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Research Note

Immobilization of Penicillin G Acylase on Non-Porous Ultrafine Silica Particles

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In this paper, immobilization of penicillin G acylase onto non-porous ultrafine silica particles has been studied. The amount of penicillin G acylase immobilized was increased by increasing the free enzyme concentration and, at 0.45 mg/ml concentration of the free enzyme, 80% of the enzyme was immobilized. The optimum pH for immobilization was found to be 7.0, close to the pI of the enzyme. Although immobilization of the enzyme on ultrafine silica particles with and without glutaraldehyde showed almost the same activities, the enzyme immobilized with glutaraldehyde retained its initial activity much longer during 40 cycle-repeated batches with a half life of 163.2 h.

INTRODUCTION

Penicillin G acylase (EC 3.5.1.11) catalyses the hydrolysis of the linear amide bond in penicillin molecules to produce the β-lactam nucleus, 6-aminopenicillanic acid (6-APA) and the corresponding carboxylic acid. The intermediate 6-APA can be produced either by chemical or enzymatic hydrolysis. However, the economic, environmental and operational advantages of the enzymatic process over the chemical route have now been realized and enzymatic hydrolysis has attracted significant attention. It can be assumed that almost all 6-APA is produced today by immobilized enzymes. For the industrial application of penicillin acylase, its immobilization, which allows the condition of repeated use of the enzyme, has been intensively investigated.

Penicillin acylase has been immobilized by several physical and chemical methods, namely, adsorption [1,2], fiber entrapment [3,4], microencapsulation [5], cross-linking [6], covalent attachment [7,8] and copolymerization [9]. However the carriers used were mainly of an insoluble organic nature, like polymeric resins, natural polymeric derivatives, organic gels and fibers, with limited capacity to reuse the immobilization matrix and, therefore, creating problems regarding the disposal of organic materials. In contrast, although inorganic carriers are more expensive than organic ones, they have the advantage of being reusable, which, in some circumstances, can decrease the immobilization support cost [10]. Moreover, one of the most important points in the consideration of enzyme immobilization, is the mass transfer limitation of substrates and products [11-13]. Such a limitation dramatically affects the kinetic parameters and, ultimately, the activity of the immobilized enzymes. Thus, the maximum reaction rate and the Michaelis constant determined for an immobilized enzyme might differ from those of its free state. Unfortunately, less attention has been paid to the problem of mass transfer limitations in most papers hitherto published on penicillin acylase immobilization. In this work, to overcome the problem of internal mass transfer limitation, non-porous silica particles were used for the first time as enzyme carriers for penicillin G acylase immobilization. The element of novelty in this paper is the use of this particular type of silica as a carrier for the penicillin G acylase enzyme. It has been shown that these particles are effective as enzyme carriers in bioconversions in aqueous two-phase systems [14-16] and this work presents the adsorption of partially purified penicillin G acylase on ultrafine silica particles with and without a covalent cross-linking agent.

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MATERIALS AND METHODS

Microorganism, Chemicals and Culture Conditions

Penicillin G potassium salt was obtained from the Jaber Iben-e-hayan Pharmaceutical Co., Tehran, Iran. The ultrafine silica particles, Snowtex 30, were a gift from Kobe University, Japan. Snowtex 30 (SiO_2: 30.4 wt%) is a colloidal silica solution made by dispersing negatively charged, amorphous silica particles in water. OH ions exist on the surface of the particles with an electric double layer formed by alkali ions. The particles are spherical in shape and the suspension is clear to opalescent with a viscosity and a density of 10 mPa.s and 2.15 g/ml at 25°C, respectively. The average diameter of these ultrafine particles was measured by the Dynamic Light Scattering method and found to be 14.8 ± 0.2 nm. Other chemicals were of an analytical grade and purchased from Merck AG, Fluka AG, Switzerland and Sigma Chemical Ltd., USA. Escherichia coli (E.coli) ATCC 11105 was grown in a culture medium consisting of the following components: Phenyl acetic acid 0.2% (w/v), yeast extract 0.5% (w/v), NH_4Cl 0.1% (w/v), K_2HPO_4 0.1% (w/v), KH_2PO_4 0.01% (w/v) and $MgSO_4$ 0.02% (w/v). The pH was adjusted to 7.00 with NaOH. Aliquots (100 mL) of the culture medium were dispensed into 500 mL conical flasks with lint caps and sterilized at 121°C for 20 min. The flasks were inoculated with E.coli and incubated on a rotary shaker (220 rpm) at 25°C for 46 hrs. Cells were harvested by centrifugation and washed and incubated in a 10 mM sodium phosphate buffer (pH = 7.8). Subsequently, cells were ruptured using an ultrasonicator Bondlin-HD 200 at cycle 90 for 90 sec. Cell debris was removed by centrifugation (8000 x g for 30 min of 0-4°C). The enzyme solution was then partially purified using ammonium sulfate salt and the fraction precipitated between 30% and 60% of ammonium sulfate saturation was collected by centrifugation (12000 x g for 40 min at 0-4°C), dissolved in a 10 mM sodium phosphate buffer (pH = 7.8) and dialyzed against the same buffer. No penicillinase activity was detected in the partially purified enzyme solution.

Enzyme Immobilization

Two kinds of enzyme immobilization were studied: Physical adsorption with and without use of glutaraldehyde as a covalent cross-linking agent. An enzyme solution (4 mL) at the desired concentration was mixed with an equal volume of the suspension of ultrafine silica particles. The immobilization was carried out in a shaker for 2 h at 30°C. Ultrafine silica particles with the adsorbed enzyme were then recovered by centrifugation (14000 x g, 40 min, 0-4°C) and resuspended in the 10 mM sodium phosphate buffer (pH = 7.5). In another investigation, the covalent cross-linked enzyme was adsorbed on ultrafine silica particles. As in the previous procedure, 4 mL of the desired concentration of enzyme solution, 0.1 ml of 1% glutaraldehyde solution and 4 mL of the suspension of ultrafine silica particles, were mixed. The immobilization was carried out in the presence of phenylacetic acid as a competitive inhibitor of penicillin G acylase to protect the active site, for 2h at 30°C. The ultrafine silica particles with covalently cross-linked enzyme were then recovered by centrifugation (14000 x g for 40 min at 0-4°C). These immobilized enzyme molecules were washed and resuspended in the 10 mM sodium phosphate buffer (pH = 7.5). In both cases, the amount of immobilized enzyme was calculated from the differences between the initial and equilibrated protein concentration.

Determination of Enzyme Activity and Protein Content

The enzyme activity was measured by the pH-stat method. This method is based on the titration of phenylacetic acid formed during the hydrolysis of penicillin G potassium [7]. The initial Penicillin G concentration in the thermostated reaction vessel was 5% (w/v). Temperature, pH and ionic strength were 37°C, 8.0 and 0.01, respectively. One unit of enzyme activity is defined as the amount of the enzyme producing 1 μmole of 6-APA per minute at 37°C and pH = 8.0 from a 5%(w/v) aqueous solution of penicillin G potassium salt.

The protein concentration was measured by the Lowry method using bovine serum albumin as the standard [17].

Reactor Condition

The schematic diagram of the reactor used for analyzing the stability of the surface-immobilized enzyme in repeated batch cycles is presented in Figure 1. 20 mL of 8% (w/v) aqueous solution of penicillin G potassium salt in 10 mM of sodium phosphate buffer (pH = 8.0) was used and the reaction was started by the addition of the immobilized enzyme. Each cycle was performed for 60 min and then the immobilized enzyme was recovered by centrifugation (14000 x g for 40 min at 0-4°C), washed and resuspended in 10 mM of sodium phosphate buffer (pH = 7.5).

RESULTS AND DISCUSSION

Enzyme Immobilization

Figure 2 shows the amount of adsorbed penicillin G acylase on ultrafine silica particles with and without

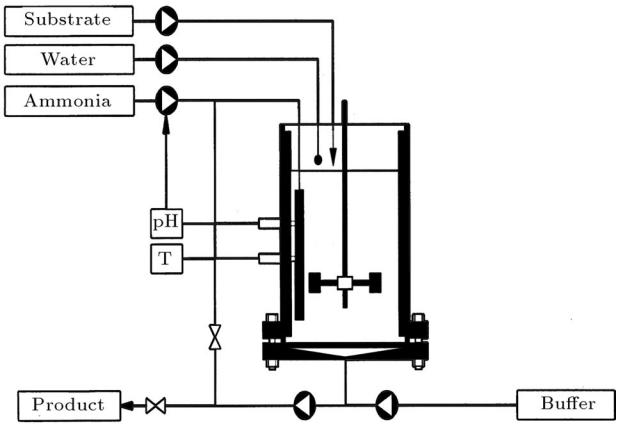
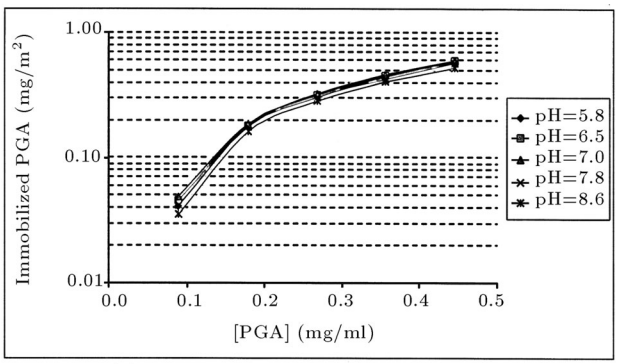
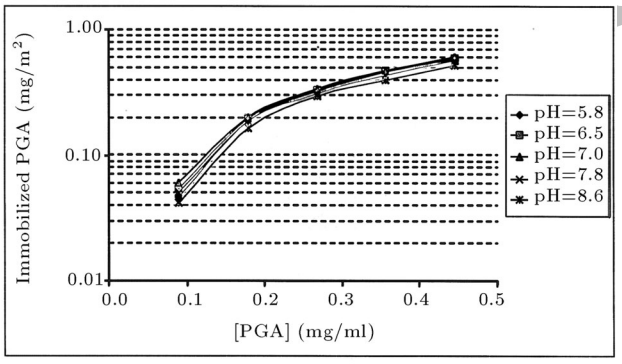


Figure 1. The schematic diagram of the reactor used for analyzing the stability of surface-adsorbed enzyme.



(a) Without covalent cross-linking reagent

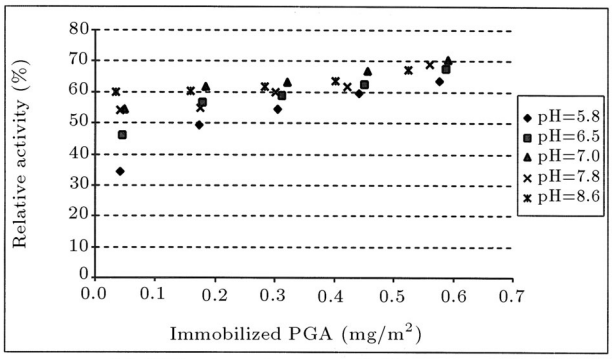


(b) With covalent cross-linking reagent

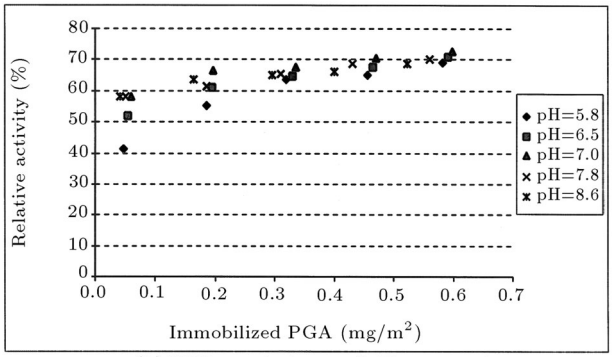
Figure 2. Amount of penicillin G acylase adsorbed on ultrafine silica particles.

glutaraldehyde as a covalent cross-linking reagent at various pH values. The amount of immobilized enzyme, which is defined as the weight of immobilized protein per unit of surface area, is plotted against the initial protein concentration. In both cases, the initial slopes and plateau values were affected by pH. The plateau values exhibited a maximum near pH = 7.0, which is known as the isoelectric pH of this enzyme and decreased when pH was increased above the pI value. Probably, a reduction in electrostatic repulsion between protein molecules near the isoelectric pH would yield a higher packing density on the particle surface. On the other hand, an increase in the electrostatic repulsion between protein molecules and negatively charged surfaces reduces the enzyme immobilization amount at pH values above the isoelectric pH. The amount of the immobilized enzyme, with or without a covalent cross-linking agent, increased with an increase in the concentration of the enzymatic solution and reached 0.6 mg/m^2 at 0.45 mg/ml of the initial enzyme concentration.

Figure 3 presents the effect of the immobilization amount on the relative activity of the enzyme, which is defined as the ratio of the specific activity of immobilized enzyme to that of a free enzyme at the same pH. In both cases, while the relative activities of immobilized penicillin G acylase were only slightly dependent on pH, they increased with an increase in the immobilization amount and reached to 70% of the free enzyme activity at 0.45 mg/ml of the initial enzyme concentration. This result is consistent with Kondo et al.'s observation, where an increase in the immobilization amount of enzyme molecules on ultrafine silica particles decreased the extent of conformational changes and activity reduction in the immobilized enzyme [14-16]. As the amount of adsorption increases, interaction between surfaces and penicillin G acylase



(a) Without covalent cross-linking reagent



(b) With covalent cross-linking reagent

Figure 3. Relative activities of penicillin G acylase adsorbed on ultrafine silica particles.

molecules is weakened by a reduction of the area available for adsorption and an increase in the repulsive force between adsorbed enzyme molecules.

Repeated Use of Immobilized Penicillin G Acylase

To investigate the possibility of repeated use of penicillin G acylase immobilized on ultrafine silica particles, the residual activity of the immobilized enzyme was measured after each reaction cycle during 40 batch cycles. Figure 4 shows the residual activities for both kinds of the immobilized enzyme. The adsorbed enzyme on ultrafine silica particles had a lower stability and, after each cycle, there was a 15-20% loss of activity. Desorption of the adsorbed enzyme is probably the main reason for this problem. On the other hand, the adsorbed covalent cross-linked enzyme showed a good stability after each cycle and only less than 20% activity loss was observed after 40 repeated batches.

Figure 5 shows the inactivation of different kinds of surface-immobilized penicillin G acylase versus operational time. The first order kinetics of inactivation appeared to be applicable to the deactivation of immobilized penicillin G acylase on ultrafine silica particles. The first-order inactivation rate constant decreased by factors of 0.007 and 0.153 for the surface-immobilized enzyme with and without glutaraldehyde, respectively, when compared with that of the unmodified enzyme. The covalent cross-linking agent increased the surface-immobilized enzyme half-life by roughly a factor of 22.7, as compared with the half-life of the adsorbed enzyme on ultrafine silica particles.

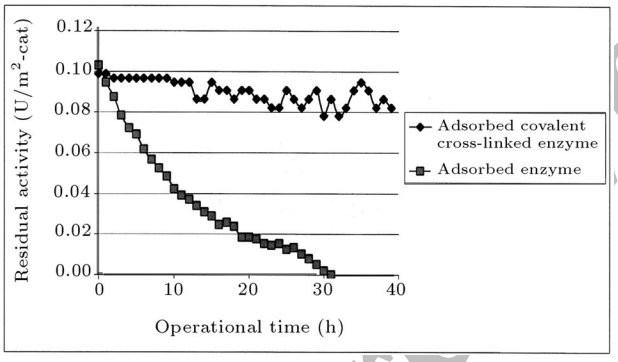


Figure 4. Stability of adsorbed enzyme during the same repeated batch cycles.

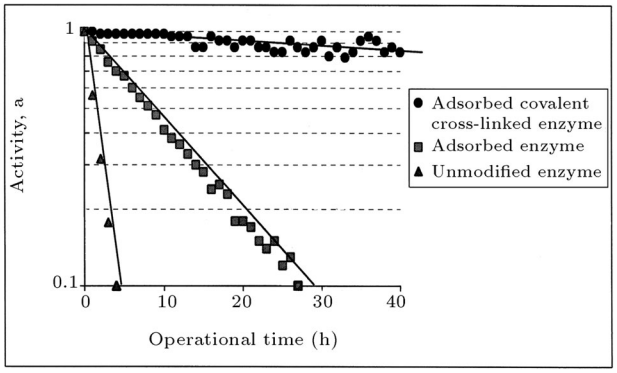


Figure 5. First-order inactivation of immobilized penicillin G acylase.

CONCLUSIONS

Immobilization should, in general, constrain the flexibility of an enzyme, thus, lowering the specific activity of the initial enzyme state when compared with the unmodified enzyme. This constrained atmosphere should also hinder the unfolding of the enzyme, thus, stabilizing the enzyme. Two kinds of immobilized enzyme were studied: The physical adsorption with and without use of glutaraldehyde as a covalent cross-linking agent. In both cases, the amounts of adsorption were high at a pH around pI of penicillin G acylase and decreased with increasing pH. Meanwhile, a higher activity at higher amount of adsorbed enzyme was observed. The lower amount of enzyme adsorption at higher pH can be attributed to the electrostatic repulsion between enzyme molecules and negatively charged particle surfaces. On the other hand, a higher activity at higher amounts of adsorbed enzyme can be attributed to smaller conformational changes upon enzyme adsorption, because the interaction between particle surfaces and enzyme molecules is weakened by a reduction of the area available for adsorption and an increase in the repulsive force between adsorbed enzyme molecules as the amount of adsorption increases. Adsorbed covalent cross-linked penicillin G acylase on ultrafine silica particles retains good activity and appears to be stable as a function of time. Therefore, these particles, lacking the internal mass transfer limitations, can be a suitable candidate for industrial application.

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