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Immobilization of GL-7-ACA Acylase for the Production of 7-ACA

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Abstract—Glutaryl-7-aminocephalosporanic acid(GL-7-ACA) acylase is an important enzyme for the production of 7-ACA (7-aminocephalosporanic acid). For an efficient immobilization of GL-7-ACA acylase, various carriers were tested. A high-porous hydrophilic carrier (FPHA) among various carriers tested was found to be the best for the immobilization of GL-7-ACA acylase. In order to develop an effective immobilization method of GL-7-ACA acylase, the parameters that affect the immobilization of GL-7-ACA acylase were also investigated under different conditions of buffer solution and different concentrations of glutaraldehyde. The highest value of GL-7-ACA acylase activity (70 Unit/g-matrix) was obtained when immobilized with 1% glutaraldehyde in a 0.1 M Tris buffer (pH 8.0). Also, in order to enhance the activity of the immobilized GL-7-ACA acylase, unreacted aldehyde groups were quenched by reaction with a low molecular weight agent such as L-lysine after immobilization. The highest activity of immobilized GL-7-ACA acylase was obtained at 0.1% of L-lysine. The immobilized GL-7-ACA acylase was tested for long-term stability and it was found that the activity was retained at about 62% of the initial value after 72 times of reuse at 25 °C.

Key words: GL-7-ACA Acylase, 7-ACA, Immobilization, FPHA

INTRODUCTION

7-ACA is an important starting material for the synthesis of semisynthetic Cephalosporins. 7-ACA is suitable for synthesis of new derivatives because it has highly reactive groups such as 3-acetoxymethyl group and 7-amino group. Enzymatic transformation of CPC into 7-ACA can be performed by a two-step process consisting of the oxidative deamination of CPC to GL-7-ACA catalyzed by a D-amino acid oxidase and the subsequent hydrolysis catalyzed by GL-7-ACA acylase [Alfani et al., 1997; Bianchi et al., 1997; Ezio et al., 1998; Roberto et al., 1998]. The production of 7-ACA using microorganisms or purified enzymes has many problems in that the biocatalyst must be removed from the product and cannot be reused. Recently, enzyme production has been improved and the purification of enzymes has been facilitated by genetic engineering. So enzymatic transformation of 7-ACA is recommended for use in the immobilization method for purified enzyme. Currently, an enzymatic method using a two-step process with immobilized D-amino oxidase and GL-7-ACA acylase is being used for the commercial production of 7-ACA [Tsuzuki et al., 1989].

There has been a great interest in immobilized enzymes and cells [Kim and Choi, 1983; Kwon and Rhee, 1984]. There are several reasons for the preparation and use of immobilized enzymes. In addition to a more convenient handling of enzyme preparations, the two main targeted benefits are easy separation of the enzyme from the product, and reuse of the enzyme. Easy separation of the enzyme from the product simplifies enzyme applications and supports a reliable and efficient reaction technology. In addition, reuse of enzymes provides cost advantages which are often an essential prerequisite for establishing an enzyme-catalyzed process in the first place [Wilhem et al., 1999]. The 7-ACA produced by immobilized GL-7-ACA acylase is a useful intermediate of great commercial interest for the preparation of semisynthetic cephalosporin antibiotics. However, research activities dealing with immobilization of GL-7-ACA acylase are lacking. Therefore, the objective of this study is to develop a suitable immobilization method for GL-7-ACA acylase by covalent coupling to the resin.

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MATERIALS AND METHODS

1. Materials

Glutaraldehyde (Fluka Co.), ρ-dimethylaminobenzaldehyde (KANTO Chemical Co. INC) glutaric anhydride (Aldrich Co.) and 3-aminopropyltriethoxysilane (Aldrich Co.) were used. The three resins used for immobilization in this study, primary amino polysiloxane carrier (PAP), polysiloxane ethylene diamine function carrier (DAP III) and high-porous hydrophilic carrier (FPHA), were supplied by the Chong Kun Dang Pharmaceutical Corp. L-Lysine and 7-ACA were also supplied by the Chong Kun Dang Pharmaceutical Corp. Ultrafiltration membrane (15659-00-1) was purchased from Sartorius.

2. Preparation of GL-7-ACA Acylase

GL-7-ACA acylase was obtained from the genetically engineered *E. coli* BL21 that cloned the GL-7-ACA acylase gene of *Pseudomonas* sp. KAC-1 (supplied by the Chong Kun Dang Pharmaceutical Corporation). 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). Afterward, the suspension was collected. And the precipitate obtained after the addition of ammo-

nium sulfate (40% saturation) was resuspended in 100 mM phosphate buffer (pH 8). The solution was dialyzed and concentrated by an ultrafiltration membrane (cutoff M.W 30,000). All purification procedures were performed at 4 °C unless stated otherwise. The solution was stored at −20 °C and used for the immobilization.

3. Synthesis of GL-7-ACA

GL-7-ACA was prepared in the laboratory following the procedure of Shibuya et al. [1981]. First 15.2 g of glutaric anhydride 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 and extracted with 120 ml of ethyl acetate, and quickly filtered by suction. The combined ethyl acetate layers from three such runs were filtered through a Millipore filter (0.45 μm) 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 50ml of ethyl acetate.

4. Assay of GL-7-ACA Acylase Activity

The activities of the immobilized enzyme were measured by using GL-7-ACA. 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-dimethylaminobenaldehyde (PDAB, 0.5% w/v in methanol) was added to the mixture. The products in the supernatant were determined by a spectrophotometer at 415 nm. One Unit of acylase activity was defined as the amount of enzyme that produced 1 μmol of 7-ACA per min at 37 °C, pH 8.

5. Calculation of Immobilization Yield

Yield of immobilization was calculated as the ratio of the amount of protein bound on the carrier to the initial amount of protein. Yield was expressed in a percentage. The amount of protein was determined by the Folin-Lowry method [Lowry et al., 1951]. The amount of protein bound to the carriers was determined by the difference between initial and residual protein concentrations.

6. Immobilization Method of GL-7-ACA Acylase

An aqueous solution (1%, v/v) of glutaraldehyde was added to the suspended resins mentioned in the Materials section in a 100 mM phosphate buffer (pH 8) at 20 °C. After stirring (150 rpm) for 2 hr, the suspension was filtered and the solid washed with water. The activated resin (0.1 g) was then resuspended in 100 mM phosphate buffer. A solution of GL-7-ACA acylase was added, and the suspension was stirred at 20 °C for 2 hr. The immobilized GL-7-ACA acylase recovered by filtration was washed and resuspended in 100 mM phosphate buffer. L-Lysine was added in the resuspended immobilized enzyme to remove the unreacted aldehyde group. After stirring for 1 hr, the immobilized GL-7-ACA acylase was recovered and washed with 1 M NaCl, and the solid was filtered and resuspended in a 100 mM phosphate buffer.

7. Conversion

The immobilized enzyme reacted with Gl-7-ACA (1%, w/v) in the 100 mM a Phosphate buffer (pH 8) at 25 °C and 37 °C. The progress of the hydrolytic reaction was monitored by HPLC utilizing a 20 mM ammonium acetate pH 5.5/2% (v/v) acetonitrile as eluent at a flow rate of 1 ml/min on a μ Bondapak C18 column (3.9×300 mm).

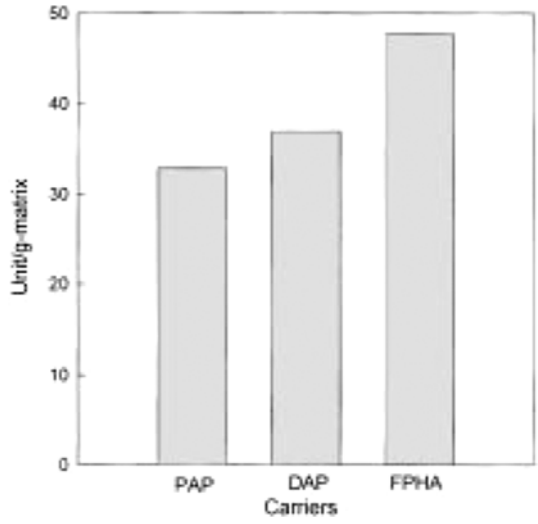

Fig. 1. Comparison of activity of GL-7-ACA acylase among various carriers on the immobilization.

RESULTS AND DISCUSSION

1. Determination of Suitable Carrier and Crosslinking by Glutaraldehyde

Immobilization is often accompanied by some changes in enzymatic activity, optimum pH, affinity for the substrate, and stability. The extent of these changes depends on the enzyme and carrier support and on the immobilization conditions. Among these, the support is the most important [Tumuturk et al., 2000]. Thus, in this work, various carriers were tested in order to determine the most suitable carrier for the immobilization of GL-7-ACA acylase. As shown in Fig. 1, the best result for the activity of immobilized GL-7-ACA acylase among the various carriers tested in this study was obtained by the GL-7-ACA acylase immobilized on the FPHA carrier. This value was 47.70 Unit/g-matrix. According to above result, FPHA was chosen as the most suitable carrier among the various carriers tested and therefore used in further studies. One of the most important techniques for enzyme immobilization is the attachment of amino groups on carriers because amino groups are susceptible to coupling reaction. Modification of the carrier surface for the attachment of amino groups has usually been introduced in the immobilization of enzymes. However, when FPHA is used as the immobilization carrier, modification of FPHA surface is not required because FPHA possesses amino groups. Thus, the use of FPHA as an immobilization carrier can simplify the immobilization process by abbreviating the modification phase of the carrier surface.

Glutaraldehyde plays a role as a space arm of the carriers [Wilhem et al., 1999]. In this work, the effect of glutaraldehyde on the activity of immobilized GL-7-ACA acylase was investigated. As shown in Fig. 2, the activity of the immobilized GL-7-ACA acylase increased up to 1% of the glutaraldehyde concentration. Beyond this concentration, the activity gradually decreased with increasing glutaradehyde concentrations. Such a decrease in activity at above 1% of glutaraldehyde is believed to be caused by the adverse effect of glutaraldehyde to the immobilized GL-7-ACA acylase [Park et al., 2001]. When used in excess, the unused glutaraldehyde seemed to attack the amine group of the enzyme and therefore affect the

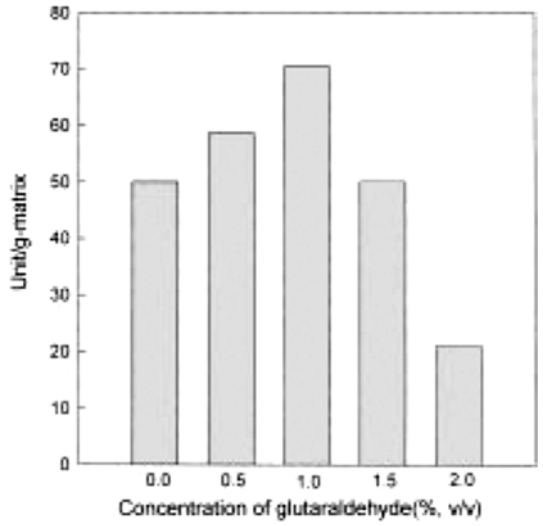

Fig. 2. Effect of glutaraldehyde concentration on immobilization using FPHA.

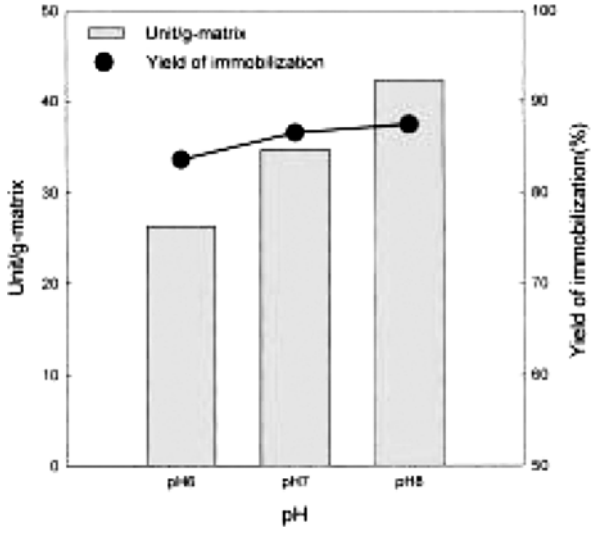

Fig. 4. Effect of pH on the immobilization by using FPHA.

stability of the immmobilized enzyme.

2. Determination of Optimal Conditions of Immobilization

One of the most important parameters that affect the immobilization of the enzyme is the buffer condition used. It is generally accepted that the activity of immobilized enzyme is affected by buffer conditions such as solution type, and the pH in the immobilization procedure [Moly Eldin et al., 2000; Alfani et al., 1999]. In this work, various buffer solutions such as Tris, phosphate and borate buffer were tested in order to determine a suitable buffer for the immobilization of GL-7-ACA acylase on FPHA. As shown in Fig. 3, the highest value of activity of immobilized GL-7-ACA acylase was obtained with a 100 mM Tris buffer (pH 8), and this value was 2-3 fold greater than those from other buffers.

The enzyme has a quaternary structure and is stabilized at a suitable pH. If the pH is changed, enzyme conformation is changed and becomes unstable, thus reducing activity. Therefore, in this study, the influence of pH on the immobilization was investigated with a 100 mM Tris buffer, which was chosen as the most suitable buffer solution as discussed above. As shown in Fig. 4, the optimal pH for immobilization was pH 8. The activity of immobilized GL-7-ACA acylase and immobilization yield was 42.33 U/g-matrix and 87.4%, respectively. According to the above results, a Tris buffer of pH 8 was chosen as the most suitable buffer for the immobilization of GL-7-ACA acylase.

Coupling time can have a considerable influence on enzyme stability. Thus, determination of the coupling time for the immobilization of GL-7-ACA acylase is very important. Fig. 5 shows the effect of coupling time on the immobilization of GL-7-ACA acylase. Activity recovery and immobilization yield improved with an increase in coupling time up to 4 hr, but decreased thereafter. The maximum values for immobilization yield and activity were 86.70% and 41.44 Unit/g-matrix, respectively, at 4 hr. Coupling time must affect stabilization as the activity of immobilized enzymes decreased with increase in coupling time from 4 to 20 hr.

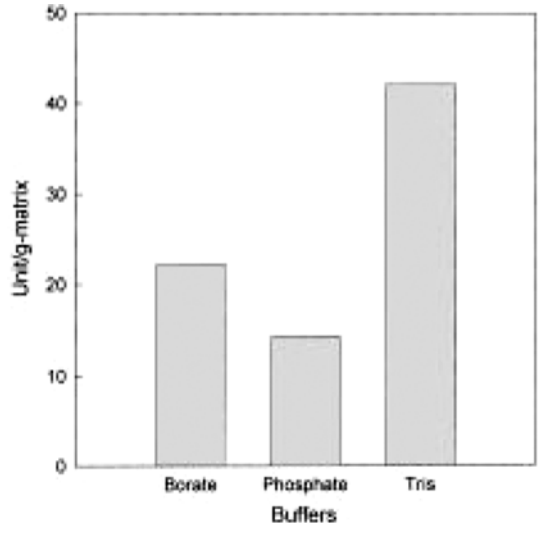

Fig. 3. Effect of various buffers on immobilization with FPHA.

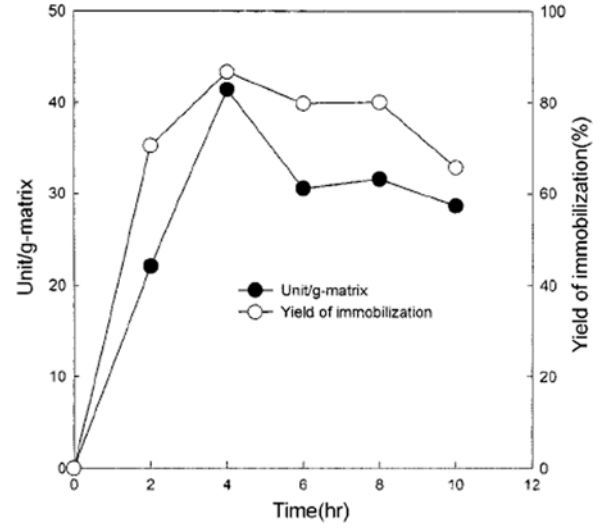

Fig. 5. Effect of coupling time on immobilization of GL-7-ACA acylase.

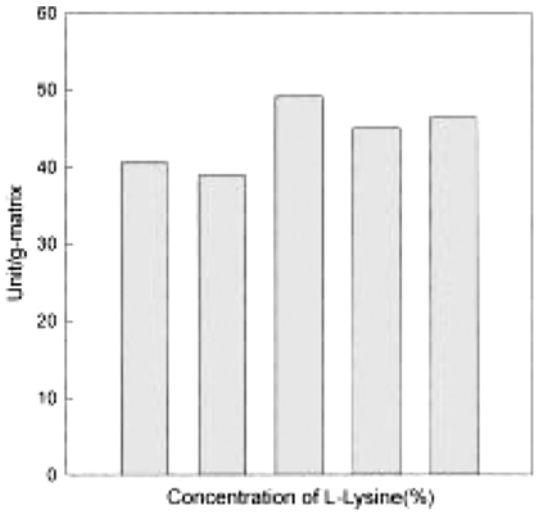

Fig. 6. Effect of L-lysine on the immobilization of GL-7-ACA acylase.

If the functional groups of glutaraldehyde are not occupied by the amine groups of the enzyme, the unbound groups can randomly bind with amine groups of the enzymes, affecting enzyme activity [Chae et al., 1998]. In this work, unreacted aldehyde groups are quenched by reaction with a low molecular weight material such as L-lysine after immobilization in order to enhance the activity of immobilized GL-7-ACA acylase. When different concentrations (0.05-0.3%, v/v) of L-lysine were added, the highest activity of immobilized GL-7-ACA acylase was obtained at 0.1% of L-lysine (Fig. 6). This result suggested that the activity of the immobilized enzyme increased by about 23% because unreacted aldehyde groups are removed by reaction with L-lysine.

Although the enzyme is bound to resin activated, weakly bound enzyme exists in the resin and contaminants are also bound to the resin because a partially purified enzyme was used. Therefore, weakly bound enzymes and contaminants must be removed because activity of the immobilized enzyme is overestimated and weakly bound enzymes can interfere with the accessibility of the substrate to the normally bound enzyme. In order to remove them, the immobilized enzyme preparation was washed with 1 M NaCl after treatment with L-lysine. As shown in Fig. 7, the activity of immobilized GL-7-ACA acylase treated with 1 M NaCl increased about 15% compared to that obtained from immobilized GL-7-ACA acylase untreated with 1 M NaCl. This result suggests that the weakly bound enzyme and contaminants interfered with the accessibility of the substrate to the normally bound enzyme.

3. Reuse of Immobilized Enzyme on the FPHA Resin

Increased operational stability of immobilized enzymes is essential for achieving cost benefits. The long-term stability of immobilized GL-7-ACA acylase at 25 °C and 37 °C was investigated by assaying it for repeated use. Immobilized GL-7-ACA acylase reacted with GL-7-ACA (1%, w/v) as substrate in a 0.1 M Tris buffer (pH

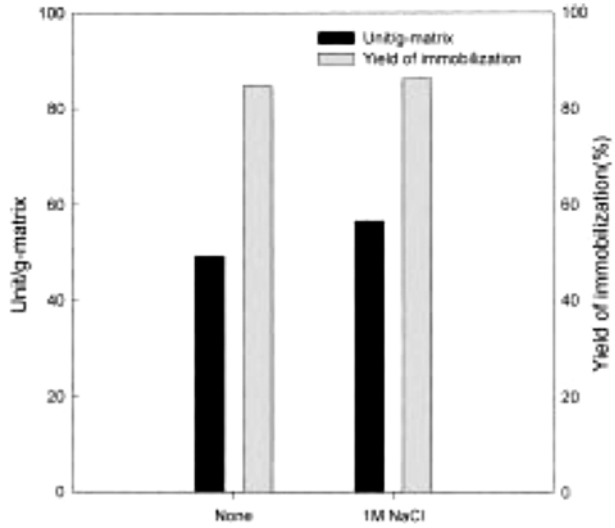

Fig. 7. Effect of NaCl treatment on the immobilization of GL-7-ACA acylase.

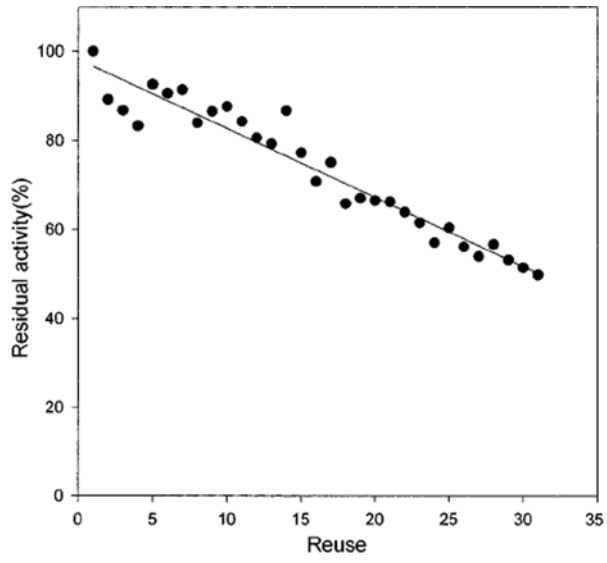

Fig. 8. Operational stability of immobilized GL-7-ACA acylase on FPHA resin at 37 °C.

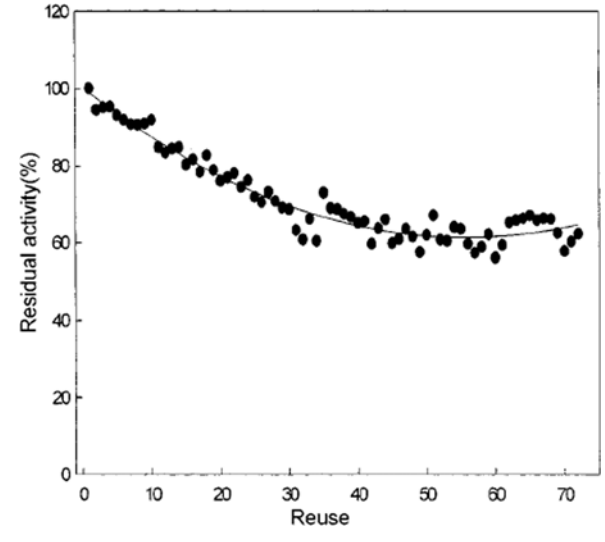

Fig. 9. Operational stability of immobilized GL-7-ACA acylase on FPHA resin at 25 °C.

8.0) for 10 min, and the reaction was repeated under the same conditions. As shown in Fig. 8, 51.32% of the activity of the immobilized enzyme at 37 °C remained after 32 times of reuse. On the other hand, 62% of the activity of the immobilized enzyme remained after 72 times of reuse at 25 °C (Fig. 9). Operational stability of the immobilized enzyme at 25 °C was much better than at 37 °C.

Bianchi et al. [1997] reported on the GL-7-ACA acylase immobilized on amberlite XAD7, a poly (methacrylic ester) resin modified by a bifunctional aliphatic amine. However, long-term stability following reuse was not investigated in their study although the enzyme showed a complete retention of activity after 100 hr of continuous operation.

4. Conversion of GL-7-ACA into 7-ACA

Fig. 10 shows the conversion of GL-7-ACA acylase immobilized on FPHA into 7-ACA at 37 °C. Initial activity of the immobilized enzyme was 49.28 Unit/g when reaction temperature was 37 °C. About 85.08% of the conversion of GL-7-ACA was obtained after 120 min and 15.32 mM of 7-ACA was produced. But the production rate of 7-ACA was decreased after 40 min. This assumed that the immobilized enzyme was inhibited by product. Fig. 11 shows the conversion of GL-7-ACA into 7-ACA at 25 °C. Initial activity of the immobilized enzyme was 48.01 Unit/g when the reaction temperature was 25 °C. About 76.98% of the conversion of GL-7-ACA was obtained after 230 min and 14.16 mM of 7-ACA was produced. But the production rate of 7-ACA decreased after 190 min. In the case of 25 °C, the production rate of 7-ACA was slower than that of 37 °C because enzyme activity was affected by temperature.

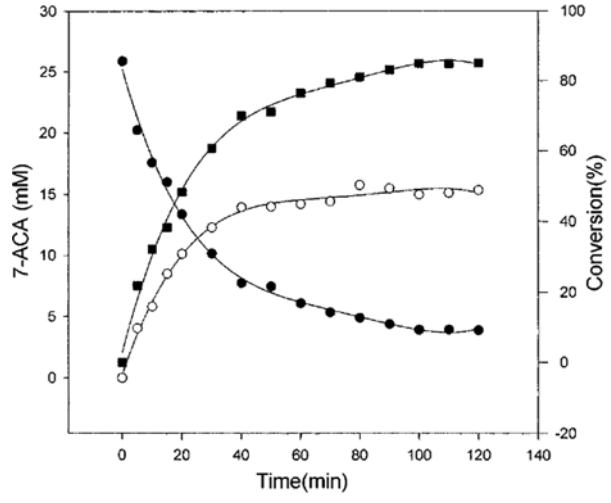

Fig. 10. Enzymatic transformation of GL-7-ACA by GL-7-ACA acylase immobilized on FPHA resin at 37 °C.
GL-7-ACA (●), 7-ACA (○), Conversion (■)

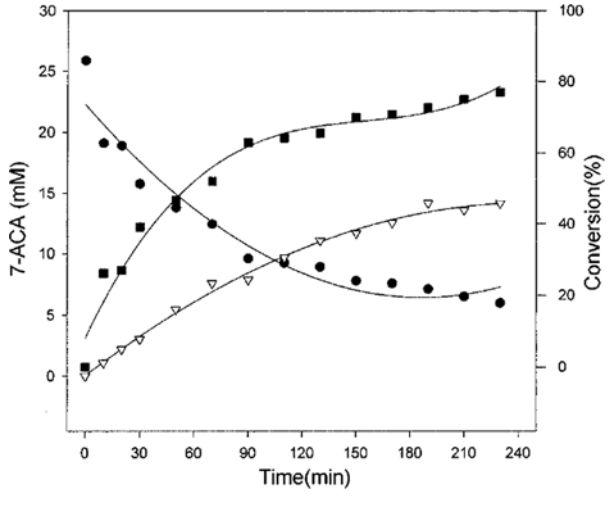

Fig. 11. Enzymatic transformation of GL-7-ACA by GL-7-ACA acylase immobilized on FPHA resin at 25 °C.
GL-7-ACA (●), 7-ACA (▽), Conversion (■)

CONCLUSION

The objective of this study is to develop a suitable immobilization method for GL-7-ACA acylase by covalent coupling to resin. A high porous hydrophilic carrier (FPHA) was selected as a suitable resin for the immobilization of GL-7-ACA acylase. Immobilization could be successfully carried out with 1% (v/v) glutaraldehyde, 0.1% (w/w) L-lysine and 100 mM Tris buffer (pH 8). Operational stability was also examined by repeated use of the immobilized enzyme under the optimal conditions. The activity of the immobilized enzyme on FPHA remained at about 60% of the initial activity after 72 times of reuse at 25 °C. The immobilization developed in the present study should be applicable to the mass production of 7-ACA in large scales.

ACKNOWLEDGEMENT

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