Penicillin Acylase Catalysed Synthesis of Ampicillin in Hydrophilic Organic Solvents

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Abstract: Penicillin acylase (EC 3.5.1.11) from *Alcaligenes faecalis*, immobilised as a cross-linked enzyme aggregate (CLEA), catalysed the synthesis of ampicillin in water-miscible organic solvents at low water concentrations. Below 4% water (v/v) no reaction was observed, showing the crucial role of water in maintaining the activity of penicillin acylase. The initial value of S/H was strongly affected by the nature of the solvent, but the effect of the water content was slight in the studied range of 4 to 15%. A reaction in acetonitrile containing 8% water afforded ampicillin in up to 86% yield.

Keywords: ampicillin synthesis; cross-linked enzyme aggregate; enzyme catalysis; immobilisation; organic solvents; penicillin acylase

Introduction

The industrial synthesis of penicillins and cephalosporins is undergoing a transition from predominantly chemistry-based procedures to biological and biocatalytic ones.[1,2] Thus, the chemical hydrolysis of penicillin G into the key building block 6-aminopenicillanic acid (6-APA) has largely been replaced by an enzymatic procedure,[3] which utilises the unique chemoselectivity of penicillin acylase (EC 3.5.1.11). In contrast, the industrial transformation of the β-lactam intermediates 6-APA and 7-deacetoxycephalosporanic acid into semisynthetic penicillins and cephalosporins still largely involves chemical procedures, although there is a definite trend towards their replacement by cleaner biocatalytic procedures.[4]

The enzymatic synthesis of a β-lactam antibiotic such as ampicillin is not trivial, since a simple condensation of D-phenylglycine (D-PG) and 6-APA is thermodynamically unfavourable.[5,6] In consequence, all viable synthesis schemes involve an activated side-chain donor, such as D-phenylglycine amide (D-PGA), which acylates 6-APA in the presence of penicillin acylase in a kinetically controlled reaction;[7] water is the reaction medium of choice. A major problem of such schemes is the competing irreversible hydrolysis of the acyl donor as well as the product, leading to unreactive D-PG (see Figure 1).

One approach to reducing the competing hydrolysis is, obviously, to replace the water by an organic solvent. Improved yields of ampicillin were indeed obtained when the reaction was performed in glycol-water (50:50).[8] Removing all of the water, as is routinely done in lipase-catalysed amine acylation,[9] would obviate the hydrolysis problem entirely. However, such reaction conditions are not tolerated very well by penicillin acylase, although it still has acyl transfer activity in toluene at reduced water activity ($a_W = 0.5$).[10] We have recently demonstrated the facile immobilization of *E. coli* penicillin acylase as a cross-linked enzyme aggregate (CLEA), which mediated the synthesis of ampicillin in various organic solvents in the presence of 5% (*v*/*v*) of water.[11]

In our continued investigations of the enzymatic synthesis of ampicillin in organic media at low water activity we turned our attention to the relatively unknown penicillin acylase from *Alcaligenes faecalis*[12,13] on account of its stability[13] and pH tolerance.[14] We now report the effects of the nature of the solvent and the water content on the course of the enzymatic coupling of D-PGA and 6-APA using a CLEA of *A. faecalis* penicillin acylase. Our goal was to increase the synthesis/hydrolysis ratio by reducing the amount of water in the system, thereby providing a more economically viable process.

Results and Discussion

We investigated the synthesis of ampicillin from D-PGA and 6-APA in the presence of an *A. faecalis* pencillin acylase CLEA[15] in three water-miscible organic solvents: *tert*-butyl alcohol, 1,2-dimethoxyethane (DME) and acetonitrile (ACN). *tert*-Butyl is a standard solvent

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for the lipase-mediated acylation of strongly hydrophilic reactants, such as carbohydrates; DME and ACN were chosen because they gave the best results in the synthesis of ampicillin in the presence of a CLEA of *E. coli* penicillin acylase.[11] The effects of water were measured by adding various amounts, ranging from 2 – 15% (v/v); estimated values of the water activity (a_w) are included in Table 1.

To assess the kinetic performance of the biocatalyst, the initial values of the synthesis and hydrolysis rate were measured (see Table 1). From these, the initial synthesis/hydrolysis ratio (S/H, the molar ratio of ampicillin and D-PG formed), which is a convenient yardstick for measuring the efficiency of a kinetically controlled process,[4] was calculated.

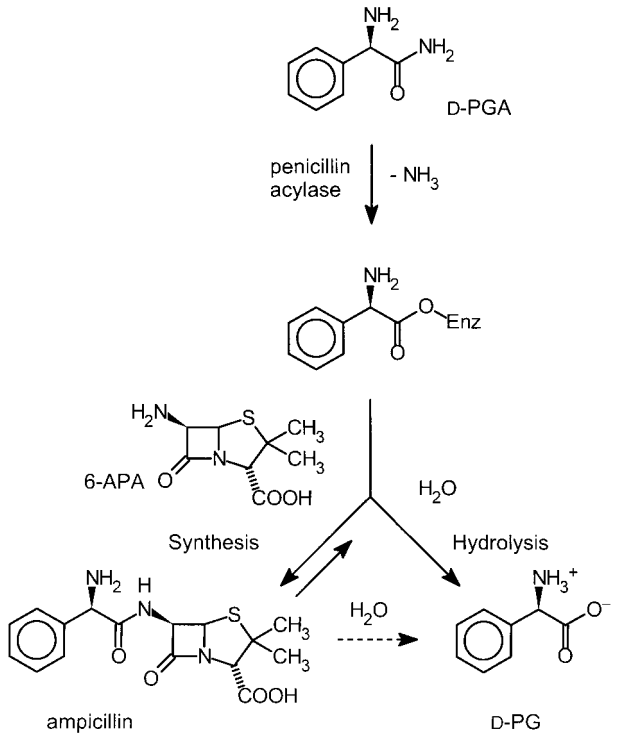

Figure 1. Kinetically controlled enzymatic synthesis of ampicillin.

Ampicillin synthesis took place in all of these solvents if the water content was at least 4% (v/v), but no reaction took place at 2% water. The presence of water is clearly crucial for the catalytic action of *A. faecalis* penicillin acylase as was also observed in the case of *E. coli* penicillin acylase,[10b] but, contrary to expectations,[16] there is no obvious relationship with the water activity. In fact, the minimum water concentration required for activity is very similar in the solvents used, although the corresponding water activities diverge widely. We can only speculate on the cause of these phenomena. The loss of the bridging water molecule that activates the catalytic serine[17] could possibly be involved. We also note that, even in 80% (v/v) methanol, water does not mix at a molecular level but mainly is present as strings or clusters of molecules.[18] Possibly the required 4% water corresponds with a transition from oligomeric water into a molecular dispersion in these media.

The nature of the solvent markedly influenced the rate of ampicillin synthesis. At $\leq$ 8% water the synthesis rate increased in the order *tert*-butyl alcohol < DME < ACN. The influence of the water content on the rate was dependent on the solvent used. The synthesis and hydrolysis rates in *tert*-butyl alcohol increased strongly when the water content was increased from 4 to 15%. In contrast, the reaction rates in DME medium were hardly affected by the water concentration, whereas in ACN the synthesis rate passed through a maximum at 8% water.

The nature of the solvent strongly affected the initial value of S/H, which increased from 0.6 in *tert*-butyl alcohol to 2 in DME and 4 in ACN *tert*-butyl alcohol.[19] We have previously observed a similar effect of the solvent on S/H with the penicillin acylase from *E. coli*.[11,21] There is no obvious relationship with any molecular property of these solvents, such as logP[22] or the polarity index[23] (see Table 2). Only the relatively

Table 1. Effect of the medium on the enzymatic synthesis of ampicillin[a]

	tert-Butyl alcohol				DME				ACN			
H_2O [vol %]	a_w	v_{synth} [μmolU^{-1}h^{-1}]	v_{hydrol} [μmolU^{-1}h^{-1}]	S/H	a_w	v_{synth} [μmolU^{-1}h^{-1}]	v_{hydrol} [μmolU^{-1}h^{-1}]	S/H	a_w	v_{synth} [μmolU^{-1}h^{-1}]	v_{hydrol} [μmolU^{-1}h^{-1}]	S/H
2	0.3	0.00	0.00	–	0.1	0.00	0.00	–	0.4	0.00	0.00	–
4	0.5	0.09	0.23	0.4	0.2	0.37	0.23	1.6	0.7	0.64	0.23	2.7
6	0.6	0.09	0.23	0.4	0.3	0.60	0.28	2.1	0.8	0.71	0.17	4.2
8	0.7	0.23	0.38	0.6	0.4	0.66	0.31	2.1	0.9	0.87	0.24	3.6
10	0.8	0.47	0.85	0.5	0.5	0.60	0.30	2.0	0.9	0.40	0.10	4.0
15	0.9	0.70	1.40	0.5	0.6	0.60	0.30	2.0	1.0	0.30	0.075	4.0

[a] Initial rates. Water activities have been estimated using the UNIFAC computer programme and are not corrected for water uptake by the reagents. Reaction conditions: 150 mM D-PGA, 50 mM 6-APA and 130 U immobilised penicillin acylase in 20 mL solvent at 0 °C.

high dielectric constant of ACN[24] could be significant in this respect, as it affects the balancing of coulombic and hydrophobic interactions in the enzyme. In contrast, the influence of the water content on S/H was slight. A lower water content would be expected to favour synthesis over hydrolysis but in fact S/H even tended to decline at the lowest water content. A very similar observation has been made in the comparable kinetically controlled alcoholysis of disaccharides in the presence of glycosidases.[25] It also is relevant to note that *Candida antarctica* lipase, for example, avidly reacts with even trace amounts of water, which may cause an appreciable hydrolytic side-reaction even under nearly anhydrous conditions.[26]

We next considered the issue of the limited solubility of the nucleophile 6-APA in the reaction media. In a saturated solution the thermodynamic activity of 6-APA will be at a maximum value, equal to that in the crystalline phase. If the 6-APA is completely dissolved its thermodynamic activity will be lower, which could result in a lower S/H. We found that the initial 6-APA concentration, 50 mM, exceeded its solubility in DME or ACN containing $<$ 8% water, whereas at higher water concentrations all of the 6-APA was dissolved. No corresponding effect on v_{syn} or S/H became apparent, however (see Table 1).

Table 2. Some properties of the solvents.

Solvent	ε_0[a]	Log P[b]	E_T^N[c]
tert-Butyl alcohol	12.47	0.35	0.39
1,2-Dimethoxyethane	7.20	− 0.21	0.16
Acetonitrile	35.94	− 0.34	0.46

[a] At 25 °C, data taken from ref.[24]
[b] See ref.[22]
[c] Normalised polarity index (water = 1.00, tetramethylsilane = 0.00) according to Reichardt.[23]

We subsequently examined the synthetic potential of our reaction system by allowing the reaction to proceed to maximum conversion. These experiments were performed using DME and ACN, which had given the best results at lower conversions. A maximum yield of 86% was obtained in ACN containing 8% water; the yield was 82% in DME containing 4% water (see Figure 2).

Analogous to what is observed in aqueous systems, the value of S/H declined as the reaction proceeded.[27] This latter effect is ascribed to the accumulation of ampicillin in the reaction medium, causing an increasing competition of ampicillin with D-PGA for the active site of penicillin acylase. Moreover, the concentration of the nucleophile (6-APA) declines in the course of the reaction, depressing the synthesis rate versus the primary hydrolysis rate (see Figure 1).

A slow net hydrolysis of ampicillin took place after its maximum concentration had been reached, in particular when the reaction was performed in ACN. This is characteristically observed in a kinetically controlled synthesis and shows that the biocatalyst is still active after 300 min in ACN medium. In DME medium the hydrolysis of ampicillin, as well as that of D-PGA, was much less pronounced in the final stage of the reaction. Inactivation of the biocatalyst was not involved as we found, in an independent experiment, that the enzymatic hydrolysis rate of D-PGA in DME-water (96:4) in the absence of 6-APA was comparable to the low rate found in the synthetic experiment after reaching maximum ampicillin concentration (data not shown). It would seem that, in DME medium, hydrolysis of the acyl-enzyme complex requires the binding of 6-APA in the active site, which is a well-recognised kinetic pathway.[28] Consequently, the hydrolysis is slow at low 6-APA concentration. A very similar effect was observed in the enzymatic synthesis of ampicillin in 50% aqueous 1,2-propanediol or 1,3-butanediol.[8] We also showed that the penicillin acylase could be recycled: more than 80% of the initial activity was recovered upon rehydration of the catalyst (see Table 4 in the Experimental Section).

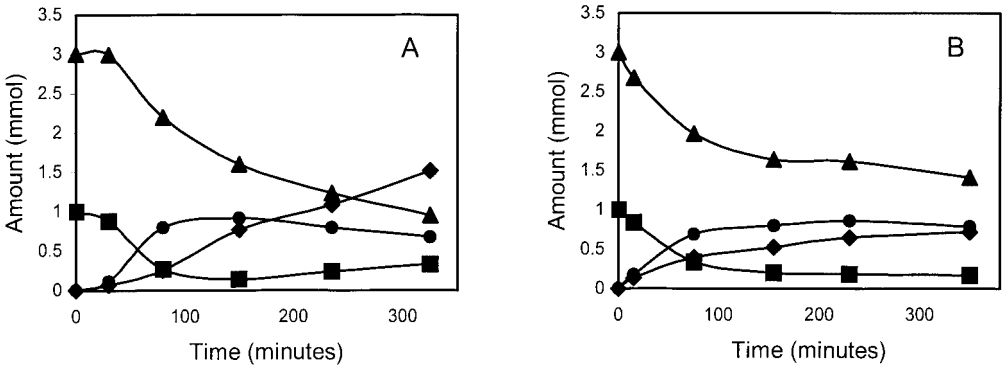

Figure 2. Penicillin acylase catalysed synthesis of ampicillin in organic solvents. A: ACN-water (92:8, *v*/*v*); B: DME-water (96:4, *v*/*v*). Reaction conditions: 800 U penicillin acylase, further as described below Table 1. Legend: D-PGA: ▲; 6-APA: ■; ampicillin: ●; D-PG: ◆.

Table 3. The catalytic efficiency of penicillin acylase in several systems

Reaction	Source	Formulation	Solvent	Temp. [°C]	pH	Turnover rate [s^{-1}]	Ref.
Pen G hydrolysis	*A. faecalis*	native	water	25	7.5	54	[14]
Pen G hydrolysis	*E. coli*	native	water	25	7.5	50	[14]
Ampicillin synthesis	*E. coli*	Assemblase[a]	water	10	6.3	0.6	[29]
Ampicillin synthesis	*E. coli*	CLEA	water	10	7	2.3	
Ampicillin synthesis	*A. faecalis*	CLEA	ACN-water (92:8)	0	–	1	[30]
Phenylacetyl transfer	*E. coli*	on celite rods	toluene-water (a_w 0.48)	30	–	0.3	[10c]

[a] A covalently bound preparation of penicillin acylase on gelatin-chitosan.[15]

As regards the biocatalyst turnover rate, it is commonly observed that enzymes exhibit low activity in organic media. To put this issue into perspective, we have compared (see Table 3) the turnover rate of the penicillin acylases from *A. faecalis* and *E. coli* in a number of reactions, each at optimum conditions. Thus, the activity of the penicillin acylases in the hydrolysis of penicillin G indeed is much higher than that of the *A. faecalis* pencillin acylase CLEA in the synthesis of ampicillin from D-PGA in an organic solvent. However, the latter is comparable with that in an aqueous medium using a carrier-bound *E. coli* enzyme,[29] but a CLEA of the same enzyme was four times as active.[30] We attribute this latter effect mainly to diffusion limitations in the carrier. Finally, a celite-immobilised penicillin acylase in low-water toluene medium was much less active than our CLEA preparation in ACN-water (92:8). We tentatively conclude that the catalytic efficiency of a penicillin acylase CLEA in the synthesis of ampicillin in organic media is competitive with that in an aqueous medium.

Conclusion

Penicillin acylase from *A. faecalis* is active in water-miscible solvents at low water content. The enzyme catalysed the synthesis of ampicillin in these solvents at a rate that is comparable with the same reaction in water. Comparison of several solvents and water contents showed that the ratio between ampicillin synthesis and formation of the hydrolytic side product D-PG varied strongly with the nature of the solvent but the water content had almost no effect on this ratio. The discovery that a suitable immobilised penicillin acylase is active in low-water organic media provides possibilities for new applications of this readily available enzyme.

Experimental Section

Chemicals

Penicillin acylase solution from *A. faecalis* and 6-aminopenicillanic acid were obtained from (DSM Anti-Infectives Delft, The Netherlands). D-(−)-Phenylglycine amide was provided by DSM (Geleen, The Netherlands). The cross-linking agents dimethyl adipimidate dihydrochloride and 25% glutaraldehyde solution in water were obtained from Aldrich. A suspension of *A. faecalis* penicillin acylase CLEA (483 U mL^{-1}) was prepared according to a published procedure.[15]

Penicillin Acylase Assay

The activity of penicillin acylase was assayed in the hydrolysis of a 2% solution of penicillin G potassium salt in 0.1 M phosphate buffer at 34 °C and pH 8.0, which was maintained by automatic titration with NaOH. One unit (U) of penicillin acylase will liberate one μmol of phenylacetic acid per min.

Activity Recovery of the Biocatalyst

Table 4. Recovery of the hydrolytic activity of *A. faecalis* penicillin acylase CLEA after 2 h in the reaction mixture.

Reaction medium	Recovered activity[a] [%]
tert-Butyl alcohol	93
DME	82
ACN	78

[a] In the standard hydrolytic assay after rehydration in 0.1 M phosphate buffer pH 7.

Enzymatic Ampicillin Synthesis

To 20 mL of an organic solvent-water mixture 6-aminopenicillanic acid (6-APA, 1 mmol), D-(−)-phenylglycine amide (3 mmol) (PGA) and of penicillin acylase CLEA (130 U) were added. The reaction mixture was stirred in a closed reaction vessel at 0 °C.

Analysis

Aliquots of the reaction mixture were taken during the reaction, dissolved in water-ethanol (3:1, v/v) and acidified with phosphoric acid; 1,3-dimethoxybenzene was added as internal standard. Samples were analysed by HPLC using a Waters M6000 pump, a 5 μ, 3 × 150 mm Nucleosil C18 column and a Shimadzu SPD 6A UV detector at 215 nm. The eluent

was prepared by adjusting a 0.68 g L^{-1} solution of KH_2PO_4 in acetonitrile-water (30:70, v/v) containing 0.68 g L^{-1} sodium dodecyl sulphate to pH 3.0 with phosphoric acid. The flow rate was 1.0 mL min^{-1}.

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A. faecalis enzyme: initial S/H = 0.96, maximum conversion 28%;
E. coli enzyme: initial S/H = 2.7, maximum conversion 43%.

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