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Enzymatic hydrolysis of penicillin and *in situ* product separation in thermally induced reversible phase-separation of ionic liquids/water mixture

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ABSTRACT

Enzymatic hydrolysis of penicillin G to produce 6-aminopenicillanic acid, key intermediate for the production of semisynthetic β-lactam antibiotics, is one of the most relevant example of industrial implementation of biocatalysts. The hydrolysis reaction is traditionally carried out in aqueous buffer at pH 7.5–8. However, the aqueous rout exhibits several drawbacks in enzyme stability and product recovery. In this study, several ionic liquids (ILs) have been used as media for enzymatic hydrolysis of penicillin G. The results indicated that hydrophobic ILs/water two-phase system were good media for the reaction. In addition, a novel aqueous two-phase system based on the lower critical solution temperature type phase changes of amino acid based ILs/water mixture was developed for *in situ* penicillin G hydrolysis and product separation. For instance, hydrolysis yield of 87.13% was obtained in system containing 30 wt% [TBP][Tf-ILe] with pH control (pH 7.6). Since the phase-separation of this medium system can be reversible switched from single to two phases by slightly changing the solution temperature, enzymatic hydrolytic reaction and product recovery were more efficient than those of aqueous system. In addition, the ILs could be reused for at least 5 cycles without significant loss in hydrolysis efficiency.

1. Introduction

6-Aminopenicillanic acid (6-APA) is a key intermediate for the production of semisynthetic β-lactam antibiotics such as ampicillin, amoxicillin, oxacillin and carbenicillin [1]. It is commercially produced by the hydrolysis of penicillin G catalyzed by the enzyme penicillin acylase (also known as penicillin amidase), with phenylacetic acid (PAA) is by-product (Fig. 1) [2]. Naturally, the enzymatic hydrolysis of penicillin G was carried out in aqueous buffer at pH 7.5–8. However, this system have several drawbacks such as (1) requirement to adjust the pH of reaction media due to the continuously production of acidic by-product PAA that led to the formation of waste salts and (2) reduction of enzyme activity due to inhibition effect of penicillin, 6-APA, and PAA [3].

Several hydrolysis processes integrated with the *in situ* product separation have been developed to eliminate the product inhibition. In general, these methods could be classified into membrane separation and liquid–liquid extraction. In the membrane separation method, hydrolysis reaction was carried out in separated membrane compartment and the produced PAA was continuously removed by electrodialysis [4–6] or perstraction [7] into another solvent phase or membrane compartment. On the other hand, additional salts or organic solvents were introduced to induce or form a separated phase to extract products from the hydrolysis mixture in the liquid–liquid extraction approach. For instance, Anderson and Hahn-Hagerdal developed integrated separation process using PEG/KH_2PO_4 aqueous two-phase (ATP) system. They found that the two products, 6-APA and PAA were extracted into polymer-rich phase, while the enzyme was remained in salt-rich phase, which significantly reduced the product inhibition [8,9]. Diender et al. [10] and Farreira et al. [11] utilized butyl acetate to extract PAA, while the penicillin was hydrolyzed in aqueous phase in butyl acetate/water two-phase system. The advantage of this system is that it was operated at pH around 3.5–4.4, which is close to the isoelectric point of 6-APA, thus allowed the spontaneous precipitation and separation of 6-APA. As a result, the reaction equilibrium was shifted toward the product side [12]. However, this system also showed several shortcomings such as low enzyme activity and stability at low pH and environmental concerns of using volatile organic solvents [10].

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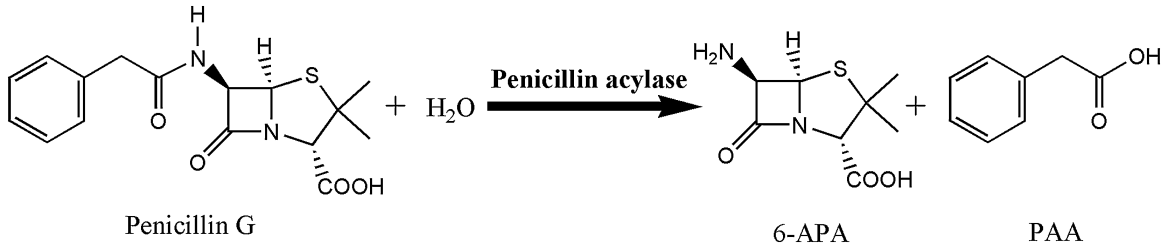

Fig. 1. Enzymatic hydrolysis of penicillin G.

Recently, penicillin hydrolysis in ionic liquids (ILs) containing system has been investigated [13–16]. It is widely acknowledged that ILs are liquid salts at room temperature. They have been extensively used as potential alternatives to toxic, hazardous, flammable and highly volatile organic solvents due to their unusual and useful properties in various application fields such as extraction, biotransformation, separation, *etc.* [17]. The enzyme penicillin acylase showed greater stability in ILs than in organic solvents. For example, a haft-life time of 23 h was observed in 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide ([Emim][Tf_2N]), which was about 200-fold higher than that in isopropanol [14]. In addition, integrated processes for penicillin hydrolysis in ILs have also been proposed. For instance, Jiang et al. [15] have developed mixed ILs/water ATP system for separation, recovery and hydrolysis of penicillin. In their study, penicillin from aqueous stream was first extracted into IL-rich phase using ILs aqueous two-phase (ILATP) system based on hydrophilic 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]) IL and NaH_2PO_4. A hydrophobic 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]) IL was then introduced into the IL-rich phase of ILATP system containing penicillin and converted it into mixed IL/water (MILW) system. Penicillin was hydrolyzed by penicillin acylase in the water phase of MILW system at pH 5. The by-product PAA was partitioned into the ILs mixture phase, while the intended product 6-APA was precipitated at the operated pH, thus benefited reaction equilibrium and enhanced the hydrolysis efficiency.

Despite the effectiveness of the above methods in minimizing enzyme inhibition and improving hydrolysis yield, they require additional equipment or reagents, which inevitably increase the operational cost and hamper the purification of product [15]. In the present work, the enzymatic hydrolysis of penicillin G was evaluated in ILs/water mixture and, of particular, in aqueous mixture of ILs that can be reversibly switched between single or two-phase by slightly changing mixture temperature. The ILs used in this study, which were developed by Fukumoto and Ohno [18], are derived from amino acids and exhibited phase separation with a lower critical solution temperature (LCST) characteristic (the solubility of ILs in water decreased as increasing temperature). For instance, tetra-*n*-butylphosphonium trifluoromethanesulfonyl leucine ([TBP][Tf-Leu]) shows phase separation with water at 25 °C, but is miscible at 20 °C [19]. The phase change temperature of these mixtures depends on the ion structures, and water content. In addition, it is lowered as increasing the hydrophobicity of ILs [18,20]. The interesting characteristics of the LCST ILs have been applied in various applications including extraction of biopolymers such as protein [19].

2. Materials and methods

2.1. Materials

Potassium penicillin G, 6-APA, PAA and penicillin amidase from *Escherichia coli* immobilized on Eupergit® C with activity of 100 U/g (1 U, according to the manufacture, corresponds to the amount of enzyme which hydrolyzes 1 μmol benzylpenicillin per minute at pH 7.6 and 37 °C) were purchased from Sigma–Aldrich. Hydrophilic ILs: 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]), 1-butyl-3-methylimidazolium trifluoromethanesufonate ([Bmim][TfO]), and hydrophobic ILs: 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]), 1-butyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide ([Bmim][Tf_2N]) with residual chloride content less than 10 ppm were provided by C-tri company (Korea). Tetra-*n*-butylphosphonium trifluoromethanesulfonyl isoleucine ([TBP][Tf-ILe]) was kindly provided by professor Hiroyuki Ohno (Tokyo University of Agriculture and Technology, Japan) [18]. All the ILs were dried in vacuum oven at 80 °C for 24 h before use. All other chemicals used in this work were of analytical grade and used without further purification.

2.2. Enzyme reaction

Penicillin G hydrolysis reactions were carried out in 5 mL glass vial containing 1 mL reaction media at 37 °C and stirring speed of 100 rpm in reaction block (Variomag, USA). The single or two-phase aqueous ILs solutions were prepared by dissolving ILs in 50 mM phosphate buffer pH 7.6. In a typical hydrolysis experiment, 50 mg potassium penicillin G was dissolved in 1 mL reaction media at 37 °C and reactions were started by adding 10 mg penicillin amidase. As [Bmim][Tf_2N], [Bmim][PF_6] and [TBP][Tf-ILe] (50 wt%) is immiscible with water at reaction temperature, hydrolysis reactions were performed in ILs/water ATP system where the enzymes are present in the ILs phase and potassium penicillin G is dissolved in water phase. Aliquot samples (20 μL) were taken at predetermined period and diluted with HPLC mobile phase. The concentration penicillin, 6-APA, and PAA were measured with HPLC. The activity of enzyme and conversion yield of the reaction was determined based on the concentration of penicillin G hydrolyzed. All the experiments were duplicated.

2.3. Enzymes and ionic liquids recovery

The enzymes were covered using centrifugation reaction media after hydrolysis. The enzymes were then washed with hexanes and dry under vacuum condition at room temperature for reuse.

For the recovery of ILs, [Bmim][Tf_2N] was recovered by decantation after hydrolysis reactions. The temperature of the [TBP][Tf-ILe]/water was raised up to 42 °C, allowed the clear phase separation, and [TBP][Tf-ILe] was recovered by decantation. The products in ILs were extracted by excess amount of hexane. ILs were dried under vacuum condition at room temperature before reuse.

2.4. Analytical methods

The partition coefficient of penicillin, 6-APA, and PAA in IL/water were determined by adding 10 mg of each compound in 1 mL mixture containing equal volume of ILs and water. The solution was stirred and incubated at 37 °C for 5 min in reaction block. The mixtures were centrifuged and the concentrations of each compound in ILs and water phase were determined by HPLC. The distribution coefficient, *K*, of each compound was calculated as follows

$$K = \frac{\text{solute concentration in IL phase}}{\text{solute concentration in aqueous phase}}$$

Concentration of penicillin, 6-APA, and PAA were determined using Shimadzu HPLC system equipped with Water symmetric C18 column (5 μm, 4.6 mm × 250 mm) and UV detector at 254 nm. The mobile phase was composed of acetonitrile: 20 mM phosphate buffer at pH 3.0 (60:40 v/v) and operated at flow rate of 0.6 mL/min at 20 °C.

3. Results and discussion

Fig. 2 shows the time course of enzymatic hydrolysis of penicillin G in aqueous and ILs containing media. The reactions quickly reached equilibrium within an hour in both aqueous buffer media and ILs containing media. However, the enzyme activity in media containing 50 wt% ILs were lower than that