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The Catalytic Performance of *Penicillin G Acylase* in Different Organic Solvents

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Abstract. This work investigated the catalytic performance of PGA engineered with a variety of functional groups in organic solvents at room temperature for different interval, such as n-alkanes, chlorinated methane, alcohols and acids. The major research highlights are listed as: ① With the extension of time, as for hydrophobic organic solvent, catalytic activity retention rate (C_r) decreased and achieved equilibrium gradually, in a series of solvent, the smaller log P, the lower C_r; as for hydrophilic organic solvent, C_r increased first and then decreased to an equilibrium value, in a series of solvent, the smaller log P, the higher C_r. ②With the increasing of the solvent molecular size (l), equilibrium C_r achieved showed different trends: when $l < 4.4$ Å, equilibrium C_r achieved was fast and not affected by l; when 4.4 Å $< l < 6.9$ Å, it was slow and increased with the increasing of l, when $l > 6.9$ Å, it was fast and decreased with the increasing of l. ③ In the series solvents, PGA treated with polyalcohol, especially glycerine, its C_r reached maximum and maintained stability.

Introduction

As one of the most important industrial biocatalysts, *penicillin G acylase* (PGA, EC 3.5.1.11) has been used widely in the production of 6-aminopenicillanic acid (6-APA) [1-3]. The property of immobilized PGA preparation is primarily governed by the property of the carrier material. Numerous carriers, organic polymeric carriers are the most widely studied materials because of the presence of rich functional groups, which provide essential interactions with PGA. For example, Eupergit C, oxirane acrylic beads, has been commercially used as an PGA carrier. At present, the researches on the performance of immobilized PGA are still focused on the catalytic activity and stability through immobilizing them on various carriers, which has made remarkable achievements. However, there has been no breakthrough in the facilitating catalyst recovery of immobilized PGA, the main reason is that no suitable carrier microenvironment maintaining the catalytic activity of PGA. It is well known that the catalytic activity of PGA arises from its protein quaternary structure, any factors induced the structure can effect its catalytic activity [4]. And the structure of PGA is influenced easily by carrier microenvironment during PGA immobilization. Nevertheless, for immobilized PGA, it was so difficult to characterize the functional group and its distribution

in solid phase microenvironment, with the result that the regularity theoretical issues of the relationship between carrier microenvironment and catalytic performance of PGA were hard to be studied. Compared with in solid phase environment, the catalytic performance of PGA in liquid phase environment is studied more easily and accurately. However, in the field of liquid phase environment, there are only limited reports being focused mainly on the catalytic activity of PVA or the syntheses of ß-lactam antibiotic catalyzed by PGA or immobilized PGA [5-9]. The catalytic performance of PGA in organic solvents was also rarely investigated [5].

Therefore, in this study, the goal is to obtain the functional group that is favorable to retain the catalytic activity of PGA selected from different series of organic solvents containing various functional groups, such as n-alkane, chlorinated methane, monoalcohol, polyalcohol, monoacid and polyacid. Subsequently, use the result to guide the design of carrier microenvironment and the synthesis of immobilized PGA carrier with high catalyst recovery in the future. To the best of our knowledge, this is the first study of the catalytic performance of PGA in different organic solvents. Based on the special structure of PGA hydrophobic pocket in the catalytic reaction and properties of organic solvent molecules such as hydrophobicity, hydrophilicity, polarity, molecular size, and so on, catalytic activity retention rate of PGA (C_r) and equilibrium time (E_t) as investigated indexes, the catalytic performance of PGA treated with various solvents for given time intervals in the reaction of catalyzing penicillin G to produce 6-APA was investigated systemically.

Materials and Methods

Materials

Penicillin G acylase (PGA, the original activity of free PGA was 886 U/g), penicillin G (PG, 98 % HPLC), 6-aminopenicillanic acid (6-APA, 98 % HPLC), phenylacetic acid (PAA, AR) and p-dimethylaminobenzaldehyde (PDAB, AR) were purchased from Hubei Blue Sky Pharmaceutical Co., Ltd. (Hubei, China); sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$, AR), disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$, AR) and phosphoric acid (H_3PO_4, AR) were obtained from Tianjin Hengxing Chemical Reagent Co., Ltd. (Tianjin, China); all organic reagents (AR) were provided by Tianjin Guangfu Science and Technology Development Co., Ltd. (Tianjin, China). Distilled water was provided by our laboratory; pH = 7.00 of 0.04 mol/L Na_2HPO_4 /NaH_2PO_4 buffer solution was prepared by us; 1.0 % (vol %) free PGA solution, 10.0 % (m/v %) PG solution, 5.0 % (m/v %) PAA solution and 0.35 mM 6-APA solution were all prepared with the Na_2HPO_4/NaH_2PO_4 buffer solution as the solvent; 4.0 % (m/v %) PDAB chromogenic reagent was prepared with the mixture of ethyl alcohol and phosphoric acid (v: v = 93: 7) as the solvent.

Methods

Determination of Standard Curve

A series of 6-APA solutions with the concentrations range from 0.05 to 0.35 mM were prepared. 0.50 mL 6-APA solution was added into a 4-mL cuvette loaded with 3.50 mL of PDAB chromogenic reagent. After standing the cuvette in a 25 ^oC environment for 3 min, the absorbency of 6-APA was determined using UV-752N spectrophotometer at 420 nm. The average absorbance at a given concentration was calculated with at least seven data point, and

the relationship between concentration (C) and average absorbance (A) (A = 1.78357C—0.00871, R^2 = 0.99891, S = 0.12 %, n = 7) was plotted as shown in Fig. 1.

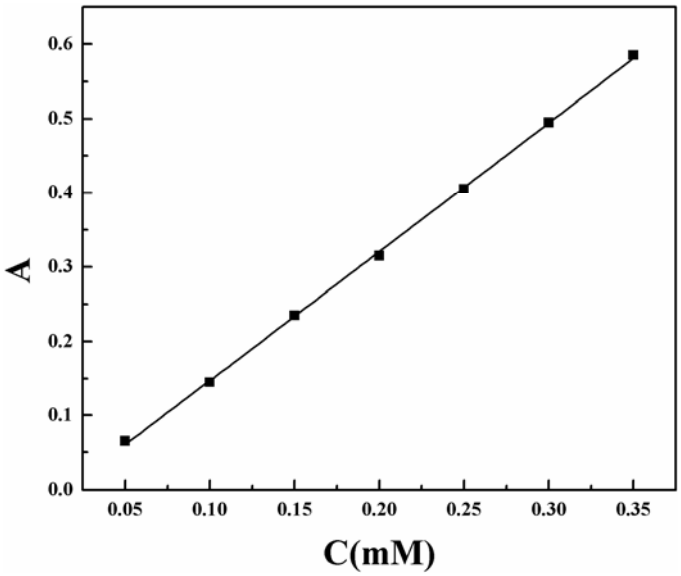

Figure 1. The influence of 6-APA concentration on absorbency.

Determination of Catalytic Activity and Catalytic Activity Retention Rate of PGA

The flask loaded with 10.00 mL of 1.0 % (vol %) free PGA solution and 10.00 mL of organic solvent was fixed in the 37 °C thermostatic bath equipped with stirrer. After stirring for given time intervals, 1.00 mL of the water phase contained free PGA was pipetted into a test tube with 3.00 mL of 10.0 % (m/v %) PG solution, and the catalytic hydrolysis reaction kept for 5 min at 37 °C under agitation. Subsequently, the absorbance of the solution was determined with the method of the section of *Determination of Standard Curve* at 420 nm. The catalytic activity (a_v) and the catalytic activity retention rate (C_r) were calculated with Eq. 1 and Eq. 2.

$$a_v = \frac{C \times V}{V_0 \times t} \tag{1}$$

$$C_r = \frac{a_v}{a_{v0}} \times 100\% \tag{2}$$

Where, av denotes the quantity of activity of units free PGA (U/mL), one activity unit is defined as the amount of PGA needed to catalyze PG hydrolyzing into 1 μmol of 6-APA in 1 min. C stands for the concentration of 6-APA in the catalytic hydrolysis reaction system (mM), V is the volume of the reaction system in mL, V0 is the volume of free PGA in mL and t is the reaction time in min. av0 denotes the original activity of free PGA treated with Na2HPO4/NaH2PO4 buffer solution, the value is 709.3 U/mL.

Determination of Molecular Size and Log P of Organic Solvents

The molecular size of organic solvents was calculated by Gaussian 03 B3LYP hybrid functional method with the STO-3G basis sets, while the molecular polarity coefficient (log P)

of them was calculated using Chemoffice 2006 software (Table 1). Where, log P presents the molecular polarity, smaller log P means greater polarity.

Table 1. The Log P and molecular size of organic solvents.

Solvent	Log P	Molecule size(Å)	Solvent	Log P	Molecule size(Å)
n-pentane	2.58	6.9	ethanol	0.07	4.1
n-hexane	3.00	8.1	n-propanol	0.55	5.4
n-heptane	3.42	9.3	n-butanol	0.97	6.7
n-octane	3.84	10.8	n-pentanol	1.39	7.9
n-decane	4.67	13.3	ethylene	-0.79	5.1
n-dodecane	5.51	15.8	glycerol	-1.33	5.7
n-tetradecane	6.34	18.0	ethanoic acid	-0.31	3.6
dichlorometha	1.62	4.4	n-propionic	0.35	5.1
trichlorometha	2.30	3.7	n-butyric acid	0.76	6.4
tetrachloromet	2.86	2.9	oxalic acid	-0.53	4.2
methanol	-0.27	2.9	citric acid	-1.68	7.8

Results and Discussion

Effect of N-alkane on the Catalytic Activity Retention Rate of PGA

Fig. 2 presents the effect of molecular chain length of n-alkane on the catalytic activity retention rate (C_r) of PGA. It could be found that C_r decreased till an equilibrium value with the prolonging of time. Besides, at the same interval, C_r increased with the increase of molecular chain length, while equilibrium time (E_t) was shorten. It is well known that there have quantities of essential water inside and outside PGA matrix, and they interact with the functional groups of amino acid residues by hydrogen-bond. Any factors that can induce PGA matrix losing water would lead to the change of three-dimensional space conformation and the decrease of catalytic activity of PGA [4]. With the extension of time, n-alkane molecules diffused into PGA matrix gradually. Due to the strong hydrophobicity of n-alkane [10], they could interacted with the hydrophobic chain segment of PGA by hydrophobic interaction. It would lead to the deformation of PGA matrix, the loss of water, the remaining essential water being compressed and the distortion of active center. As a result, the catalytic activity of PGA declined, C_r declined correspondingly. When n-alkane molecules diffused into PGA matrix at a certain extent, the compressed essential water was transformed into a relatively continuous phase to hinder the continuing diffusion of n-alkane molecules completely. Naturally, the catalytic activity of PGA reduced to an equilibrium value, C_r also reached equilibrium. Besides, as shown in Table 1, the molecular chain length of n-alkane is proportional to its log P and molecular size. With the increase of molecular chain length, the steric hindrance n-alkane molecules becomes more severe, which would lead to the diffusion rate of n-alkane molecules in PGA matrix becomes slow and the PGA matrix area where n-alkane molecules remains small. It shows that decrease of the catalytic activity of PGA was small, so C_r increased correspondingly. Simultaneously, the continuous phase of essential water formed earlier, as a result, equilibrium C_r arrived at a faster rate.

Effect of Chlorinated Methane on the Catalytic Activity Retention Rate of PGA

The influence of the number of chlorine atom in chlorinated methane on C_r is shown in Fig. 3. It shows that C_r declined dramatically in the beginning 4 h, then declined slowly till an equilibrium value, and equilibrium time (E_t) was almost equal. Additionally, at the same interval, C_r declined with the decrease of the number of chlorine atoms. Notably, there have quantities of essential water inside and outside PGA matrix. The active centre is located at the entrance of hydrophobic pocket, it contains Serβ-1 and other amino acid residues with functional groups, such as -COOH and -NH_2 , and water molecules that play bond bridge role interact with the negative charged functional groups by hydrogen-bond [4]. Due to the polar chemical nature of chlorinated methane molecules, the carbocation and electrophilicity would produce relatively [10]. Naturally, solvent molecules would compete with essential water molecules to interact with functional groups, leading to decreasing in quantities of essential water and deforming PGA matrix. Consequently, the catalytic activity of PGA declined dramatically. Meanwhile, compared with other solvents, the molecular size of chlorinated methane is relatively smaller (Table. 1), the steric hindrance therefore decreased, which would facilitate diffusion of chlorinated methane molecules and augment deformation degree of PGA matrix, especially hydrophobic pocket. Based on both above thermodynamics and kinetics reasons, C_r decreased dramatically. When chlorinated methane molecules were distributed throughout PGA matrix, the catalytic activity tended to be stable, C_r achieved equilibrium eventually. Moreover, it could be found that the number of chlorine atoms is proportional to the log P from Table 1. It means the molecule with fewer chlorine atoms possesses higher electrophilicity, competes functional groups stronger, and deforms PGA matrix more seriously. Thus, C_r decrease order should be understood easily. As for the almost same equilibrium time, it could be ascribed to the levelling effect of steric hindrance [10]. When the maximum molecular size of chlorinated methane (the mean square end-to-end distance is 4.4 Å) was smaller than the minimum pore diameter of PGA matrix, they would diffuse into PGA matrix with almost the same speed and were distributed throughout PGA matrix at the same time. Besides, based on the analysis of n-alkane and chlorinated methane, it was concluded that the pore diameter of PGA matrix was in the range of [4.4, 6.9 Å].

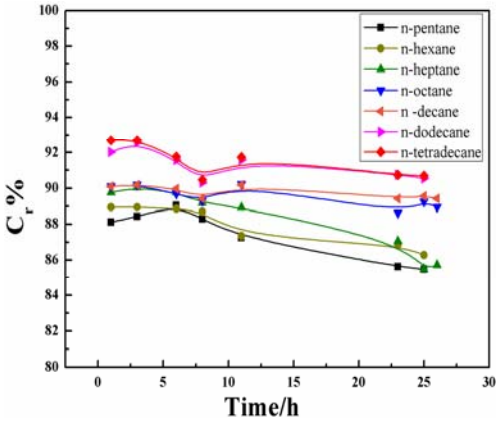

Figure 2. The relationship between Cr of PGA treated with n-alkane and time.

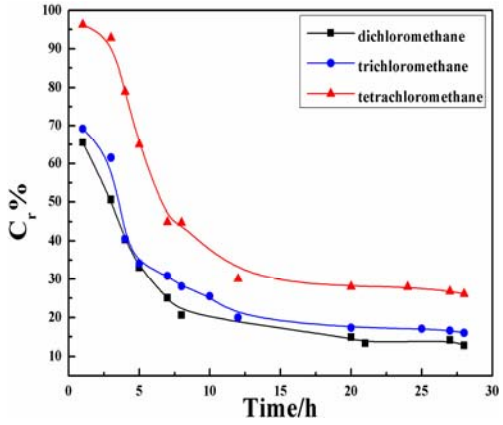

Figure 3. The relationship between Cr of PGA treated with chlorinated methane and time.

Effect of Monoalcohol on the Cataleytic Activity Retention Rate of PGA

Fig. 4 presents the relationship between molecular chain length of monoalcohol and C_r. It shows that C_r reduced to a low value immediately, then increased to the maximum value and then reduced gradually to reach equilibrium. Moreover, C_r declined with the decrease of molecular chain length of monoalcohol. While the variation of E_t is complex, the equilibrium time of methanol and ethanol was almost equal, that of n-propanol was longest, and that of n-butanol and n-pentanol was gradually shortened in turn.

As monoalcohol molecules diffused into PGA matrix, because of the molecular polarity and hydrogen-bond, they would compete with essential water to interact with the adjacent function groups through high coordination number to keep itself stability [12]. As a result, quantities of essential water was lost, and PGA matrix, especially hydrophobic pocket, was constricted seriously. Under this condition, the entrance of PG substrate molecules into PGA matrix became difficult, leading to the catalytic hydrolysis reaction couldn't run smoothly. Thus, C_r was low relatively. However, with the prolonging of time, more monoalcohol molecules diffused into PGA matrix gradually under the impellent of osmotic pressure. Part of the later entered monoalcohol molecules would compete the functional groups coordinated with the former ones, which resulted in gradual decrease of coordination number. Sequentially, the constricted matrix and hydrophobic pocket would enlarge gradually again to allow PG to enter, C_r increased naturally. When the coordination number reduced to a status that all residues changed to almost their naturally stretch form, the quantity of PG molecules entered would increase to a maximum value. Consequently, C_r arrived maximum correspondingly. But, with the further prolonging of time, as the coordination number continued to reduce, the functional groups interacted with monoalcohol molecule were fewer and fewer, even only one functional group. It would lead to the three-dimensional conformation of PGA changed to rigid linear structure gradually, C_r decreased correspondingly. When the coordination number reduced to a minimum value, PGA changed into irreversible rigid linear structure completely, C_r achieved equilibrium. As for C_r decreased with the decrease of molecular chain length of monoalcohol, which could be ascribed to the molecular polarity and steric hindrance. Additionally, the variation of equilibrium time could be attributed to the levelling, distinguishing as well as size exclusion effect of steric hindrance [10].

Effect of Polyalcohol on the Catalytic Activity Retention Rate of PGA

Fig. 5 shows the effect of the number of hydroxyl on C_r. It could be found that the relationship between C_r and time of polyalcohol was the same as that of monoalcohol, it is only not obvious. Moreover, C_r and E_t declined with the decrease of the number of hydroxy. C_r reduced to a relative small value, and then increased to a maximum value, which also resulted from the coordination number. Subsequently, C_r showed a long time interval platform, which could be ascribed to the formation of stable ring structure between polyalcohol molecules and functional groups. According to the literature [13], as ethylene glycol molecule was introduced into PGA matrix, it would interact with functional groups and form a six-element ring, which would stabilize the conformation of PGA and crystallize it. Thus, we could conclude reasonably that as glycerol molecules was introduced, the similar six-element ring or five-element ring would be formed. Thus, a long time interval platform of C_r presented naturally. Compared with polyalcohol, monoalcohol could not form a stable ring, so C_r showed a lower value at maximum state, and the range of maximum was also narrower.

However, as superabundant amount of polyalcohol molecules diffused into PGA matrix, the stability of ring decreased due to the stronger competition among them to interact with function groups, so C_r reduced slightly.

It could be also found that, compared with in buffer solution, the activity of PGA in polyalcohol solvents was higher, which resulted from the strong hydration of hydroxyl [10,13]. As for the fact that C_r of PGA treated with glycerol was higher than ethylene glycol, we predict that, although hydration was possible, the more important reason should be the increased probability of forming stable six-element ring. Because glycerol has three hydroxyls, and the structure of arbitrary adjacent two hydroxyls is similar to that of hydroxyls in ethylene glycol, thus, the probability that glycerol forms six-element ring would be much higher than ethylene glycol, which was more favourable to maintain stable active conformation of PGA, the higher C_r could be obtained naturally. While E_t was shortened with the decrease of the number of hydroxyl, which could be ascribed to the distinguishing effect of steric hindrance.

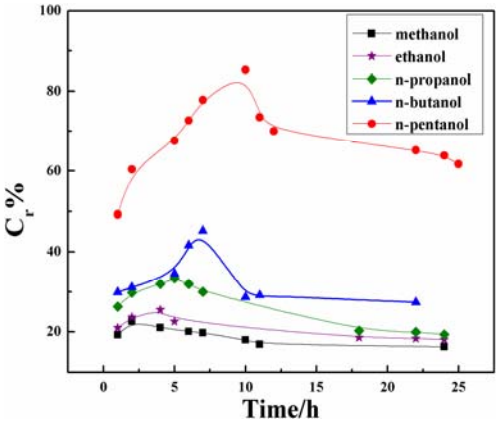

Figure 4. The relationship between Cr of PGA treated with monoalcohol and time.

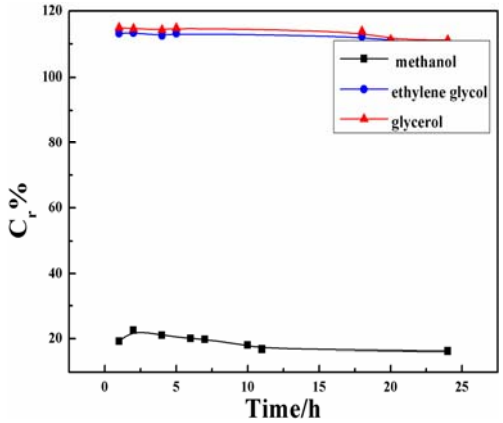

Figure 5. The relationship between Cr of PGA treated with polyalcohol and time.

Effect of Monoacid on the Catalytic Activity Retention Rate of PGA

The effect of molecular chain length of monoacid on C_r is shown in Fig. 6. It shows that C_r reduced to a low value immediately, then increased and showed a long time interval platform. Subsequently, it reduced dramatically to achieve equilibrium. Moreover, C_r declined with the increase of molecular chain length of monoacid. Besides, E_t was shortened with the decrease of molecular chain length of monoacid. Similar to the polyalcohol, we could also conclude reasonably that monoacid molecule interacted with functional groups to form similar five-element ring structure due to it has carboxyl. Thus, the relationship between C_r of PGA treated with monoacid and time could be explained by the above discussion about polyalcohol. In addition, because the hydration was related to the ratio of hydrophilic functional groups, the order of C_r of monoacid could be understood naturally. As for the E_t was shortened with the decrease of molecular chain length of monoacid, which could be ascribed to the distinguishing effect of steric hindrance.

Effect of Polyacid on the Catalytic Activity Retention Rate of PGA

Fig. 7 shows the relationship between C_r of PGA treated with polyacid and time, and the reason was also similar as the explanation of polyalcohol. Besides, the order of C_r of polyacid could be ascribed to the hydration, that is, the ratio of carboxyl. While E_t was shortened with

the decrease of the number of carboxyl, which is related to the distinguishing effect of steric hindrance.

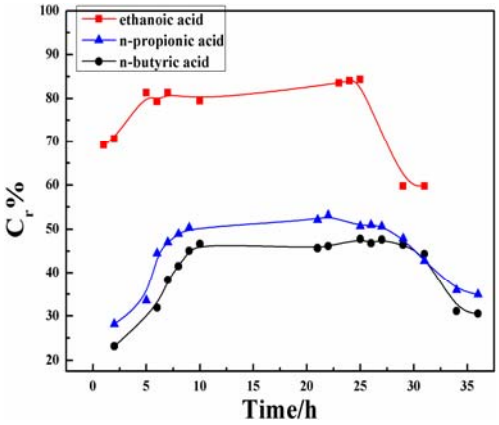

Figure 6. The relationship between Cr of PGA treated with monoacid and time.

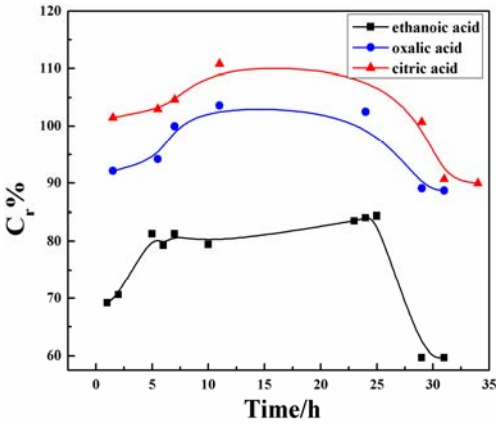

Figure 7. The relationship between Cr of PGA treated with polyacid and time.

Conclusions

The catalytic performance of PGA treated with different series of organic solvents containing various functional groups was investigated to obtain the functional group that be in favour of retaining the catalytic activity of PGA. The results can be concluded as following highlights: ①As for hydrophobic organic solvent, the smaller log P, the lower C_r; while as for hydrophilic organic solvent, in a series of solvent, the smaller log P, the higher C_r. ② With the increasing of l, equilibrium C_r achieved showed different changing trends: when $l < 4.4$ Å, equilibrium C_r achieved was fast and not affected by l; when 4.4 Å $< l < 6.9$ Å, it was slow and increased with the increasing of l, when $l > 6.9$ Å, it was fast and decreased with the increasing of l. ③C_r of ethylene glycol and glycerol reached up to 112 % and 115 % respectively and maintained stability, that were excellent solvents to promote the catalytic performance of PGA for building carrier microenvironment.

Acknowledgements

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