Impact of Polybasic Alcohols on Biocompatibility and Selectivity of Penicillin G Acylase for Kinetically Controlled Synthesis

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Abstract

The enzyme catalyzed synthesis of cephalexin (CEX) from 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) and D-α–phenylglycine methyl ester (PGM) by Penicillin G acylase (PGA) is a model for kinetically controlled synthesis. The parallel hydrolysis of PGM, the activated acyl donor, is the principle competing pathway in this reaction, limiting the synthetic yield and reaction efficiency. To improve the performance of PGA catalyzed CEX synthesis, the biocompatibility and selectivity of various co-solvents were investigated. Polybasic alcohols such as ethylene glycol, glycerol and PEG400 did not cause deleterious changes to the enzyme, whereas monobasic alcohols, such as butyl alcohol, disrupted the PGA activity. Compared with the reaction in aqueous medium, the use of ethylene glycol as a co-solvent was found to have good selectivity in order to facilitate CEX synthesis and significantly minimize PGM hydrolysis. The pH of ethylene glycol medium was also optimized. The mechanism of the enhanced effect of polybasic alcohols as co-solvents on both biocompatibility and selectivity of enzymatic kinetically controlled synthesis is suggested.

Keywords: Penicillin G acylase; cephalexin; kinetically controlled synthesis; co-solvent; polybasic alcohols; biocompatibility; selectivity

Abbreviations: PGA, Penicillin G acylase; **CEX**, Cephalexin**; 7-ADCA**, 7-amino-3-deacetoxycephalosporanic acid; **PGM**, D-α–phenylglycine methyl ester; **PG**, D-α–phenylglycine

1 Introduction

Penicillin G acylase (PGA, EC 3.5.1.11) was originally used to catalyze the hydrolysis of natural penicillins to yield 6-amino-penicillinianic acid (6-APA)， a starting point for as the common nucleus of a range of semi-synthetic penicillins. As penicillin acylase activity is associated with a phenylacetyl moiety, a variety of phenylacetylated derivatives of anilines, alcohols, amino acids and β-lactam antibiotics can be recognized as substrates. PGA has also been employed in many valuable reactions including the kinetically controlled synthesis of β–lactam antibiotics from an activated side chain and β–lactam nucleus, the resolution of racemic mixtures by stereo-selective hydrolysis of L-enantiomer nucleophile substrates, as well as the use of activated acyl donors as protective groups in the production of optically active and protected amino compounds [1-3].

PGA is a large (86-kD) a heterodimeric protein comprising two peptide subunits known as A (23-kDa) and B (63-kDa). The mechanism of action of PGA is thought to involve attack on the carbonyl of the peptide bond by the N-terminal serine of the B subunit. PGA belongs to the N-terminal nucleophilic (Ntn) hydrolase superfamily, undergoes post-translational modification and is produced by intein-mediated auto-splicing of a 92-kDa precursor [4-5]. Members of the Ntn hydrolase superfamily typically consist of a four-layered catalytically active fold, with an αββα core structure. Apart from PGA, a range of other enzymes such as proteasome, aspartylglucosaminidase and glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase share a similar structure to members of the Ntn hydrolase

superfamily [6]. Protein engineering or directed evolution studies have been used to understand the structure-function relationship and to improve PGA properties such as activity, substrate specificity and stability [7-16].

There are two main methods of the enzymatic synthesis of β-lactam antibiotics. Thermodynamically controlled synthesis uses the side-chain of semisynthetic β-lactam antibiotics as an acyl donor, which can couple with the β-lactam nucleus. The yield of this reaction is dependent on the thermodynamic equilibrium, which is independent of the enzyme properties. Kinetically controlled synthesis uses an activated acyl donor, ester or amide form of a side chain of semisynthetic β-lactam antibiotics, which can be coupled with the β-lactam nucleus giving a higher yield with the enzyme playing a crucial role [17].

The PGA catalyzed synthesis of cephalexin (CEX) from 7-ADCA and D-α–phenylglycine methyl ester (PGM) is an example of kinetically controlled synthesis [18-22]. The enzymatic synthesis of β-lactam antibiotics as described above is accompanied by two side reactions, (1) hydrolysis of the activated acyl donor (PGM) and (2) hydrolysis of the resultant antibiotics (CEX), leading to loss of the activated acyl donor and limiting the product yield. Practical and effective strategies to increase the selectivity of the PGA catalyzed synthesis to hydrolysis reaction have mainly focused on changing in kinetic parameters and chemical properties of the reaction medium components. An acidic pH favors the selectivity ratio of synthesis to hydrolysis but decreases the solubility of substrates [23-26]. Numerous studies have shown that the use of co-solvents, such as polyols, can improve the yield of the final

product [27-35], however, the tolerance of PGA to such co-solvents needs to be addressed in detail as it appears to be important for enzyme engineering. Immobilization methods have also been pursued in the recent past to improve the synthetic performance of PGA [7, 36]

In this study, we wanted to see if we could improve the performance of PGA for CEX synthesis by medium engineering using co-solvents and optimizing pH conditions. The non-toxic effect of a co-solvent on the function of an enzyme in a specific reaction medium has been studied. In contrast to the stability, which is used to describe the length of time during which an enzyme remains actives, biocompatibility is associated with the denaturing effect of the co-solvent on the performance of an enzyme.

2 Materials and Methods

2.1 Enzyme

A PGA solution prepared from *Bacillus megaterium* was kindly provided by the Beijing Institute of Microbiology, Chinese Academy of Sciences. Enzymatic activity was determined by the hydrolysis of CEX. One activity unit is defined as the amount of enzyme capable of hydrolyzing 1 μmol CEX upon addition of a 2% solution of CEX at pH7.5 and 30℃.

2.2 Chemicals

7-ADCA and CEX were kindly provided by Northeast China Pharmaceutical Group Co., Ltd. PG and PGM were obtained from Shanghai Industrial Chemical Co., Ltd.

Ethylene glycol and glycerol were purchased from Beijing Yili Fine Chemical Co., Ltd. Polyethylene glycol 400 (PEG400), n-butanol and DMF (N,N-dimethylformamide) were purchased from Sinopharm Medicine Holding Co., Ltd.

HPLC grade methanol was purchased from Tianjin Siyou Biomedicine Technology Co., Ltd. Other chemicals were of analytical grade.

2.3 Reaction of penicillin acylase-catalyzed synthesis of cephalexin

Enzymatic reactions were carried out in a stirred batch reactor in 0.1M potassium phosphate at 30℃ by the addition of 0.2 ml (3.3×10^6U) enzyme solution and 10ml substrate mixture, with an initial ratio of PGM to 7-ADCA of approximately 2:1. The enzyme concentration was 3.3×10^5 U/ml, with, substrate concentrations were 0.5% (w/v) 7-ADCA and 1% (w/v) PGM respectively. After the desired amounts of 7-ADCA and PGM were dissolved, the pH was adjusted to the indicated value using 2M ammonium hydroxide. Organic co-solvents listed in Table were selected by studying the synthesis reaction within a concentration of 10%~40%. The reaction was monitored by HPLC to measure the concentration of CEX, 7-ADCA, PGM and PG.

2.4 Analytical method

The concentration of CEX, 7-ADCA, PGME and PG was determined by high performance liquid chromatography (HPLC) using a Waters 600E chromatography system with an UV detector (model 486) at 220nm with an YWG-C18 column (10μm; 4.6×300). The mobile phase was mixture of 70% methanol and 30% 0.02M potassium phosphate (KH_2PO_4), adjusted to a finial pH5.5 by the addition of tri-ethylamine. At a

flow rate of 1ml/min, the retention time was 3.4min for PG, 3.8min for 7-ADCA, 7.7min for CEX and 10.7min for PGM. Identification and evaluation of the concentration of substrates and products was performed in comparison with authentic standard samples.

2.5 Reaction yield, reaction time and selectivity ratio of synthesis to hydrolysis

The reaction time was evaluated when the highest concentration of CEX in the reaction system was achieved. The reaction yield was calculated based on 7-ADCA conversion yield. The selectivity ratio of synthesis to hydrolysis is referred to as product CEX to byproduct PG at the end of reaction.

3 Results and Discussion

3.1 Kinetics of penicillin G acylase catalyzed synthesis of cephalexin

The proposed mechanism of PGA-catalyzed kinetically controlled synthesis is shown in Figure 1. PGA catalyzes the conversion of esters and amides via an acyl-enzyme intermediate. Either the amino group of an added external nucleophile (such as 7-ADCA) or water can attack the acyl-enzyme intermediate, yielding either the desired acyl-transfer product (CEX) or the hydrolyzed acyl donor (PGM). The proportion of product CEX to byproduct PG is used as an important parameter to evaluate the synthetic performance of PGA. Here we examined the selectivity ratio of synthesis to hydrolysis to reveal competition between 7-ADCA and water attacking the acyl-enzyme intermediate. The product yield, based on conversion of 7-ADCA, and reaction time, based on how long the highest concentration of CEX in the reaction

system was achieved, were also used to evaluate the performance of PGA catalyzed synthesis of CEX.

From the course of the reaction (HPLC figures as shown in the of supporting information), CEX synthesis from 7-ADCA and PGM can be seen as well as the hydrolysis of PGM and CEX leading to the byproduct PG, which is in agreement with the proposed mechanism of PGA catalyzed synthesis of CEX. Most of the PG may be derived from the hydrolysis of PGM, as CEX is present at a low concentration when compared with PGM concentration in the reaction mixture. The reaction yields, the selectivity ratio of CEX synthesis to PG hydrolysis and the complete reaction time are shown in Table 1. A reaction yield of 54.3% was achieved at pH7.0 and the selectivity ratio of CEX to PG was 0.70. Increasing the ratio of PGM to 7-ADCA will result in high 7-ADCA conversion yield, but this is inefficient for CEX synthesis as high concentration of PG would accumulate as PGM concentration rises. Here we have focused on using co-solvents to improve the selectivity ratio of CEX synthesis to PG hydrolysis.

3.2 Screening co-solvents for penicillin G acylase catalyzed synthesis of cephalexin

Several polybasic alcohols were examined as co-solvents to see if they could improve the performance of PGA catalyzed CEX synthesis as shown in Table 1. Ethylene glycol, glycerol and PEG 400 were found to be as good as an aqueous medium, however, DMF and n-butanol inhibited the synthesis of reaction. This result suggests that ethylene glycol, glycerol and PEG 400 have good biocompatibility with

enzymes. Specifically, ethylene glycol exhibited a 20% increase (from aqueous 0.7 to ethylene glycol 0.83) in the selectivity ratio of CEX synthesis to PG hydrolysis compared with aqueous medium and was chosen it to optimize the reaction.

3.3 Effect of pH on penicillin G acylase catalyzed synthesis of cephalexin

We evaluated the reaction yields, the ratio of CEX synthesis to PG hydrolysis and the complete reaction time between pH 6.5 and 8.0 (Table 2). Reaction time decreased from 180 min at pH6.5 to 45min at both pH7.5 and pH8.5, indicating that the reaction rate rises with increasing pH. In contrast to the reaction rate, the CEX yield increased with decreasing pH and the reaction yield achieved its highest value (54.26%) at pH7.0. However, the selectivity ratio of CEX synthesis to PG hydrolysis also rose with decreasing pH and showed highest value 0.862 at pH6.5.

The effect of pH on the selectivity ratio of CEX synthesis to PG hydrolysis, the reaction yield and the complete reaction time was examined when using 10% ethylene glycol added to the aqueous medium (Table 3). The optimized pH shifted from pH6.5 to pH7.5 with the addition of 10% ethylene glycol to the aqueous medium, for the selectivity ratio of CEX synthesis to PG hydrolysis from 0.87 in aqueous medium to 0.89 in 10% ethylene glycol. The reaction requires 45min to complete and the product yield was nearly 50%. The results of the selectivity ratio of CEX synthesis to PG hydrolysis, the reaction yield and the complete reaction time in 10% ethylene glycol medium are comparable to those obtained in the aqueous medium. However, the addition of ethylene glycol increasing optimized pH from 6.5 to 7.5 should favor the dissolution of substrates, thus contributing to improved performance of PGA catalyzed CEX synthesis.

3.4 Enhancement effect of ethylene glycol on penicillin G acylase catalyzed kinetically synthesis of cephalexin

After determining the optimum pH, the effect of ethylene glycol concentration was investigated (Table 4). When ethylene glycol concentration increased from 0 to10%, 20% or 40%, both the selectivity ratio of CEX synthesis to PG hydrolysis and the yield of product increased. When 40% ethylene glycol concentration is compared with 10%, the reaction time was extended from 45min to 150min, however, the reaction yield increased from 49.67% to 55.24% and the ratio of CEX synthesis to PG hydrolysis increased from 0.89 to 1.23, which indicates significant enhancement of selectivity on CEX synthesis. Moreover, as PGA was tolerant to up to 40% ethylene glycol, this indicats that ethylene glycol has very high biocompatibility with enzymes.

3.5 Mechanism of the complex of polybasic alcohols and water on Penicillin G acylase catalyzed kinetically synthesis of Cephalexin

As water plays a critical role in the hydrolysis of PGM and CEX into PG, a rational strategy to decrease hydrolysis of PGM and CEX is to reduce water concentration in the reaction medium. However, as enzymes are generally inactivated and lose stability in the presence of non-aqueous medium, it is necessary to select biocompatible co-solvents. Many alcohols can serve as co-solvents due to their ability to form hydrogen bonds with water. Hydroxyl groups at the end of each polybasic alcohol allow a hydrogen atom to act as a hydrogen bond donor and oxygen atom is a hydrogen bond acceptor. When a monobasic alcohol was compared with a polybasic alcohol as co-solvent, quite a different result was obtained. Polybasic alcohols have desired biocompatibility when employed in the PGA catalyzed kinetically controlled synthesis of CEX. As monobasic alcohols have only one hydroxyl group on one end of the molecule to form hydrogen bonds with water, whereas the hydrophobic end of monobasic alcohols are exposed, which is believed to lead to deactivation of enzymes [37]. Polybasic alcohols, on the other hand, have at least two polar O-H bonds in

hydroxyl groups at the end of each molecule, enabling them to form hydrogen bonds with water, with hydrophobic patches are enclosed in the interior. Of the co-solvents ethylene glycol, glycerol and polyethylene glycol, ethylene glycol behaves most similarly to the hydrogen-bonding ability of water and demonstrates an enhancement effect on PGA.

Recent studies using both FTIR and UV-V's spectroscopies suggest that a likely complex of three ethylene glycol molecules to four water molecules are formed at the maximal excess molar volume in an ethylene glycol-water mixture (EGW) [38]. EGW replacing water in the medium could reduce the water concentration, which most likely explains why ethylene glycol has both high biocompatibility and selectivity effects on PGA catalyzed synthesis.

4 Conclusion

This study shows that polybasic alcohols, such as ethylene glycol, glycerol and PEG-400, may serve as suitable co-solvents to enhance the PGA catalyzed synthesis of CEX from 7-ADCA and PGM, when compared with mono-basic alcohol, such as butyl alcohol, with in terms of their selectivity and biocompatibility. The high solubility of polybasic glycols in water, owing to their ability to form hydrogen bonds with water, make these compounds ideal co-solvents for PGA catalyzed kinetically controlled synthesis reaction. Our results clearly demonstrate that the hydrogen-bond complex between ethylene glycol and water has good biocompatibility with PGA. As the complex of ethylene glycol and water significantly reduce water concentration in the medium, the selectivity of CEX synthesis to PG hydrolysis by PGA was enhanced. Moreover, pH conditions combined with ethylene glycol were found to be optimal at

basic pH favoring the dissolution of substrates and increased PGA catalyzed reaction rate. Our study may be particularly useful for the application of PGA to a large scale manufacture of semisynthetic β-lactam antibiotics.

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Legends of tables and figures

Table 1 Effect of organic co-solvents on penicillin G acylase catalyzed synthesis of cephalexin from PGM and 7-ADCA.

Table 2 Comparison of the effect of pH on penicillin G acylase catalyzed synthesis of cephalexin from 7-ADCA and PGM.

Table 3 Effect of pH on penicillin G acylase catalyzed synthesis of cephalexin from PGM and 7-ADCA with the addition of 10% ethylene glycol.

Table 4 Comparison of the effect of ethylene glycol concentration on Penicillin G acylase catalyzed synthesis of cephalexin from PGM and 7-ADCA at pH7.5.

Figure 1 Mechanism of Penicillin G acylase catalyzed kinetically controlled synthesis.

Table 1 Effect of organic co-solvents on penicillin G acylase catalyzed synthesis of cephalexin from PGM and 7-ADCA

Solvent （10% organic cosolvent）	Reaction yield （%）	Ratio of synthesis/hydrolysis [CEX]/[PG]	Reaction time (min)
aqueous	54.3	0.70	75
DMF	0		
n-Butanol	0		
Ethylene Glycol	50.8	0.83	80
Glycerol	56.5	0.57	60
PEG400	56.9	0.55	60

Reaction conditions: temperature 30℃; enzyme concentration 70U/ml; pH7.0; substrate concentration: 0.5% 7-ADCA (7-amino-3-deacetoxycephalosporanic acid), 1% PGM (D-α-phenyglycine methylester).

Table 2 Comparison of the effect of pH on penicillin G acylase catalyzed synthesis of cephalexin from 7-ADCA and PGM

pH	Reaction yield (%)	Ratio of synthesis/hydrolysis [CEX]/[PG]	Reaction time (min)
6.5	52.1	0.86	180
7.0	54.3	0.70	75
7.5	50.0	0.67	45
8.0	45.7	0.53	45

Reaction conditions: temperature 30℃; enzyme concentration 70U/ml; substrate concentration: 0.5% 7-ADCA (7-amino-3-deacetoxycephalosporanic acid), 1% PGM (D-α-phenyglycine methylester).

Table 3 Effect of pH on penicillin G acylase catalyzed synthesis of cephalexin from PGM and 7-ADCA with the addition of 10% ethylene glycol

pH	Reaction yield (%)	Ratio of synthesis/hydrolysis [CEX]/[PG]]	Reaction time (min)
6.5	50.2	0.80	150
7.0	50.8	0.83	80
7.5	49.7	0.89	45
8.0	41.7	0.71	45

Reaction conditions: temperature 30℃; enzyme concentration 70U/ml; substrate concentration: 0.5% 7-ADCA (7-amino-3-deacetoxycephalosporanic acid), 1%PGM (D-α-phenyglycine methylester); co-solvent: 10% ethylene glycol.

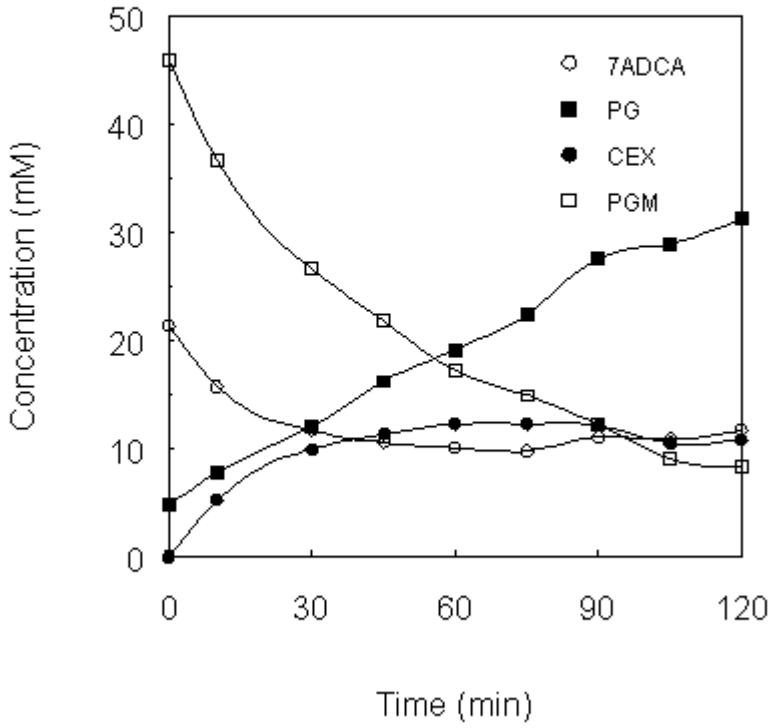
Table 4 Comparison of effect of ethylene glycol concentration on penicillin G acylase catalyzed synthesis of cephalexin from PGM and 7-ADCA at pH7.5

Concentration of ethylene glycol （% volume）	Reaction yield (%)	Ratio of synthesis/hydrolysis [CEX]/[PG]]	Reaction time (min)
0	50.1	0.69	45
10	49.7	0.89	45
20	50.3	0.90	90
40	55.2	1.23	150

Reaction conditions: temperature 30℃; enzyme concentration 70U/ml; pH 7.5; substrate concentration: 0.5% 7-ADCA (7-amino-3-deacetoxycephalosporanic acid), 1% PGM (D-α-phenyglycine methylester); co-solvent: ethylene glycol.

Fig. 1. （**A**）Penicillin acylase catalyzed synthesis of cephalexin from 7-ADCA and PGM at pH7.0. Reaction conditions: temperature 30℃，enzyme concentration 70U/ml. Symbols: PG（D-α-phenyglycine）, CEX（Cephalexin）, PGM（D-α-phenyglycine methyl ester）（**B**）Mechanism of penicillin G acylase catalyzed kinetically controlled synthesis. Symbols: **EH**: Enzyme (penicillin G acylase); **X-CO-OR**: activated acyl donor PGM (D-α-phenyglycine methyl ester); **ROH**: byproduct methanol; **X-CO-E**: acyl-enzyme intermediate; **X-COO$^-$**: byproduct PG (D-α-phenyglycine); **Y-NH$_2$**: acyl acceptor 7-ADCA（7-amino-3-deacetoxycephalosporanic acid）; **X-CO-NH-Y**: acyl-transfer product CEX（cephalexin）.

(A)



(B)