Enantioselective biocatalytic conversions of epoxides
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Chapter 4

A tandem enzyme reaction to produce optically active halohydrins, epoxides and diols
Microorganisms provide us with a variety of enzymes that have evolved to help the organism deal with the presence of synthetic compounds in their environment. In a single organism multiple enzymes can be involved in the degradation of xenobiotics. Examples can be found in bacteria that metabolize epoxides or haloaliphatics.\textsuperscript{1} If such enzymes are enantioselective, they can be useful for the preparation of optically active intermediates. In the fine chemicals industry, optically pure epoxides are used as building blocks for various pharmaceutical products. Several biocatalytic methods to produce optically active epoxides using enzymes involved in epoxide degradation or formation have been reported.\textsuperscript{2} Examples are direct epoxidation of alkenes by mono-oxygenases or kinetic resolution of racemic epoxides. Kinetic resolutions have been carried out by means of lipases, using another nearby functionality such as an ester, or by using epoxide hydrolases for the direct resolution at the epoxide ring.\textsuperscript{3} Outstanding examples of the synthesis of optically pure epoxides by kinetic resolution with metal containing salen complexes have recently been published by Jacobsen et al.\textsuperscript{4,5}

Halohydrins can be considered as direct precursors of epoxides. Ring closure of an optically pure halohydrin generally yields an optically pure epoxide. Aromatic halohydrins such as 2-chloro-1-phenylethanol can be obtained optically pure by microbial reduction of $\alpha$-haloacetophenones or by kinetic resolution using lipases.\textsuperscript{6,7}

Optically pure halohydrins can also be produced using halohydrin dehalogenases.\textsuperscript{8} These enzymes catalyze the ring closure of a vicinal halohydrin to an epoxide and the reverse reaction. Several organisms capable of dehalogenating halohydrins have recently been isolated. From Corynebacterium sp. two halohydrin dehalogenases were characterized and the genes were cloned and sequenced.\textsuperscript{9} Recently, a halohydrin dehalogenase from Arthrobacter erithii H10a has been characterized.\textsuperscript{10} This enzyme could be used to synthesize optically active epihalohydrins from 1,3-dichloro-2-propanol by enantioselective dehalogenation and halogenation.\textsuperscript{11}

\begin{equation}
\begin{array}{cccc}
\text{Cl} & \text{Cl} & \text{OH} & \text{OH} \\
\text{OH} & \text{OH} & \text{Cl} & \text{Cl} \\
\text{a} & \text{b} & \text{c} & \\
+H_2O & -HCl & +H_2O & \\
\end{array}
\end{equation}

Figure 1  \textit{Degradation route of epichlorohydrin to glycerol by Agrobacterium radiobacter AD1. Epoxide hydrolase (step a and c) and halohydrin dehalogenase (step b).}

We have isolated several organisms that can degrade chlorinated aliphatic compounds, including bacteria that could grow on halohydrins and epichlorohydrin. A halohydrin dehalogenase was purified and characterized from the 3-chloro-1,2 propanediol-
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utilizing bacterium *Arthrobacter* strain AD2. Another organism, *Agrobacterium radiobacter* AD1, degraded the toxic epichlorohydrin to glycerol by an epoxide hydrolase and a halohydrin dehalogenase (Figure 1).

Recently, we have cloned and overexpressed a gene that encodes the epoxide hydrolase of *A. radiobacter* AD1. With this enzyme optically pure aromatic epoxides could be obtained by kinetic resolution although the E-values were only moderate. Styrene oxide, for example, was hydrolysed with an E-value of 16. Non-aromatic epoxides such as epichlorohydrin were hydrolysed with low enantioselectivity (E<2). We have also cloned and overexpressed the second enzyme involved in the degradation pathway, the halohydrin dehalogenase. Research with halohydrin dehalogenases has so far mainly been focussed on C3 epoxides and halohydrins, such as 2,3-dichloro-1-propanol and 1,3-dichloro-1-propanol. One paper has been published describing the conversion of aromatic halohydrins by resting cells of *Flavobacterium rigense* but the enantioselectivity was very low.

The goal of our research was to establish the enantioselectivity of the halohydrin dehalogenase from *A. radiobacter* AD1 towards a variety of substrates, including halohydrins with an aromatic moiety. Since the application of epoxides and halohydrins as precursors for drugs requires them to be available in optically pure form it was the objective to obtain the investigated halohydrins with an enantiomeric excess (e.e.) of higher than 99%.

### Results and discussion

The halohydrin dehalogenase from *A. radiobacter* enantioselectively dehalogenated 2,3-dichloro-1-propanol 1 and 2,3-dibromo-1-propanol 2. The conversion of 1 proceeded with a very high enantioselectivity (E>100) for the (R)-enantiomer, leaving the (S)-enantiomer unreacted. Initially the (R)-enantiomer was converted with a specific activity of 0.9 µmol.min⁻¹.mg⁻¹, but the reaction gradually slowed down and after 20 h an e.e. of 96% was reached. The yield of the remaining (S)-enantiomer was higher than 49.5% (maximum theoretical yield is 50.0%). Instead of enantiomerically pure epichlorohydrin, racemic epichlorohydrin and prochiral 1,3-dichloro-2-propanol were formed. This can be explained by the fact that the enzyme also catalysed the reverse reaction, the cleavage of the epoxide ring by an halide (Figure 2).

The favoured attack of the chloride in the halogenation reaction was at the β-position of epichlorohydrin to yield 1,3-dichloro-2-propanol (step 4a) instead of α-attack to yield 1 (step 1). This prochiral 1,3-dichloro-2-propanol was then converted to both enantiomers of epichlorohydrin (steps 4a and 4b). The specific activity for 1,3-dichloro-2-propanol was 3.9 µmol.min⁻¹.mg⁻¹, which is more than 4-fold higher than the activity for 1. Because of these side reactions the conversion of 1 was inhibited during the course of the reaction.
**Figure 2**  Conversion of 2,3-dichloro-1-propanol 1 by halohydrin dehalogenase and epoxide hydrolase. Epoxide hydrolase (steps 2 and 3) and halohydrin dehalogenase (steps 1, 3, 4a and 4b).

**Figure 3**  Kinetic resolution of 1 with halohydrin dehalogenase (■, □), with addition of excess epoxide hydrolase (●, ○).(R)-1: (●, ■) and (S)-1: (○, □).
To overcome the unwanted side reactions (steps 4a and 4b), an excess of recombinant epoxide hydrolase from *A. radiobacter* AD1 was added. In this way the formed (S)-epichlorohydrin was converted immediately to (S)-3-chloro-1,2-propanediol (step 2). This was then further converted via glycidol to glycerol (step 3). In this case, formation of 1,3-dichloro-2-propanol and epichlorohydrin were not observed. The enantiomeric excess of (S)-3-chloro-1,2-propanediol was dependent on the amount of the epoxide hydrolase that was added due to competition with chemical hydrolysis and the reverse reaction. The maximum measured e.e. was 91%. In the presence of epoxide hydrolase, the conversion of (R)-1 was accelerated and a maximum e.e. of higher than 99% for (S)-1 was reached in less than 5 h (Figure 3). The analytical yield of 1 was higher than 49.5%.

Another method to circumvent the reversibility of a halohydrin dehalogenase was recently described by Assis et al.\textsuperscript{11} They studied the conversion of 1,3-dichloro-2-propanol to (R)-epichlorohydrin, which could be obtained with an e.e. of 89.5%. By adding an excess of bromide, only the contaminating (S)-epichlorohydrin was halogenated and the (R)-epichlorohydrin was obtained with an e.e. of higher than 95%, but only in a 10.7% yield.

![Figure 4](https://example.com/figure4.png)

**Figure 4**  *Halohydrins used as substrates by the halohydrin dehalogenase from A. radiobacter AD1.*

Surprisingly, halohydrins containing an aromatic group such as halohydrin 3 and 5 were also converted, with E-values of 73 and 23 respectively (Table 1). This high activity and enantioselectivity of a halohydrin dehalogenase towards aromatic halohydrins has not been described before. In the case of the kinetic resolution of halohydrin 3, (R)-styrene oxide was formed with an e.e. of 90%. Because of the reversibility of the reaction the conversion was incomplete and the e.e. of (S)-3 reached a maximum of only 92%. Formation of halohydrin 4 was not observed during the reaction. With addition of an excess of epoxide hydrolase, resulting in removal of the inhibiting epoxide, the conversion was completed and an e.e. of the (S)-enantiomer of higher than 99% was reached.

The diols produced by hydrolysis of the aromatic epoxides had a lower e.e. than the corresponding epoxides. This was mainly due to the regioselectivity (β-attack versus α-attack) of the epoxide hydrolase. When, in a separate experiment, optically pure (R)-styrene oxide or (R)-m-chlorostyrene oxide were hydrolysed by epoxide hydrolase, the (R)-diols were formed with an e.e. of respectively 85% and 90%.
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Table 1  The kinetic resolution of halohydrins with recombinant halohydrin dehalogenase and epoxide hydrolase.

<table>
<thead>
<tr>
<th>halohydrin</th>
<th>specific activity$^a$</th>
<th>e.e. halo-hydrin (%)</th>
<th>analytical yield (%)</th>
<th>abs.config.$^b$</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9</td>
<td>&gt;99</td>
<td>&gt;49.5$^d$</td>
<td>(S)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2</td>
<td>26.6</td>
<td>&gt;99</td>
<td>&gt;49.5$^d$</td>
<td>nd</td>
<td>&gt;100</td>
</tr>
<tr>
<td>3</td>
<td>14.1</td>
<td>&gt;99</td>
<td>47.0</td>
<td>(S)</td>
<td>73</td>
</tr>
<tr>
<td>4</td>
<td>8.7</td>
<td>&gt;99</td>
<td>26.5</td>
<td>(R)</td>
<td>&gt;50$^e$</td>
</tr>
<tr>
<td>5</td>
<td>4.5</td>
<td>&gt;99</td>
<td>39.5</td>
<td>(S)</td>
<td>23</td>
</tr>
</tbody>
</table>

$^a$) In Tris-SO\(_4\) buffer, pH = 7.5, 30 °C, substrate concentrations 5 mM, activity in µmol.min$^{-1}$.mg$^{-1}$.  
$^b$) Absolute configuration of the remaining enantiomer.  
$^c$) The exact E-ratio could not be determined due to the high chemical instability of the halohydrin.  
$^d$) The maximum analytical yield is 50%.

The kinetic resolution of halohydrin 4 was strongly influenced by the high chemical conversion of the substrate in the buffer solution (42% degradation per h in buffer pH=7.5, T= 30°C) leading besides the epoxide to major side products. This was probably due to dimerisation of the halohydrin.\(^{18}\) When the kinetic resolution of 4 was performed without the addition of epoxide hydrolase, \((R)\)-styrene oxide was formed with an e.e. of 89%, indicating an E-value of at least 50. The chemical instability limits a practical application of the kinetic resolution of this halohydrin. No significant chemical conversion was observed with halohydrins 1, 2, 3 and 5.

Table 2  The kinetic resolution of halohydrins with recombinant halohydrin dehalogenase, with and without epoxide hydrolase.

<table>
<thead>
<tr>
<th>halohydrin</th>
<th>without addition of epoxide hydrolase</th>
<th>with addition of epoxide hydrolase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>e.e. halo-hydrin (%)</td>
<td>e.e. epoxide (%)</td>
</tr>
<tr>
<td>1</td>
<td>96</td>
<td>&lt;5</td>
</tr>
<tr>
<td>3</td>
<td>92</td>
<td>90</td>
</tr>
<tr>
<td>5</td>
<td>95</td>
<td>72</td>
</tr>
</tbody>
</table>

The importance of the addition of epoxide hydrolase was substrate dependent as shown in Table 2. In case of the conversion of halohydrin 1, without epoxide hydrolase the kinetic resolution was strongly inhibited due to the side reactions shown in Figure 2. The formed optically active epichlorohydrin rapidly racemised. In the case of the aromatic halohydrins 3 and 5 no side-reactions of the epoxide to other products occurred. The importance of epoxide hydrolase was dependent on the ratio of the concentrations of epoxide
and halohydrin at equilibrium. Without the addition of epoxide hydrolase, (S)-3 was obtained with a lower e.e. than (S)-5 because with the latter the equilibrium was positioned further towards the epoxide. It is important to state that the excess epoxide hydrolase was only added to draw the kinetic resolution of the halohydrins to completion by removing the inhibiting epoxide. The epoxide hydrolase itself has no influence on the enantioselectivity of the conversion.

These results are an example of a tandem enzyme reaction. The combined use of these enzymes was developed by evolution in Nature for the degradation of xenobiotic compounds. We used this combination of biocatalysts to produce optically active halohydrins, epoxides and diols. Since the genes for both enzymes were cloned and the enzyme was brought to overexpression, application on an industrially interesting scale may become feasible.

**Experimental**

**General**

The enantiomeric excess (e.e.) and the yields of the halohydrins, epoxides and derivatised diols were determined with a Hewlett-Packard 5890 gas chromatograph equipped with a FID-detector, using a Chiraldex G-TA capillary column (col I, 50 meters, Astec), Chiraldex B-TA capillary column (col II, 30 meters, Astec) or β-dex 120 (col III, 30 meters, Supelco) all of 0.25 mm inside diameter. NMR-spectra were recorded in CDCl$_3$. Halohydrin 1 was purchased from Merck, halohydrin 2 from Fluka, halohydrin 3 from Lancaster, $m$-chlorostyrene oxide was a gift from DSM. All other chemicals were purchased from Aldrich.

**Synthesis of halohydrins**

Halohydrins 4 and 5 were synthesised by treating the corresponding epoxides with 36% aqueous HCl according to described methods.$^{19}$ To a solution of styrene oxide (1.0 g, 8.3 mmol) in CHCl$_3$ (80 ml), 36% aqueous HCl was added (25 ml) and stirred for 1 h. After separating, the organic phase was washed with saturated aqueous NaHCO$_3$ and water, dried with MgSO$_4$, and removed by a rotary evaporator giving halohydrin 4 in 90% yield as a colourless oil. $^1$H NMR, δ: 2.2-2.4 (s,1H), 3.89 (dd, 2H), 4.98 (t, 1H), 7.3 (m, 5H); $^{13}$C NMR δ: 62.25, 65.34, 125.01, 126.30, 126.37, 135.48.

To a solution of $m$-chlorostyrene oxide (0.75 g, 4.8 mmol) in acetonitrile (5 ml) were added LiClO$_4$ (0.80 g, 7.5 mmol) and NH$_4$Cl (0.40 g, 7.5 mmol) and the resulting mixture was stirred for 4 h at 80°C. The reaction mixture was diluted with water and extracted with ether. The ether was washed with water, dried with MgSO$_4$, and removed by a rotary evaporator yielding a colourless oil. Flash chromatography on silica 60 H using petroleum ether/ether (ratio 7:3) as eluent yielded pure halohydrin 5 in 25 % yield. $^1$H NMR, δ: 2.6 (s, 1H), 3.55 (dd, 1H), 3.65 (dd, 1H), 4.85 (dd, 1H), 7.2-7.3 (m, 3H), 7.36 (s, 1H); $^{13}$C NMR δ: 48.12, 70.86, 121.71, 123.79, 126.08, 127.42, 132.13, 139.38.
Production of halohydrin dehalogenase and epoxide hydrolase

A gene library of *A. radiobacter* AD1 was constructed in the cosmid vector pLAFR3. After in vitro packaging, the library was transduced to *E. coli* HB101. Transconjugants were screened for dehalogenase activity with 1,3-dichloro-2-propanol. The halohydrin dehalogenase gene, designated hheC, was sequenced and subsequently amplified by PCR and cloned behind the T7 promoter of the expression vector pGEF, yielding pGEFhheC. The halohydrin dehalogenase gene was overexpressed up to 30% of soluble protein by introduction of pGEFhheC in *E. coli* BL21(DE3).

For the described kinetic resolutions purified enzyme was used. Plasmid DNA was transformed by electroporation to competent *E. coli* BL21 (DE3) cells, which were then plated out on LB medium containing tetracycline and incubated overnight at 30 °C. A preculture was started by inoculating 100 ml of LB medium containing tetracycline with the transformants from a plate to an initial OD₆₀₀ of 0.1. The culture was incubated at 30 °C until an OD₆₀₀ of 1-2 was reached, diluted in 1 l of LB medium containing tetracycline and incubated overnight at 20 °C. The cells were subsequently centrifuged, washed and resuspended. A crude extract was prepared by ultrasonic disruption and centrifugation of the cells. This was followed by a purification step with a Resource Q column. The purified recombinant epoxide hydrolase from *A. radiobacter* AD1 was prepared as described before.

Kinetic resolution with halohydrin dehalogenase and epoxide hydrolase

A screw-capped bottle containing 30 ml Tris buffer (100 mM, pH 7.5) was incubated in a shaking waterbath at 30 °C. The halohydrin was added to a final concentration of 5 mM. The reaction was started by the addition of the appropriate amount of purified halohydrin dehalogenase. In experiments where epoxide hydrolase was used, this enzyme was added immediately afterwards. The reaction was monitored by periodically taking samples from the reaction mixture. The samples were extracted with diethylether containing mesitylene or 1-chlorohexane as an internal standard. Prior to analysis by chiral GLC, the samples were dried over a short column containing MgSO₄. The diols were analysed after derivatisation to their acetonides. The diethylether was vaporized under a stream of nitrogen and the sample was redissolved in 0.5 ml 2,2-dimethoxypropane. The solution was then shaken for 1 h with 200 mg amberlite IR-120 (H⁺). After addition of 50 mg NaHCO₃ the organic phase was analysed by chiral GLC.

Calculation of enantioselectivity (E) and activity

The enantioselectivity of the kinetic resolutions with the halohydrin dehalogenase was determined in the presence of an excess of epoxide hydrolase. The enantioselectivity of the kinetic resolutions of halohydrins 3 and 5 was calculated using the enantiomeric ratio which is defined by equation (1), where *V*ₘₐₓ and *K*ₘ represent the Michaelis-Menten parameters of both enantiomers. To estimate the kinetic parameters, the equations that describe competitive Michaelis-Menten kinetics were fitted by numerical integration to the data, as described
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before. With the obtained kinetic parameters or in cases where no unique solutions were obtained, with the lumped parameter $V_{\text{max}}/K_m$, the E-value was calculated using equation (1).

$$E = \frac{V^S_{\text{max}}/K^S_m}{V^R_{\text{max}}/K^R_m}$$

(1)

The exact enantioselectivity of the conversions of halohydrins 1 and 2 could not be determined because no significant decrease of the remaining enantiomer could be measured, indicating an E-value of at least higher than 100. With halohydrin 4, the conversion rate of slow reacting enantiomer was approximately equal to the chemical conversion rate, indicating an E-value of at least 50. The activities as presented in Table 1 were calculated from the substrate depletion at a concentration of 5 mM. In the case of halohydrin 4 the initial activity was corrected for chemical conversion.

Analysis of chiral compounds

The data from chiral GLC were as follows. Halohydrin 1 on col II: temp. prog. 60°C for 2 min, increase with 3°C per min to 90°C, 20 min on 90°C, $t_r =$ 20.2 min and 20.8 min, halohydrin 2 on col I: temp. 105°C, $t_r =$ 53.4 min and 55.0 min, halohydrin 3 on col III: temp. 140°C, $t_r =$ 26.8 min and 28.2 min, halohydrin 4 on col III: temp. 140°C, $t_r =$ 25.4 min and 26.0 min, halohydrin 5 on col III: temp. 170°C, $t_r =$ 21.8 min and 22.4 min. Styrene oxide on col I: temp. 110°C, $t_r =$ 15.3 min and 18.0 min, m-chlorostyrene oxide on col I: temp. 110°C, $t_r =$ 20.2 min and 31.1 min, epichlorohydrin on col II: temp. prog. 60°C for 2 min, increase with 3°C per min to 90°C, 20 min on 90°C, $t_r =$ 7.8 min and 8.2 min, derivatised 3-chloro-1,2-propanediol on col I: temp. 70°C. $t_r =$ 42.1 min and 43.4 min, derivatised phenylethanediol on col III: temp. 130°C, $t_r =$ 15.1 min and 16.3 min, derivatised m-chlorophenylethanediol op col III: temp. 150°C, $t_r =$ 18.4 min and 19.7 min.

The absolute configurations of the halohydrins were determined by basic ring closure of the remaining enantiomers to the corresponding epoxides. The absolute configuration of epichlorohydrin, styrene oxide and m-chlorostyrene oxide was determined by co-injection of the commercially available optically pure epoxides. The absolute configurations of 3-chloro-1,2-propanediol and phenylethanediol were determined by co-injection of the derivatised commercially available optically pure diols. The absolute configuration of m-chlorophenylethanediol was determined by enzymatic hydrolysis of (R)-m-chlorostyrene oxide and analysis of the formed and derivatised diol.
References

2. Faber, K. *Biotransformations in Organic chemistry 3rd ed.*; Springer-Verlag New York, **1997**