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ORIGINAL RESEARCH PAPER

Enantioselective resolution of racemic styrene oxide at high concentration using recombinant *Pichia pastoris* expressing epoxide hydrolase of *Rhodotorula glutinis* in the presence of surfactant and glycerol

Seung Sik Yoo · Sunghoon Park · Eun Yeol Lee

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Abstract The reaction medium was optimized to accomplish epoxide hydrolase-catalyzed, batch enantioselective hydrolysis of racemic styrene oxide at high initial substrate concentrations. The recombinant *Pichia pastoris* containing the epoxide hydrolase gene of *Rhodotorula glutinis* was used as the biocatalyst. Enantiopure (*S*)-styrene oxide with 98% *ee* was obtained with 41% yield (maximum yield = 50%) from 1.8 M racemic styrene oxide at pH 8.0, 4°C in the presence of 40% (v/v) Tween 20 and 5% (v/v) glycerol.

Keywords Enantioselective hydrolysis · Epoxide hydrolase · Glycerol · Surfactant

Introduction

Enantiopure epoxides provide useful synthetic intermediates for the production of chiral compounds. Enantiopure epoxides can be prepared by hydrolytic kinetic resolution of racemic epoxides by epoxide hydrolase (EH) (Archelas and Furstoss 2001). EH is the enzyme that hydrolyzes one specific enantiomer of racemic epoxide substrate into the corresponding diol (Lee and Shuler 2007). As a result, remaining enantiomer can be obtained in an entiomerically enriched form.

The theoretical yield of hydrolytic kinetic resolution can never exceed 50% (Strauss et al. 1999). In spite of this drawback, the kinetic resolution is still one of the major industrial methods for the production of chiral compounds. To date, there have been some attempts to enhance the yield and volumetric productivity of EH-catalyzed kinetic resolutions at high substrate concentration (Cleij et al. 1998). Various organic solvents have been employed to prevent a spontaneous degradation of epoxide substrates such as epichlorohydrin (Choi et al. 1999a). The solubility of epoxide substrates was increased by employing hydrophobic organic solvents (Gong and Xu 2005). However, organic solvents decreased the activity and stability of EHs in a two-liquid-phase system due to molecular toxicity and interfacial toxicity (Baldascini and Janssen 2005). The toxicity of organic solvents decreased the catalytic efficiency mainly due to an increase in the K_m value (Karboune et al. 2006). The diol product inhibition can limit the application of EHs to kinetic resolution at high epoxide substrate concentration. An aqueous/organic two-phase cascade membrane bioreactor was employed to prevent the organic solvent toxicity and diol product inhibition (Choi et al. 1999b). Even though (*S*)-1,2-epoxyhexane (>98% *ee*) was successfully produced at high concentration greater than 1 M, the practical

S. S. Yoo · S. Park
Department of Chemical Engineering, Pusan National University, Busan 609-735, South Korea

E. Y. Lee (✉)
Department of Chemical Engineering, Kyung Hee University, Gyeonggi-do 446-701, South Korea
e-mail: eunylee@khu.ac.kr

application of membrane bioreactor as a commercial production system is rather limited due to the high cost.

Recently, we have reported that the simple batch kinetic resolution of racemic styrene oxide at 526 mM by using the recombinant *Pichia pastoris* expressing the EH gene of *Rhodotorula glutinis* as the whole-cell biocatalyst (Lee et al. 2004). In general, whole-cell biocatalysts are more stable than the isolated enzymes when epoxides at high concentrations constitute a separate phase by itself. In order to perform an efficient batch kinetic resolution of racemic styrene oxide at up to 1.8 M, we investigated the effect of the addition of surfactants and glycerol on the enantioselective biohydrolysis reaction catalyzed by the recombinant whole-cell *P. pastoris*.

Materials and methods

Media and culture conditions

The recombinant *P. pastoris* was cultivated on a BMGY medium containing 10 g yeast extract/l and 10 g peptone/l at 30°C (Kim et al. 2006). The expression of EH gene was induced by adding 1% (v/v) methanol for 72 h. The cells were harvested, and then washed with Tris/HCl buffer (100 mM, pH 8).

Enantioselective resolution of racemic styrene oxide

The given amount of the harvested cells ranging from 43 to 92 mg (dry cell wt, DCW) was resuspended in 10 ml 100 mM KH_2PO_4 buffer in a 50 ml screw-cap bottle. The enantioselective hydrolysis reaction was initiated by adding the given concentration of racemic styrene oxide. The reactions were carried out in a shaking incubator at 30°C and 250 rpm, and samples were periodically withdrawn to follow the reaction.

Effect of surfactants and glycerol on the enantioselective resolution of racemic styrene oxide at high concentration

Various concentrations of surfactants and glycerol were added to biohydrolysis reaction medium at the given concentrations and the mixtures were preincubated for 5 min. After the given amount of cells and racemic styrene oxides were added, the reaction started and the progression of batch kinetic resolutions were periodically followed by the analysis of samples by chiral GC.

Analyses

The enantiomeric excess (*ee*) and yield for enantiopure epoxides were determined by chiral GC with a fused silica capillary beta-DEX-250 column (60 m length, 0.25 mm ID, and 0.25 μm film thickness, Supelco Inc.) and a FID detector. The column, injector, and detector temperatures were 100, 220, and 220°C, respectively. The enantiomeric excess was determined by the following equation, $ee\ (\%) = [((S)\text{-epoxide} - (R)\text{-epoxide})/((S)\text{-epoxide} + (R)\text{-epoxide})] \times 100$.

Results and discussion

Effect of surfactant on the enantioselective resolution of racemic styrene oxide at high concentration

Recently, the recombinant *P. pastoris* harboring the EH gene of *R. glutinis* was constructed and used for the kinetic resolution of racemic styrene oxides (Lee et al. 2004). Chiral (*S*)-styrene oxide with 98% *ee* and 36% yield was obtained from 526 mM racemic substrate for 16 h reaction. When the substrate concentration was further increased above 0.6 M, the recombinant cells could not efficiently catalyze the kinetic resolution. One reason might be diol product inhibition. To analyze the effect of diol concentration on the kinetic resolution, we carried out the enantioselective hydrolysis for 100 mM racemic styrene oxide in the presence of 0–500 mM racemic phenyl-1,2-ethanediol. There was no inhibition up to 250 mM and about 20% reduction in initial hydrolysis rate in the presence of 500 mM (data not shown). Therefore, diol inhibition appeared not to be the main reason for poor resolution by itself.

Surfactants such as crown ether, Triton X-100, Tween 20 and Tween 80 enhance the performance of EHs (Gong et al. 2003, Kronenburg and de Bont 2001). However, these results were obtained for the kinetic resolution of racemic epoxides by isolated EH enzymes. In order to investigate the stimulatory effect of surfactant on enantioselective resolutions by the recombinant whole-cell biocatalyst at high substrate concentration, a kinetic resolution of 877 mM racemic

Table 1 Enantioselective resolution of racemic styrene oxides by the recombinant *P. pastoris* in the presence of Tween 20 (Reaction condition: 4°C, 250 rpm, 43–92 mg DCW/ml)

Tween 20 conc. % (v/v)	Initial racemic styrene oxide conc. (mM)	Reaction time (h)	*ee* (%)	Yield (mM)
0	877	22.5	68.0	422
3	877	22.5	86.3	416
5	877	22.5	90.4	412
10	877	22.5	95.2	402
13	877	22.5	98.0	396
15	877	22.5	98.0	374
20	877	22.5	99.1	319
20	1,052	24.5	89.1	479
25	1,052	24.5	90.8	459
30	1,052	24.5	98.1	413
30	1,315	28.5	91.7	539
35	1,315	28.5	94.2	523
40	1,315	28.5	95.1	511

styrene oxide in the presence of 0–20% (v/v) Tween 20 was performed (Table 1). In the absence of Tween 20, the enantiopurity of (*S*)-styrene oxide was below 70%. When 10 and 20% Tween 20 were added, (*S*)-styrene oxides with enantiopurity more than 95 and 98% *ee* were obtained, respectively. Enantiopurity of 98% *ee* was obtained in the presence of 30% Tween 20 at 1,052 mM. For 1,315 mM, 95% enantiopurity was obtained in the presence of 40% Tween 20. When Tween 20 concentration was further increased, more than 40%, the enantiopurity was not further enhanced. The yields were 38 and 39% for 1,052 and 1,315 mM, respectively.

Effect of glycerol on the enantioselective resolution of racemic styrene oxide at high concentration

To obtain enantiopure (*S*)-styrene oxide from racemic styrene oxide at high concentration more than 1.3 M, the effect of glycerol addition on the enantioselective hydrolysis was investigated. The hydrolysis rates at 3 h were 46 and 41 nmol min^{-1} mg^{-1} in the presence and absence of 5%(v/v) glycerol, respectively. The hydrolysis rate after 9 h in the presence of glycerol was maintained relative higher than that in the absence of glycerol. We obtained (*S*)-styrene oxide with enantiopurity more than 98% *ee* from

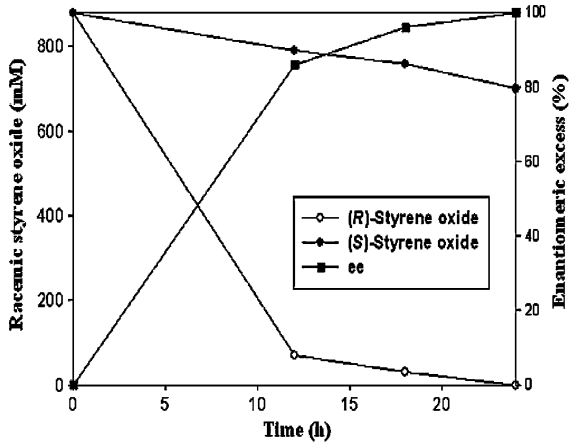

Fig. 1 Enantioselective resolution of 1.8 M racemic styrene oxide by the recombinant *P. pastoris* in the presence of 40% (v/v) Tween 20 and 5% (v/v) glycerol. Reaction conditions: 4°C, 250 rpm, 92 mg DCW/ml

1,315 mM racemic styrene oxide in the presence of 5% glycerol. When the initial concentration was further increased up to 1,754 mM, the recombinant whole-cell biocatalysts successfully resolved racemic substrate in enantiomeric fashion. The enantiopure (*S*)-styrene oxide with 98% *ee* was prepared with 41% yield (theoretical yield = 50%) in the presence of 40% Tween 20 and 5% glycerol (Fig. 1).

Biocatalyst stability is of importance to perform a successful kinetic resolution at high substrate concentration. Surfactant and glycerol are known to stabilize the enzyme activity (Marcozzi et al. 1998, Bradbury and Jakoby 1972). The presence of surfactant and glycerol seem to preserve the structures of enzymes or induce conformational changes for a prolonged stability. The exact mechanisms of stabilization of the enzyme by surfactant and glycerol are not yet determined. The activity of the partially purified EH from *Rhodotorula glutinis* was enhanced by adding detergents such as sucrosemonolaurate to stabilize membrane-associated EH (Kronenburg and de Bont 2001). The addition of 0.5% Tween-80 increased the apparent activity and *E*-value of the EH of *Bacillus megaterium* ECU1001 (Gong et al. 2003). All these stimulatory effect of surfactants were observed for purified enzymes not whole cells. Herein, we employed the recombinant whole-cell *P. pastoris* as the biocatalyst, and obtained similar stimulatory effects of surfactant and glycerol. Even though the whole-cell biocatalysts were used, a successful kinetic resolution could be carried out at high

substrate concentration, making it a useful way to produce chiral epoxides. More detailed investigation is needed to elucidate mode of action of the stimulatory effect of surfactant and glycerol for recombinant whole-cell-catalyzed enantioselective hydrolysis. A possible problem for this reactor system is that high concentrations of surfactant and glycerol used to enhance productivity may significantly cause the difficulty in design of a product recovery process. Recently, kinetic resolution of an azole antifungal key synthons has been successfully carried out in so-called salt-free process, employment of unbuffered water as the reaction medium to allow a simplified recovery of the product (Monfort et al. 2004). Compared to this salt-free process, our system has a disadvantage when used in preparative-scale reactions since we used a rather complex reaction medium. Therefore, it needs to be investigated to select proper surfactants permitting easier recovery of the enantiopure products.

Conclusion

The batch enantioselective resolution of 1.8 M racemic styrene oxide by recombinant *P. pastoris* harboring the *R. glutinis* EH gene was performed in the presence of 40% (v/v) Tween 20 and 5% (v/v) glycerol. The enantiopurity of (*S*)-styrene oxide was 98% *ee* and obtained with 41% yield after 25 h reaction. This study demonstrated that chiral (*S*)-styrene oxide could be readily prepared using the recombinant whole-cell biocatalysts based on simple reaction medium engineering.

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