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Enzymatic Conversion of Glutaryl 7-Aminocephalosporanic Acid to 7-Aminocephalosporanic Acid with an Immobilized Glutaryl 7-Aminocephalosporanic Acid Acylase

SHIN, HAN-JAE[2], SEUNG-GOO LEE[2], WANG-SIK LEE[3], AND KI-HONG YOON[1]*

[1]*Department of Food Science, Woosong Sanup University, San 6-7, Jayang-Dong, Dong-Gu, Taejon 300-100, Korea*
[2]*Korea Research Institute of Bioscience and Biotechnology, Taejon 305-600, Korea*
[3]*Biotechnology Laboratory, Chong Kun Dang Corp., Seoul, Korea*

Glutaryl 7-aminocephalosporanic acid acylase of *Pseudomonas* sp. SY-77-1 was immobilized with oxiran acrylic beads for the production of 7-aminocephalosporanic acid (7-ACA) from glutaryl 7-aminocephalosporanic acid (GL 7-ACA). The immobilized enzyme maintained its activity at a constant level for 7 days, but lost 30% of its activity after 20 days. Optimal reaction conditions for the synthesis of 7-ACA were found to be 30°C and pH 8.0 using the immobilized enzyme. For the economic production of 7-ACA, substrate and enzyme concentrations were optimized to 60 mM and 0.5 g wet weight per 10 ml of reaction volume, respectively. Under optimized conditions, 50 mM 7-ACA was produced from 60 mM GL 7-ACA within 8 h, resulting in a conversion yield of 83%.

7-Aminocephalosporanic acid (7-ACA) is an essential intermediate for production of various clinically important semi-synthetic cephalosporins. It has been produced from cephalosporin C (CPC) by chemical reactions using nitrosyl chloride (8) or imino ether (4). To overcome the environmental and safety problems occurring in the chemical processes, bioconversion processes which convert CPC into 7-ACA, have recently been attempted using cephalosporin acylase as a biocatalyst (7, 11). However, almost all cephalosporin acylases are known to be less active on CPC than 7-β-(glutarylamido) cephalosporanic acid (GL 7-ACA). GL 7-ACA was prepared by either the oxidative deamination of CPC with chemical oxidation or by enzymatic reaction with D-amino acid oxidase (D-AAO) (5, 6, 12). Hence, a two-step enzymatic deacylation of CPC, which includes the oxidative deamination of CPC to GL 7-ACA by D-AAO and a subsequent deacylation of GL 7-ACA to 7-ACA by GL 7-ACA acylase, showed promise for the commercial production of 7-ACA (9, 14). Enzymatic transformation has been carried out with immobilized enzymes or microorganisms producing the D-AAO (3). Recently, a coimmobilized enzyme system of both D-AAO and GL 7-ACA acylase was also tried for one-step production of 7-ACA (10).

In a previous work, we reported on the immobilization of GL 7-ACA acylase from *Pseudomonas* sp. SY-77-1 using Eupergit C as a carrier (13). Here, the enzymatic reaction was optimized using the immobilized GL 7-ACA acylase for the efficient production of 7-ACA in a repeated-batch reactor.

*Corresponding author
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MATERIALS AND METHODS

Chemicals

The cephalosporin C and GL 7-ACA were obtained from Chong Kun Dang Co. (Korea). 7-ACA was purchased from Sigma (USA). Eupergit C is a trade name for an oxirane-acrylic resin of Rohm Pharma (Germany). All other reagents were commercial products of analytical grade.

Preparation of Immobilized GL 7-ACA Acylase

The GL 7-ACA acylase was partially purified from cell-free extract of *Pseudomonas* SY-77-1 by successive chromatographies on DEAE Sepharose CL-6B and Phenyl Sepharose CL-4B as described previously (13). The partially purified GL 7-ACA acylase with a specific activity of 0.92 U per mg protein was immobilized on Eupergit C as follows; Eight mililiters of 1 M photassium phosphate buffer (pH 8.0) containing 27.8 mg of purified enzyme was mixed with 1 g dry weight of Eupergit C beads and gently stirred at room temperature for 5 min and left to stand at 20°C for 24 h. Immobilized enzyme beads were then washed three times with the same buffer. The immobilized GL 7-ACA acylase was

stored at 4°C. Immobilized GL 7-ACA acylase with a specific activity of 2.27 U per g wet Eupergit C was used for converting GL 7-ACA into 7-ACA in this work.

Operation of the Immobilized Enzyme Reactor

The enzyme reaction was carried out at 30°C in a nitrogen-atmosphere in a 30 ml reactor. The reaction was started by adding the immobilized GL 7-ACA acylase beads to a GL 7-ACA solution (10 ml) with magnetic stirring. The pH of the reaction mixture was controlled to 8.0 with a titrator (Cole-parmer, U.S.A) using 1.5 M NaOH as a base. Aliquots of the reaction mixture were taken up at time intervals, followed by the measuring of the amount of GL 7-ACA and 7-ACA. In repeated-batch operation, each cycle was continued for 9 h. After each cycle of enzyme reaction, the aqueous phase of the reaction mixture was withdrawn, and replaced with fresh GL 7-ACA solution.

Assay of Enzyme Activity

The activities of both soluble and immobilized GL 7-ACA acylase were determined by measuring the amount of 7-ACA by the colorimetic method using *p*-dimethylaminobenzaldehyde (1). A reaction mixture containing 20 mM GL 7-ACA and the enzyme in a phosphate buffer (pH 8.0) was incubated for 10 min at 30°C with shaking. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of 7-ACA per minute. Protein concentrations were determined by the Bradford method (2).

HPLC Analysis of 7-ACA and GL 7-ACA

In the reaction mixture, amounts of 7-ACA and GL 7-ACA were determined by HPLC using a μ-Bondapak RP C_{18} column (Waters, U.S.A). The mobile phase was 0.03% disodium phosphate solution supplemented with 20% methanol and the flow rate was 1 ml min^{-1}. Column eluent was detected at 250 nm.

RESULTS AND DISCUSSION

Characteristics of GL 7-ACA Acylase Immobilized on Eupergit C

The effect of temperature on GL 7-ACA acylase activity was investigated by measuring enzyme activity at temperatures ranging from 20 to 60°C. The maximal activity of the immobilized enzyme was found at about 45°C, which was slightly lower than the optimal temperature for the soluble enzyme (Fig. 1).

From a practical viewpoint, one of the most serious problems in the enzymatic process seems to be coupled with the inactivation of the biocatalyst. Therefore, we investigated the stability of the immobilized enzyme at different temperatures for 7 days and found that the enzyme was dramatically inactivated at temperatures higher than 30°C. At 30°C, the immobilized enzyme maintained its initial activity constantly for 7 days, but lost 30% of its activity after 20 days, while the soluble enzyme lost 50% of its activity after 7 days (Fig. 2).

The effect of pH on enzyme acivivity was also investigated at pHs ranging from 5 to 11. The optimal pH for the immobilized enzyme was 8.0, which was similar to that for the soluble enzyme (Fig. 3).

Optimization of GL 7-ACA Concentration and the Immobilized Enzyme Content

In order to optimize substrate concentration, the reaction rate was measured at various concentrations of GL 7-ACA. As shown in Fig. 4, the activity of the immobilized enzyme increased until 100 mM GL 7-ACA concentration while the specific activity of the soluble enzyme reached the maximum reaction rate at 20 mM

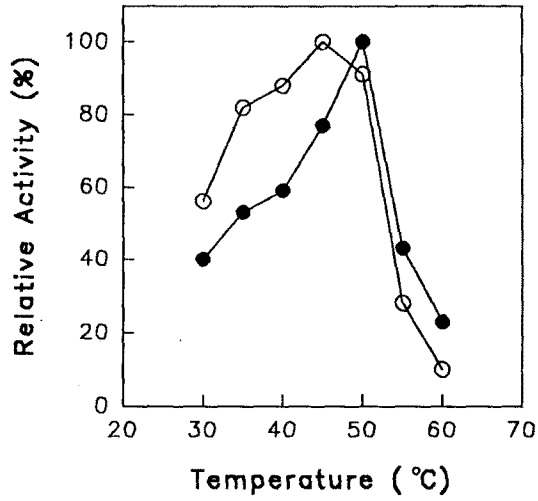

Fig. 1. Effect of temperature on the emzyme activity of soluble (●—●) and the immobilized (○—○) GL 7-ACA acylase.

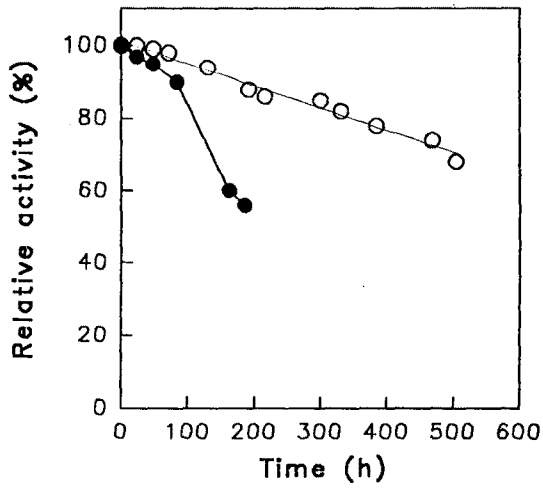

Fig. 2. Stability of the GL 7-ACA acylase.
The soluble (●—●) and the immobilized (○—○) GL 7-ACA acylase were incubated at 30°C without mixing.

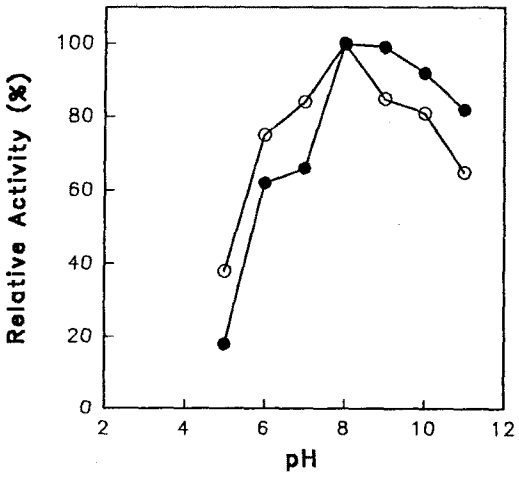

Fig. 3. Effect of pH on the enzyme activity of soluble (●—●) and immobilized (○—○) GL 7-ACA acylase.

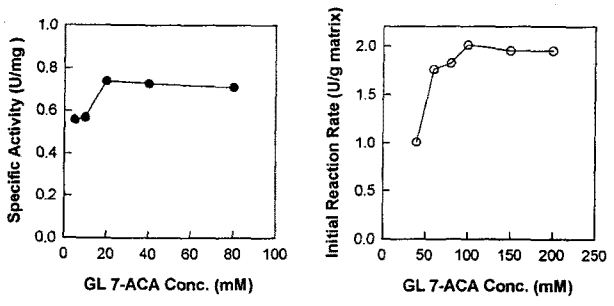

Fig. 4. Effect of substrate concentration on the enzyme activity of the soluble (●—●) and the immobilized (○—○) GL 7-ACA acylase.

GL 7-ACA, showing that the concentration of substrate should be as much over 60 mM as possible.

In order to optimize the concentration of GL 7-ACA, the effect of substrate concentration was examined in terms of % conversion yield of GL 7-ACA to 7-ACA. Batch reactors containing different concentrations of substrate were operated for 15 h according to the conditions described in Materials and Methods. As shown in Table 1, the conversion yield was maintained above 77% in cases of 40 and 60 mM of GL 7-ACA, but it dropped to 70% at 80 mM of GL 7-ACA. On the basis of comparisons of the reaction rate and conversion yield, the optimal concentration of substrate was determined to be 60 mM. It was also necessary to determine the most effective amount of immobilized enzyme for producing 7-ACA. In the presence of 60 mM of GL 7-ACA, the hydrolysis rate of GL 7-ACA was measured with various amounts of immobilized enzyme. As shown in Fig. 5, the productivity of 7-ACA increased proportionally to the amount of immobilized GL 7-ACA acylase up to 0.5 g of wet weight per 10 ml of reaction mixture. Little increase in productivity of 7-ACA was observed at volumes higher than 0.5 g of wet weight, indicating that 0.5 g of the immobilized GL 7-ACA acylase was sufficient to efficiently convert GL 7-ACA to 7-ACA.

Table 1. Conversion yield of GL 7-ACA to 7-ACA at different substrate concentrations.

GL 7-ACA (mM)	Conversion yield (%)	7-ACA (mM)
40	78	30
60	77	45
80	70	56

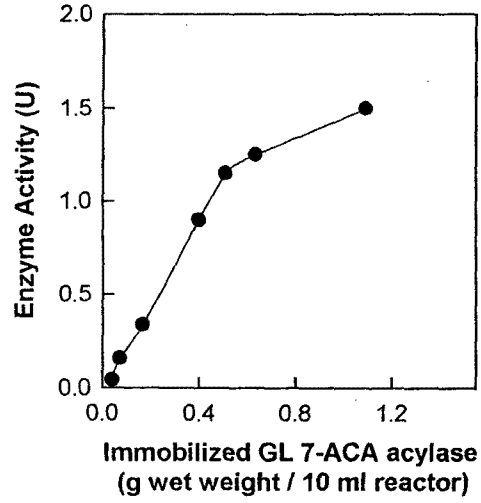

Fig. 5. Effect of immobilized enzyme amount on the production of 7-ACA.

Repeated Batch Production of 7-ACA by Immbilized GL 7-ACA Acylase

The production of 7-ACA was carried out in a repeated batch reactor containing 0.5 g (wet weight) of the immobilized enzyme and 10 ml of GL 7-ACA solution (60 mM). For the first cycle, both the 7-ACA formation and GL 7-ACA consumption were determined as shown in Fig. 6. The 7-ACA was produced in an approximately equimolar ratio to the hydrolysis amount of GL 7-ACA during 9 h of reaction time. It was found that 10 mM of GL 7-ACA remained in the reaction solution after 9 h of reaction. At that moment, the concentration of 7-ACA was 50 mM and the conversion yield of GL 7-ACA was calculated to be 83%.

In order to produce 7-ACA using the immobilized enzyme, repeated batch operations were conducted under optimized conditions. As shown in Fig. 7, the activity of the immobilized enzyme decreased to approximately 60% of its original activity over ten successive operations. By comparing the residual activity of the repeatedly used enzyme with that of the enzyme incubated at 30°C (Fig.

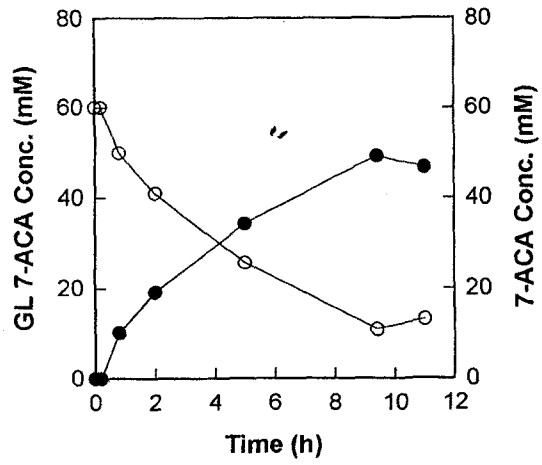

Fig. 6. Time course of 7-ACA production in a batch operation.
Immobilized enzyme beads 0.5 g of wet weight were added to 10 ml of 60 mM GL 7-ACA solution, and then the mixture was incubated for 10 h at 30°C in STR. Symbols are: ●—●, 7-ACA concentration; ○—○, GL 7-ACA concentration.

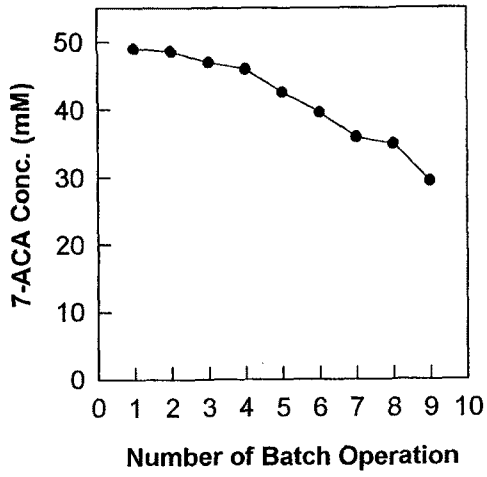

Fig. 7. Repeated batch production of 7-ACA from GL 7-ACA in a repeated batch reactor.
Each cycle was continued for 9 h and the concentrations of 7-ACA were determined by HPLC analysis.

2), it is apparent that the activity of the reused enzyme is lower than that of the incubated enzyme because of the abrasion of Eupergit C.

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