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Polyacrylamide Gel Immobilization of Porcine Liver Esterase for the Enantioselective Production of Levofloxacin

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Abstract Porcine liver esterase was immobilized in polyacrylamide gel for the enantioselective production of levofloxacin from ofloxacin butyl ester. The initial activity of immobilized esterase was found to be significantly affected by the polyacrylamide gel composition. The optimum concentrations of monomer and crosslinker were determined to be 20% and 8.3%, respectively. The activity of immobilized esterase was 55.4% compared to a free enzyme. Enantiomeric excess was maintained at 60%, almost the same level as that of free enzyme. In addition, the immobilized esterase could be used repeatedly up to 10 times without experiencing any severe loss of activity and enantioselectivity.

Keywords: immobilized enzyme, levofloxacin, ofloxacin, polyacrylamide gel, porcine liver esterase

INTRODUCTION

Since each stereoisomer has its own physiological characteristics, the absorption, utilization, and receptor binding properties vary for each stereoisomer in a living system. For medical utilization, the separation of a specific stereoisomer is an important factor [1]. In general, one component of a mixture of enantiomers is active while the other component is regarded as impure because it lowers the level of activity [2]. Therefore, the selective isolation of the active isomer is necessary. A great deal of research is presently being conducted using enantioselective enzymes or cells expressing enzymes since the isolation is feasible under mild conditions with greater stereospecificity than possible with chemical methods [3,4]. Recently, Jaeger and Reetz reported the use of directed evolution technology for enantioselective enzymes [5]. In addition to kinetic (biocatalytic) resolution, which is one of the most useful techniques for the development of chiral compounds, stereoselective assimilation from the racemate for optical resolution has also been reported [6]. For enzymatic chiral resolution, enzymes such as lipase, esterase, and protease are usually employed. These enzymes are easily obtained from various sources and their applications are also possible in organic solvents as well as aqueous solution [7,8]. In spite of these advantages in chiral resolution, the enzyme cost remains a bottleneck for commercialization. Therefore, enzyme immobilization is inevitable in order to reduce production costs. Since expensive enzymes can be used repeatedly and are easily separated after the reaction, immobilization has been used in various areas [9].

Much attention has been focused on ofloxacin, one of the fluoroquinolone antibiotics, as the next generation antibiotic. Levofloxacin, the L-form of ofloxacin, is more effective than D-ofloxacin or ofloxacin itself [10]. Therefore, levofloxacin has much higher commercial value than D-ofloxacin. Levofloxacin can be produced selectively from ofloxacin butyl ester using porcine liver esterase [11].

Usually, the optimum pH and temperature of immobilized enzymes is shifted compared to those of free enzymes and this shift may possibly lead to an increase in productivity [12]. In addition, improvements in stability through immobilization have been well documented [13]. The entrapment method using polyacrylamide gel is a typical method of enzyme immobilization. In polyacrylamide gel formation, concentrations of monomer (acrylamide) and crosslinker affect both gel strength and enzymes stability [14]. Excessive formation of pores in the gel may induce enzymes leakage. On the contrary, insufficient pore formation may limit the mass transfer and reduce the enzymatic reaction. Therefore, optimization of the gel composition is necessary for the efficient use of immobilized enzymes.

In this study, the concentrations of acrylamide and crosslinker were optimized for the enhanced production of levofloxacin from ofloxacin butyl ester using immobilized porcine liver esterase in polyacrylamide gel. The

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feasibility of repeated use of the polyacrylamide gel, without loss of enzyme activity and enantioselectivity, was also investigated.

MATERIALS AND METHODS

Chemicals and Enzyme Reaction

Porcine liver esterase and all the chemicals, such as acrylamide, *N,N'*-methylenebisacrylamide (BIS), ammonium persulfate, and *N,N,N',N'*-tetramethylethylenediamine (TEMED), were purchased from Sigma Chemical Co. For enzyme reaction, 2 g/L of porcine liver esterase and 5 g/L of ofloxacin butyl ester were added into 0.1 M phosphate buffer at pH 6.8. The enzyme reaction was carried out with 20 mL of the reaction solution in a 100-mL flask using a rotary incubator at 200 rpm and 30°C.

Enzyme Immobilization

For the immobilization in polyacrylamide gel, 0.04 g of the enzyme dissolved in 1.25 mL of 0.5 M Tris/HCl buffer (pH 6.8) was added to 3.33 mL of stock solution containing 0.275 g/L acrylamide and 0.025 g/L BIS. After mixing, 0.42 mL of distilled water was added to top up to a final volume of 5 mL. To start the polymerization reaction, 25 μL of ammonium persulfate stock solution (0.1 g/mL) and 2.5 μL of TEMED were added. Gelling of acrylamide was performed on a 1 mm wide glass plate. After gel formation, the gel was cut into 2 × 2 × 1 mm for the experiment. All the immobilized enzyme reactions were performed in 0.1 M phosphate buffer at pH 6.8 for 48 h. The immobilized enzymes were collected using Whatman No. 1 filter papers and washed with excess 0.1 M phosphate buffer for re-use.

Analytical Method

Enzyme activity was measured as follows. The reaction in a 0.1 mL sample was stopped in a hot water bath for 5 min. After the addition of an equal amount of methanol, the mixture was centrifuged at 5,000 rpm for 10 min and 0.1 mL of the supernatant was obtained to quantify the amount of product.

Levofloxacin quantitative analysis was performed using an HPLC system consisting of Vintage 2000LC pump (Orom Co., Korea) and M720 UV detector (Youngin Scientific Co., Korea) fixed at 330 nm. As a stationary phase, a Capcell pak column (4.6 × 250 mm, Shiseido Co., Japan) was used. The mobile phase was a mixture of distilled water and methanol at the ratio of 85:15 with the addition of 1.21 g/L L-isoleucine and 1.07 g/L $CuSO_4 \cdot 5H_2O$. The flow rate was 1.0 mL/min.

RESULTS AND DISCUSSION

In our previously reported work, it was found that the addition of ofloxacin butyl ester as the substrate, at the concentration of 5 g/L, was desirable to avoid product inhibition and to maintain porcine liver esterase activity for up to 72 h [11]. The porcine liver esterase used in this study reacted with L-ofloxacin butyl ester and D-ofloxacin butyl ester at a ratio of 4:1 and eventually produced an enantiomeric excess of 60%. Enantiomeric excess is defined as [(L-form - D-form)/(L-form + D-form)] × 100.

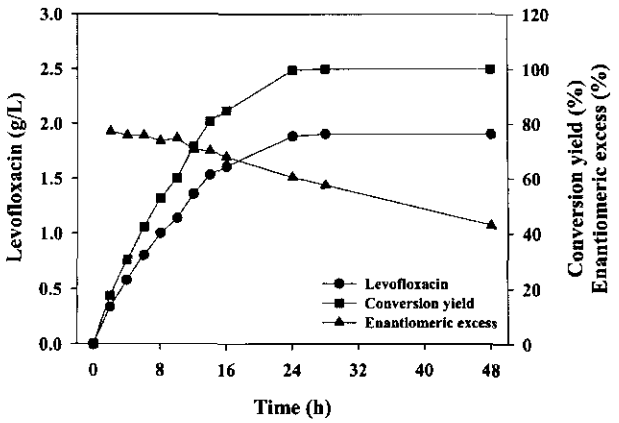

Fig. 1. Profiles of levofloxacin production, enantiomeric excess, and conversion yield during the enzyme reaction with 2 g/L of free porcine liver esterase.

Time course profiles of levofloxacin production, enantiomeric excess, and conversion yield during the reaction with free enzyme are shown in Fig. 1. Since the substrate, ofloxacin butyl ester, is a 1:1 mixture of L-form and D-form, conversion yield was calculated as the amount of levofloxacin produced at a given time relative to the maximum value of levofloxacin obtainable theoretically. After 24 h of enzyme reaction, 1.88 g/L of levofloxacin was produced at a conversion yield of 99.4%, indicating that the reaction was almost completed. From an initial value of 77%, the enantiomeric excess decreased to 60% after 24 h, possibly due to the increase of D-ofloxacin formation after the completion of the reaction to levofloxacin.

When the porcine liver esterase entrapped in polyacrylamide gels was used, the relative activity (the activity of immobilized enzyme relative to the activity of free enzyme) was 84.7% with an enantiomeric excess of 68.6% at the end of the enzyme reaction. Therefore, it was evident that more than 80% of the enzyme could be entrapped using polyacrylamide gels and that the maintenance of high enantioselectivity was possible. Sánchez *et al.* [15] reported that the enantiomeric excess was increased proportionally to the concentration of substrate in an enantioselective reaction using lipase and that this phenomenon was more evident when using immobilized enzyme rather than free enzyme. High substrate concentration could have increased the chance of contact between the enzyme and substrate. In addition, immobilization influenced the microenvi-

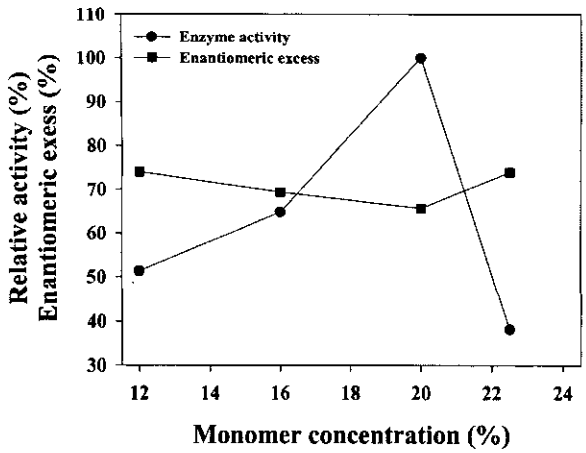

Fig. 2. Effect of total monomer concentration on the relative activity and enantiomeric excess of immobilized enzyme. Relative activity at a given monomer concentration is the activity relative to that of the immobilized enzyme when the monomer concentration is 20% (w/v).

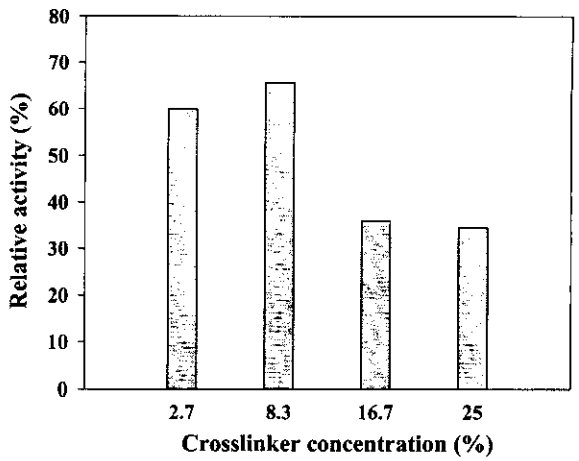

Fig. 3. Effect of crosslinker concentration on the relative activity and enantiomeric excess of immobilized enzyme. Total monomer concentration was fixed at 20%. Relative activity is the activity of immobilized enzyme relative to the activity of free enzyme.

ronment of the enzyme reaction such as spatial and diffusional limitations.

In polyacrylamide gel formation, the ratio of monomer (acrylamide) to crosslinker (*N,N'*-methylenebisacrylamide, BIS) affected the physical characteristics such as hardness and porosity of the gel [16]. The effect of monomer concentration on the relative activity and enantiomeric excess of the immobilized enzyme is shown in Fig. 2. While the percentage ratio of BIS to acrylamide was fixed at 2.7% (w/w), the acrylamide concentrations were varied (12, 16, 20, and 22.5% (w/v)). As the monomer concentration was increased from 12 to 20% (w/v), a proportional increase of enzyme activity was observed. However, a sharp decrease of activity was noticed at 22.5% (w/v). As for enantiomeric excess, no significant changes were found in spite of the large variation in relative activity. Das *et al.* [17] also reported a similar proportional increase of immobilized enzyme activity in polyacrylamide gel up to a certain monomer concentration, followed by a sudden decrease at high monomer concentrations. They assumed that this sudden decrease was due to the toxicity of high acrylamide concentration. However, the structural changes caused by the increase of monomer concentration as well as the changes and spatial limitation between the enzyme and substrate could represent other reasons for the low enzyme activity. From these findings/data, it is clear that there is an optimum value of monomer concentration.

On the basis of the above experiment, monomer concentration was fixed at 20% (w/v) and the crosslinker concentration was optimized. The results are shown in Fig. 3. When the percentage of BIS to monomer was high at 16.7% or 25% (w/w), enzyme activity relative to free enzyme decreased significantly. However, at 8.3% (w/w), the highest relative activity (65.6%) was achieved. With repeated use of the immobilized enzyme, a more drastic reduction of enzyme activity was noticed at 16.7% and 25% (w/w), while at 8.3% and 2.7% (w/w) the reduction was less severe (data not shown). Pizarro *et al.* [18] also reported that the immobilized enzyme activity varied with increasing amounts of crosslinker. With small amounts, the enzyme activity decreased due to the enzyme loss. However, as the amount of added crosslinker became excessive, contact between the enzyme and substrate was limited due to the hardness of the gel.

From the above two experiments, the optimum concentrations of monomer and crosslinker were determined to be 20% (w/v) and 8.3% (w/w), respectively. As the advantage of using immobilized enzyme is that it allows re-use of the expensive enzymes, the possibility of repeated enzyme use was examined under these optimized conditions. As mentioned in the Materials and Methods section, the immobilized enzymes were collected after the reaction using Whatman No. 1 filter paper, washed with excess 0.1 M phosphate buffer, and placed again in reaction buffer with substrate for re-use.

The relative activity compared to that of free enzyme, as well as the levofloxacin production, conversion yield, and enantiomeric excess, over 10 repeated batches, are summarized in Table 1. The initial relative activity was 55.4%, and the final value after 10 repeated batches only decreased to 51.2%, confirming the possibility for re-use of the immobilized enzyme without significant loss of activity. From the 2nd to 6th batches, enzyme activity even showed a significant increase relative to the initial value. Such an increase is either due to the retention of the produced levofloxacin in the gel during the washing and reaction processes, or due to the improved contact between the enzyme and substrate resulting from the internal structural changes

Table 1. Repeated use of immobilized enzyme

Batch number	1	2	3	4	5	6	7	8	9	10
Relative activity (%)	55.4	83.4	68.2	72.6	68.9	64.5	53.8	50.2	45.2	51.2
Levofloxacin (g/L)	1.05	1.57	1.29	1.37	1.30	1.22	1.02	0.95	0.85	0.97
Conversion yield (%)	55.5	83.1	68.3	72.5	68.8	64.6	54.0	50.3	45.0	51.3
Enantiomeric excess (%)	70.2	57.0	63.9	61.4	59.4	61.6	66.0	66.6	67.8	67.0

occurring during the repeated use. Considering that the 57.0% value of enantiomeric excess at the 2nd batch was considerably lower than the initial value (70.2%), it could be concluded that the 2nd batch enzyme reaction was much better. This suggests that the enzyme activity increase noted above was most likely due to the improved contact between the enzyme and substrate.

In conclusion, the immobilization of porcine liver esterase in polyacrylamide gel for the enantioselective production of levofloxacin was successfully performed in this study. The immobilization conditions were optimized and repeated use of the immobilized enzyme was found to be possible up to 10 times without significant loss of activity.

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