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Ampicillin Synthesis Using a Two-Enzyme Cascade with Both α -Amino Ester Hydrolase and Penicillin G Acylase

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Abstract

The current enzymatic production of semisynthetic β -lactam antibiotics requires isolation and purification of the intermediate 6-aminopenicillanic acid which adds cost and complexity to the manufacturing process. In this work, we took advantage of the unique substrate specificity of α -amino ester hydrolases to perform a purely aqueous one-pot production of ampicillin from penicillin G and D-phenylglycine methyl ester, catalyzed by α -amino ester hydrolase and penicillin G acylase. The synthesis was performed in both a one-pot, one-step synthesis resulting in a maximum conversion of 39%, and a one-pot, two-step process resulting in a maximum conversion of 47%. The two-enzyme cascade reported in this paper is a promising alternative to the current enzymatic two-step, two-pot manufacturing process for semisynthetic β -lactam antibiotics which requires intermittent isolation of 6-aminopenicillanic acid.

Keywords

amino esters; antibiotics; enzyme catalysis; hydrolases; lactams

Introduction

Semisynthetic β -lactam antibiotics, which include penicillins and cephalosporins, are the most prescribed class of antibiotics in the world.[1] Their four-membered β -lactam ring is the crucial moiety to combat bacterial infections because it inhibits bacterial cell wall synthesis.[2] These compounds are classified as semisynthetic because their β -lactam moiety is obtained from the enzymatic hydrolysis of a natural fermentation product and their acyl side chain is obtained from a chemical or chemoenzymatic synthesis. The β -lactam moiety for all penicillins, 6-aminopenicillanic acid (6-APA), is produced on an industrial scale through the hydrolysis of either penicillin G (penG) using penicillin G acylase (PGA, EC 3.5.1.11) or penicillin V using penicillin V acylase (EC 3.5.1.11). Chemical coupling of a β -lactam moiety with an acyl side chain has dominated the industrial production of semisynthetic β -lactam antibiotics since their discovery in the early 1960s even though such a process requires low temperatures, highly reactive reagents, large volumes of solvents, low temperatures, and generates large amounts of waste.[3] Enzymatic coupling of a β -lactam moiety with an acyl side chain can be accomplished in an environmentally benign process at

ambient temperature, that does not require toxic or hazardous reagents or solvents, and thus minimizes waste generation.[3] DSM Anti-infectives BV (Delft, Netherlands) is currently manufacturing amoxicillin, cephalexin, and cefadroxil with an enzymatic process that utilizes PGA.[4] A less investigated enzyme, α -amino ester hydrolase (AEH, EC 3.1.1.43), can also be employed for the coupling reaction if the acyl side chain features an amino group in the α -position.[5–11]

Cascade conversions, which combine multiple reactions without intermediate recovery steps, are increasingly studied to render syntheses more environmentally benign and economically advantageous. Replacing a multistage synthesis with a cascade process eliminates the need for isolation and purification of intermediates and therefore results in smaller reactor volumes, shorter cycle times, higher volumetric and space time yields, and decreased amount of waste produced.[12,13] Cascade conversions can combine multiple biocatalytic steps, multiple chemocatalytic steps, or can combine both biocatalytic and chemocatalytic steps. Typically, it is easiest to combine multiple biocatalytic steps as most enzymes have similar operating conditions.[12] There have been several reports of utilizing cascade processes for semisynthetic β -lactam antibiotic synthesis. Wegman et al. combined the synthesis of the acyl side chain *D*-phenylglycine amide from *D*-phenylglycine nitrile utilizing nitrile hydratase and the enzymatic coupling of *D*-phenylglycine amide with the β -lactam nucleus 7-aminodesacetoxycephalosporanic acid utilizing PGA to synthesize cephalexin in a one-pot synthesis.[14] Fernandez-Lafuente et al. reported a chemoenzymatic synthesis of cefazolin that started from the naturally occurring cephalosporin C and involved three biocatalytic transformations in fully aqueous medium.[15,16] Finally, Du et al. and Wu et al. employed PGA in partially organic media to catalyze both the hydrolysis of penG to the β -lactam nucleus 6-APA and the enzymatic coupling of 6-APA with *D*-phenylglycine methyl ester (*D*-PGME) or *D*-hydroxyphenylglycine methyl ester to synthesize ampicillin (AMP)[17] or amoxicillin,[18] respectively, in a one-pot system.

We examined the feasibility of utilizing a cascade conversion with two biocatalytic reactions in fully aqueous medium to synthesize AMP (Scheme 1). In the first reaction, 6-APA was produced from the thermodynamically-controlled hydrolysis of penG with immobilized penicillin G acylase (iPGA). The byproduct from this reaction, phenylacetic acid (PAA), is a known inhibitor of PGA with a $K_I=70\text{ }\mu\text{M}$. [19] In the second reaction, AMP was produced in a kinetically-controlled coupling of 6-APA with *D*-PGME using either iPGA or AEH.[6] As AEHs are unique in their specificity toward α -amino groups on the acyl moiety, they cannot catalyze the hydrolysis of penG to yield 6-APA and are not inhibited by PAA,[7] thus their advantage in this cascade. In addition to the desired coupling reaction, both PGA and AEH catalyze the undesired primary hydrolysis of the activated acyl side chain, *D*-PGME, and the secondary hydrolysis of the antibiotic, AMP. These two side reactions negatively affect yield.[3]

We investigated both a one-pot, one-step (1P1S) and one-pot, two-step (1P2S) scheme. In the 1P1S scheme, a batch process, we added *D*-PGME, penG, and either iPGA or both iPGA and AEH at the beginning of the experiment. In the 1P2S scheme, we first added penG with iPGA and allowed the reaction to proceed near completion to produce 6-APA. Next, we added *D*-PGME and either AEH or additional iPGA to the reaction mixture. We investigated the effect of different relative enzyme loadings on the overall yield of AMP for both schemes.

Results and Discussion

We evaluated both the 1P1S and 1P2S systems over a range of iPGA and AEH concentrations as shown in Table 1. In this cascade, enzyme concentrations have a large

effect on the overall yield and the degree of secondary hydrolysis observed. Typical reaction profiles for both configurations are shown in Figure 1.

It has been previously shown that the initial ratio of D-PGME to 6-APA concentrations is an important parameter in optimizing the coupling reaction for semisynthetic antibiotics.[20] In our experiments, we targeted a D-PGME/6-APA ratio of 60 mM:20 mM which has been demonstrated as the optimal ratio for both iPGA[21] and AEH-catalyzed syntheses.[6]

The two-enzyme 1P1S system resulted in AMP yields between 6% and 39%, as shown in Table 1 and Figure 2A. The system performed poorly with low iPGA enzyme loading (22 UPenG) and high AEH enzyme loading (11 UAmp). AEHs have excellent D-PGME hydrolytic activity ($k_{cat}=982\text{ s}^{-1}$),[6] thus the majority of the D-PGME was hydrolyzed prior to the production 6-APA that is necessary for synthesis. Increased iPGA enzyme loading (99 UPenG) and decreased AEH enzyme loading (between 1.1 UAmp–5.5 UAmp) improved the AMP yields. The optimal configuration resulted in a 39% yield and was observed when 99 UPenG iPGA and 4.4 UAmp AEH were utilized. This configuration gave a ratio of 7.5 mol D-PGME per mol of AMP consumed at the maximum product concentration ($([D-PGME]_{t=0}-[D-PGME]_{t=AMP_{max}})/[AMP]_{t=AMP_{max}}$). In the one-enzyme 1P1S system with iPGA, the reactions only achieved a maximum conversion of 10% after 24 h. The reduced reaction yield using iPGA alone was expected, due to the strong inhibition of *E. coli* PGA with the intermediate PAA and the preference of *E. coli* PGA for penG ($K_M=0.013\text{ mM}$) over D-PGME ($K_M=12.5\text{ mM}$).[22,23]

The two-enzyme 1P2S system resulted in AMP yields between 27% and 47% as shown in Table 2 and Figure 2B. Several configurations of enzyme loadings led to yields around 47%, which is equivalent to the yields when catalyzing the synthesis reaction with AEH directly from 6-APA and D-PGME.[6] In the 1P2S system, the enzyme loading of AEH mostly impacted the secondary hydrolysis and decreased AEH loadings (between 1.1 and 4.4 UAmp) reduced the amount of secondary hydrolysis. The optimal configuration resulted in a 46% yield with minimal secondary hydrolysis and was observed when 99 UPenG iPGA and 4.4 UAmp AEH was utilized. This configuration gave a ratio of moles of D-PGME consumed per moles of AMP at the maximum product concentration of about 6. Similar to the 1P1S configuration, the single enzyme systems using iPGA resulted in low yield with a maximum conversion of 15% after 23 h.

To investigate the impact of the excess iPGA on the secondary hydrolysis in the system, we conducted a one-pot, two-step, two-stage (1P2S-2S) scheme where iPGA was removed by filtration prior to the addition of AEH to the system in the second step. The removal of iPGA did not reduce the secondary hydrolysis of AMP, and therefore was not deemed beneficial to the 1P2S scheme.

The 1P1S system required fewer manipulations and had an overall faster cycle time but resulted in a lower overall yield when compared to the 1P2S system. The lower yields were likely due to the lower initial 6-APA nucleophile concentrations as 6-APA was generated at the same time it was consumed. The 1P2S step system required higher cycle times but resulted in higher overall yields and allowed for the most control of the system parameters, including the D-PGME/6-APA ratio, when compared to the 1P1S system. One challenge for the cascade syntheses is that the ratio of moles of D-PGME consumed per mole of AMP at the maximum product concentration is elevated when compared to the ratio of the direct synthesis from 6-APA and D-PGME. For the 1P1S system, this ratio was approximately 7.5 and for the 1P2S system, this ratio was approximately 6. The direct syntheses with iPGA or AEH gave values of <2 and about 4, respectively.

Conclusions

We have demonstrated the first purely aqueous cascade system toward AMP using a two-enzyme system with both AEH and iPGA. The 1P1S and 1P2S systems resulted in optimum AMP yields of 39 and 46%, respectively. At such conditions, the 1P1S configuration required 7.5 moles of *D*-PGME per mole of AMP at the maximum product concentration, compared to only 6.2 for the 1P2S scheme. Maximum conversions were achieved in one to two hours, significantly reducing the reaction times previously observed in the systems that used iPGA and ethylene glycol.[17,18] In all cases, the two-enzyme system with iPGA and AEH outperformed the systems that used only iPGA, thus demonstrating the clear advantage of using AEH. While the 1P1S system resulted in slightly lower yields, it could be advantageous due to its operational ease and faster cycle times. In the 1P2S system, higher conversion was achieved and secondary hydrolysis was minimized by adjusting the relative enzyme loadings. These reaction schemes could be scaled up and incorporated with enzyme reuse, which has been previously demonstrated for iPGA.[13,24] However, further optimization is still required to improve yields and reduce ester usage for these processes.

Experimental Section

Materials

6-Aminopenicillanic acid, (*D*)-phenylglycine, ampicillin, (*D*)-phenylglycine methyl ester hydrochloride, penicillin G, phenylacetic acid, and Eupergit-immobilized penicillin G acylase from *Escherichia coli* were all procured from Sigma Aldrich (St. Louis, MO). Soluble amino ester hydrolase from *Xanthomonas campestris* *pv. campestris* was prepared in our laboratory as described in Blum et al.[6]

One-Pot, One Step Synthesis: PenG (15 mL of 20 mM) and *D*-PGME (60 mM in 100 mM phosphate buffer, pH 7) were added to a round bottom flask along with iPGA or iPGA and purified *X. campestris* *pv. campestris* AEH (Table 1). The reactions were stirred using a magnetic stir plate and carried out at room temperature (22 °C–25°C).

One-Pot, Two-Step Synthesis: PenG (7.5 mL of 40 mM) in phosphate buffer (100 mM, pH 7) was added to a round bottom flask along with iPGA (Table 2; 124 UPenG per gram of carrier), where 1 UPenG is defined as one μ mol of penicillin G hydrolyzed per minute. The reactions were stirred using a magnetic stir plate and carried out at room temperature (22 °C–25°C). After the reaction reached near completion, as determined by HPLC, *D*-PGME (7.5 mL of 120 mM) was added. The pH was adjusted with NaOH from approximately 6.4 to 7.0 and *X. campestris* *pv. campestris* AEH was added (Table 2; 79 UAmp mg⁻¹ protein), where UAmp is defined as one mmol of AMP hydrolyzed per minute under saturation conditions. Additional experiments were conducted in which pH was controlled between 7.0 $\pm$ 0.1; the pH control had no effect on the results of the experiment. In reactions where iPGA was used in both steps, we replaced the AEH with equivalent AMP synthesis units of iPGA based on initial synthesis rate data from 6-APA and *D*-PGME using only AEH[6] and only iPGA[21] where 1 UAmp of AEH $\approx$ 1 UAmp of iPGA $\approx$ 6.8 UPenG of iPGA.

One-Pot, Two-Step, Two-Stage Synthesis: These experiments were conducted analogously to the 1P2S schemes, with the exception that after the completion of the first step, the iPGA was removed from the reaction using filtration.

HPLC Assay

All analyses were conducted using high performance liquid chromatography complete with a Shimadzu-LC-20AT pump, Beckman Coulter Ultrasphere ODS 4.6 mm $\times$ 25 cm column, and SPD-M20A prominence diode array detector (PDA) monitored at 215 nm. Samples (100

μL) were diluted 10 $\times$ into 900 μL of HPLC quench buffer (75% methanol, 25% 0.02 M potassium phosphate, pH 6.0). The sample (2 μL) was loaded onto the column. A step change method was used with a 1 mL min⁻¹ flow rate. The initial mobile phase was 20% methanol and 80% 0.02 M phosphate buffer (pH 7). From 5.5–25 min the methanol was increased to 35%. At 25 min, the methanol was returned back to 20% for the duration of the method of 35 min. All components, D-PG, PAA, 6-APA, D-PGME, AMP and penG were detected using this method. Results were normalized based on the penicillanic ring mass balance.

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Scheme 1.

One-pot, two-enzyme direct conversion of penicillin G to ampicillin using iPGA and AEH. Undesired side reactions, primary hydrolysis of D-PGME to D-PG, and secondary hydrolysis of AMP are shown.

Figure 1.

Reaction profile of the enzymatic conversion of penicillin to ampicillin using 99.2 UPenG of iPGA and 2.2 UAmp AEH. Both the A) 1P1S and the B) 1P2S profiles are shown. D-PG (+), 6-APA (●), PAA (◆), AMP (■), D-PGME (▲), PENG (*).

Figure 2.

Ampicillin conversion profiles for both the A) 1P1S and B) 1P2S systems. In the 1P2S reaction profiles, there was no ampicillin until the second reaction step was initiated 60–140 min into the reaction. 24.8 UPenG iPGA, 11 UAmp AEH (▲), 99.2 UPenG iPGA, 1.1 UAmp AEH (●), 99.2 UPenG iPGA, 2.2 UAmp AEH (◆), 99.2 UPenG iPGA, 4.4 UAmp AEH (■), 99.2 UPenG iPGA, 5.5 UAmp AEH (*), 1P2S-2S 99.2 UPenG iPGA, 5.5 UAmp AEH (+).

Table 1

Conversion results from the one-pot, one-step (1P1S) reaction configuration.

Enzyme loading ^[a]		<i>t</i> ^[b] [min]	Moles of D-PGME per mole of AMP at max conv. [mol mol ⁻¹]	Maximum conversion ^[c] [%]
iPGA [UPenG]	AEH [UAmp]			
24.8	11	20	48	6
99.2	1.1	360	8.7	23
99.2	2.2	300	6.3	38
99.2	4.4	60	7.5	39
99.2	5.5	60	11	30
99.2	none	360	31	3
114	none	1500	21	10
129	none	1500	20	9
136	none	360	25	5

^[a] In ampicillin synthesis reactions starting from 6-APA and D-PGME, 1 UAmp of AEH $\approx$ 6.8 UPenG of iPGA.

^[b] Time at which maximum conversion was obtained.

^[c] Conversions are based on the moles of ampicillin produced per mole of penicillin G starting material. All concentrations are based on analytical measurements, not isolated yields.

Table 2

Conversion results from the one-pot two-step (1P2S) reaction configuration.

Step 1 Enzyme loading ^[a] iPGA [UPenG]	Step 2 Enzyme loading ^[a] iPGA [UPenG]	AEH [U/Amp]	Step 1 t [min]	Step 2 t [min]	Total t [min]	Moles of D-PGME per mole of AMP at max conv. [mol mol ⁻¹]	Maximum conversion ^[c] [%]
24.8	none	1.1	145	15	160	6.0	47
99.2	none	1.1	60	300	360	6.9	27
99.2	none	2.2	60	180	240	6.3	35
99.2	none	4.4	60	90	150	6.2	46
99.2	none	5.5	60	30	90	6.1	47
24.8 ^[d]	none	1.1	130	20	150	6.1	45
24.8	74	none	130	410	540	15	6
99.2	15	none	60	1290	1350	17	12
99.2	30	none	60	1290	1350	15	14

^[a] In ampicillin synthesis reactions starting from 6-APA and D-PGME, 1 U/Amp of AEH $\approx$ 6.8 UPenG of iPGA.

^[b] Time at which maximum conversion was obtained.

^[c] Conversions are based on the moles of ampicillin produced per moles of penicillin G starting material. All concentrations are based on analytical measurements, not isolated yields.

^[d] iPGA removed from the second step using filtration in the one-pot, two-step, two-stage process.