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Separation of Phenylacetic Acid from 6-Aminopenicillanic Acid via Cloud-Point Extraction with *N*-Decyltetra(ethylene Oxide) Nonionic Surfactant

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ABSTRACT

The partitioning behavior of phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA) in the nonionic surfactant *N*-decyltetra(ethylene oxide) ($C_{10}E_4$)-based aqueous two-phase system has been investigated. The two-phase system is formed by raising the solution temperature above the cloud-point temperature of $C_{10}E_4$. PAA shows a high affinity toward the surfactant-rich top phase. The partition coefficient of PAA is much greater than unity and strongly affected by pH. The partition coefficient decreases significantly from 82.3 to 3.8 when the pH is increased from 3.3 to 8.0 in the system with 1% (w/w) $C_{10}E_4$ at 30°C. The partition coefficient of PAA also increases with temperature and the surfactant concentration. 6-APA, on the other hand, has a partition coefficient smaller than unity. It prefers to stay in the surfactant-lean bottom phase. A high degree of separation of PAA from 6-APA is achieved when penicillin-G hydrolysate is subjected to cloud-point extraction. The separation factor, defined as the ratio of the partition coefficient of PAA to that of 6-APA, is about 200 in the system with 2% (w/w) $C_{10}E_4$ at pH 8 and 30°C. Simultaneous enzymatic reaction and extraction is also carried out to reduce the inhibition effect of PAA toward enzyme penicillin-G acylase. However, the enzymatic reaction rate is not enhanced in this two-phase system.

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INTRODUCTION

When nonionic surfactant molecules are dissolved in water beyond a threshold concentration known as the critical micelle concentration (cmc), they spontaneously form aggregate structures known as micelles in the bulk aqueous solution. As the temperature is increased, the micellar solution separates into the surfactant-rich phase and the dilute aqueous phase. The temperature corresponding to the onset of phase separation, or the onset of turbidity, is called the cloud-point temperature. The cloud-point phase separation behavior of some nonionic surfactant solutions has been used as a liquid–liquid extraction step for the separation, concentration, and purification of a variety of substances including metal ions (1), organic substances (2), and proteins (3). One of the advantages of using cloud-point extractions is that only a small amount of nonionic surfactant is required to generate the two phases, which obviates the need to handle the usually large volumes of organic solvent required in traditional liquid–liquid extractions. The potential explosion and fire hazards, along with the cost, of the cloud-point extraction system are less than those of an organic–aqueous extraction system. Also, cloud-point extraction system can provide a mild and friendly environment to biomolecules since nonionic surfactants usually do not denature biomolecules (4, 5).

6-Aminopenicillanic acid (6-APA) is a very important intermediate of semisynthetic antibiotics such as ampicillin, amoxycillin, etc. It is usually produced from penicillin-G using the enzyme penicillin-G acylase (PGA) as shown in Fig. 1. Phenylacetic acid (PAA) is produced along with 6-APA as a by-product. Usually, 6-APA is recovered from the hydrolysate solution by precipitation at its isoelectric point, while PAA is removed by *n*-butyl acetate solvent extraction. Instead of using a conventional solvent extraction, in this paper we demonstrate the separation and concentration of PAA using cloud-point extraction. *N*-Decyltetra(ethylene oxide) ($C_{10}E_4$) is used as the nonionic surfactant because of the low temperature required for cloud-point formation

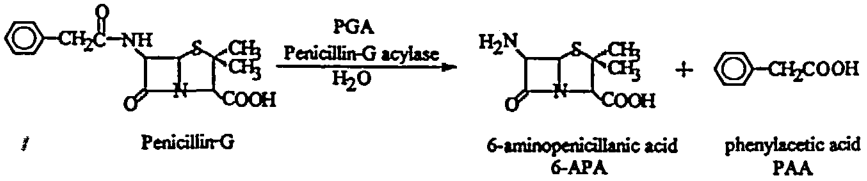

FIG. 1 Penicillin-G acylase catalyzes the hydrolysis of penicillin-G for the production of PAA and 6-APA.

(18°C) and its very low UV absorbance, since the concentration of analytes is determined with a UV spectrophotometer. The effects of pH, salt concentration, and surfactant concentration on the partitioning behavior of PAA, 6-APA, and penicillin-G are also evaluated.

PAA and 6-APA are known as inhibitors to the enzyme penicillin-G acylase (6). The hydrolysis rate of penicillin-G by enzyme PGA will be reduced in the presence of PAA or 6-APA. In order to alleviate the inhibition effect and enhance the hydrolysis rate, the techniques of polymer aqueous two-phase extraction (7) and electrodialysis (8) have been employed to remove PAA or 6-APA during the penicillin-G hydrolysis reaction. In this work we also perform simultaneous enzymatic reaction and cloud-point extraction to study the effect of cloud-point extraction of PAA on the PGA-catalyzed penicillin-G hydrolysis rate.

MATERIALS AND METHODS

Materials

N-Decyltetra(ethylene oxide) ($C_{10}E_4$) was purchased from Nikkol Chemical Co. (Kyoto, Japan). Phenylacetic acid and 6-aminopenicillin acid were obtained from Sigma Co. (St. Louis, MO, USA). Penicillin-G potassium salt was obtained from Nacalai Tesque (Kyoto, Japan). Immobilized penicillin-G acylase (PGA) was obtained from Recodati Co. (Milan, Italy). All other chemicals used were the best analytical grades available.

Cloud-Point Extraction

For the partitioning behavior study, the sample solutions were prepared by dissolving PAA, 6-APA, and penicillin-G separately in distilled water. For the study of separating PAA from 6-APA, the PGA-hydrolyzed penicillin-G solution was employed. The pH of the sample solutions was adjusted with HCl and NaOH using an autotitrator. In a typical extraction, a specified amount of surfactant was added into 5 g of a thermally equilibrated sample solution and shook vigorously for 1 minute. The micelle-containing solutions were then left in a water bath at various temperatures for 24 hours in order to have two separated homogeneous phases. Since the cloud-point extraction was carried out in a 10-mL graduated cylinder, the phase volume was determined by reading the scale of the graduated cylinder. An aliquot of solution was taken separately from each phase for determining analyte concentrations. The sample taken from the surfactant-rich phase, the top phase in this case, was properly diluted with distilled water to obtain a homogeneous solution if the UV-Vis absorbance method was employed for concentration determination. The partition coefficient is defined as the ratio of the analyte concentration in the top phase to that in the bottom phase. Since $C_{10}E_4$ has a very low absorbance at 280 nm, the concentrations of PAA and penicillin-G were determined by measuring absorbance at 280 nm using a UV-visible spectrophotometer (Shimadzu model 160A). The concentration of 6-APA was determined using the *p*-dimethylamino-benzaldehyde (PDAB) calorimetric method (9).

Simultaneous Enzymatic Reaction and Cloud-Point Extraction

The penicillin-G solution of 0.5% (w/v) was prepared in pH 8.0, 0.05 M phosphate buffer. The reaction solution was prepared by mixing 0.4 g nonionic surfactant with 18.6 g of penicillin-G solution in a 30°C water bath. Immobilized penicillin-G acylase of 0.1 g (wet weight) was added into the thermally equilibrated reaction solution to initiate the penicillin-G hydrolysis. A magnetic stirrer was employed to stir the heterogeneous reaction solution. The pH of the reaction solution was continuously maintained at 8.0 via an autotitrator (Kyoto Electronics, Model AT-200) using 0.05 N NaOH solution. For the control, where no surfactant is added, an additional volume of distilled water equal to that of the surfactant volume was inputted.

RESULTS AND DISCUSSION

Cloud-Point Extraction of PAA

The temperature versus surfactant concentration phase diagram of the $C_{10}E_4$ system was determined by Liu et al. (10) using the cloud-point method. The coexistence curve is a concave-upward bell shape and the lower critical point is located at 18.8°C and 2% (w/w) surfactant. When the temperature and surfactant concentration are within the coexistence curve, the micellar solution separates into two isotropic phases. The top phase is surfactant-rich and the bottom is surfactant-lean. The volume ratio of the two phases and the PAA partitioning behavior in this micellar two-phase system are shown in Table 1. The volume of top phase is much smaller than that of the bottom phase. It was observed that the volume ratio is not affected to a great extent by pH and temperature. But it increases significantly with the surfactant concentration. The partition coefficient of PAA, defined as the ratio of the PAA concentration in the top phase to that in the bottom phase, is much larger than unity. This indicates that PAA preferentially partitions into the surfactant-rich top phase. Its partition coefficient is strongly affected by pH, as shown in the 1% $C_{10}E_4$ system. The partition coefficient is 82.3 at pH 3.3 but decreases significantly to 4.4 and 3.8 as the pH increases to 6.4 and 8.0, respectively. The pK_a of PAA is about 4.25; in other words, only 10% of the PAA will be dissociated at pH 3.3 as its anionic acetate form. On the other hand, 99%

TABLE 1
Partitioning Behavior of PAA in the $C_{10}E_4$-Based Aqueous Two-Phase Systems[a]

$C_{10}E_4$ concentration, % (w/w)	pH	Temperature (°C)	Volume ratio, $G = V_T/V_B$	Partition coefficient, $K = C_T/C_B$
1	3.3	30	0.031	82.3
1	6.4	30	0.031	4.4
1	8.0	30	0.031	3.8
2	3.3	20	0.053	82.5
2	3.3	30	0.053	158.8
2	3.3	40	0.053	366.9
2	3.3	50	0.053	545.5
3	3.3	30	0.087	362.4
4	3.3	30	0.136	618.6

[a] Initial PAA concentration was 0.25% (w/v).

of the PAA will be dissociated at a pH higher than 6.4. The higher partition coefficient of PAA at a lower pH indicates that the neutral protonated PAA binds very well to the nonionic micelle-rich phase compared to the anionic form of PAA.

Theoretically, the initially loaded surfactant concentration will only influence the phase volume but not the concentration of surfactant in the two phases at equilibrium as long as the temperature is kept constant in the temperature-induced cloud-point extraction system. The partition coefficient of PAA should change with the equilibrium surfactant concentration in the two phases but not with the initially loaded surfactant concentration. However, it was observed that the partition coefficient of PAA increases from 82.3 to 618.6 when the initial surfactant concentration is increased from 1 to 4% (w/w) at 30°C. It was also observed that the volume of the micelle-rich phase decreases in the presence of PAA (data not shown). This fact indicates that PAA itself will affect the nature of the micelle-rich phase. This unusual change of partition coefficient with initial surfactant concentration may be attributed to the influence of PAA on surfactant concentration and the physicochemical properties of the micelle-rich phase. This influence will decrease the partition coefficient of PAA, and the extent of influence decreases with an increase of the initial surfactant concentration. When PAA concentration is decreased, the extent of influence of PAA on the nature of the micelle-rich phase will also decrease. As a consequence, the partition coefficient of PAA will increase. Figure 2 shows the effect of PAA concentration on its partition coefficient in a system with 1% (w/w) surfactant at 30°C. The partition coefficient of PAA increases from ca. 50 to 1500 when the PAA concentration decreases

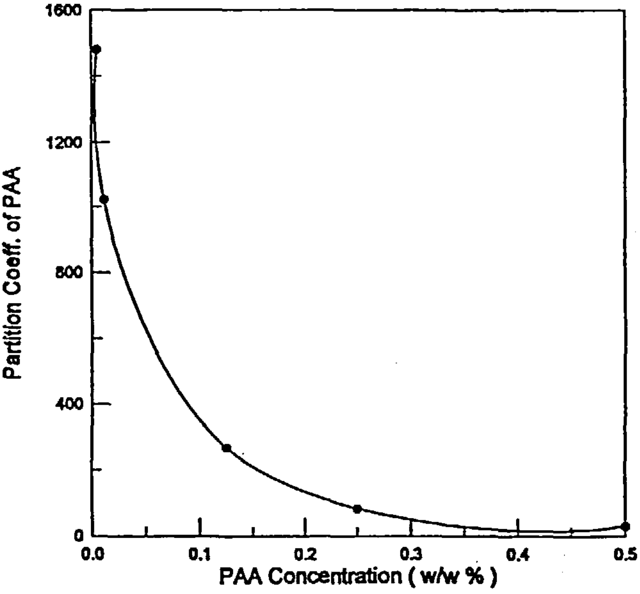

FIG. 2 Effect of PAA concentration on the partition coefficient of PAA in 1% (w/w) $C_{10}E_4$ system at pH 3.3 and 30°C.

from 0.5 to 0.005%. This shows that the partition coefficient of PAA is strongly affected by the ratio of initial PAA and surfactant concentration in the system.

The cloud-point phase separation of a nonionic surfactant is induced by increasing temperature. Temperature will affect the phase composition of the two-phase system. Based on the phase diagram of the $C_{10}E_4$ system, the top phase becomes richer and the bottom phase becomes leaner in $C_{10}E_4$ as the temperature is increased. The increase of micelle concentration will enhance the amount of PAA molecules being partitioned into the micelle phase and results in an increase of the partition coefficient. As shown in Table 1, the partition coefficient increases 7-fold from 82.5 to 545.5 as the temperature increases from 20 to 50°C in the system of pH 3.3 and 2% $C_{10}E_4$. The increase of the partition coefficient of PAA with temperature can also be attributed to the increase in hydrophobic interaction, since it is generally known that hydrophobic interaction can be enhanced by increasing temperature. Hydrophobic interaction between PAA and the micelle will be enhanced by an increase of temperature. Therefore, the binding capacity of a micelle for PAA increases with increasing temperature. The higher binding capacity of each micelle for PAA will result in an increase of the partition coefficient of PAA. The hydrophobic interaction can also be enhanced by water structure-making salts such as sodium sulfate (11). Figure 3 shows the effect of sodium sulfate concentration on the partition coefficient of PAA. The partition coefficient increases significantly with sodium sulfate concentration. This again shows the importance of the hydrophobic interaction between PAA and the micelle for determining the partition coefficient of PAA in the cloud-point extraction system.

Cloud-Point Extraction of PAA from PGA Hydrolyzed Penicillin-G Solution

It was found that nonionic surfactants do not bind to biomolecules to a significant extent and hence do not denature proteins (4, 5). Consequently, the $C_{10}E_4$-based aqueous two-phase extraction system can provide a mild and friendly environment for the enzyme penicillin-G acylase. In addition, the byproduct PAA, which is an inhibitor to penicillin-G acylase, can be effectively removed to the micelle-rich phase as shown earlier. Therefore, we expect the penicillin-G hydrolysis rate catalyzed by immobilized PGA will be enhanced

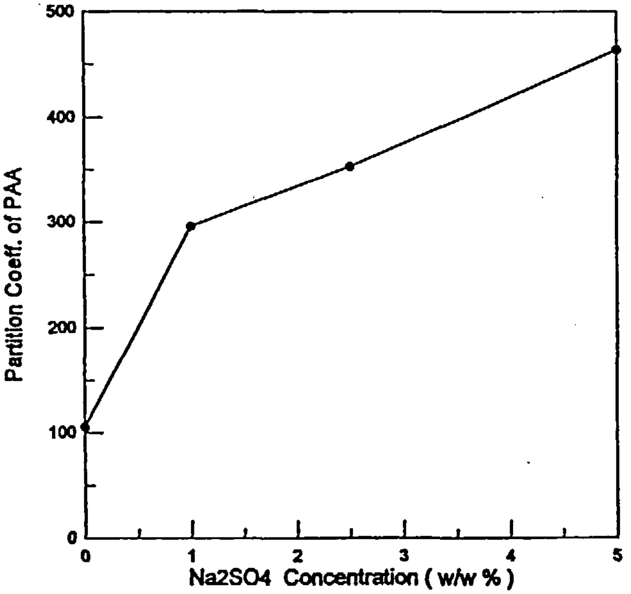

FIG. 3 Effect of sodium sulfate concentration on the partition coefficient of PAA at a constant PAA concentration of 0.25% (w/v) in the 1% (w/w) $C_{10}E_4$ system at pH 3.3 and 30°C.

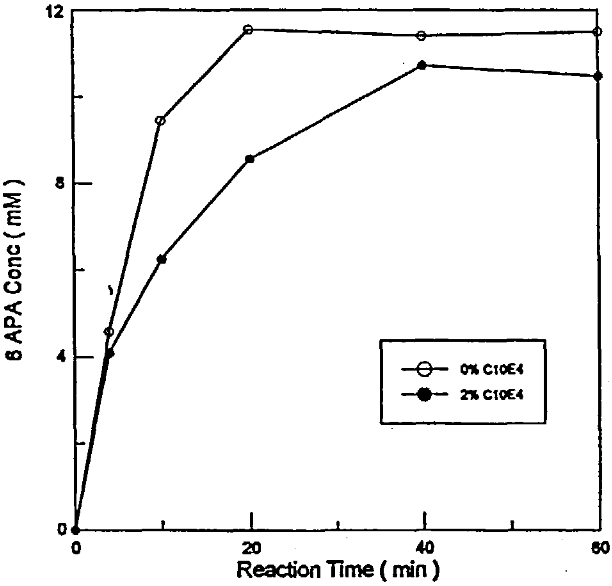

FIG. 4 6-APA production by the penicillin-G acylase catalyzed hydrolysis of penicillin-G in the presence and absence of $C_{10}E_4$. Initial penicillin-G concentration was 0.5% (w/v).

in the cloud-point extraction system. Figure 4 shows the effect of $C_{10}E_4$ addition on the 6-APA production rate in an immobilized PGA reaction system. In the absence of $C_{10}E_4$, the reaction is complete in 20 minutes. In the presence of $C_{10}E_4$, the reaction stops after 40 minutes. Obviously, the addition of $C_{10}E_4$ to initiate cloud-point extraction does not enhance the penicillin-G hydrolysis rate. The partitioning behavior of reactant penicillin G and product 6-APA in the system may affect the PGA reaction rate. As shown in Table 2, penicillin G and 6-APA have partition coefficients of 1 and 0.4, respectively, at pH 8.0. This indicates that 6-APA prefers the bulk aqueous phase rather than micelle-rich phase. The 6-APA concentration in the bulk aqueous phase, in which the immobilized PGA hydrolysis reaction takes place, will be higher in the reaction system with the addition of $C_{10}E_4$. 6-APA is also known as a noncompetitive inhibitor to the enzyme PGA (6). The increase of 6-APA concentration will inevitably slow down the PGA reaction rate. Besides, immobilized PGA was employed in this reaction system in which the PGA enzyme is immobilized inside a porous carrier. The aggregated micelle may block the diffusion path of reactant toward the active sites of immobilized PGA during reaction. As a consequence, the PGA reaction rate is reduced.

TABLE 2
Partition Behavior of Penicillin-G, 6-APA, and 0.5% (w/v) Penicillin-G Hydrolysate[a]

$C_{10}E_4$ concentration, % (w/w)	pH	Pure component		Hydrolysate	
		K_{PenG}	K_{6APA}	K_{PAA}	K_{6APA}
1	3.3	—	0.59	—	—
1	8.0	1	0.4	—	—
2	8.0	—	—	111.51	0.56

[a] Initial penicillin-G and 6-APA concentrations: PenG = 1% (w/v); 6-APA = 0.32% (w/v).

Cloud-point extraction has also been carried out in the penicillin-G hydrolysate solution to separate the product 6-APA from the by-product PAA. When the hydrolysis of 1% (w/v) penicillin G has been completed by immobilized PGA, extraction is carried out at 30°C by employing 2% (w/w) $C_{10}E_4$ in the hydrolysate. Table 2 shows the partition behavior of PAA and 6-APA in the system. Although the pH of the hydrolysate is at 8.0, PAA still has a partition coefficient of 111.5. The partition coefficient of 6-APA is 0.56. In other words, PAA prefers to stay in the surfactant-rich top phase and 6-APA, which is hydrophilic in nature, likes to stay in the water-rich bottom phase. The degree of separation of PAA and 6-APA in this cloud-point extraction system is very high as indicated by its separation factor of 200. The separation factor is defined as

$$\alpha = \frac{(C_t/C_b)_{PAA}}{(C_t/C_b)_{6\text{-}APA}} = \frac{K_{PAA}}{K_{6\text{-}APA}}$$

where C_t and C_b are the top- and bottom-phase concentrations, respectively.

The separation factor may be enhanced further by lowering the pH of the hydrolysate, since the partition coefficient can be increased 20-fold for PAA but only 1.5-fold for 6-APA as the pH is decreased from 8.0 to 3.3. The high separation factor makes this nonionic surfactant cloud-point extraction a very attractive technique for the separation and recovery of 6-APA from penicillin-G hydrolysate.

CONCLUSION

A cloud-point extraction system formed by raising the temperature of a $C_{10}E_4$ nonionic surfactant solution can be used to recover PAA from an aqueous solution. PAA prefers to partition into the surfactant-rich top phase. The partition coefficient increases as the pH of the solution decreases. By increasing the temperature, the partition coefficient of PAA can also be increased due to the surfactant concentration increase in the micelle-rich phase and the thermal-enhanced hydrophobic interaction. The salt-enhanced hydrophobic interaction in the system with sodium sulfate also increases the partition coefficient of PAA. The partition coefficient of PAA also strongly depends on the ratio of initial PAA and surfactant concentration. When the temperature is kept constant, the PAA partition coefficient increases with the decrease of the PAA-to-surfactant ratio. In contrast to PAA, 6-APA prefers to stay in the surfactant-lean bottom phase and hence shows a partition coefficient smaller than unity. When immobilized PGA-catalyzed penicillin-G hydrolysate is subjected to cloud-point extraction for the separation of 6-APA from PAA, a high degree of separation is achieved. The separation factor is about 200 for a 2% (w/w) $C_{10}E_4$ system at pH 8.0 and 30°C. Simultaneous PGA reaction and PAA extraction in a $C_{10}E_4$ two-phase system does not improve the penicillin-G hydrolysis rate.

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