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Biocatalytic resolution of glycidyl aryl ethers by *Trichosporon loubierii*: cell/substrate ratio influences the optical purity of (*R*)-epoxides

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Abstract

Glycidyl aryl ethers (**1a–1e**) were resolved by using lyophilized cells of *Trichosporon loubierii* ECU1040 having epoxide hydrolase activity. The activity and enantioselectivity depended on the structure of the aryl group. Different cell/substrate ratios also influenced the optical purity of remaining substrate. An additional stability test of the whole-cell enzyme suggests that rapid deactivation of the epoxide hydrolase was the potential reason. (*R*)-Epoxides were prepared in gram amounts with optical purity of 87%–99% ee.

Introduction

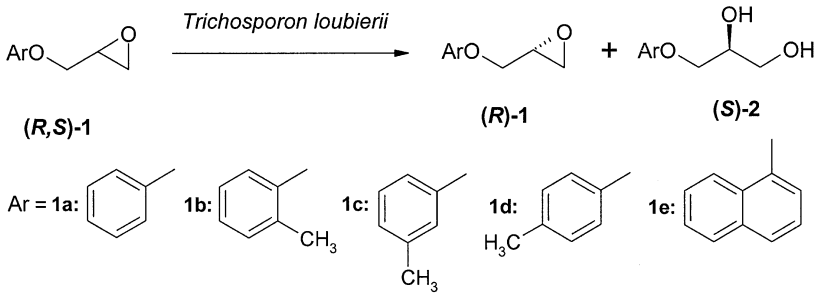
Due to their chemical versatility and high reactivity, chiral epoxides and vicinal diols are important in the preparation of various bioactive products (Steinreiber & Faber 2001). Both chemical and biological methods have been explored for the synthesis of enantio-pure epoxides and diols. Heavy metal-based catalysts have been developed to synthesize the epoxide in enantiopure forms via either direct epoxidation of an olefin or kinetic resolution of racemic epoxides (Ready & Jacobsen 2001, Konishi *et al.* 1992). A biochemical approach using epoxide hydrolases (EHs) has also drawn much attention for using mild condition and being environmentally friendly (Archelas & Furstoss 2001, Genzel *et al.* 2002, Hellstrom *et al.* 2001). Moreover, EHs are cofactor-independent enzymes and, depending on the substrate, may exhibit high activity and enantioselectivity. Their use will therefore allow organic chemists to prepare enantiopure epoxides in a simple way from cheap racemic epoxides.

Aryl glycidyl ethers and their related compounds are potentially useful intermediates for the synthesis of chiral amino alcohols (Kamal *et al.* 1992) and β-blockers (Aboul-Enein & Wainer 1997). Nevertheless, only very few of this type of compounds have been investigated regarding their kinetic resolution by EHs (Spelberg *et al.* 1998, Choi *et al.* 1998, Tang *et al.* 2001). Recently, a yeast, *Trichosporon loubierii* ECU1040, was screened in our laboratory, which could preferentially hydrolyze the (*S*)-enantiomer of phenyl glycidyl ether and its analogs, yielding (*R*)-epoxides and (*S*)-diols with good selectivity (Pan & Xu 2003). The present paper focuses on the kinetic resolution of five glycidyl aryl ethers (**1a–1e**) using whole cells of *Trichosporon loubierii* (Scheme 1). In addition, the relationship between the cell/substrate ratio (w/w) and the optical purity of finally resultant epoxides has been investigated. (*R*)-Epoxides have been prepared at the gram scale.

Materials and methods

General

All the chemicals were obtained commercially and of analytical grade. Glycidyl phenyl ether (GPE, **1a**) was purchased from Acros Co. Ltd. Glycidyl 2-methylphenyl ether (**1b**), glycidyl 3-methylphenyl



Scheme 1. The enantioselective hydrolysis of (*R*,*S*)-glycidyl aryl ethers (**1a-1e**) catalyzed by lyophilized cells of *Trichosporon loubierii* ECU1040.

ether (**1c**), glycidyl 4-methylphenyl ether (**1d**) and glycidyl naphthyl ether (**1e**) were synthesized from corresponding sodium phenolates and epichlorohydrin. Typical yields of glycidyl phenyl ethers were 20%∼44% depending on the structure of phenols.

Organism, growth and lyophilization

Trichosporon loubierii ECU1040 was cultivated as previously described (Pan & Xu 2003), on an optimized medium containing (per liter): glucose, 7.5 g; glycerol, 22.5 g; peptone, 18 g; yeast extract, 12 g; NH_4Cl, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.5 g; KH_2PO_4, 2.7 g; GPE (**1a**), 1.5 g. Cells were grown to the late growth phase (28∼32 h), and then the culture was centrifuged, resuspended in KH_2PO_4/KOH buffer (50 mM, pH 8), re-centrifuged and lyophilized. Typical yields of lyophilized cells ranged from 8–12 g l^{-1}.

Enantioselective hydrolysis of chiral epoxides **1*a*** **– 1*e***

Lyophilized cells were rehydrated in 60 ml of KH_2PO_4/KOH buffer (100 mM, pH 8) for 30 min on a shaker (160 rpm, 30 °C). Then 6.7 ml of DMSO containing 500 mg substrate was added and the mixture was agitated at 30 °C. The progress of the reaction was monitored by TLC or HPLC. When the degree of hydrolysis reached ca. 50%, the reaction mixture was extracted with ethyl acetate, condensed under vacuum, and purified by silica gel column chromatography using ethyl acetate/hexane (3:2v/v) as eluent, affording (*R*)-epoxides and (*S*)-diols.

Effect of cell/substrate ratio

Different weights of lyophilized cells (100, 300 and 500 mg) were suspended in 9 ml of KH_2PO_4/KOH buffer (100 mM, pH 8) and pre-incubated on a shaker (160 rpm, 30 °C) for 10 min. The reaction was started by adding 1 ml dimethyl sulphoxide (DMSO) containing 100 mg epoxide. Samples were taken periodically. The conversion degree and enantiomeric excess of the substrates (ee_s) were monitored by HPLC.

Stability of Trichosporon loubierii *whole-cell enzyme*

To several test tubes containing 1.8 ml of 100 mM potassium phosphate buffer (pH 8), was added each 50 mg of lyophilized cells. These tubes were shaken on a shaker (160 rpm, 30 °C). At different times, one tube was taken and 0.2 ml 100 mM GPE (**1a**) in DMSO was added to give 10 mM. The reaction was conducted on a shaker (160 rpm, 30 °C) for 20 min before adding 6 ml methanol to stop the reaction. Samples were centrifuged and filtrated with micro-pore membrane before analysis with HPLC.

Analytical methods

The substrate and product concentrations were determined by HPLC using a reverse phase C_{18} column and methanol/water (60/40, v/v) as the mobile phase (0.8 ml min^{-1}). α-Naphthol was used as an internal standard. The enantiomeric excesses of the epoxides and diols were determined by HPLC using a chiral column (Chiralcel OD, 250 mm × ∅4.6 mm, Daicel Co., Japan). The mobile phase was hexane/isopropanol (90/10 v/v for **1a**, **1c** & **1e**; 95/5 v/v for **1b** & **1d**), at 1 ml min^{-1} and the detector was set at 254 nm.

Table 1. Biocatalytic preparation of optical active (*R*)-glycidyl aryl ethers and corresponding (*S*)-diols.

Substrate[a]	Cell/substrate (g g^{-1})	Reaction time (h)	(*R*)-Epoxide		(*S*)-Diol		E^{b}
			ee (%)	yield (%)	ee (%)	yield (%)	
1a	5/1	4.5	>99	35	60	35	20
1b	7/1	6	>99	40	72	54	41
1c	7/1	11	95	32	71	46	21
1d	7/1	6	88	45	72	51	17
1e	15/1	7	97	26	59	36	15

[a]Reaction conditions: as described in the experimental section.
[b]*E*-value was calculated according to the reference (Rakels *et al.* 1993).

Results and discussion

*Effect of cell/substrate ratio on the optical purity of remaining substrate (*ee$_s$*)*

In the kinetic resolution process of epoxides using an epoxide hydrolase, one of the most important goals is to get the chiral epoxide in high enantio-purity. The optical purity of remaining epoxide will increase continually with prolongation of the reaction time and can reach nearly 100% as long as sufficient time is given. In principle under ideal conditions, this means that there is no inhibition by substrate or product and no loss of enzyme activity under the resolution conditions. In a practical process, however, all the above-mentioned phenomena happen frequently which may influence the optical purity of resultant epoxides. It is necessary to know which factor is the most important and how to get the desired epoxide in higher optical purity.

In the hydrolysis of glycidyl phenyl ether using lyophilized cells of *Trichosporon loubierii* ECU1040, the cell/substrate ratio was an important factor that influenced the enantiomeric excess of the remaining substrate (ee_s). As shown in Figure 1, the final ee_s value (at 17 h) was only 23% when the cell/substrate ratio (w/w) was 1:1. As the ratio was increased up to 3:1 and 5:1, the final ee_s values were enhanced up to 95% and >99% respectively. On the other hand, the ratio of cell/substrate also affected the conversion of glycidyl phenyl ether in a similar way. In addition, variation of the cell/substrate ratio changed the enantioselectivity too. A 2-fold enhancement of enantioselectivity ($E = 22$ vs. $E = 10$) was observed when increasing the cell/substrate ratio from 1 to 3, though additional increase in cell/substrate ratio (3 → 5) had little effect on the enantioselectivity

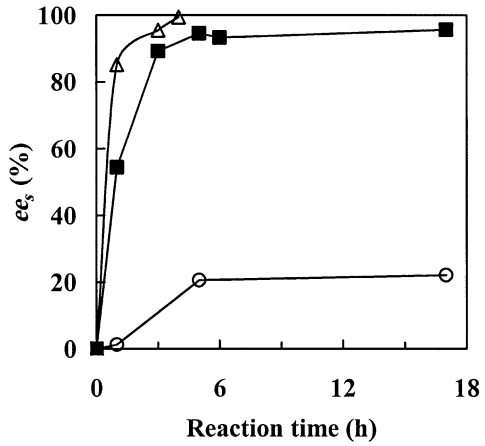


Fig. 1. Effect of cell/substrate ratio on the final optical purity of remaining substrate. Reaction conditions: temperature, 30 °C; reaction volume, 60 ml; substrate, 100 mg; lyophilized cells, 100, 300 or 500 mg, corresponding to cell/substrate ratios of 1/1 (○), 3/1 (■) and 5/1 (△), respectively.

($E = 25$ vs. $E = 22$). Hellström *et al.* (2001) also found a nearly 2-fold selectivity enhancement upon doubling of the biocatalyst and a further increase had no significant influence.

For glycidyl naphthyl ether, the larger substituent than that in glycidyl phenyl ether caused a lower activity of epoxide hydrolase. So a higher cell/substrate ratio was needed (Table 1). For example, the ee_s was only 75% when the cell/substrate ratio was 7:1 after 21 h and the ee_s was 80% after 45 h. When the ratio was 10:1, the ee_s reached 95% at 21 h (data not shown).

The reason why different substrate/cell ratios gave different optical purities of the remaining substrate (ee_s) was considered to be the stability of the lyophilized cells of *Trichosporon loubierii*. As shown in Figure 2, the epoxide hydrolase activity of the cells

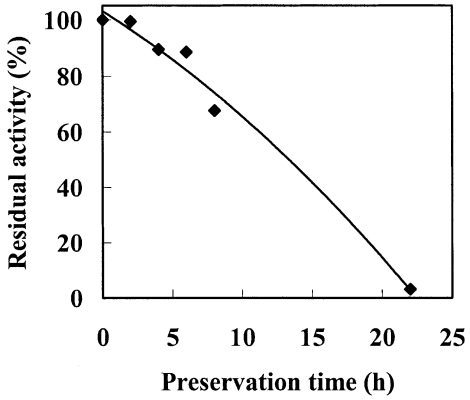


Fig. 2. Variation of the lyophilized cell activity with preservation time (100% = 9 μmol min^{-1} g^{-1} dry cell). To several test tubes containing 1.8 ml of 100 mM potassium phosphate buffer (pH 8), was added 50 mg of lyophilized microbial cells. These tubes were incubated on a shaker (160 rpm, 30 °C). At a certain time, one of the tubes was taken and 0.2 ml of 100 mM GPE (**1a**) in DMSO was added, giving a final concentration of 10 mM. The reaction was conducted on a shaker (160 rpm, 30 °C) for 20 min before adding 6 ml of methanol to stop the reaction. Samples were centrifuged and filtrated with micro-pore membrane before analysis with HPLC.

decreased rapidly with time and the cells were deactivated almost completely after 24 h. Deactivation of the cells is thus the main reason for the incomplete resolution of chiral epoxides with a lower ratio of active cells to epoxides (e.g., 1:1, in Figure 1).

*Preparation of (*R*)-epoxides (***1a–1e***) and (*S*)-diols (***2a–2e***) by using lyophilized cells of* Trichosporon loubierii

Based on the above results, a preparative scale experiment was carried out with 500 mg racemic epoxides. After the degree of hydrolysis was around 50%, the residual epoxide and produced diol were extracted by ethyl acetate and then separated by flash chromatography, giving (*R*)-epoxide and (*S*)-diol. As shown in Table 1, the activity and enantioselectivity of cells changed with respect to different structures of the aryl group in the epoxide substrates (**1a–1e**). The activity of epoxide hydrolase decreased significantly when the size of the aryl group increased. Among the five epoxides, phenyl glycidyl ether (**1a**) was the fastest reaction substrate, while naphthyl glycidyl ether (**1e**) was the slowest one. On the other hand, the enantioselectivity increased as the methyl group in the phenyl ring was shifted from 4-position (**1d**) to 2-position (**1b**). The resolution of **1a–1e** was also reported (Choi *et al.* 1998) using whole cells of *Aspergillus niger* with epoxide hydrolase activity. However, no preparative scale experiment was reported and the substrate concentration of the epoxides was very low (10–20 mM). Though they found the epoxide hydrolase activity was not affected by the presence of methyl side chain and its location in the phenyl group (**1a–1d**), the kinetic resolution of epoxide **1e** was extremely unsatisfactory in their case (only 1% yield after 5 h of reaction).

In conclusion, the biocatalytic resolution of epoxides (**1a–1e**) was accomplished by using lyophilized cells of *Trichosporon loubierii* ECU1040 with an epoxide hydrolase activity, yielding (*R*)-epoxides and (*S*)-diols. The activity and enantioselectivity of the *Trichosporon loubierii* cells depended on the structure of aryl group. It was also found that appropriate ratio of cell to substrate should be selected for achieving higher optical purity of the remaining epoxides due to the poor stability of the lyophilized cells of ECU1040. The immobilization should be an effective method for improving the stability of the whole-cell enzyme. Preliminary results showed more than 3-fold stability enhancement when the cells were entrapped in alginate gel (data not shown) and the corresponding work is still under investigation.

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