to pH 1.5 with concentrated hydrochloric acid and diluted with 2.5 ml of ethanol (0.01% of trifluoroacetic acid). The biocatalyst was removed by centrifugation. A mobile phase composed of acetonitrile, water and 0.1% trifluoroacetic acid was used according to a gradient (organic phase increased from 15 to 50% in 10 min).

2.7. Spectroscopic characterisation

2.7.1. Ampicillin

^1H NMR (200 MHz, D_2O) δ (ppm): 1.42 (s, 3H, C**H**$_3$), 1.54 (s, 3H, C**H**$_3$), 4.12 (s, 1H, N–C**H**–COOH), 4.73 (s, 1H, C**H**–NH_2), 5.40 (dd, 1H, NH–CH–C**H**–S), 5.48 (dd, 1H, NH–C**H**–CH–S), 7.38 (m, 5H, C_6**H**$_5$).

ES–MS *m/z*: 350 (M^+).

2.7.2. Cephalexin

^1H NMR (200 MHz, D_2O) δ (ppm): 2.94 (s, 3H, C**H**$_3$), 3.14 (d, 1H, S–C**H**H), 3.24 (d, 1H, S–CH**H**), 4.79 (dd, 1H, NH–CH–C**H**–S), 4.94 (s, 1H, C**H**–NH_2), 5.38 (dd, 1H, NH–C**H**–CH–S), 7.24 (m, 5H, C_6**H**$_5$).

ES–MS *m/z*: 348 (M^+).

3. Results and discussion

3.1. State of the art

From a kinetic study by Janssen and co-workers it emerged that, for the synthesis of ampicillin, a saturation of the ratio of the maximum rates of synthesis over hydrolysis (Vs/Vh) was observed at increasing 6-APA concentrations, indicating that

Table 2
Composition of the highly condensed systems at the beginning of the reaction and at the maximum CEX conversion

Entry	Reagents (mmol)				Products (mmol) (conversion (%))[a]			Composition
	PGA-450 (U)[b]	PGM	7-ADCA	PGM/7-ADCA	CEX	PG	PG–PGM	CEX_{max}/PG
9	261	2.126	0.463	4.6	0.083 (18)	0.829 (39)	0.013 (0.6)	0.10
10	52	2.126	0.463	4.6	0.090 (20)	0.291 (14)	0.017 (0.8)	0.31
11	261	1.060	0.463	2.3	0.088 (19)	0.146 (14)	0.008 (0.8)	0.60
12	52	1.060	0.463	2.3	0.128 (28)	0.134 (13)	0.014 (1.3)	0.96
13	522	10.601	4.625	2.3	1.388 (30)	0.954 (9)	–[c]	1.45

[a] Conversion of CEX is calculated as millimoles of cephalexin over the initial millimoles of 7-ADCA, conversions of PG and PG–PGM are calculated over the initial millimoles of PGM.
[b] PGA-450 (initial water content of 62.3%) was dehydrated to a final water content of 27% (specific activity of 453 U/g_{dry}).
[c] No formation of dipeptide was detected.

when 6-APA binds the acyl-enzyme to form the tetrahedral intermediate, water is not excluded from the active site and it is still able to deacylate the enzyme [11]. Competition between water and 6-APA for the acyl-enzyme is strongly dependent on the type of acyl group and the nucleophilic attack of 6-APA is more favoured in the case of the phenylacetylated enzyme as compared to the phenylglycinated enzyme.

When 7-ADCA is used as nucleophile, instead of 6-APA, with phenylglycine as acyl donor, a higher Vs/Vh is observed at all concentrations of 7-ADCA, finding a linear dependence of Vs/Vh on the concentration of 7-ADCA. The explanation of such behaviour lays on the different binding interactions of the two nuclei in the active site of the enzyme. Since Vs/Vh ratio in a synthesis reaction can be improved by using high concentrations of β-lactam nuclei this synthetic process can be designed working in saturated systems.

Švedas and co-workers have reported some interesting studies on the synthesis, catalysed by native penicillin G amidase, of ampicillin and cephalexin in "aqueous solution–precipitate" systems and highly condensed aqueous systems, studying the effect of high substrate concentration and supersaturation [17,20]. They reported an increased efficiency of the enzymatic acyl transfer upon increasing the initial 6-APA concentration to saturation. Working with a suspension of 6-APA, an optimal acyl transfer efficiency can be guaranteed for a longer reaction time and the entire amount of a nucleophile, present in the homogeneous supersaturated solution over its thermodynamic saturation level, can take part in enzymatic synthesis [25–28].

Starting from these considerations, in the present work the aim was to verify whether it is possible to operate at the limit of the experimental conditions, namely in the absence of an aqueous liquid phase. However, the acyl transfer cannot be performed in diluted organic solvents due to the unreactivity of the zwitterionic β-lactam nucleus. Moreover, we verified that phenylglycine and its derivatives are not accepted as acyl donors in organic solvents (see Section 2.3). However, we have previously demonstrated that in some cases enzymes suspended in reaction systems consisting mainly of solid reactants dispersed in a micro-liquid phase formed by the other reactant show different substrate selectivity due to the high substrate concentration [29].

3.2. The highly condensed system

To solve the problem of the hydrolysis of both activated acyl donor and product a process where any liquid aqueous phase in the reaction systems is avoided would be the ideal answer. Nonetheless, this solution is difficult to obtain in practice due to the need to solubilize, at least partially, the β-lactam nucleus and the acylating agent and due to the fact that PGA requires water to maintain its activity. It has been demonstrated that glutaryl acylase, a hydrolase whose catalytic mechanism is similar to PGA, can be used in reaction systems consisting mainly of solid reactants suspended in a micro-liquid phase formed by the other reactant [29]. In such a system the water present in the reaction mixtures was supplied by controlling the residual hydration of the immobilised biocatalyst.

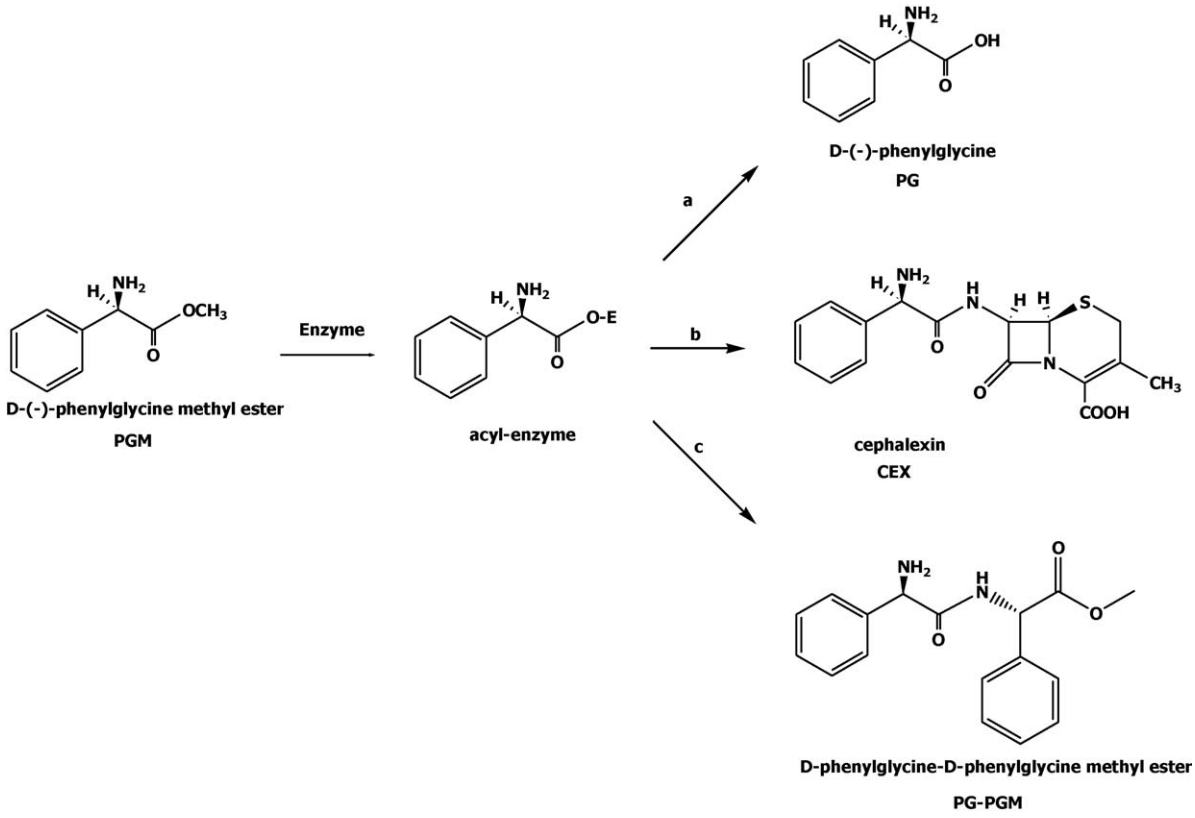
Penicillin G amidase, to display its activity, requires a minimum amount of water that is guaranteed by operating at controlled water activity (a_w comprised between 0.4 and 0.8) [30,31] and by operating in these conditions the degree of hydrolysis of products and reagents is limited [32–36]. In our experiments the optimal hydration of the PGA covalently immobilised on 300 μm diameter polymeric beads (PGA-450 from Roche) was guaranteed by the water retained by the polymer. The enzymatic preparation was previously dehydrated to achieve an optimal hydration by using a simple and mild process already reported [23]. Following the same approach that was used for glutaryl acylase [29], reactions were started by adding the immobilised enzyme to a mixture formed by the β-lactam nucleus partially dissolved in the liquid phenylglycine methyl ester. It must be underlined that phenylglycine methyl ester is a liquid only at temperatures above 20–25 °C. We observed that PGM solidifies at temperatures between 4 and 20 °C only when it is highly pure and minor traces of impurities are sufficient to inhibit the transition of state. Therefore, once the β-lactam nucleus was suspended at room temperature in the liquid PGM it was possible to carry out the reaction at 4 °C.

In order to evaluate the effect of viscosity and enzyme immobilisation on mass diffusion, the system was not stirred.

The composition of the reactions and the molar ratios of PGM on 6-APA are reported in Table 1.

Initial experiment (entry 1) was carried out by using excesses of PGM over 6-APA (4.6 excess of PGM) and by using larger amounts of enzyme. At maximum product conversion (12%, determined by HPLC), hydrolysis of PGM to PG was observed (6%, referred to the initial PGM). When the same experiment was performed in the presence of a lower amount of biocatalyst (entry 2) an improvement of the maximum conversion was observed (22% maximum conversion), whereas the hydrolysis of PGM was maintained to similar values (5%). This translates into an improvement in the ratio of AMP_{max} on formed PG (from 0.43 to 0.95), that is a relevant parameter in the industrial workup for the recovery of the final antibiotics. As it has been widely reported for the kinetically controlled synthesis of β-lactam antibiotics, by decreasing the excess of the activated reagent (PGM) over the β-lactam nucleus (6-APA) the maximum conversion of AMP was expected to decrease. Our results show that, surprisingly, the decrease of the PGM/6-APA ratio (from 4.6 to 1.9, entries 2 and 7) exerts a beneficial effect on AMP_{max}/PG (from 0.95 to 1.34).

The negative effect of the biocatalyst amount is observable also at low PGM/6-APA ratios (see entries 5 and 7) and this can be ascribed to the different amount of water supplied to the system by the immobilised enzyme in the two cases (49 mmol of water when using 261 U against 10 mmol when using 52 U). Most probably, a higher amount of immobilised biocatalyst favours hydrolytic reactions and at the same time also increases mass transfer limitations. We then attempted the synthesis of AMP in the presence of equimolar amounts of 6-APA and PGM (entry 8): despite an increased viscosity of the reaction system due to the precipitation of the product the maximum conversion significantly increased up to 35% with a ratio AMP_{max}/PG of 0.99.

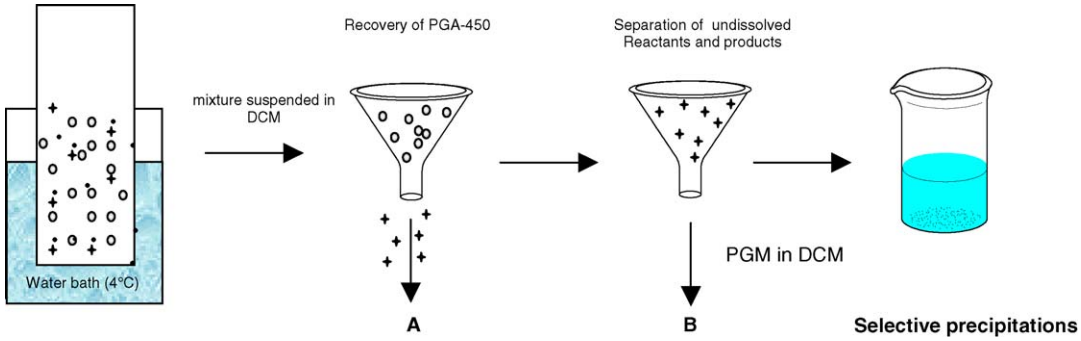


Scheme 1. Formation of products in the reaction catalysed by PGA-450 at 4 °C in the presence of 7-ADCA and PGM ((a) H_2O, (b) 7-ADCA and (c) PGM).

The same process was applied to the synthesis of cephalexin, as reported in Table 2. Also in this case beneficial effects were obtained by reducing both the immobilised biocatalyst (compare entries 9 and 10 or 11 and 12) and the excess of PGM over 7-ADCA (from 4.6 to 2.3). In both cases an increased conversion and a reduced side hydrolysis of PGM was observed. Entry 13 in Table 2 indicates that it is possible to perform reactions also in a larger scale.

Results reported in Tables 1 and 2 show that in almost all cases the formation of the phenylglycine–phenylglycine methyl ester dipeptide was observed, thus meaning that PGM was used by the enzyme at the same time as acyl donor and as nucleophile.

It has been widely reported that enzymes working in organic solvent or, in general, in non-conventional media show altered substrate and/or enantioselectivity. As a matter of fact, PGA is highly enantioselective towards L-phenylglycine so that D-PGM is not accepted as nucleophile in aqueous media (*E* values higher than 1000 have been reported for the hydrolysis of D,L-*N*-phenylacetyl-phenylglycine) [25], whereas in organic solvent PGA shows a "relaxed enantioselectivity" that



Scheme 2. Process for the recovery of β-lactam antibiotics at the maximum conversion. The reaction mixture was composed of PGA-450 (grey circles), the mainly undissolved β-lactam nucleus and products (black stars) and liquid PGM .The recovery of products and reagents proceeded through the suspension of the reaction mixture in DCM followed by a first selective filtration (A) using a porosity 0 glass filtration system to separate PGA-450, then by a second filtration (B) using a porosity 4 glass filtration system to separate the undissolved reagents and products (PG, 7-ADCA or 6-APA, products and PG–PGM) from the PGM dissolved in DCM. The remaining reagents and products were recovered through selective precipitations at different pH (see Section 2).

has been exploited for the acylation of many D-amino acids, as D-phenylglycine [37]. Similar results were also found for glutaryl acylase that showed different substrate selectivity passing from a diluted solvent system to a saturated system [29].

The experimental data show that PGA, in highly condensed systems, accepts D-PGM as nucleophile, and that D-PGM can compete with water and the β-lactam nucleus in the deacylation of the acyl-enzyme (Scheme 1), so that up to 5% of dipeptide formation (PG–PGM) was observed.

3.3. The workup of the highly condensed system

The workup of the reaction was performed as described in Scheme 2.

At the maximum of conversion the unreacted reagents, the immobilised enzyme and the reaction product were all recovered and analysed. The enzyme and the unreacted PGM were recovered by selective filtrations, whereas the reaction products (AMP or CEX) and the unreacted β-lactam nucleus (6-APA or 7-ADCA) were recovered by selective precipitations (pH shift).

4. Conclusions

We have studied the behaviour of PGA in the enzymatic synthesis of AMP and CEX by operating in systems where no aqueous liquid phase was present. The water present in these highly condensed systems with undissolved substrates is limited to the water hydrating the support on which the enzyme is covalently immobilised.

Obtained data demonstrate that, despite the high viscosity of the system, an immobilised penicillin G amidase can be used even in the absence of a stirring system.

PGA is active also in this highly condensed systems with substrates mainly undissolved but the enzyme shows a selectivity that is different from that displayed either in organic solvent or in aqueous medium.

The highly condensed system was applied to the synthesis of both ampicillin and cephalexin, and can be performed also using equimolar amounts of reagents. A simple workup procedure enables the recovery of the unreacted reagents and the immobilised biocatalyst.

This study is intended as a starting point for exploring the possibility of developing synthetic processes in such non-conventional conditions so reducing competing hydrolytic reactions. Since diffusion limitations are the main factor affecting the reaction efficiency, appropriate reactor configuration and enzyme formulation are under investigation.

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