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Enzymatic Hydrolysis of Penicillin for 6-APA Production in Three-Liquid-Phase System

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Abstract A dodecane/thermosensitive polymer/water three-liquid-phase system was introduced for enzymatic hydrolysis of penicillin G (Pen G) for 6-aminopenicillanic acid (6-APA). The enzyme was covalently attached to the terminal of PEO–PPO–PEO polymer (L63), which would be transferred into a polymer coacervate phase at high temperature above its "cloud point". 6-APA was primarily resided in the aqueous phase due to its zwitterionic nature. More than 70% phenylacetic acid (PAA) was transferred into the organic phase using trioctylmethylammonium hydroxide and trihexyl-(tetradecyl)phosphonium bis 2,4,4-trimethylpentylphosphinate ionic liquids (Cyphos IL-104) mixture at pH 5.5, while most of Pen G resided in water. As a result, high operational pH was permitted in three-liquid-phase system, which leads to higher enzymatic activity (120 IU at 40°C) and stability (enzymatic half-time up to 55 h at 60°C) in comparison with the value in butyl acetate/water two-phase system. On the other hand, two products in three-liquid-phase system might be automatically separated from the enzyme sphere into different phases at the same time, which facilitated the reaction equilibrium towards the product's side with 6-APA productivity of 80% at 42°C, pH 5.5.

Keywords Three-liquid-phase system · Covalent attachment · PEO–PPO–PEO polymer (L63) · Ionic liquids (Cyphos IL-104) · Penicillin G acylase · 6-aminopenicillanic acid (6-APA)

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Introduction

6-aminopenicillanic acid (6-APA) is an important biomaterial for manufacturing semisynthetic antibiotics, such as ampicillin, oxacillin and carbenicillin [1–3]. Usually, it is produced from the hydrolysis of Pen G with penicillin G acylase (PGA) in water at pH 7–8 [4] (Fig. 1). The purification procedure for 6-APA follows extraction with MIBK (methyl isobutyl ketone) at pH 2, concentration at pH 8, and precipitation of product 6-APA at pH 4 (the isoelectric point, IEP) [5]. There were several drawbacks existing in the aqueousv 6-APA route: The byproduct PAA would acidify the pH, which led to low enzymatic activity and stability in water [6]. If pH-adjustment was employed to maintain pH at 7–8, waste salt will produce. Secondly, Pen G, PAA and 6-APA were strong inhibitors for PGA [7], which leads to a low product yield during the reaction [8].

Different strategies have been developed to "*in situ*" separate the products from the enzyme solution to overcome these drawbacks in single-phase water system. Gaidhani and Pangarkar used long-chain tertiary amine to extract PAA during the enzymatic reaction [9]. Rindfleisch developed a hollow fiber membrane instrument to separate 6-APA from the enzymatic solution [10]. Bora used Aliquat-336 in butyl acetate phase for recovery of 6-APA from the aqueous solution [5]. Wyss employed liquid-core capsules dispersed in water to extract PAA during the reaction [11]. Wang has built a Triton X-114/water aqueous two-phase system and separated PAA from the catalytic aqueous phase continuously [12]. Liao employed polymer-salt aqueous two-phase system to hydrolyze Pen G with cell-immobilized PGA, in which the enzyme distributed in the salt-rich phase, while the products 6-APA and PAA were automatically separated into the polymer-rich phase [13]. Despite obvious advantages in these "*in situ*" systems, they could only separate one kind of products from the enzyme phase, leaving another product resided in enzyme phase. Therein, the enzymatic activity and 6-APA yield were greatly inhibited by the coexisting product, and further separation between the product and enzyme was required.

To efficiently separate the reaction products from the enzymatic solution simultaneously, Diender developed a butyl acetate (BA)/water two-phase system [14]. In that system, Pen G was hydrolyzed by PGA in aqueous bottom phase, while the by-product PAA was transferred into butyl acetate phase. The operational pH in this system was around 3.5–4.4, which protonated the acidic product PAA into the organic phase while maintained pH constant in water [15]. The two-phase system exhibits excellent ability to "*in situ*" separate 6-APA from the aqueous phase at pH close to IEP [16, 17]. Taking advantage of the efficient separation of 6-APA and PAA from the enzymatic solution, the reaction equilibrium in butyl acetate/water two phase system could be shifted towards the product's side, lowering the inhibition in enzyme solution [18].

Despite evident advantages for BA/water system, it also exhibited several shortcomings. Low pH was employed for 6-APA precipitation, which might lead to low enzymatic activity and stability [19]. It was reported that PGA lost its 60% activity at pH below 4 [11].

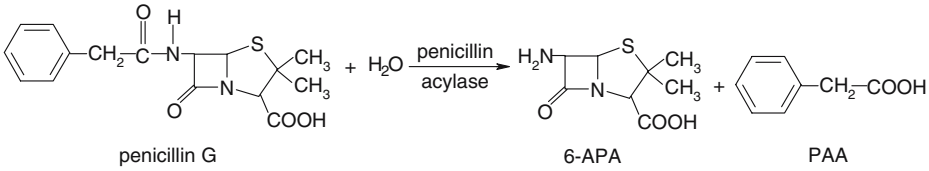

Fig. 1 Enzymatic hydrolysis of penicillin G

Secondly, low pH reduced the substrate's stability. Reschke observed that the half-life of Pen G was 32 h at pH 6, while it reduced to 18 min at pH 3 [20]. To increase the stability of Pen G in BA/water system, low temperature at 25°C had to be employed [14], which resulted in a relatively low 6-APA yield [21]. Thirdly, for applications, optimum pH required for this two-phase system was not feasible because over 30% PAA resided in the aqueous phase under the physical extraction condition, which might lower the system pH (less than 3.5), while reducing catalytic activity. In addition, the thermodynamic equilibrium of Pen G hydrolysis would be shifted to β-lactam antibiotics synthesis at a low pH [22], thereby lowering the 6-APA productivity.

A novel three-liquid-phase system was proposed in this research to decrease the product's inhibition and increase the operational pH in enzyme solution. The three-liquid-phase system comprised of a top dodecane phase, a middle aqueous phase and a bottom PEO–PPO–PEO polymer-rich phase (Fig. 2). Since catalyst was covalent attached to the terminal of thermo-reversible PEO–PPO–PEO chain [23], it could be immobilized in the polymer-rich phase at temperature above the "cloud point" of polymers [24] (Fig. 2). Shimoboji has linked an endoglucanase 12A with temperature-sensitive *N*-Ndimethylacrylamide/*N*-4-phenylazo-phenylacrylamide copolymers (DMAAm). The system exhibited similar activities below and above the "cloud point" of polymer-conjugated enzyme [25]. Chen modified β-D-glucosidase with a thermo-reversible *N*-isopropylacrylamide (NIPAAm) oligomer, which showed very high retention activity and improved thermal stability compared to native enzyme [26]. On the other hand, 6-APA was resided in water phase in a wide pH range, which could be due to its zwitterionic nature. Another product PAA was extracted into the dodecane phase using of 20% trioctylmethylammonium hydroxide and 80% Cyphos IL-104 mixture at pH 5.5 (Fig. 3). The result is in line with previous experiments that the strong interactions existed within trioctylmethylammonium cation and PAA^- anion via ion metathesis at neutral pH [27]. To reduce the co-extraction of

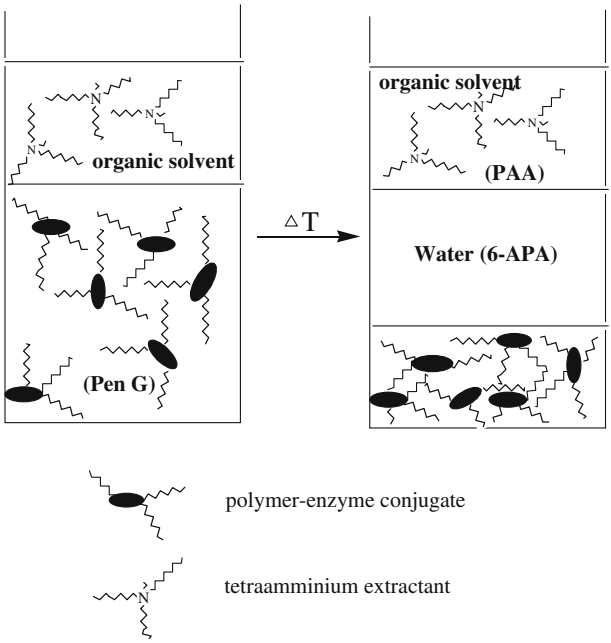

Fig. 2 Formation of three-liquid-phase system at high temperature

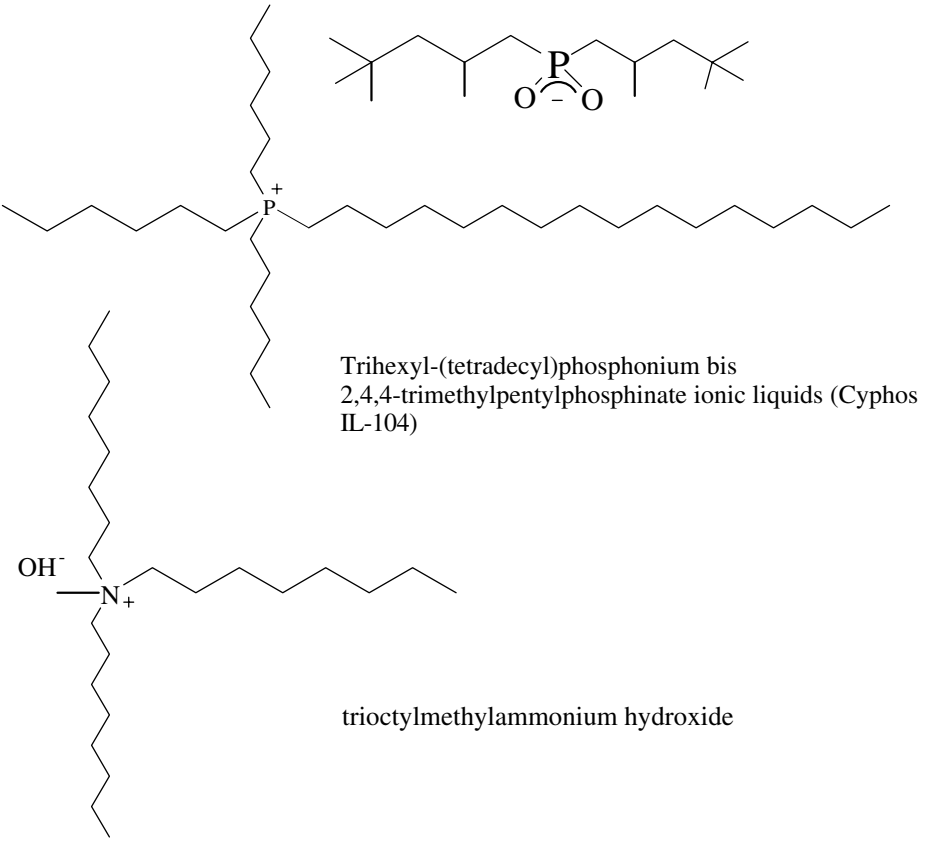

Fig. 3 The structure of trioctylmethylammonium hydroxide and Trihexyl-(tetradecyl)phosphonium bis 2,4,4-trimethylpentylphosphinate ionic liquids (Cyphos IL-104)

Pen G^- anion into the organic phase by trioctylmethylammonium hydroxide [28], neutral ionic liquids Cyphos IL-104 was introduced as coextractants, which was capable of extracting uncharged molecules into the organic phase, leaving most of charged compounds in water [29]. The combination of trioctylmethylammonium hydroxide and Cyphos IL-104 could provide efficient aid for separating PAA into the organic phase, while leaving most of Pen G in water (see the Supporting Information). As a result, the selectivity between PAA and Pen G in three-liquid-phase system was greatly improved, which facilitated the reaction equilibrium shift towards the product's side.

Three-liquid-phase system is a newly developed medium in the fields of separation and catalysis. Shen has employed a three-liquid-phase system to separate Pen G from its fermentation broth [30]. He found that PAA and Pen G had different affinities for the polymer and organic phases, respectively. Lee built an oil/nonionic amphiphile/water three-liquid-phase system, and measured the conductivity under various conditions [31]. Hu has successfully employed TBP/kerosene/H_2SO_4 three-liquid-phase to prepare ultrafine powder of TiO_2 [32]. In this work, a three-liquid-phase system was employed for carrying out the enzymatic catalysis of Pen G for production of 6-APA. The novel system might present several advantages over the single aqueous phase or BA/water two-phase system. Firstly, enzyme, 6-APA and PAA were separated into different phases, whereby preferentially

decreasing the product inhibition and shifting the reaction equilibrium toward the product's side. Secondly, relatively high pH was permitted in three-liquid-phase system for extracting PAA from the enzymatic phase with the aids of ionic liquids extractant, which could result in higher enzymatic activity and stability in comparison with that in the BA/water system. In addition, the OH^- anion, which is dissociated from trioctylmethylammonium hydroxide, could neutralize the H^+ ion dissociated from PAA molecule, as a result keeping pH constant in three-liquid-phase system without pH-adjustment.

Materials and Methods

Materials

L63 PEO–PPO–PEO polymer, MW=5,000, purity >99%, was purchased from BASF company; trioctylmethylammonium hydroxide, purity >95%, purchased from Chuangqi Chemical Company, Beijing. Trihexyl-(tetradecyl)phosphonium bis 2,4,4-trimethylpentyl-phosphinate (Cyphos IL-104), purity>95%, was from Sigma-Aldrich company. P-dimethylaminobenzaldehyde (PDAB), A.R., was from Beijing Chemical Company. Pen G salt was a gift from North China Pharmaceutical Group. PGA with activity of 180 IU/ml, protein concentration 28 mg/ml, was purchased from Haider Biochemical, Zhejiang province. One IU of enzyme was defined as the amount of enzyme that converts 10^{-6} mol per min of 1% (*w*/*v*) Pen G in a 50 mM phosphate buffer at pH 7.2 and 37°C.

Preparation of L63-PGA Conjugation

The structure of PGA has been known for long time [33]. It has 41 Lys groups (36 of them exposed to the medium) [34], which might be employed as a matrix for immobilizing PEO–PPO–PEO polymer through a 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) spacer. The input molar ratio of L63 to PGA was 5:1. In a typical experiment, 6 ml enzyme solution was dissolved in 8 ml of 50 mM, pH 6.5 sodium phosphate buffer containing 50% (*v*/*v*) dimethylsulfoxide (DMSO), followed by centrifugation at 3,000 rpm for 5 min 0.1 g L63 PEO–PPO–PEO was added into 11 ml of the above enzyme solutions, followed by centrifugation at 3,000 rpm for 5 min. Then 20 mg of EDC was added into 10 ml of the supernatant. After reaction at room temperature for 3 h, the solution was dialyzed with 50 mM, pH 7.0 sodium phosphate buffer for 24 h to remove residual reagents. The unconjugated PGA was removed by ultrafiltartion (MWCO=100 k) using Microcon Centrifugal Filter Devices (Millipore). The amount of conjugated and unconjugated protein was determined by BCA method [35], according to which the conjugation yield was determined. The results suggest that each PGA molecule was modified with 4–5 polymers.

Phase Separation of Pen G, 6-APA, PAA and L63-PGA in Three-Liquid-Phase System

About 0.1 g Pen G, 0.058 g PAA, 0.043 g 6-APA and 0.1 g L63-PGA were dissolved into 10 ml aqueous solution, which was in equilibrium with 5 ml dodecane containing 0.15 g trioctylmethylammonium hydroxide and 0.6 g Cyphos IL-104. The initial pH in this system was adjusted using 0.1 mol/l phosphate acid solution as determined by Metrohm pH meter. As temperature raised above 40°C, three-liquid-phase system was formed. The system was stirred and centrifugated for 10 min until phase reached equilibrium. Volume of each phase

was carefully observed. The 6-APA concentration in each phase was measured by PDAB method [36]. The Pen G and PAA concentration in different phases were measured using HPLC (HP1100 system, Agilent corporation equipped with 250×4 mm Zorbax SB-C18 column and DAO detector at 254 nm, the mobile phase was methanol/0.05 M phosphate at pH 3 at 36:64 v/v, flow rate at 1 ml/min). The mass fractions of compounds in each phase as obtained from Eq. 1:

$$w_{j:i} = \frac{C_{j:i}V_{j:i}}{\sum_j \left(C_{j:i}V_{j:i}\right)} \times 100\% \tag{1}$$

where i denotes compounds such as Pen G(1), PAA(2), 6-APA(3) and conjugated enzyme (4). j denotes the different phases in this system. $w_{j:i}$ represented the mass fraction of compound i in the j phase. $C_{j:i}$ and $V_{j:i,}$ represented the concentration and phase volume for compound i in the j phase, respectively.

Enzymatic Activity and Stability in Single, Two-Phase and Three-Liquid-Phase Systems

L63-PGA (0.1 g) was incubated in 10 ml aqueous solution at equilibrium with 5 ml dodecane containing 0.1 g trioctylmethylammonium hydroxide and 0.4 g Cyphos IL-104. Phosphate acid solution of 0.1 mol/l was used to adjust the pH. The temperature was increased to 40°C to form the three-liquid-phase system. 0.1 g Pen G was introduced into the system and the reaction was started. After 5 min, the reaction was stopped and the volume in each phase was carefully measured. The 6-APA concentration in each phase was measured by PDAB method [36]. The enzymatic activity was calculated from the overall 6-APA amount obtained during the first 5 min [37].

To measure the overall 6-APA productivity in three-liquid-phase system, similar procedure was repeated as described above, but the reaction time was prolonged to 200 min for accomplishing the reaction. The pH value in three-liquid-phase system was continuously monitored during the course. The 6-APA productivity was calculated using Eq. 2.

$$Y = \frac{\sum_j C_{j:3}V_{j:3}}{w_1^0 M_3/M_1} \times 100\% \tag{2}$$

where Y referred to the productivity of 6-APA, w_1^0 was the initial weight of Pen G. M_1 and M_3 were the molar weight of Pen G and 6-APA.

To measure the half-life of enzyme in the three-liquid-phase system, 10 ml aqueous solution containing 0.1 g L63-PGA was in equilibrium with 5 ml dodecane containing 0.15 g trioctylmethylammonium hydroxide and 0.6 g Cyphos IL-104. The system was incubated at certain temperature for 30 min, and the relative activity at the beginning and the end of the incubation time was determined. The half-life of enzyme was calculated according to Eq. 3 [38].

$$t_0 = -\ln 2{*}0.5 \Big/ \ln\left(\frac{A_{30}}{A_0}\right) \tag{3}$$

where t_0 was the half-life (hour) of enzyme in three-liquid-phase system. A_0 and A_{30} were the enzymatic activities at the beginning and after 30 min of reaction.

To measure the enzymatic activity, stability and 6-APA productivity in single aqueous phase system (or BA/water two-phase system), 0.1 g Pen G and 8 μl enzyme solution were

added into 10 ml water containing 0.05 mol/l pH 7 phosphate buffer (or 10 ml water at pH 4 in equilibrium with 5 ml butyl acetate). No pH-adjustment was used in these systems.

Kinetic Parameters in Single, Two-Phase and Three-Liquid-Phase

To measure the kinetic parameters in three different systems, 10 ml single aqueous phase with 0.05 M phosphate buffer (pH 7), 10 ml aqueous solution (pH 4) in equilibrium with 5 ml butyl acetate (two-phase), and 10 ml aqueous solution (pH 5) in equilibrium with 5 ml dodecane containing 0.1 g trioctylmethylammonium hydroxide and 0.4 g Cyphos IL-104 (three-liquid-phase) were prepared. 80 μl enzyme solution was added into the single or two-phase system, while 0.1 g L63-PGA (containing the same amount of enzyme) was used in three-liquid-phase system.

Parameters K_m and V_{max} were measured by varying the substrate concentration from 5 to 30 mmol/l, while substrate concentration from 0.2 to 0.5 mol/l was employed for determining the parameter K_{is}. The enzymatic activity was measured at different substrate concentrations by the preliminary method. The kinetic parameter was obtained from Eqs. 4 and 5 using the enzymatic activity during the initial 5 min [39].

$$\upsilon_1 = \frac{V_{max} S}{K_m + S + S^2/K_{is}} \tag{4}$$

$$\frac{1}{\upsilon_1} = \frac{K_m}{V_{max}} \frac{1}{S} + \frac{1}{V_{max}} + \frac{S}{V_{max} K_{is}} \tag{5}$$

υ_1 was the initial reaction ratio, S was the substrate concentration, K_m was the Michaelis constant, referring to the binding ability of enzyme to the substrate; V_{max} was defined as the maximum enzymatic reaction rate; K_{is} was the inhibition constant of substrate.

Results

Extraction Behavior of Pen G, 6-APA, PAA and L63-PGA Conjugate in Three-Liquid-Phase System

Figure 4 showed the mass fractions of Pen G, PAA, 6-APA and L63-PGA conjugates in different phases of three-liquid-phase system. It was found that Pen G preferentially stayed in organic phase at pH below 4. In the pH range of 4~6, Pen G equally distributed in the three equilibrium phases. When pH raised above 7, most of Pen G partitioned into the water phase. The distribution of PAA in three-liquid-phase is in similar manner as that of Pen G, despite its favorable pH to reside in organic phase was extended to 5–7. Such different partitioning behaviors would be attributed to the higher pKa of PAA molecule compared with Pen G, which would be preferably accumulated in hydrophobic organic phase at pH 5.5 [30]. In contrast, 6-APA primarily resided in water due to its zwitterionic nature [6], which facilitated its separation from PAA and the catalyst after the reaction. The covalent linkage between catalyst and polymer enabled itself to be immobilized in polymer coacervate phase in a wide pH range, which resulted in a low inhibition from the two products. The slight increase of L63-PGA concentration in organic phase at acidic pH may be due to the protein emulsion in the W/O interface [40].

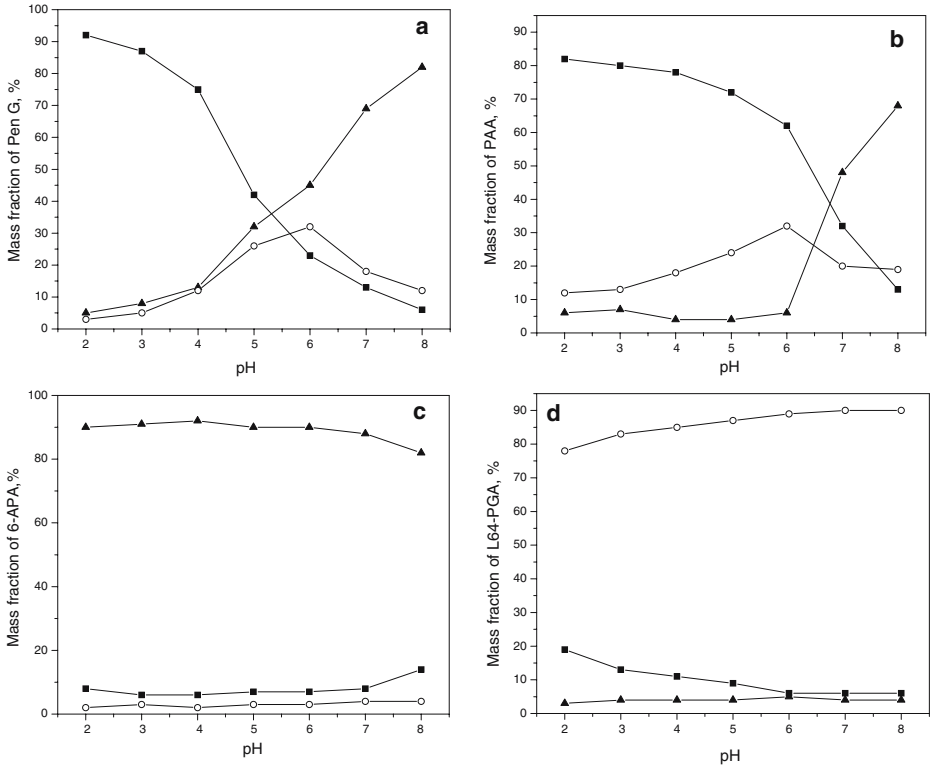

Fig. 4 Mass fractions of Pen G (**a**), PAA (**b**), 6-APA (**c**), and L63-PGA (**d**) in the three-liquid-phase system. *Filled square*, top organic phase; *filled triangle*, middle water phase; *open circle*, bottom polymer phase

Due to more than 90% 6-APA and 70% PAA were separated from the enzyme phase at pH 5.5, the product inhibition and reaction equilibrium in three-liquid-phase system were greatly improved as compared to that in single water phase or BA/water two-phase system.

Enzymatic Activity of L63-Conjugated Enzyme

Figure 5 shows the enzymatic activity observed in the single aqueous phase at pH 7, the BA/water system at pH 4 and the three-liquid-phase system at pH 5.5, respectively. Native PGA was employed in the two pervious systems, while L63-PGA conjugate was used in three-liquid-phase system. It was found that the enzymatic activity in single aqueous system increased proportionally with increasing temperature, and reached its optimal value 200 IU at 50°C. After that, the enzymatic activity decreased rapidly, which may be due to the distortion of enzymatic structure at high temperature [41]. In BA/water two-phase system, the enzymatic maximum activity was 50 IU/g at 30°C, and the activity decreased continuously with increasing temperature. This relatively low activity at operational temperature (30°C) in BA/water system might be related to the destroyed enzymatic structure at acidic environment. The enzymatic activity in three-liquid-phase system increased proportionally with temperature, and reached its maximum 120 IU at 40°C. After that, the enzymatic activity decreased slowly. The turnover of L63-PGA activity at 40°C

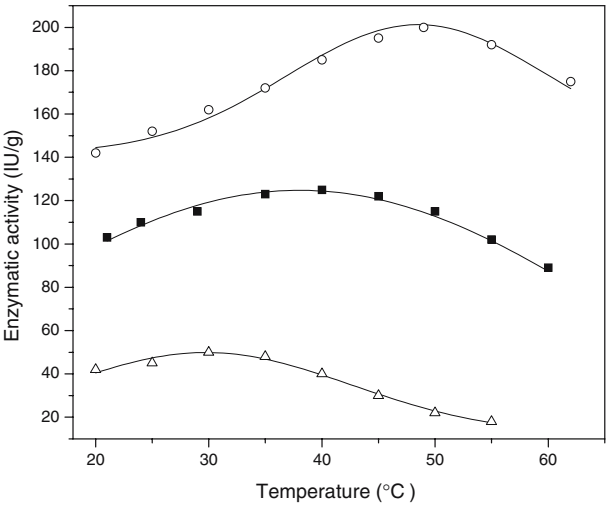

Fig. 5 Comparison of enzymatic activities in three systems. *Open circle*, native PGA in water at pH 7; *open triangle*, native PGA in BA/water system at pH 4; *filled square*, PEG-modified PGA in three-liquid phase at pH 5.5

might be due to the phase separation of polymer-enzyme conjugates from the substrate solution at high temperature, which increased the diffusional resistance of the substrate into the catalytic center surrounded by a layer of polymer [42].

Enzymatic Stability of L63-Conjugated Enzyme

Figure 6 explained the comparative enzymatic stability in the three systems mentioned above. In single aqueous phase, the enzymatic stability remained nearly constant at temperature

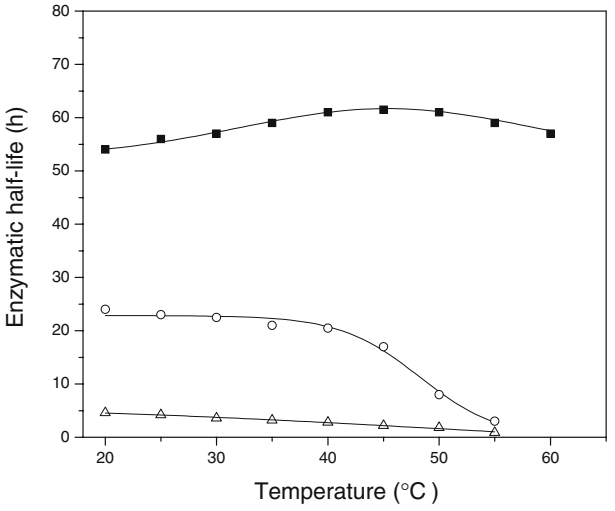

Fig. 6 Enzymatic stabilities in three systems. *Open circle*, native PGA in water at pH 7; *open triangle*, native PGA in BA/water system at pH 4; *filled square*, L63-PGA conjugate in three-liquid phase at pH 5.5

below 40°C. After that, the half-life of enzyme decreased dramatically from 20 to 2.8 h when increasing the temperature to 55°C. In BA/water system, over-low pH resulted in a low level of enzymatic stability in a wide spectrum of temperature. The L63-PGA conjugates in three-liquid-phase system exhibited significant improvement of enzymatic stability comparing with its native counterpart. This might be due to the polymer layer immobilized on the enzyme's surface [43], which protected the catalyst from the negative effect of organic solvent [44].

Effect of Pen G Concentration

The effect of substrate concentration on the enzyme activity was explored in Fig. 7. It was observed that the enzymatic activity in single aqueous phase decreased dramatically when Pen G concentration exceeded 8% [6]. While in BA/water system, the concentration limitation of Pen G for enzymatic inhibition extended to 15%. In three-liquid-phase system, stable enzymatic activity was observed when Pen G concentration is below 23%. The random distribution of Pen G in three separated phases might be the primary reason for low substrate inhibition in three-liquid-phase system during this process (Fig. 4).

Kinetic Parameter of Polymer-Modified Enzyme

Table 1 showed the kinetic parameters for Pen G hydrolysis in three systems. Native PGA in single aqueous phase had the highest reaction rate (V_{max}) and strongest binding ability for substrate (K_m), but it suffered from the strong substrate inhibition, which led to a low activity at 8% substrate concentration or more. BA/water two-phase system preserved 25% enzymatic activity comparing with that in single aqueous phase, while three-liquid-phase system maintained 65% residual activity. The difference of enzyme activity was in line with the sequential decrease of pH in the three systems: single aqueous phase (pH 7)>three-liquid-phase (pH 5.5)>BA/water (pH 4).

The binding ability of PGA for substrate (K_m) in BA/water two-phase system is similar as that in single aqueous system, while it decreased evidently in three-liquid-phase system,

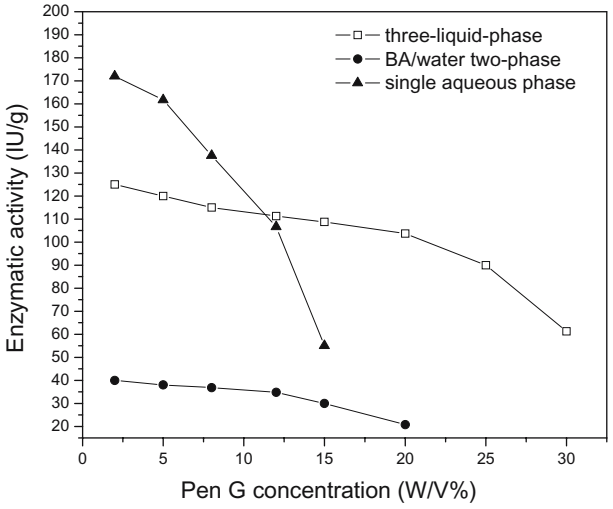

Fig. 7 Effect of initial Pen G concentration on the enzymatic activities in the three systems

Table 1 Comparison of the dynamic constant for penicillin acylase in different systems.

System	V_{max}(μ M/min)	K_m(mM)	K_{is}(mM)
Single aqueous phase	186	0.5	89
BA/water two-phase	43	0.8	42
Three-liquid-phase	126	1.9	23

which may be due to the diffusional resistance of substrate in the attached polymer shell on the enzyme [45].

The substrate inhibition in BA/water system was 50% lower than that in the single aqueous system, while the residual inhibition leave only 30% as that in single aqueous phase. The decrease in substrate inhibition in two-phase or three-phase system may be due to the random distribution of Pen G in the equilibrium phases, which preferentially reduced the substrate concentration in the enzyme phase [46].

The Yields of 6-APA

Figure 8 showed the 6-APA production efficiency in four different systems. In single aqueous system containing 0.2 mol/l phosphate buffer (original pH is 7.3 without pH-adjustment during the reaction). The 60% productivity was reached after 100 min. The unsatisfying yield may be due to the accumulation of acidic PAA in aqueous phase, which decreased pH and shorten the enzyme life-span (Fig. 9). Although 6-APA yield increased to 80% in BA/water system after 160 min, the initial rate was smaller than that in single aqueous system. The primary reason may be that the residual PAA in water caused the fluctuation of pH from 4.3 to 3.6 (Fig. 9), and led to a low enzymatic activity [18]. In contrast, the improved initial rate and final 6-APA yield in three-liquid-phase system may be due to the high system efficiency for transferring PAA into the organic phase at pH 5.5

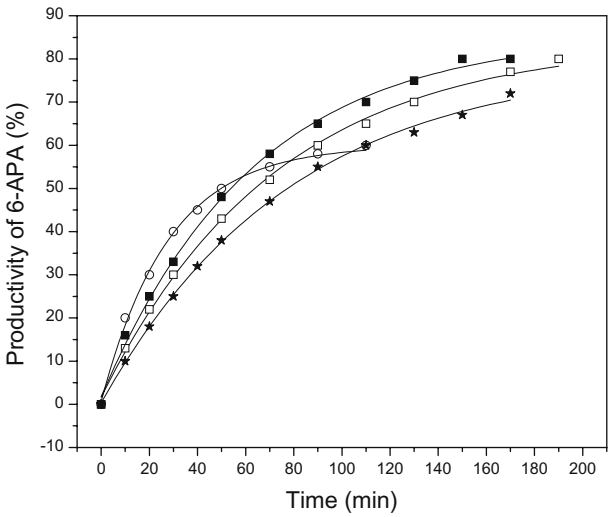

Fig. 8 6-APA productivity in four systems (1% *w*/*w* Pen G). *Open circle*, native PGA in water at pH 7 (37°C); *open square*, native PGA in BA/water at pH 4 (37°C); *filled square*, L63-PGA conjugate in three-liquid-phase at pH 5.5 (42°C); *open square*, L63-PGA conjugate in three-liquid-phase (42°C) without organic phase

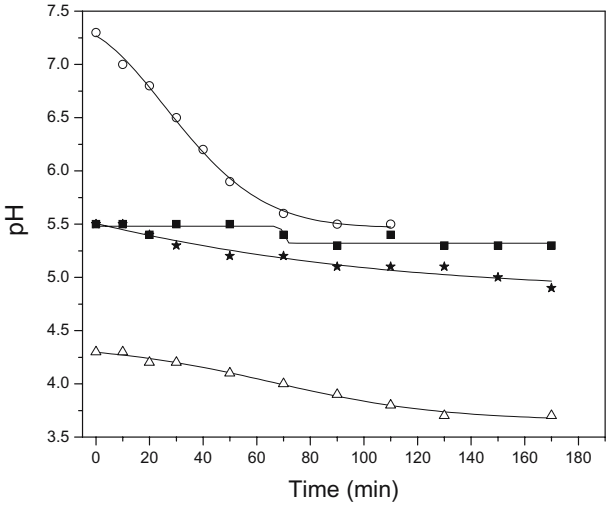

Fig. 9 Final pH in the four systems. *Open circle*, native enzyme in water; *open triangle*, native enzyme in BA/water; *filled square*, L63-PGA conjugate in three-liquid-phase; *filled star*, L63-PGA conjugate in three-liquid-phase without organic phase

(Fig. 4), thereby reducing the pH fluctuation in water and endowing a higher enzymatic activity as compared to that in singled aqueous phase and BA/water two-phase system. The ion metathesis of OH^- from trioctylmethylammonium hydroxide with the H^+ ion in water help to maintain a constant pH in water. It is interesting to note that when the organic phase was absent from the three-liquid-phase system, the initial rate and final productivity were rapidly decreased. This may be because of the unfavorable accumulation of PAA in the polymer-rich phase, which reduced pH and increased the product's inhibition in comparison with that of three-liquid-phase system [12] (Fig. 9).

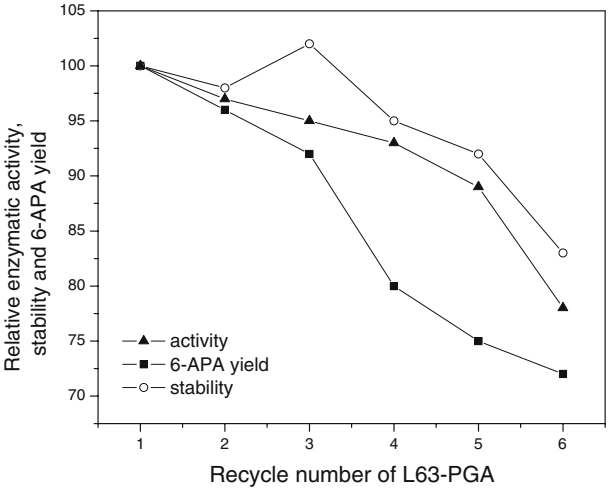

Fig. 10 Relative activity, stability and 6-APA yield using recyled L63-PGA (the 100% relative activity is 120 IU/g, 100% relative stability is 27 h, and 100% relative yield of 6-APA is 82%)

Recovery and Reuse of L63-PGA

Due to the thermo-reversible ability of PEO–PPO–PEO polymer attached on the enzyme surface, the catalyst could be recycled and reused from the reaction medium. Figure 10 compared the relative activity, stability and 6-APA yield using recycled L63-PGA. It's found that the L63-PGA conjugates preserved 78% enzymatic activity, 72% stability, and 83% 6-APA yield after six turns. This result indicated that the polymer conjugate can be used for separation, recovery and recycle of an enzyme from three-liquid-phase system by applying small temperature changes to the reaction medium.

Economical Evaluation

Table 2 gives the economical evaluation of the 6-APA production in three hydrolytic systems. It's found that in single aqueous phase, the cost of the substrate and enzyme is \$1.7/l, and its profit is \$0.43/l. While in BA/water two-phase system, the total cost of substrate and enzyme is \$2.8/l, and its profit is \$1.54/l. While in three-liquid-phase system, the cost of substrate and enzyme increases to \$4.3/l, and the cost of Cyphos IL-104 is \$64 if it is used only once. In considering cost balance in three-liquid-phase system, the recycling of expensive Cyphos IL-104 is required. If 25 turns of Cyphos IL-104 is employed, the cost balance in three-phase system for production of 6-APA is realized. In fact, the practically feasible system would be used for producing other expensive semi-synthetic drugs, such as 7-ADCA and 7-ACA.

Conclusion

A three-liquid-phase system was proposed for carrying out the enzyme hydrolysis of Pen G for 6-APA production. Since different phases were created for enzyme, PAA and 6-APA, respectively, in this three-liquid-phase system, low pH strategy for 6-APA precipitation in BA/water system was not necessary. PAA could be extracted into the organic phase at relatively high pH using mixed extractants. At this high pH, the enzymatic performance was improved comparing with the more denature BA/water system. On the other hand, two products were separated from the enzyme phase spontaneously, which might favorably shift the reaction equilibrium towards the product's side, and lead to higher productivity of 6-APA in three-liquid-phase system in comparison with that in single-phase aqueous phase or BA/water two-phase system.

Table 2 Economical evalution of the 6-APA production in three hydrolytic systems.

	Substrate	Enzyme	Cyphos IL-104	Total cost	6-APA	Profit
Single phase	1.1	0.6		1.7	2.13	0.43
BA/water two-phase	2.2	0.6		2.8	4.34	1.54
Three-liquid-phase	3.3	1	64/25 turns		6.47	2.37

Where the volume of single aqueous phase is 1L, the volume of organic and aqueous phase in BA/water two-phase system is 1L and 1L, respectively. The volume of organic and aqueous phase in three-phase system is 1L and 1L, respectively, and the substrate concentration in single aqueous phase, BA/water two-phase system and three-liquid-phase system are 0.25 mol/l, 0.5 mol/l and 0.75 mol/l, respectively (respect to the volume of aqueous phase). The unit of cost is dollar.

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