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Lipase Immobilization on Silica Gel Using a Cross-linking Method

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Abstract: The objective of this study was to develop a suitable method for immobilizing lipase. Although many investigators have studied lipase immobilization, detailed optimization of each step for lipase immobilization using cross-linking methods has been lacking. Therefore, we focused on establishing and optimizing each step of the immobilization procedure to improve procedural efficiencies. Lipase produced from *Rhizopus oryzae* was immobilized on silica gel using a cross-linking method. The immobilization procedures, such as the pretreatment of silica gel, silanization, crosslinking, glutaraldehyde modification, and elimination of unreated aldehyde groups, were all optimized. Furthermore, the stability of the immobilized lipase and its suitability for reuse were also evaluated.

Keywords: *lipase, immobilization, silanization, glutaraldehyde, reuse*

Introduction

Lipase (triacyl glycerol ester hydrolase, EC 3.1.1.3) is an enzyme that catalyzes the hydrolysis of triacylglycerols to fatty acids, mono- and di-acylglycerols, and glycerol [1]. It also catalyzes various other reactions, such as esterification, interesterification, and transesterification. Lipase is a versatile enzyme with many potential industrial applications; it has been used for the modification of fats and oils and the synthesis of flavor esters and food additives. Because lipase is also enantioselective, it can be used for the resolution of chiral compounds and the synthesis of high-value pharmaceutical intermediates [2].

The high cost of lipase, however, makes enzymatically driven processes economically unattractive. The use of immobilized lipase is a possible solution to this problem because the enzyme can be recovered from the product and reused [3]. The reuse of lipase provides cost advantages that are often an essential prerequisite for establishing a lipase-catalyzed process [4,5]. Furthermore, easy separation of lipase from the product simplifies lipase applications and provides the basis for a reliable and efficient technology.

Among the various immobilization methods available, immobilization using covalent bonding has been most widely studied [6]. Covalent bonding provides a powerful link between the lipase and its carrier matrix. Lipase immobilized through covalent bonding can be reused more often than other available immobilization methods, such as adsorption and entrapment.

Inorganic materials have been successfully used for the immobilization of enzymes [7]. Silanization to activate supports and subsequent covalent binding of an enzyme to the carrier by using a coupling reagent, e.g., glutaraldehyde, is a common method used for immobilization [8]. Silica gel is an amorphous inorganic polymer composed of siloxane groups (Si-O-Si) in the inward region and silanol groups (Si-OH) distributed on its surface. Chemical modifications that can occur with this polymer are related to the presence of the deposed silanol groups in its surface. Surface modifications are usually achieved with silanization using appropriate organosilane agents [9]. Glutaraldehyde has been used as a crosslinker for immobilization of enzymes in which the amino groups of a protein are expected to form a Schiff base with the glutaraldehyde.

The objective of this study was to develop a suitable method for immobilizing lipase onto silica gel by using a cross-linking method. Many investigators have studied lipase immobilization, but detailed optimization of each step for lipase immobilization using cross-linking methods has been lacking. Therefore, we focused on establishing

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and optimizing each step of the immobilization procedure to improve procedural efficiencies. The stability of the immobilized lipase and its suitability for reuse were also evaluated.

Experimental

Materials

3-Aminopropyltriethoxysilane (3-APTES), 3-aminopropyltrimethoxysilane (3-APTMS), 3-(trimethoxysilyl)propylethylendiamine (3-TMSPEDA) and ninhydrin reagent were purchased from Sigma (USA). Glutaraldehyde was purchased from the Fluka (Switzerland) and silica gel was obtained from the Grace Davison (USA). The ultrafiltration membrane (15659-00-1) was purchased from Sartorius (Germany).

Preparation of Lipase

Lipase was produced using the strain of *Rhizopus oryzae* KCCM 11970. The culture broth of *R. oryzae* was centrifuged at 10,000 rpm for 15 min and the supernatant was prepared as a crude lipase solution. Ammonium sulfate was added to 60 % saturation to the crude lipase solution and the resulting suspension was centrifuged at 10,000 rpm for 15 min to obtain the supernatant. The precipitate was suspended in 0.05 M phosphate buffer (pH 7) and the solution was concentrated using an ultrafiltration membrane and stored at 4 ^{o}C.

Lipase Immobilization Method

For silica gel pretreatment, 1 g of dry silica gel was mixed with 15 % 3-APTES in 20 mL acetone and incubated at 50 ^{o}C for 2 h with constant mixing. The treated silica gel was then washed with water and dried at 60 ^{o}C for 2 h. Glutaraldehyde was modified in aqueous solution at 64 ^{o}C for 20 min; the treated silica gel was suspended in 0.05 M phosphate buffer (pH 7). Thereafter, the modified glutaraldehyde was added to the silica suspension and the suspension was filtered after stirring at 20 ^{o}C for 2 h. The activated silica gel produced was then washed with 0.05 M phosphate buffer (pH 7) and resuspended in the same buffer. To immobilize lipase to the activated silica, 2 mL of lipase solution was added to the suspension and stirred at 20 ^{o}C for 2 h. The immobilized lipase was recovered by filtration, washed with water, and resuspended in 0.05 M phosphate buffer (pH 7). The product was set aside for storage at 4 ^{o}C.

Modification of Glutaraldehyde

To reduce the toxicity of the glutaraldehyde, glutaraldehyde was heated in aqueous solution at different temperatures and times, as described by S. W. Park and coworkers [10]. The specific conditions used for this process were governed by the design of the experiment.

Assay of Immobilized Lipase Activity

The enzymatic activity of the immobilized lipase was determined as follows: 10 mL of isooctane containing 10 % (w/v) olive oil was added to 10 mL of 0.05 M phosphate buffer (pH 7) containing 1 g of the immobilized lipase. The reaction mixture was heated with shaking in a water bath at 37 ^{o}C and 150 rpm for 30 min. The reaction was stopped by adding 1 mL of 6 N HCl and then the mixture was agitated vigorously for 30 sec. A portion (2 mL) of the upper layer was then transferred to a test tube wherein a cupric acetate-pyridine reagent (0.5 mL) was added; the free fatty acids liberated and dissolved by the isooctane were quantified by UV spectrophotometry at 715 nm [11]. One unit of lipase activity was defined as the amount of the enzyme required to liberate 1 mol of free fatty acid per min.

Determination of the Degree of Silanization

The degree of silanization of the silica gel surface was determined using the ninhydrin method [12]. To 1 mL of 3-APTES solution in distilled water, 1 mL of different concentrations of ninhydrin agent was added. The solution was heated in a water bath at 100 ^{o}C for 20 min. The reactants were then cooled below 30 ^{o}C in a cold water bath and diluted with 5 mL of 50 % (v/v) ethanol/water. The absorbance at 570 nm was measured using a UV spectrophotometer.

Determination of the Amount of Protein Bound to Silica Gel

Protein was quantified using the Bradford method [13]. The amount of protein bound to silica gel was determined from the difference between the initial and residual protein concentrations.

Results and Discussion

Pretreatment of Silica Gel

The effect that pretreatment had on the degrees of silanization and protein binding was investigated in this study. The pretreatment of the silica gel was intended to eliminate its surface contaminants and activate the surface with silanol groups for reaction with the silanization agent [14]. Various chemicals were tested for the pretreatment of silica gel. A sample of 1 g of the pretreated silica gel was then silanized using 15 % (w/v) 3-APTES dissolved in 20 mL of acetone at 50 ^{o}C for 2 h. Figure 1 indicates that silanization and protein binding were highest when the silica gel was pretreated with 35 % hydrogen peroxide; as a result, this agent was chosen for subsequent pretreatment.

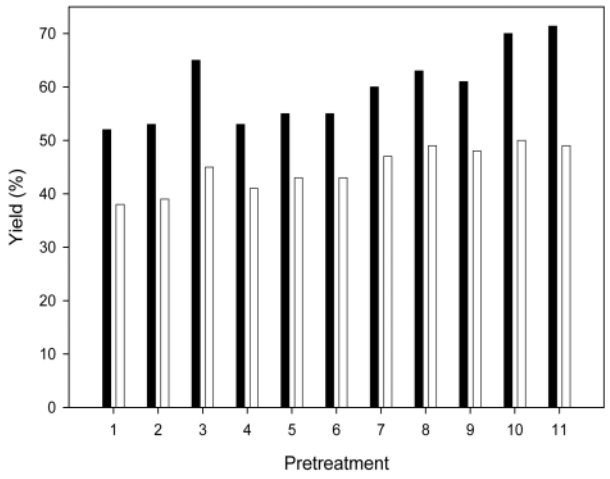

Figure 1. Effect of silica gel pretreatment on lipase immobilization: ■, silanization; □, glutaraldehyde. Experiments were conducted with (1) no pretreatment, (2) sonication at room temperature for 2 h, and mixing with (3) H_2SO_4 and H_2O_2 (5:1), (4) 5 % H_2O_2, (5) 10 % H_2O_2, (6) 15 % H_2O_2, (7) 20 % H_2O_2, (8) 25 % H_2O_2, (9) 27 % H_2O_2, (10) 30 % H_2O_2, (11) 35 % H_2O_2.

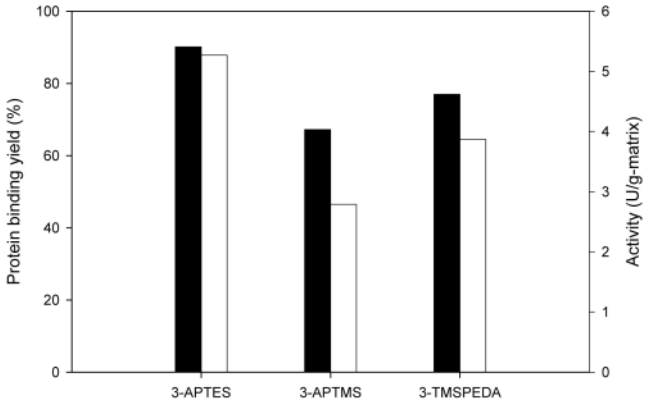

Figure 2. Effect of silanization reagent on silanization: ■, protein binding yield; □, activity of immobilized lipase.

Silanization on the Silica Gel Surface

The coupling reaction of lipase and silica gel commonly uses the amino groups of organosilanes [15]. In this study, 3-APTES, 3-APTMS, and 3-TMSPEDA were dissolved in acetone and tested for their ability to silanize a silica gel surface. Figure 2 shows that both the protein binding yield and the lipase activity were highest when 3-APTES was the silanization reagent. We also investigated the effect of the 3-APTES concentration on the silanization. Various concentrations of 3-APTES were tested and, as shown in Figure 3, when 15 % (w/v) 3-APTES was used as the silanization reagent, both the protein binding yield and the activity of the immobilized lipase were highest. Therefore, a 15 % (w/v) 3-APTES was used for subsequent silanization of silica gel surfaces.

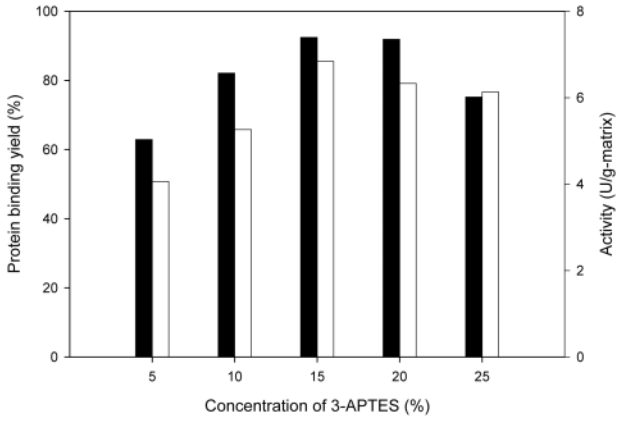

Figure 3. Effect of 3-APTES concentration on silanization: ■, protein binding yield; □, activity of immobilized lipase.

Crosslinking by Glutaraldehyde

Glutaraldehyde is a bifunctional reactive agent capable of reacting with the surface amino groups of enzymes and carriers [16]. Glutaraldehyde is toxic, however, and causes the denaturation of immobilized enzymes. This negative effect of glutaraldehyde can be reduced by its modification [10]. In this study, the glutaraldehyde modification chosen was to heat it in aqueous solution. The important parameters that affect this modification are temperature and time. Therefore, glutaraldehyde was treated at various temperatures and different times to enhance the potential immobilized lipase activity. The response surface methodology (RSM) was used to determine the optimal heating temperatures and times. The variables were normalized and the coded values x_1 and x_2 were defined as

$$x_1 = (X_1 - 60) / 20$$

where x_1 is the coded value of the heating temperature and X_1 is the real value of the heating temperature, and

$$x_2 = (X_2 - 20) / 10$$

where x_2 is the coded value of the heating time and X_2 is the real value of the heating time.

The real and coded values of the experimental factors are shown in Table 1. Experiments were conducted using a 2^2 full-factorial central composite experiment design with four star points ($\alpha = \pm 1.414$) and three replicates at the center point. The design of the experiment and the results are provided in Table 2. Statistical analysis by RSM was performed using these experimental values. The following second-order equation relating to the activity of the immobilized lipase was obtained from the SAS package (v. 9.13; SAS Institute).

Table 1. Real and Coded Values of the Factors Used in the Experimental Design to Optimize Reaction Temperature and Time for Glutaraldehyde Modification

Factors	Symbol	Coded values				
		-1.414	-1	0	1	1.414
Heating temperature (^oC)	x_1	31.7	40.0	60.0	80.0	88.3
Heating time (min)	x_2	5.86	10.0	20.0	30.0	34.1

Table 2. Experimental Design and Results to Enable Determination of Optimal Heating Temperature and Heating Time for Glutaraldehyde Modification

Runs	x_1	x_2	Activity (U/g-matrix)
1	-1	-1	3.73
2	+1	-1	5.99
3	-1	+1	2.81
4	+1	+1	6.18
5	-1.414	0	6.24
6	0	-1.414	3.78
7	+1.414	0	4.16
8	0	+1.414	4.52
9	0	0	7.21
10	0	0	6.83
11	0	0	7.02

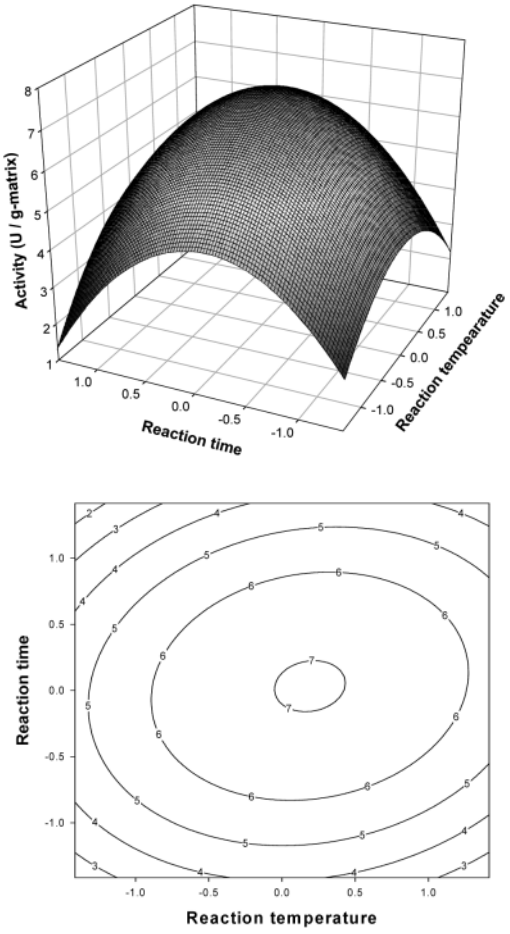

Figure 4. Effect of the heating temperature and time for glutaraldehyde modification on the activity of the immobilized lipase.

$$\text{Activity} = 7.019999 + 0.336161\ x_1 + 0.039551\ x_2 + 0.277500\ x_1x_2 - 0.909472\ x_1^2 - 1.434631\ x_2^2$$

where x_1 is the coded value of the heating temperature and x_2 is the coded value of the heating time.

Contour plots of the activities obtained from the calculated response surface are presented in Figure 4. The temperature and time required to optimize the immobilized lipase activity were 64 ^oC and 20.3 min, respectively. The maximum activity value predicted using the model was 7.12 U/g-matrix. Experiments were performed at 64 ^oC for 20 min to confirm the model prediction. The experimental value for the activity of immobilized lipase (7.05 U/g-matrix) was very similar to the predicted value.

Effect of Reaction Temperature and Time on Lipase Immobilization

We investigated the effects of the reaction temperature and time on the immobilization of lipase on the silica gel matrix. Immobilization was performed using different reaction times and temperatures. As shown in Figure 5, when immobilization was conducted at 10 ^oC, the rate of immobilization was slow, i.e., the activity of immobilized lipase after 180 min was <5 U/g-matrix. When immobilization was performed at 20 ^oC, however, the activity of the immobilized lipase reached 8.0 U/g-matrix after 120 min and did not increase thereafter. In a similarly fashion, at 30 ^oC, the activity of the immobilized lipase increased to 120 min at a slightly lower activity than that obtained at 20 ^oC. By experimentation, the optimal time and temperature for attaching lipase to

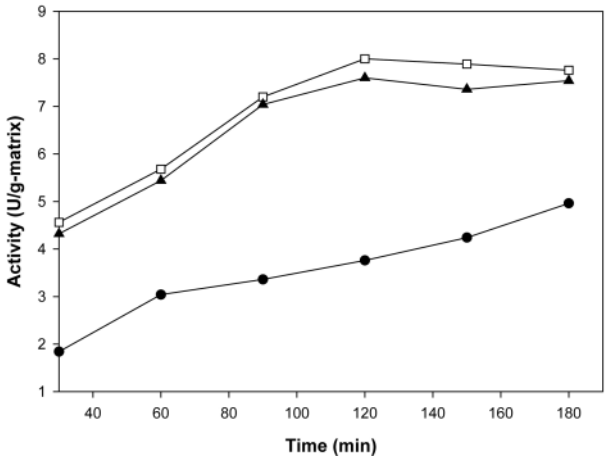

Figure 5. Effect of reaction temperature and time for the coupling reaction of lipase and silica gel on the subsequent activity of the immobilized lipase. (- ● -), 10 °C; (- □ -), 20 °C; (- ▲ -), 30 °C.

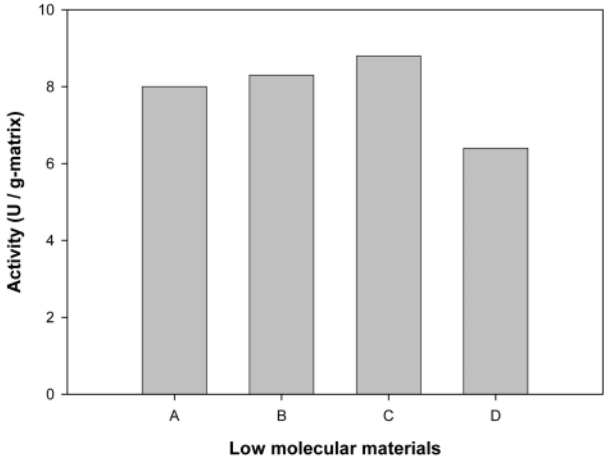

Figure 6. Effects of low molecular weight materials on the activity of immobilized lipase. (A) No treatment. Immobilized lipase was treated with 0.2 % (v/v) of (B) L-lysine, (C) ethanolamine, and (D) glycine after immobilization.

the silica gel matrix were determined to be 120 min and 20 °C, respectively.

Elimination of Unreacted Aldehyde Groups

Glutaraldehyde has a toxic effect on enzymes. If unreacted aldehyde groups remain after the coupling with an amino group in the enzyme, the aldehyde units can attach to other amino groups of the enzyme, thereby reducing the enzyme's activity. This problem reportedly can be solved by reacting any unreacted aldehyde groups with other low molecular weight materials. Park and coworkers [17] reported that the activities of immobilized GL-7-ACA acylase treated with the low molecular weight materials were higher than that of the untreated immobilized GL-7- ACA acylase. In this study, L-lysine, glycine, and ethanolamine were examined as potential

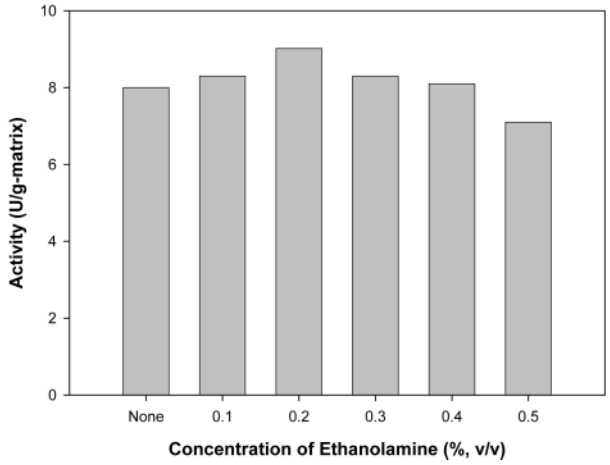

Figure 7. Effect of ethanolamine concentration on the activity of immobilized lipase.

low molecular weight materials to prevent any unreacted aldehyde groups in glutaraldehyde from attacking the immobilized enzyme. After immobilizing lipase on the silica gel, these materials (0.2 %, v/v) were added individually to suspensions containing the immobilized lipase. Reactions proceeded at 20 °C for 2 h. Figure 6 shows that each activity of immobilized lipase when treated with low molecular weight material was higher than that of untreated immobilized lipase. The highest activity of immobilized lipase achieved was obtained when using ethanolamine. Therefore, ethanolamine was selected as the low molecular weight material for the subsequent elimination of unreacted aldehyde groups. In addition, the effect of the ethanolamine concentration on the activity of the immobilized lipase was investigated. Accordingly, immobilized lipase was treated with different concentrations of ethanolamine. As shown in Figure 7, the activity of the immobilized lipase treated with 0.2 % (v/v) of ethanolamine was the highest (8.8 U/g- matrix).

Stability of Immobilized Lipase on Reuse

The stability of immobilized enzyme is extremely important for any subsequent industrial applications. Thus, in this study we investigated the long-term stability of the immobilized lipase. Isooctane containing 10 % (w/v) olive oil was added to 10 mL of 0.05 M phosphate buffer (pH 7) containing 1 g of the immobilized lipase. The reaction mixture was incubated to hydrolyze the olive oil at 37 °C and 150 rpm for 30 min. Immobilized lipase was reused 20 times, with repeated washing after each 30 min reaction. As shown in Figure 8, immobilized lipase activity was maintained at a level exceeding 80 % of the original activity after 20 reuses. Many investigators have reported reuse stabilities of immobilized lipases and, in general, the reuse stability of lipase immobilized through covalent bonding is higher than that

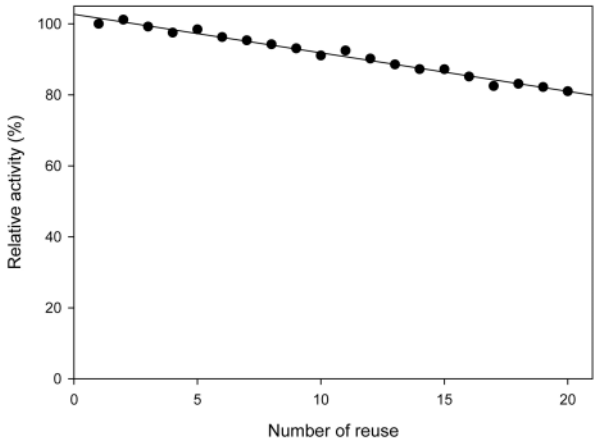

Figure 8. Stability of immobilized lipase after repeated use.

obtained using adsorption methods. Chen and lipas Hsieh [18] reported the activity of an immobilized lipase on ultrafine cellulose fibers prepared using an adsorption method was lost after five cycles of reuse. Ye and coworkers [19] studied lipase immobilization using the covalent bonding method and reported that the residual activity of the immobilized lipase was 67 % after 10 such reuses. We have demonstrated that lipase can be successfully immobilized on a silica gel matrix using a covalent bonding method, resulting in a long-term stability profile exceeding that of previous reported methods.

Conclusions

The objective of this study was to develop a suitable method for immobilizing lipase. Lipase produced from *Rhizopus oryzae* was immobilized on silica gel using a cross-linking method. Each step in the procedure was optimized to improve the immobilization efficiency. The optimal conditions for each step were as follows: pretreatment of silica gel with 35 % hydrogen peroxide, silanization of the silica gel surface with 15 % 3-aminopropyltriethoxysilane in acetone, and crosslinking using 2 % glutaraldehyde modified in aqueous solution at 65 ^{o}C for 25 min. The lipase was then immobilized on the silica gel at 20 ^{o}C for 120 min. After attaching the lipase on the silica gel, any unreacted aldehyde groups of glutaraldehyde were eliminated through treatment with 0.2 % (v/v) ethanolamine. After 20 consecutive uses of this preparation, 80 % of the enzymatic activity of the immobilized lipase was retained.

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