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# Biosynthesis of Cephalexin in PEG400-Ammonium Sulfate and PEG400-Magnesium Sulfate Aqueous Two-Phase Systems

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**Abstract** The biosynthesis of cephalexin was carried out in the aqueous two-phase systems using penicillin acylase as a catalyst, and 7-aminodeacetoxicephalosporanic acid (7-ADCA) and phenylglycine methyl ester (PGME) as substrates. 20% PEG400-17.5% $(NH_4)_2SO_4$ containing 0.5 M NaCl and 1.5 M methanol aqueous two-phase systems (ATPS) were selected as reaction medium, and 53% product yield was obtained using immobilized penicillin acylase as a catalyst. 20% PEG400-15% $MgSO_4$ ATPS was also used for the synthesis of cephalexin, and 60–62% product yield was obtained by using free penicillin acylase as a catalyst. When batch reactions were repeated in the ATPS, the cephalexin yields decreased during the reactions due to deactivation, loss, and product inhibition of penicillin acylase. The effect of different ratio of phenylglycine methyl ester to 7-ADCA on the product yield was investigated, and high cephalexin yield was obtained at a high molar ratio.

**Key words:** Aqueous two-phase systems, cephalexin, penicillin acylase, biosynthesis

Aqueous two-phase systems (ATPS) have advantages of low interfacial tension and decreasing deactivation of biomolecules. These advantages have been applied to the purification of protein. The bioconversion in aqueous two-phase systems (ATPS) has been studied since 1980s [1, 2, 3, 6, 7]. In the bioconversion, enzyme and substrates are partitioned to one side of two phases, and products to the other phase. As a result, the inhibition of products to enzyme is decreased. Enzyme stability, mass transfer, and specific productivity are improved.

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Penicillin acylase or closely related 7-ACA acylase (EC 3.5.1.11) has been widely applied to the production of 6-aminopenicillanic acid (6-APA) and the semisynthesis of β-lactam antibiotics [11]. Usually, the semisynthesis of β-lactam antibiotics is performed in a monophase aqueous solution. However, the bioconversion results in the inhibition of enzyme activity and low product yield. Odette *et al.* [12] synthesized cephalexin in ATPS composed of 100% PEG600-3 M ammonium sulfate, with a cephalexin partition coefficient of 23 and improved product yield. Previously, substances with small molecular weights such as amino acids, peptides, and antibiotics have been thought to have nearly even partition coefficient in ATPS [1, 15]. However, more and more data have indicated that uneven partition could be achieved by optimization of ATPS [9, 13]. For the synthesis of small molecular products, it is important to find a suitable ATPS to satisfy the uneven partition of enzyme, substrates, and products.

Many results have been obtained through optimum poly(ethylene)glycol (PEG)/Dextran (Dx), PEG/sodium phosphate, and PEG/ammonium sulfate ATPS [5, 8]. In the present study, the biosynthesis of cephalexin was performed in a 20% PEG-17.5% $(NH_4)_2SO_4$ ATPS containing 0.5 M NaCl and 1.5 M methanol and 20% PEG-15% $MgSO_4$ ATPS, which was defined as one of the optimum ATPS for cephalexin synthesis [8]. The higher product yields of cephalexin in ATPS than in monophase aqueous phase were observed.

## Materials and Methods

### Materials

Cephalexin, 7-aminodeacetoxicephalosporanic acid (7-ADCA), phenylglycine methyl ester (PGME), and Penicillin G acylase (PGA, EC3.5.1.11) were obtained from Sigma,

U.S.A.; PEG400 was obtained from E. Merck, Germany. The immobilized penicillin G acylase was prepared according to the Guisan method [9] and previously equilibrated with the ammonium sulfate-rich bottom phase when added into the ATPS. All other reagents were of analytical grade.

### Assay

Cephalexin, 7-ADCA, and PGME in the top and bottom phases were measured by HPLC (Japan) with Shim-Pack VP-ODS (250×4.6 mm, 5 μm) and a UV detector (248 nm). The buffer of gradient acetonitrile and sodium phosphate (pH 6.7, 50 mM) was used as an eluant at a flow rate of 1.0 ml/min at 25°C. Enzyme activity was assayed by the method of Balasinghan *et al.* [4].

### Partition of Cephalexin and 7-ADCA and Optimization of ATPS

20% PEG400-17.5% $(NH_4)_2SO_4$ ATPS and 20% PEG400-15% $MgSO_4$ ATPS were prepared by adding calculated amounts of PEG, salt, and water into 10-ml calibration tube to make 10 g of ATPS. Two aliquots of the ATPS were prepared. Cephalexin (100 mg), PGME (100 mg), and 7-ADCA (50 mg) were added into one aliquot of ATPS, and PGA (100 IU) was added into the other aliquot. The composition of ATPS was calculated by % weight/weight (w/w). The sealed tubes were shaken in a shaker of 200 rpm until all components were dissolved (about 2 h), centrifuged at 1,000 ×g for 15 min, and then the volume ratio was measured by graduation. Samples taken from the top and bottom phases, and the concentrations of PGME, 7-ADCA, and cephalexin, were assayed by HPLC. PGA activity was analyzed by Balasinghan's method.

For investigating the effect of NaCl concentration on the partition of cephalexin, the ATPS of different NaCl concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 M) were prepared by adding NaCl and cephalexin (100 mg), respectively. Then the partition coefficient of cephalexin was determined.

### Stability of Penicillin G Acylase in 20% PEG400-15% $MgSO_4$ ATPS and $MgSO_4$ Solution

One-hundred IU PGA was added into 10 ml of 20% PEG400-15% $MgSO_4$ ATPS at pH 6.5 and 15°C, and the PGA activity was measured at different times (h). In another experiment, 100 IU PGA was added into 2.5, 5, 15, and 20% $MgSO_4$ solution at 37°C, and the PGA activity was measured after 24 h of incubation.

### Biosynthesis of Cephalexin

Three aliquots of 7-ADCA (0.32 g) and PGME (0.30 g) were added to 20 ml of 20% PEG400-17.5% $(NH_4)_2SO_4$ ATPS, 20% PEG400-15% $MgSO_4$ ATPS containing 50 mM sodium phosphate (pH 6.5) and 20 ml of sodium phosphate solution (50 mM, pH 6.5, monophase system), respectively. The reaction in the 20% PEG400-17.5% $(NH_4)_2SO_4$ ATPS was initialized by addition of immobilized penicillin G acylase, and the reactions in the 20% PEG400-15% $MgSO_4$ ATPS and in the sodium phosphate solution were initialized by adding 100 IU PGA (0.4 ml). The reaction temperature was controlled at 15°C. The samples were withdrawn at different times (min), and cephalexin concentrations were analyzed by HPLC.

### Effect of Molar Ratio of PGMA to 7-ADCA on the Synthesis Yield

Substrates of different ratios of PGMA to 7-ADCA (1:1, 2:1, 3:1, 4:1) were added into 20 ml of 20% PEG400-17.5% $(NH_4)_2SO_4$ ATPS. The same procedures as mentioned above were performed, and the total yields of two phases were calculated.

### Repeat Batch Reaction

Repeat batch reactions were carried out in 20 ml of reaction solution of the 20% PEG400-15% $MgSO_4$ ATPS in 100-ml flasks at 15°C, and 0.30 g of PGMA and 0.32 g of 7-ADCA were added into the ATPS. The reaction was initialized by addition of 100 IU PGA. After the reaction was over, the top phase was removed and replaced with a new top phase, which contained the original composition of top phase with fresh substrates. The samples were withdrawn at different times (min), cephalexin concentrations were analyzed by HPLC, and the total yields of two phases were calculated.

## Results and Discussion

### Optimization of PEG400-$(NH_4)_2SO_4$ ATPS

ATPS can be formed by adding a salt to a polymer solution, and the efficiency of various salts forming ATPS follows the sequence of lyotropic series. The contribution of anion is greater than that of cation, and multivalent anions with PEG are the most effective to form ATPS. In this experiment, $MgSO_4$ and $(NH_4)_2SO_4$ were selected to investigate their effects on the partition of cephalexin, PGMA, 7-ADCA, and PGA (Table 1).

In the 20% PEG400-15% $MgSO_4$, a $K_C$ value of 6.7 was obtained. This shows that cephalexin is biased to the top phase, while PGA is partitioned to the bottom phase

**Table 1.** Partition coefficients of cephalexin, 7-ADCA, PGME, and PGA in ATPS.

| ATPS | $K_A$ | $K_P$ | $K_C$ | $K_{PGA}$ |
|---|---|---|---|---|
| 20%PEG 400-15%$MgSO_4$ | 1.2 | 1.5 | 6.7 | <0.01 |
| 20%PEG 400-17.5%$(NH)_4SO_4$ | 1.7 | 1.4 | 3.2 | 14.9 |

$K_A$, the partition coefficient of 7-ADCA; $K_P$, the partition coefficient of PGME; $K_C$, the partition coefficient of cephalexin; $K_{PGA}$, the partition coefficient of PGA.

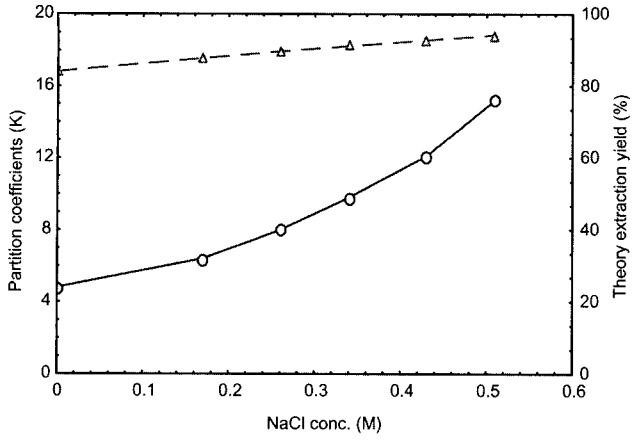


**Fig. 1.** Effect of NaCl on extraction yield and partition coefficient of cephalexin.
○, $K_c$; △, cephalexin yield.

($K_{PGA}$<0.01). 7-ADCA and PGMA biased slightly to the top phase. In that case, cephalexin is synthesized in the favorable direction, because product and enzyme are biased in different phases and product inhibition is reduced. In 20% PEG400-17.5% $(NH_4)_2SO_4$, cephalexin, 7-ADCA, PGMA, and PGA all biased to the top phase. This situation is not favorable for cephalexin, due to product and enzyme being partitioned to the same phase resulting in product inhibition. In order to change the situation, PGA was immobilized to agarose and the immobilized PGA was compelled to the bottom phase. Furthermore, NaCl was chosen to improve the $K_C$. The effect of NaCl concentration on the partition coefficient and the extraction yield of cephalexin were investigated. As shown in Fig. 1, the partition coefficient and extraction yield of cephalexin increased with the increase in concentration of NaCl, while the volume ratio of top-to-bottom phase was slightly influenced. When 20% PEG400-17.5% $(NH_4)_2SO_4$ ATPS contained 0.5 M NaCl and 1.5 M methanol, $K_C$ reached 15.2, $K_A$ was 1.8, and $K_P$ was 1.2, resulting in the 94.2% extraction yield of cephalexin. Neutral salts in ATPS could change the difference of electrical potential between two phases due to the uneven partition of ion (Donnan potential). On the other hand, neutral salts could change surface hydrophobicity of partitioned substances to influence partition behavior. The salt effects could be described by the equation (1).

$$\ln k = HF(HFS + \Delta HFS) + \frac{FZ}{RT}\Delta\varphi \quad (1)$$

where K, HF, HFS, ΔHFS, F, Z, R, T and Δφ indicate partition efficient, hydrophobic factor, hydrophobic factor of solutes, Faraday constant, electric charge number, gas constant, absolute temperature, and potential difference between two phases, respectively. The first part on the right hand side of the equation expresses the hydrophobic effect of salts and the second part expresses potential difference effect [19]. Other salts such as KCNS and $NaClO_4$ can also improve the partition coefficient of Kc, but they were not selected, considering the influence on the stability of penicillin acylase, biocompatibility, and environmental compatibility (data not shown). Although some surfactants could improve the cephalexin partition, they were not used since the surfactants resulted in a high viscosity even at very low concentration [14]. A certain amount of methanol was added into the ATPS, because methanol is one of the products of PGME hydrolysis and thus inhibits the hydrolysis reaction [10, 16, 17]. The ATPS, composed of 20% PEG400-17.5% $(NH_4)_2SO_4$, methanol (1.5 M) and NaCl (0.5 M), indicates a potential for the extractive bioconversion of cephalexin. The ATPS should contain 0.1 M phosphate to maintain the medium pH at 6.0–6.5.

## Synthesis of Cephalexin in ATPS

Figure 2 shows the process of the enzymatic synthesis of cephalexin in the 20% PEG400-17.5% $(NH_4)_2SO_4$ ATPS, 20% PEG400-15% $MgSO_4$ ATPS, and the water system. The maximum cephalexin yield significantly increased in the two ATPS. The yield of cephalexin was 53% in the PEG400-$(NH_4)_2SO_4$ ATPS, and 58–60% in PEG 400-$MgSO_4$ ATPS, while 19% in the aqueous system. The yield reflected the equilibrium of three different catalytic activities of the PGA, including the synthesis of cephalexin, hydrolysis of cephalexin previously synthesized, and hydrolysis of the activated acyl donor [16, 17]. Since cephalexin is both a substrate and an inhibitor of PGA, the hydrolysis reaction of cephalexin can be promoted and the activity of PGA may be strongly inhibited when cephalexin accumulates around the immobilized PGA. Consequently, a lower transiently maximum yield of cephalexin in the

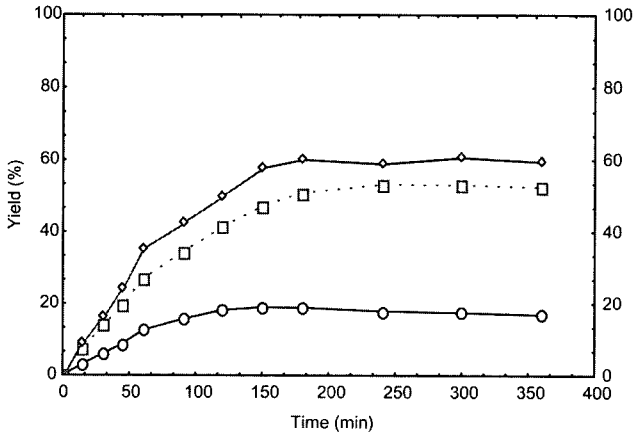


**Fig. 2.** Effect of PGME ratio on cephalexin yield in PEG-magnesium sulfate ATPS.
○, aqueous system; □, PEG400-$(NH_4)_2SO_4$ATS; ◇, PEG400-$MgSO_4$ ATPS.

aqueous system is observed. In the ATPS, cephalexin produced could be transferred into the PEG phase, therefore, the accumulation of cephalexin around the PGA (partitioned in the ammonium sulfate bottom phase) was avoided. As cephalexin was transferred from the vicinity of PGA, the reaction equilibrium shifted to favorable synthesis of cephalexin. As a result, the higher yield of cephalexin was achieved in the ATPS.

Most of the soluble enzyme would be precipitated at 17.5% ammonium sulfate concentration. Therefore, PGA immobilized on a glyoxyl-agarose was used for the bioconversion. This porous immobilized PGA was retained to reach equilibrium in the ammonium sulfate bottom phase for 10 days at 15°C, and used for the bioconversion. Odette *et al.* [12] carried out cephalexin synthesis in 100% PEG600-3 M ammonium sulfate ATPS, and similar yield was obtained, although partition coefficient of cephalexin was 23, which was higher than that of cephalexin in the 20% PEG400-17.5% $(NH_4)_2SO_4$ ATPS. When the free enzyme was used, however, the yield of cephalexin in 20% PEG400-15% $MgSO_4$ ATPS was slightly higher than 20% PEG400-17.5% $(NH_4)_2SO_4$ ATPS using the immobilized enzyme.

## Effect of Molar Ratio of PGMA to 7-ADCA on Synthesis Yield

The effect of concentration ratio of acyl donor (PGME) to the nucleophile (7-ADCA) on the maximum yields of cephalexin in the PEG400-$MgSO_4$ ATPS and in the aqueous system is shown in Fig. 3. The cephalexin yield increased with the increase in molar ratio of PGMA to 7-ADCA. The excess of acyl donor has a positive effect on improving the yields both in the ATPS and aqueous system due to it shifting the equilibrium of the reaction to the synthesis direction. At 150 mM PGME and 75 mM 7-ADCA, the yield of cephalexin in the ATPS was 85–87%,

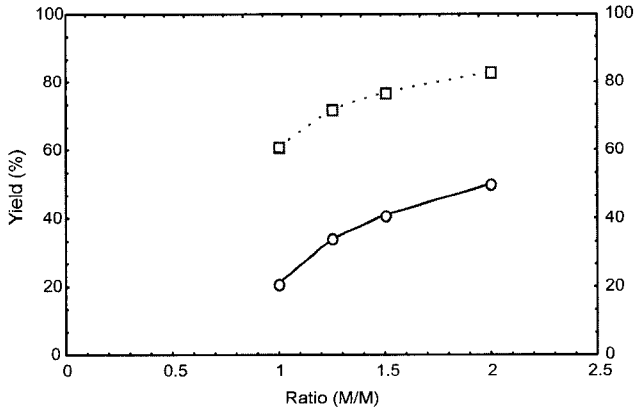


**Fig. 3.** Effect of PGME ratio on cephalexin yield in PEG-magnesium sulfate ATPS.
○, ATPS; □, aqueous system.

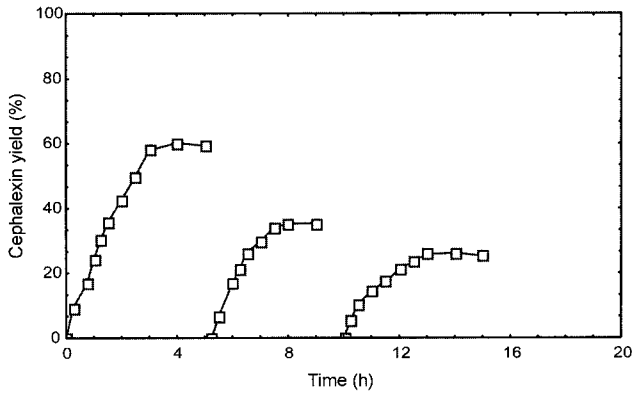


**Fig. 4.** Repeat batch synthesis of cephalexin in PEG-magnesium sulfate.

while the yield in the aqueous system was less than 50% at the same donor/nucleophile ratio (2:1). This also demonstrates that the extraction synthesis protected PGME and synthesized cephalexin from hydrolyzation. On the other hand, the excess ratio of PGMA to 7-ADCA increases the cost of cephalexin synthesis. As a fraction of PGME in solution or in ATPS is slowly hydrolyzed during the reaction, the ratio should be higher than 1:1 in order to obtain a reasonable yield of cephalexin synthesis.

## Repeat Batch Reaction

Repeat batch reactions were performed in the 20% PEG400-15% $MgSO_4$ ATPS by removing the top phase and replacing it with a new top phase, which contained the same initial composition of top phase with fresh substrates, and the result is shown in Fig. 4. Each batch bioconversion took 4 h. The maximum yield of cephalexin in the first batch was 60–62%, while that of the second and the third showed a decreased tendency. The free enzyme was probably inactivated and lost during the repeat batch bioconversions. The inactivation of PGA was demonstrated

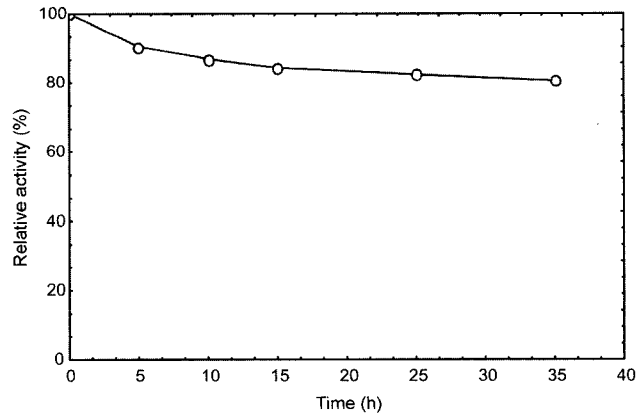


**Fig. 5.** Stability of penicillin acylase in magnesium sulfate solution.

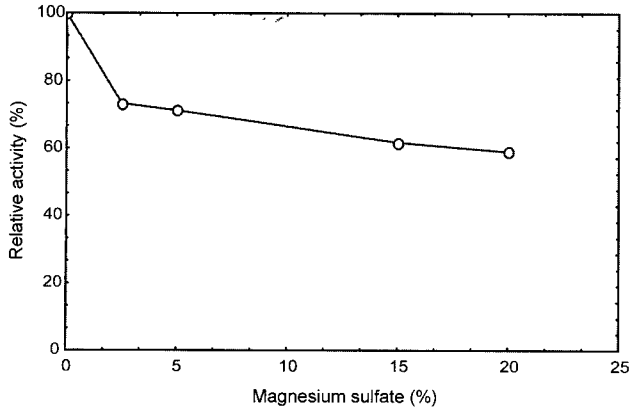


**Fig. 6.** Effect of magnesium sulfate on stability of penicillin acylase.

by following the stability experiment of PGA in PEG400-$MgSO_4$ ATPS. As shown in Fig. 5, the enzyme activity decreased as the incubation time of PGA in the ATPS increased, and 80.6% of the enzyme activity remained at 35 h (pH 6.5 and 15°C). The effect of concentration of $MgSO_4$ on PGA activity at pH 7.0 and 37°C was also studied (see Fig. 6), and the enzyme was found to be slowly inactivated in $MgSO_4$ solution. When the concentration of $MgSO_4$ increased to 20% (w/w), only 59% of the enzyme activity remained. On the other hand, the enzyme in the top phase was lost due to replacement of the fresh top phase and removal of the old top phase. Addition of new free enzyme can compensate the enzyme loss. If immobilized PGA is used, enzyme loss can be avoided [18]. Moreover, the degree of product inhibition increased for the second and the third repeated batch. Although the reaction in ATPS can reduce the product inhibition, the inhibition in the bottom phase still remained thus resulting in the decreased yield. Even though the cephalexin concentration in the bottom phase was the same as that in the aqueous phase, the cephalexin yield was higher than that in the aqueous phase due to much cephalexin transferred to the top phase.

## CONCLUSION

The biosynthesis of cephalexin was carried out in 20% PEG400-17.5% $(NH_4)_2SO_4$, 20% PEG400-15% $MgSO_4$ ATPS, and aqueous phase. The higher cephalexin yield was obtained in ATPS. An immobilized PGA has to be used in order to prevent the PGA from precipitating in the bottom phase of 20% PEG400-17.5% $(NH_4)$. In 20% PEG400-15% $MgSO_4$ ATPS, the free PGA was slowly deactivated during the repeated cephalexin synthesis. The enzyme stability would be improved if the enzyme were immobilized on a supporter.

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