Enantioselective Hydrolysis of Racemic Styrene Oxide by Epoxide Hydrolase of *Rhodosporidium kratochvilovae* SYU-08

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Abstract Enantioselective hydrolysis for the production of chiral styrene oxide was investigated using the epoxide hydrolase activity of a newly isolated *Rhodosporidium kratochvilovae* SYU-08. The effects of reaction parameters - buffer type, pH, temperature, initial substrate concentrations, phenyl-1,2-ethanediol concentrations on hydrolysis rate, and enantioselectivity - were analyzed. Optically active (*S*)-styrene oxide with an enantiomeric excess higher than 99 % was obtained from its racemate, with a yield of 38 % (theoretically 50% maximum yield) from an initial concentration of 80 mM.

Keywords: enantioselective hydrolysis, epoxide hydrolase, *Rhodosporidium kratochvilovae*, chiral styrene oxide

Chiral epoxides are important synthons in organic synthesis and can be used as key building blocks for the production of optically active compounds [1]. Various chemical and biological methods have been developed for the production of chiral epoxides [2]. Among the biocatalytic production routes, the kinetic resolution of racemic epoxides using enantioselective hydrolysis activity of epoxide hydrolase (EH) is a very promising method because chiral epoxides with high optical purity can be obtained from relatively cheap and readily available racemic epoxides [3,4].

EH is an enzyme that catalyzes the addition of a water molecule to the oxirane ring to form the corresponding diol [5-7]. As a constitutive hydrolytic enzyme that is cofactor independent, EH is a commercially potential biocatalyst. Another advantage of EH is that it is ubiquitous in nature. Kinetic analysis and molecular characterization of microsomal EH (mEH) of mammalian cells have been investigated with attention to the role of mEH in the detoxification of epoxides [8]. While mEH has a disadvantage for industrial applications due to difficulty in preparing the enzyme, microbial EH can be used as industrial biocatalysts for the production of commercially important chiral epoxides [3,4]. Generally, bacterial EHs are soluble and monomeric enzymes, whereas eukaryotic EHs are soluble and membrane-bound enzymes.

Recently, the potential of yeast EH as a biocatalyst for preparing chiral epoxides was evaluated [5,7,9]. Enantioselective hydrolysis of racemic epoxide by yeasts was first investigated for several aryl and alkyl epoxide substrates using the EH activity of *Rhodotorula glutinis* [5]. Biocatalytic resolution of 1,2-epoxyoctane using resting cells of 187 yeast strains from 25 different genera was also investigated to identify new EH activity. Only a few yeasts less than 4% from the tested strains were found to show EH activity. Furthermore, enantioselective EH was found only in very few yeasts that mainly belonged to the basidiomycetes genera, including *Rhodosporidium*, *Rhodotorula*, and *Trichosporon*. EH activity of these yeasts showed good enantioselectivity towards aliphatic epoxides such as 1,2-epoxyhexane, 1,2-epoxyoctane, and 1,2-epoxyoctene. EH activity of yeast is very useful for the kinetic resolution of racemic epoxides since yeast cells are more easy to culture and the catalytic efficiency of yeast whole cell is relatively high [7]. Although yeast EH has several advantages as an industrial biocatalyst, no detailed study on the enantioselective hydrolysis of commercially valuable aromatic epoxides, such as styrene oxide, has been reported. In this study, we have characterized the enantioselective hydrolysis of racemic styrene oxide by *Rhodosporidium kratochvilovae* SYU-08, and the reaction conditions of kinetic resolution have been optimized for the preparation of chiral styrene epoxide.

EH activity of various microorganisms was identified by the colorimetric method [10,11]. For the colorimetric assay in 96 well microtiter plates, cell suspensions in 100 mM KH_2PO_4 buffer (pH 8.0) were incubated for 2 h at 30°C to perform the enantioselective hydrolysis of racemic styrene oxide, and then 4-(*p*-nitrobenzyl) pyridine (NBP) assay and periodate-coupled fluorogenic assay were performed as described in the literatures [10,11].

R. kratochvilovae SYU-08 was cultured on the medium

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containing 20 g/L malt extract, 20 g/L glucose, and 1 g/L peptone for 48 h. Cultured cells were suspended in 5 ml 100 mM KH_2PO_4 buffer in 50 mL screw-cap bottles sealed with a rubber septum. Enantioselective hydrolysis was started with the addition of 20 mM racemic styrene oxide, and the reaction mixture was incubated in a shaking incubator at 35°C and 250 rpm. The reaction was stopped by extraction with equal volume of diethyl ether. The progression of enantioselective hydrolysis was investigated using a 100 mL glass enzyme reactor with a magnetic stirrer and followed by the analysis of samples withdrawn periodically from the reaction mixture. Commercially available racemic styrene oxide, (*R*)- and (*S*)-enantiomers of styrene oxide were all obtained from Aldrich Chemical Co., Inc. Enantiomeric excess (*ee* = 100 × (S-R)/(S+R)) and conversion yield for chiral styrene oxide were determined by chiral GC analysis. The reaction mixture was extracted with equal volume of diethyl ether, and the organic layer was analyzed by chiral GC with a fused silica capillary beta-DEX 120 column (30 m length, 0.25 mm ID, and 0.25 μm film thickness, Sulpelco Inc.) and a FID detector. The temperatures of the column, injector, and detector were 100, 220, and 220°C, respectively.

Generally, EH activity has been measured by chiral GC analysis of the reaction mixture, a rather time-consuming approach. Recently, a colorimetric assay suitable for screening EH activity was reported [10]. This assay was based on the formation of a blue dye between an epoxide and 4-(*p*-nitrobenzyl)pyridine (NBP). If a strain possesses EH activity, the concentration of epoxide in a hydrolysis reaction mixture decreases and the intensity of the blue dye also decreases compared to a control reaction without EH activity. Another screening strategy for identifying EH activity is based on the change in maximum absorption in UV spectrometry during the periodate oxidation of a diol product to aldehyde [11]. Normally, compounds with hydroxyl groups undergo oxidative cleavage to produce carbonyl groups when treated with sodium periodate. Since this reaction quantitatively occurs, the relative value of EH activity can be estimated by measuring the amounts of carbonyl products using a UV spectrometer. Based on the above simple assays, microbial strains with EH activity were screened from our laboratory culture collections and from isolated strains of various samples. First, we screened microorganisms that reduced the intensity of the blue dye. Among the several microorganisms, one microorganism, *Rhodosporidium kratochvilovae* SYU-8, was selected for further investigation as it had the lowest color intensity. Enantioselective hydrolysis of 20 mM racemic styrene oxide was performed to confirm the enantioselective EH activity. EH of *R. kratochvilovae* SYU-08 has enantio-preference for (*R*)-styrene oxide.

One major drawback of EH-catalyzed preparation of chiral styrene oxide is 50% maximum yield limitation intrinsic to kinetic resolutions, clearly indicating the need for EH-catalyzed kinetic resolutions to be optimized. The effects of reaction conditions including buffer type, pH, temperature, product concentration, and initial substrate concentration on hydrolysis rate and enantioselectivity were analyzed and optimized. Three types of aqueous solvents - Tris-HCl buffer, phosphate buffer, and plain water - were tested for their ability as solvents for enantioselective hydrolysis reaction. The highest initial hydrolysis rate was obtained with phosphate buffer, while plain water induced a hydrolysis rate of EH that was 97% of the rate by phosphate buffer (data not shown) [12]. The effects of initial pH and temperature were investigated. Since EH activity could not be accurately measured at pH below 5 due to chemical hydrolysis in acidic conditions, enantioselective hydrolysis reactions were performed in the range of pH 5-9. As shown in Fig. 1, EH activity of *R. kratochvilovae* SYU-08 exhibits a pH and temperature optimum of 8.0 and 35°C, respectively.

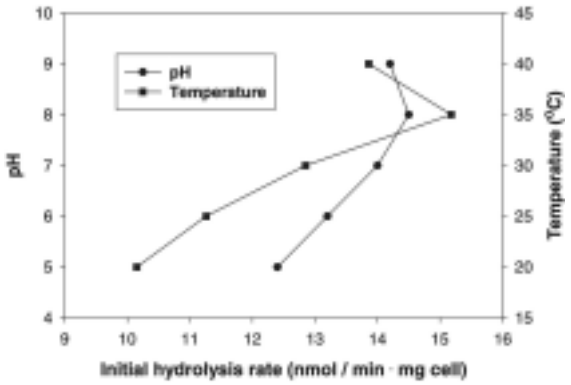

Fig. 1. Effect of initial pH and temperature on the hydrolysis rate of (*R*)-styrene oxide by *R. kratochvilovae* SYU-08.

Table 1. Effect of various phenyl-1,2-ethandiol, diol product, concentrations on hydrolysis rate, and relative enantioselectivity of *R. kratochvilovae* SYU-08-catalyzed enantioselective hydrolysis of racemic styrene oxide

Diol conc. (nM)	Intitial hydrolysis rate (nmol min^{-1} mg^{-1})
0	15.6
30	13.9
60	12.6
100	10.7
150	6.2
200	3.6

EH appeared to be sensitive to inhibition by phenyl-1, 2-ethanediol, the product of EH-catalyzed hydrolysis of styrene oxide. To analyze the effect of product concentration on hydrolysis rate and enantioselectivity, initial hydrolysis rates were determined at various initial concentrations of phenyl-1,2-ethanediol from 0 to 200 mM. As expected, the hydrolysis rate decreased with increasing initial phenyl-1,2-ethanediol concentrations (Table 1). Increasing initial phenyl-1,2-ethanediol concentrations above 100 mM led to drastic decreases in the hydrolysis rate and enantioselctivity, clearly indicating that the diol product should be removed for enhanced production of chiral styrene oxide. To investigate the effect of initial

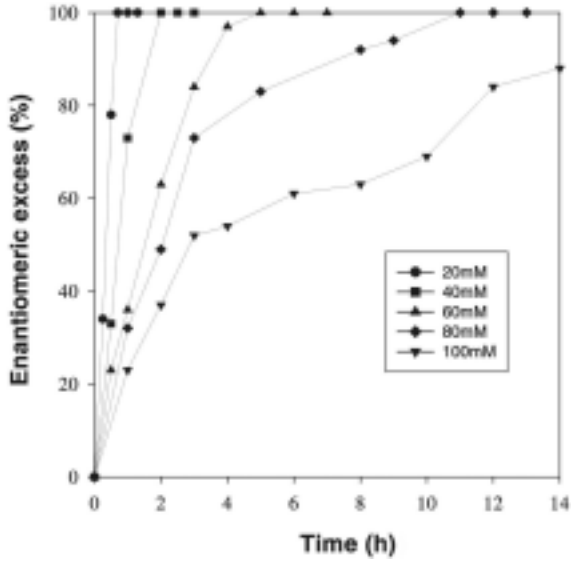

Fig. 2. Effect of initial substrate concentration on enantiomeric excess by *R. kratochvilovae* SYU-08.

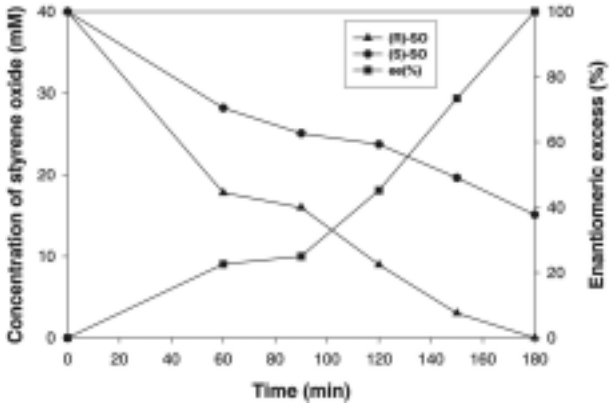

Fig. 3. Batch kinetic resolution of 80 mM racemic styrene oxide for the preparation of (*S*)-styrene oxide using enantioselective hydrolysis activity of *R. kratochvilovae* SYU-08.

substrate concentrations on the hydrolysis rate and enantioselectivity, enantioselective hydrolysis by *R. kratochvilovae* SYU-08 was performed at various initial substrate concentrations of 20, 40, 60, 80 and 100 mM. Enantiopurity of 100% *ee* was obtained up to 80 mM (Fig. 2) at reasonable reaction times.

The kinetic resolution of racemic styrene oxide to prepare enantiopure (*S*)-styrene oxide by enantioselective epoxide hydrolase activity of *R. kratochvilovae* SYU-08 was performed at optimal reaction conditions. The optimal conditions of pH, temperature, and initial substrate concentration were 8, 35°C, and 80 mM, respectively. The progression of a batch kinetic resolution is shown in Fig. 3. The enantiopurity of the remaining (*S*)-styrene oxide increased from 0 to 100% after 3 h. Racemic styrene oxide of 80 mM was resolved with almost 100% *ee* and 38% yield (theoretical yield = 50%). In conclusion, this study demonstrates that EH activity of *R. kratochvilovae*, a basidiomycete yeast, can be successfully applied to prepare commercially valuable aromatic epoxides such as styrene oxide.

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