0273-2289/03/104/0185/$20.00

Immobilization of Glutaryl-7-aminocephalosporanic Acid Acylase on Silica Gel and Enhancement of Its Stability

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Received July 1, 2001; Revised August 1, 2002; Accepted August 1, 2002

Abstract

Glutaryl-7-aminocephalosporanic acid (GL-7-ACA) acylase is an enzyme that converts GL-7-ACA to 7-aminocephalosporanic acid, a starting material for semisynthetic cephalosporin antibiotics. In this study, optimal conditions for the immobilization of GL-7-ACA acylase were determined by experimental observations and statistical methods. The optimal conditions were as follows: 1.1 *M* phosphate buffer (pH 8.3) as buffer solution, immobilization temperature of 20°C, and immobilization time of 120 min. Unreacted aldehyde groups were quenched by reaction with a low-molecular-weight material such as L-lysine, glycine, and ethanolamine after immobilization in order to enhance the activity of immobilized GL-7-ACA acylase. The activities of immobilized GL-7-ACA acylase obtained by using the low-molecular-weight materials were higher than those obtained by immobilized GL-7-ACA acylase not treated with low-molecular-weight materials. In particular, the highest activity of immobilized GL-7-ACA acylase was obtained using 0.4% (v/v) ethanolamine. We also investigated the effect of sodium cyanoborohydride in order to increase the stability of the linkage between the enzyme and the support. The effect on operational stability was obvious: the activity of immobilized GL-7-ACA acylase treated with 4% (w/w) sodium cyanoborohydride remained almost 100% after 20 times of reuse.

Index Entries: Immobilization; glutaryl-7-aminocephalosporanic acid acylase; statistical method; ethanolamine; sodium cyanoborohydride.

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Introduction

7-Aminocephalosporanic acid (7-ACA) is a key intermediate for the production of semisynthetic cephalosporin antibiotics. The traditional method for 7-ACA production involves chemical deacylation of the corresponding 7-ACA. However, the chemical method has many problems such as process complexity, use of organic solvent, super-low-temperature reaction (–70°C), and environmental issues *(1,2)*. To overcome the problems associated with the chemical synthetic route, an enzyme-mediated process for the synthesis of 7-ACA from cephalosporin C (CPC) has been proposed as an alternative route to conventional industrial chemical processes *(3)*. Enzymatic transformation of CPC into 7-ACA can be performed by a two-step process: the oxidative deamination of CPC to glutaryl-7-aminocephalosporanic acid (GL-7-ACA) catalyzed by a D-amino acid oxidase and the subsequent hydrolysis catalyzed by GL-7-ACA acylase *(4–6)*. 7-ACA production is problematic because a microorganism or purified enzyme must be removed from the product or it cannot be reused.

Immobilization has been extensively developed in industrial applications of enzymes. Advantages are facilitation of enzyme separation from the product and reuse of enzyme. Easy separation of the enzyme from the product simplifies enzyme applications and supports a reliable and efficient reaction technology *(7)*. In addition, reuse of enzymes provides cost advantages, which are often an essential prerequisite for establishing an enzyme-catalyzed process in the first place *(8)*.

Enzyme immobilization is affected by several factors such as reaction temperature on immobilization, time of immobilization, and conditions of buffer used in immobilization. In particular, pH and ionic strength of buffer used in immobilization are very important. There are numerous reports by many investigators concerning these factors *(9–12)*. However, study of the interaction between pH and concentration of buffer has not been pursued, and study of GL-7-ACA acylase immobilized on silica gel has not yet been reported although there are reports on the immobilization of GL-7-ACA acylase by different methods such as covalent attachment and ionic binding on organic and inorganic carriers *(4,5,13)*.

Thus, in the present study, GL-7-ACA acylase was immobilized on silica gel modified with 3-aminopropyltriethoxysilane, followed by glutaraldehyde for the production of 7-ACA. The objectives of the study were to optimize the conditions of immobilization for GL-7-ACA acylase, such as buffer solution, temperature, and immobilization time. The effect of sodium cyanoborohydride as reducing agent was also investigated for enhancement of operational stability for the immobilized GL-7-ACA acylase.

Materials and Methods

Chemicals and Other Materials

Glutaraldehyde was obtained from Fluka and ρ-dimethylaminobenzaldehyde from Kanto. Glutaric anhydride and 3-aminopropyl-

triethoxysilane were obtained from Aldrich Silica gel and 7-ACA were supplied by Chong Kun Dang Pharmaceutical. Ultrafiltration membrane (15659-00-1) was purchased from Sartorius.

Preparation of GL-7-ACA Acylase

GL-7-ACA acylase from genetically engineered *Escherichia coli* BL21 that contains the GL-7-ACA acylase gene of *Pseudomonas* sp. KAC-1 was supplied by Chong Kun Dang Pharmaceutical. The preparation procedure of the enzyme was as follows. Ammonium sulfate was added up to 20% saturation in the solution of cell extracts and the suspension was centrifuged (10,000 rpm for 15 min) to obtain the supernatant. Then, precipitate obtained after addition of ammonium sulfate to 40% saturation was resuspended in 100 m*M* phosphate buffer (pH 8.0). The solution was dialyzed and concentrated by an ultrafiltration membrane (mol wt cutoff = 30,000). All purification procedures were performed at 4°C unless stated otherwise. The solution was stored at –20°C and used for immobilization.

Synthesis of GL-7-ACA

GL-7-ACA was prepared by the method of Shibuya et al. *(14)*. Glutaric anhydride, 15.2 g in 10 mL of acetone, was added to a solution, which was prepared by dissolving 9.07 g of 7-ACA in 70 mL of 1.0 *M* sodium bicarbonate (pH 9.0). The reaction mixture was stirred at room temperature for 10 min and then evaporated under reduced pressure at 25°C for 10 min to remove the acetone. The solution was acidified with 5.0 *N* HCl to pH 1.5, extracted with 120 mL of ethyl acetate, and quickly filtered with suction. The combined ethyl acetate layers from three such runs were filtered through a 0.45-µm Millipore filter and concentrated to about 50 mL under reduced pressure at 25°C. Chloroform (450 mL) was added to the concentrated solution, and mixed well. The precipitates were collected by filtration and washed thoroughly with 50 mL of ethyl acetate.

Immobilization of GL-7-ACA Acylase

One gram of dry silica gel was mixed with 3-aminopropyltriethoxysilane (10% in 25 mL of dissolved water). The suspension was incubated at 80°C for 2 h with constant mixing, washed thoroughly with water before drying at 120°C for 2 h, and then 1% (v/v) glutaraldehyde was added to the silica gel suspended in 100 m*M* phosphate buffer (pH 8.0) at 20°C. After stirring (150 rpm) for 2 h, the suspension was filtered and carriers were washed with water. The activated silica gels were resuspended in 100 m*M* phosphate buffer (pH 8.0). Finally, a solution of enzyme was added. At this time, the added amount of protein was equal to about 10 mg/mL. The suspension was stirred at 20°C for 2 h, and the immobilized GL-7-ACA acylase recovered by filtration was washed. After resuspending in 100 m*M* phosphate buffer (pH 8.0), the immobilized enzyme was analyzed.

Assay of GL-7-ACA Acylase Activity

The activity of the immobilized enzyme was measured as follows. The immobilized enzyme was incubated at 37°C for 10 min in the presence of 1% (w/v) GL-7-ACA. The reaction was stopped with an aqueous solution of 20% (v/v) acetic acid and 0.05 *N* NaOH. Then ρ-dimethylaminobenzaldehyde (0.5% [w/v] in methanol) was added to the mixture. The absorbance of mixture at 415 nm was measured. One unit of acylase activity was defined as the amount of enzyme that produced 1 µmol of 7-ACA/min at 37°C, pH 8.0.

Determination of Amount of Protein Bound to Carriers

The amount of protein was determined by the Folin–Lowry method *(15)*. The amount of protein bound to the carriers was determined by the difference between initial and residual protein concentrations.

Calculation of Protein-Binding Yield

Yield of protein binding was calculated as the ratio of the amount bound on silica gels to the initial amount. Yields were expressed as a percentage.

Experimental Designs

Response surface methodology (RSM) was introduced to determine the optimal condition of interaction between pH and concentration of phosphate buffer *(16–18)*. Experiments were carried out using a 2^2 full-factorial design containing four star points and three center points *(19,20)*. The rotatability of the design depends on the α-value, the coded value for star points: in order to be rotatable, α depends on the number of points used in the factorial fraction *(21)* and is equal to 1.414 for this design. All variables have been normalized for statistical calculation. The factor X_i has been coded as x_i according to the following transformation:

$$x_i = (X_i - X_o)/\delta X$$

in which x_i is the dimensionless coded value at the factor X_i, X_i is the real value of the factor, X_o is the real value of the X_i at the center point, and δX is the step change value. Real and coded values of the experimental factors used are given in Table 1.

A second-order polynomial model for predicting the optimal points was obtained from the SAS package (SAS Institute). The polynomial model equation was expressed as follows:

$$Y = b_o + \Sigma b_i x_i + \Sigma b_{ii} x_i^2 + \Sigma b_{ij} x_i x_j$$

in which Y is the value of the response, b is the regression coefficient, and x is the coded value of the factors.

Table 1
Real and Coded Values of Factors Used in Experimental Designs

		Coded values				
Factor	Symbol	–1.414	–1	0	+1	+1.414
pH	x_1	6.6	7.0	8.0	9.0	9.4
Buffer concentration (*M*)	x_2	0.3	0.5	1.0	1.5	1.7

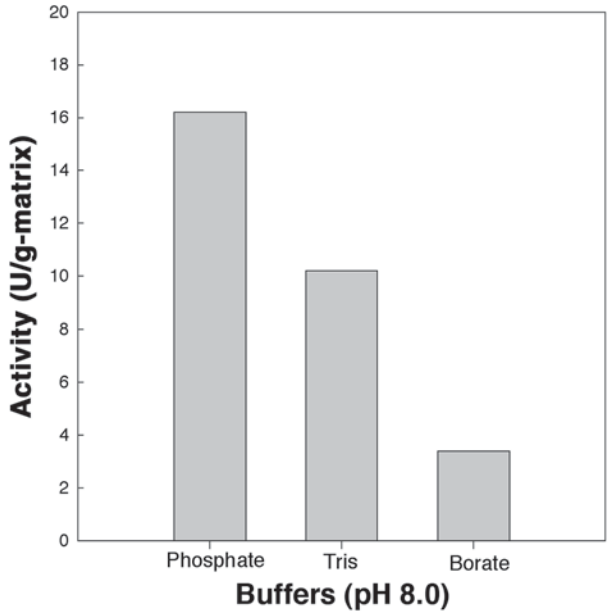

Fig. 1. Effect of buffer solutions on activity of immobilized GL-7-ACA acylase.

Results and Discussion

Effect of Buffer on Immobilization of GL-7-ACA Acylase

One of the most important parameters that affects the immobilization of enzyme is the buffer condition used. It is generally accepted that the activity of immobilized enzyme is affected by buffer conditions such as type of buffer, pH, and concentration (ionic strength) in the immobilization procedure *(9,10)*.

The influence of buffer solution on immobilization of GL-7-ACA acylase was investigated. Various kinds of buffer solution were tested in order to determine a suitable buffer for immobilization of GL-7-ACA acylase. As shown in Fig. 1, there were great differences in the activity of immobilized GL-7-ACA acylase between the buffers tested, and the highest activity of immobilized GL-7-ACA acylase was observed for phosphate buffer (pH 8.0). Therefore, phosphate buffer was chosen as a suitable buffer for further studies.

Table 2
Experimental Designs and Results for Determination of Optimal Conditions Between pH and Buffer Concentration

Run	pH	Buffer concentration (*M*)	Activity (U/g matrix)
1	–1.0	–1	19.72
2	+1.0	–1	21.56
3	–1.0	+1	20.01
4	+1.0	+1	22.25
5	–1.414	0	17.96
6	0	–1.414	21.38
7	+1.414	0	21.39
8	0	+1.414	22.25
9	0	0	23.94
10	0	0	23.24
11	0	0	22.74

Optimization of Interaction Between pH and Concentration of Buffer Solution Using RSM

The pH and concentration of buffer solution are very important in the immobilization of enzyme. Many investigators have reported on the effects of pH and concentration of buffer *(11,12)*. However, study on the interaction between pH and concentration of buffer solution has not been pursued. Therefore, in our study, optimal conditions of interactions between pH and ionic strength of phosphate buffer were investigated for enhancement of activity of immobilized GL-7-ACA acylase through experimental observations and statistical methods.

RSM, an experimental strategy for seeking optimum conditions, is an efficient technique for optimization *(22)*. Experiments were carried out using a 2^2 full-factorial central composite design experiment with four star points ($\alpha = \pm 1.414$) and three replicates at the center point. The design of this experiment is given in Table 2, together with the results of the experiments. Statistical analysis by RSM was performed on the basis of these experimental values. The statistical significance of the models was tested for adequacy by the Fisher test. The *F* value is the ratio of the mean square due to regression to the mean square due to error *(23)* and indicates the influence (significant or not) of the model tested. A more rigorous test conclusion could be obtained from the use of *p* value corresponding to *F* value, which indicates the probability that differences between both calculated and tabulated statistics are only due to random experimental error. If the *p* value is smaller than the fixed level of significance, the null hypothesis is rejected and infers that the tested models are significant. For more reliable analysis, the determination coefficient (R^2), which is a measure for this model and can be calculated by dividing the variation explained by the model by total variation *(19)*, was also used. As shown in Table 3, the *F* and *p* values for the model tested were 43.03 and 0.0004, respectively. Thus, the tested model is

Table 3
Statistical Analysis for Models of Activity of Immobilized GL-7-ACA Acylase at Different pH Levels and Buffer Concentrations

Source	Sum of squares	Degrees of freedom	Mean square	*F* Value	*p* Value
Model	29.49	5	5.90	43.03	0.0004
Error	0.68	5	0.14		
Total	30.17	10			
R^2	0.977				

statistically significant at the 1% level of significance. On the other hand, the determination coefficient (R^2) was 0.977. This value indicates that 97.7% of the variability in the response could be explained by the model. Consequently, these results indicate that the model for the activity of immobilized GL-7-ACA acylase is adequate. Using these results, the following second-order polynomial equation relating to the activity of immobilized GL-7-ACA acylase explains the experimental data:

$$Y = 27.376 + 1.116x_1 + 0.277x_2 - 1.815x_1^2 - 0.746x_2^2 + 0.101x_1x_2$$

in which x_1 is the coded value of pH, and x_2 is the coded value of the buffer's concentration.

The contour plot on the activity of immobilized GL-7-ACA acylase obtained from the calculated response surface is represented in Fig. 2. The optimum values of pH (x_1) and concentration of buffer (x_2) obtained for the activity of immobilized GL-7-ACA acylase are as follows:

$$x_1 = 0.313, \quad x_2 = 0.206$$

For calculation purposes, the normalized, coded values x_1 and x_2 were defined as follows:

$$x_1 = (X_1 - 8)/1 \quad \text{and} \quad x_2 = (X_2 - 1)/0.5$$

According to these results, optimal pH and buffer concentration for the activity of immobilized GL-7-ACA acylase were calculated to be 8.3 and 1.1 *M* phosphate buffer, respectively. The maximum value of activity predicted from the model was 23.57 U/g of matrix.

To confirm these results, experiments were carried out to recheck the performance of the model. An experiment was performed at different pHs at a buffer concentration (x_2) fixed to 1.1 *M*. As shown in Fig. 3, a good agreement between the experimental points and the values predicted by the models confirms the validity of the models for activity although a slight difference in low and high levels of pH existed. An experiment was also performed with different concentrations of buffer (x_2) at a pH value fixed at 8.3. As shown in Fig. 4, although a slight difference in low concentration of phosphate buffer existed, a similar tendency between predicted value by the model and the real value by the experiment was also observed. Thus, we confirm the validity of the models used in statistical analysis.

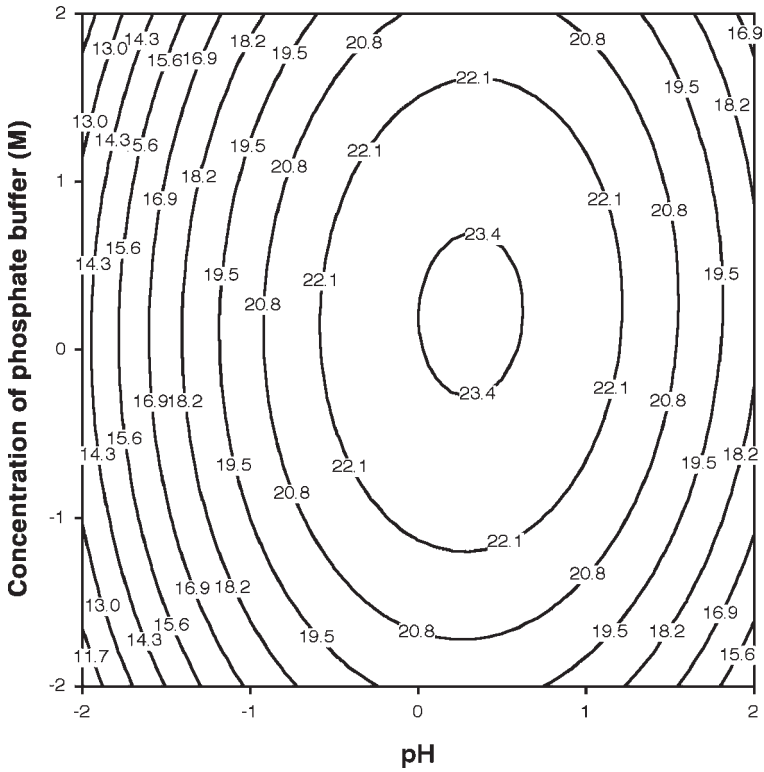

Fig. 2. Effect of pH and concentration of phosphate buffer on activity of immobilized GL-7-ACA acylase.

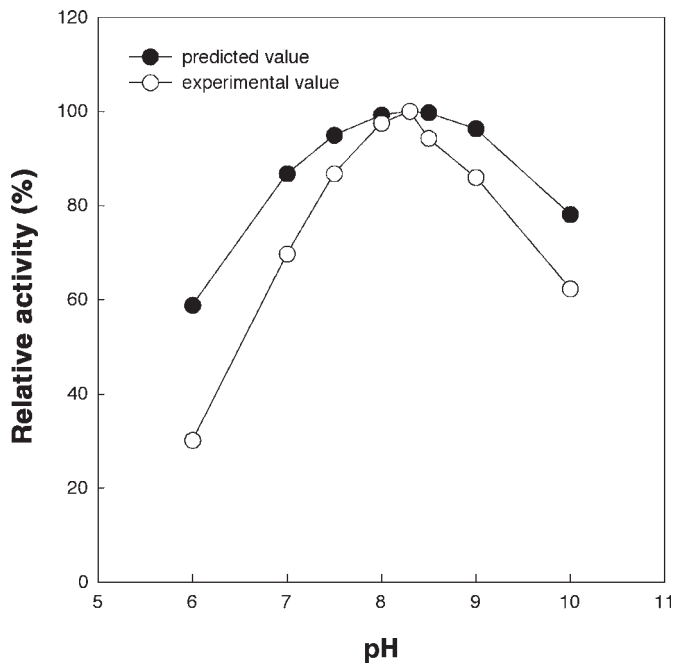

Fig. 3. Comparison of activities between predicted and experimental values. Experiments were carried out with different pHs under 1.1 *M* phosphate buffer. Relative activities are expressed as percentage of highest activity.

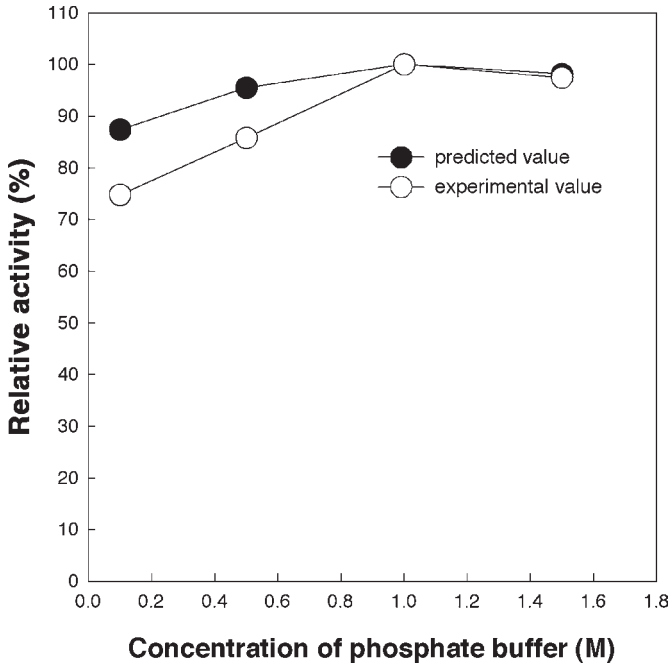

Fig. 4. Comparison of activities between predicted and experimental values. Experiments were carried out with different buffer concentrations under pH values fixed to 8.3. Relative activities are expressed as percentage of highest activity.

Effects of Reaction Temperature and Time on Immobilization of GL-7-ACA Acylase

On the basis of the preceding results, the effects of reaction temperature and time on immobilization were also investigated. Immobilization was performed with different reaction times at different temperatures. As shown in Fig. 5A, yields of protein binding at 20 and 30°C were increased up to 120 min, and then almost unchanged afterward. On the other hand, yield of protein binding at 4°C was lower than at other pHs.

In addition, the behavior of the activity of GL-7-ACA acylase immobilized at 20°C was almost the same as the yield of protein binding at 20°C (Fig. 5B), and maximum activity was obtained after 120 min. However, unlike the behavior of the activity of GL-7-ACA acylase immobilized at 20°C, the activity of GL-7-ACA acylase immobilized at 30°C slightly decreased with increasing time above 90 min (although the activity increased up to 90 min). Such a decrease in activity at 30°C is believed to be caused by the denaturation of enzyme. These results confirm the important role of temperature in immobilization of enzymes. Through these experiments, reaction temperature and reaction time were determined to be 20°C and 120 min, respectively, for immobilization of GL-7-ACA acylase on silica gel, in consideration of protein binding yield and activity of immobilized GL-7-ACA acylase.

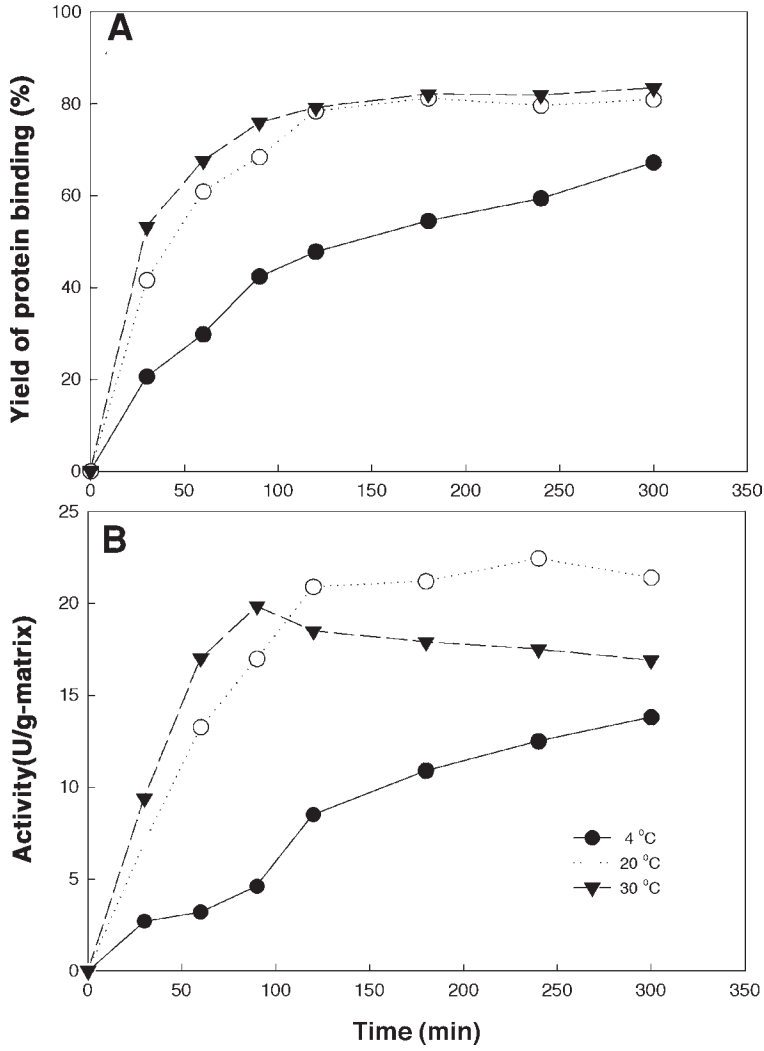

Fig. 5. Effect of reaction temperature and time on immobilization of GL-7-ACA acylase. **(A)** Yields of protein binding; **(B)** activity.

The experimental results showed that 1.1 *M* phosphate buffer (pH 8.3) as buffer solution, immobilization temperature of 20°C, and immobilization time of 120 min were the optimum conditions for immobilization of GL-7-ACA acylase on silica gel.

Elimination of Unreacted Aldehyde Groups by Reaction with Low Molecular Weight Materials

Glutaraldehyde, which was introduced in our study, has been widely used as a crosslinking agent for the immobilization of enzyme. However, toxicity of glutaraldehyde to the enzyme is a major problem *(24,25)*. If the amine groups of enzyme do not occupy the functional groups of the glut-

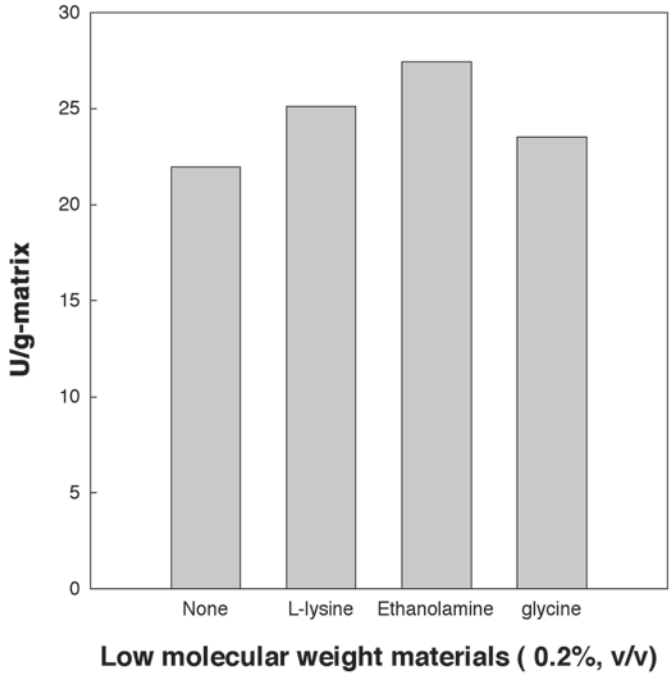

Fig. 6. Effect of low molecular weight materials on immobilization of GL-7-ACA acylase. Immobilized GL-7-ACA acylase was treated with 0.2% (v/v) low-molecular-weight materials after immobilization.

araldehyde, the unbound groups of glutaraldehyde can randomly bind with amine groups of the enzymes. This would affect enzyme activity *(26)*. In our study, unreacted aldehyde groups were quenched by reaction with a low-molecular-weight material such as L-lysine, glycine, and ethanolamine after immobilization in order to enhance the activity of immobilized GL-7-ACA acylase. As shown in Fig. 6, activities of immobilized GL-7-ACA acylase obtained by using the low-molecular-weight materials (0.2% [v/v]) were higher than that obtained by immobilized GL-7-ACA acylase not treated with low-molecular-weight materials. In particular, the highest activity of immobilized GL-7-ACA acylase was obtained with ethanolamine. Thus, ethanolamine was chosen as the low-molecular-weight material for elimination of the unreacted aldehyde groups, and the effect of ethanolamine concentration was investigated. Immobilized GL-7-ACA acylase was treated with different concentrations of ethanolamine. As shown in Fig. 7, increasing ethanolamine concentration increased the activity of immobilized GL-7-ACA acylase up to 0.4% (v/v). Beyond this concentration, the activity slightly decreased with increasing ethanolamine concentration. The activity of immobilized GL-7-ACA acylase treated with 0.4% (v/v) ethanolamine was 28.54 (U/g of matrix). This value was increased up to about 25% compared with that obtained by immobilized GL-7-ACA acylase not treated with ethanolamine.

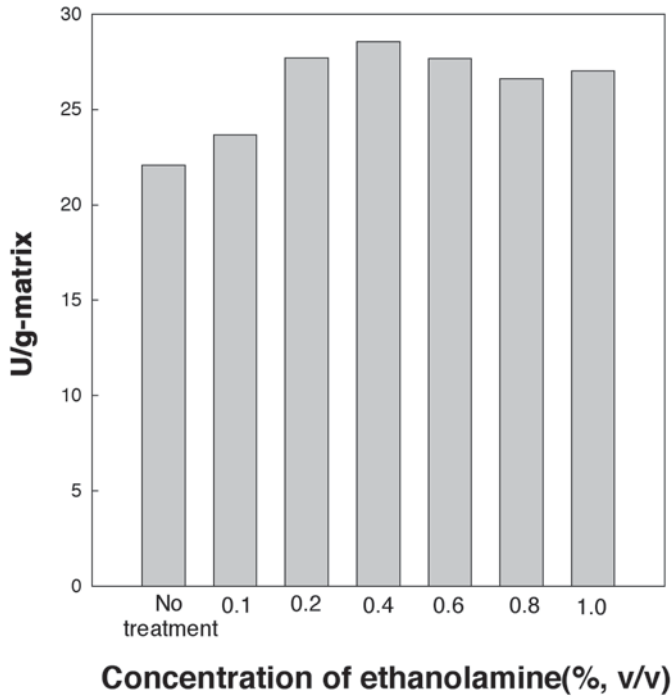

Fig. 7. Effect of ethanolamine concentration on immobilization of GL-7-ACA acylase.

Improvement in Stability of Immobilized GL-7-ACA Acylase by Treatment of Reducing Agent

To increase the stability of the linkage between enzyme and support, we investigated the effect of reducing the Schiff's bases formed in the glutaraldehyde coupling *(27)*. Sodium cyanoborohydride was selected as the reducing agent. As shown in Fig. 8, treatment with sodium cyanoborohydride did not significantly affect the activity of the immobilized enzymes until the concentration of sodium cyanoborohydride increased up to 4% (w/w, carrier), determining a loss of activity of <4%. Beyond this concentration, activity of immobilized enzyme decreased with increasing concentration of sodium cyanoborohydride because it has a strong alkali pH.

On the basis of these results, the abilities for long-term stability of the immobilized GL-7-ACA acylase treated with 2 and 4% (w/w) sodium cyanoborohydride were investigated by assaying for repeated washes. As shown in Fig. 9, the effect of sodium cyanoborohydride reduction on operational stability was obvious. In particular, the activity of immobilized GL-7-ACA acylase treated with 4% (w/w) sodium cyanoborohydride remained almost 100% after 20 times of reuse. As a consequence, if this immobilization method should be introduced in scale-up for the mass production of 7-ACA, more superior effects might be obtained.

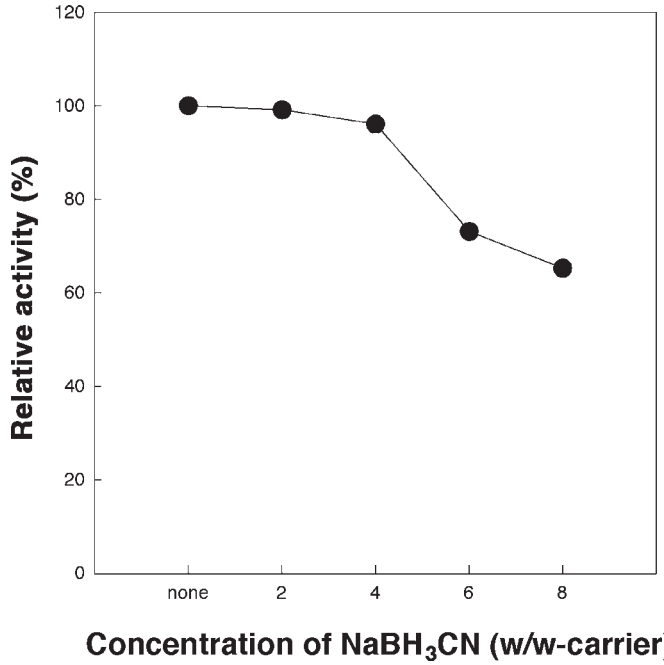

Fig. 8. Effect of $NaBH_3CN$ on immobilization of GL-7-ACA acylase. Activities are expressed as percentage of immobilized GL-7-ACA acylase untreated with $NaBH_3CN$.

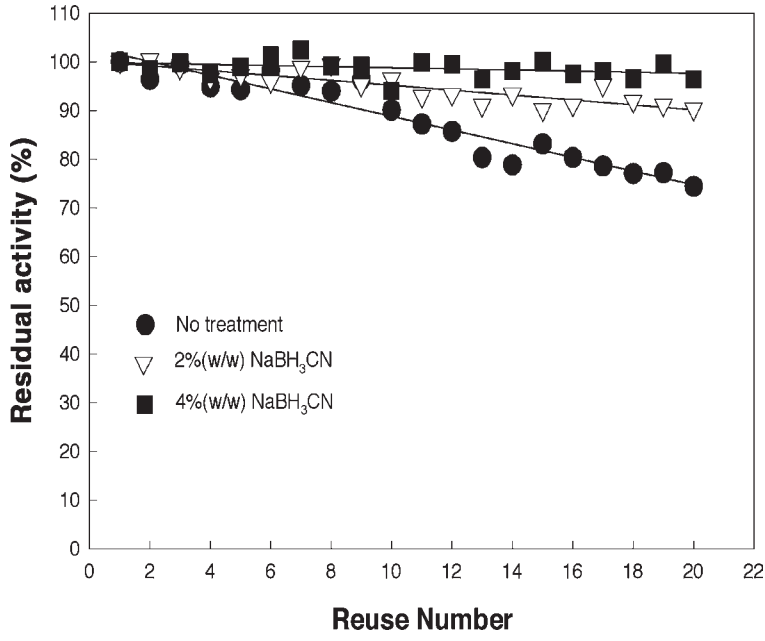

Fig. 9. Operational stability of GL-7-ACA acylase immobilized on silica gels.

Acknowledgment

This study was supported by research grants from the Korea Science and Engineering Foundation (KOSEF) through the Applied Rheology Center, an official KOSEF-created engineering research center at Korea University, Seoul, Korea.

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