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The enantioselectivity of haloalkane dehalogenases

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Abstract—Two haloalkane dehalogenases were tested for their ability to perform kinetic resolutions of a series of racemic substrates and to convert *meso* substrates enantioselectively. For the kinetic resolutions *E*-values of up to 9 were measured and, in the conversions of the *meso* substrates, products were obtained with an enantiomeric excess of up to 47%. A kinetic analysis revealed that despite modest overall chiral recognition (expressed as *E*-values), there are large differences between the K_m values (>100 fold) of two enantiomeric substrates but that these differences are compensated by correspondingly large differences in k_{cat}.

Haloalkane dehalogenases are capable of replacing a halide in an organic substrate by a hydroxyl group.[1] These enzymes have been isolated from polluted industrial sites from organisms capable of growing on haloalkanes. Such organisms can play an important environmental role in the clean-up of those sites. Haloalkane dehalogenases also hold potential in biocatalysis, since haloalkanes or haloalcohols are valuable building blocks in organic synthesis, especially when chiral and of high enantiomeric purity. To date no enantioselective conversions with haloalkane dehalogenases have been reported. We therefore initiated a search for such reactions using two prominent members of this enzyme family. The first one is a haloalkane dehalogenase designated DhlA,[2] with optimal activity for short-chain haloalkanes and originally obtained from the 1,2-dichloroethane degrading organism *Xanthobacter autotrophicus* GJ10. The second enzyme, DhaA,[3] exhibits additional activity for long-chain haloalkanes and was originally obtained from the chlorobutane-degrading *Rhodococcus rhodochrous* NCIMB 13064. For both of these enzymes the genes have been cloned and they can be obtained in large quantities by efficient overexpression in *E. coli*. This latter feature makes their practical application feasible.

We screened the two enzymes for enantioselectivity with a series of chiral and *meso* haloalkanes. Compounds were first identified as substrates by using an assay that quantifies the amount of halide released[4] over time. The degree of chiral recognition in the conversion of the compounds was determined by analyzing the reaction mixture by gas chromatography on a chiral column.[5] The enantiodiscrimination was expressed in terms of *E*-values (Table 1).[6] Most compounds tested were primary halides, although some had additional secondary halide centers. Secondary alkyl halides were found to be very poor substrates for DhlA. Although DhaA was better at converting secondary halides, 2-bromobutane was converted without observable chiral selection.

Several 1,2 and 1,3 short chain dihaloalkanes were tested (entries 1–6). They were all converted with little selectivity by both enzymes. Interestingly, in the conversion of the 1,2-dihalo compounds by DhlA, not only is the primary halide replaced, but also significant amounts of the secondary halide, although 2-bromobutane is converted very sluggishly by this enzyme. With the 1,3-dihaloalkanes only the primary halogen was replaced. In all cases *E*-values were based on the disappearance of the starting material but in several cases additional determinations were based on the e.e. of the product.[6] A small increase in enantioselectivity was seen with methyl-3-bromo-2-methyl propionate (entry 7). This compound proved to be a very good substrate for DhaA and was converted with an *E*-value of 5. Attempts were made to increase the selectivity by modifying the structure of the substrate. Changing the

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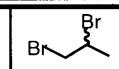
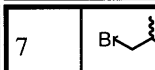
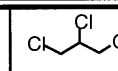
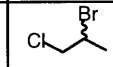
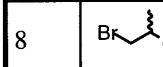
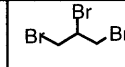
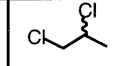
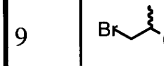
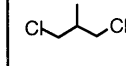
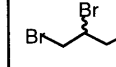
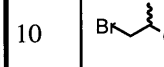
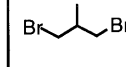
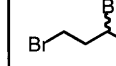
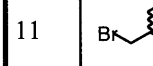
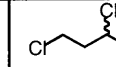
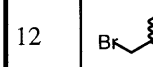
methyl ester to an ethyl ester (entry 8) resulted in a slight reduction in selectivity. Introducing a branching point by means of the isopropyl ester (entry 9) led to an almost non-selective reaction. Going from a methyl ester to a methyl amide (entry 10) had a small negative effect. In short, modifying the methyl ester seemed to have only negative effects with respect to enantioselectivity. Changing the methyl group at the chiral center to ethyl (entry 11) resulted in an increase of the *E*-value ($E = 9$), but unfortunately with the propyl group the selectivity decreased again to an *E*-value of 3 (entry 12). These results show that there is a delicate steric balance determining the enantioselectivity.

An attractive route to optically pure chiral compounds is the selective conversion of *meso* compounds. Although the maximum yield of a kinetic resolution is 50%, here the full 100% can theoretically be reached. Four *meso* substrates were studied with both DhlA and DhaA and the e.e.'s of the formed haloalcohols were determined by chiral GC (Table 1, entries 13–16). In a few cases the e.e.'s approached 50%.

The nature of the enantioselectivity of the kinetic resolution of methyl-3-bromo-2-methyl propionate (Table 1, entry 7) was studied in more detail. In a kinetic resolution the (*R*)-enantiomer was converted first leading to an enrichment of the (*S*)-enantiomer (Fig. 1A). We then performed experiments in which the progress of *separate* reactions of the individual enantiomers was monitored. Interestingly, the initial conversion rate of the (*S*)-enantiomer was more then 4 times faster than that of the (*R*)-enantiomer. This indicates that in a kinetic resolution of a racemate, the (*R*)-enantiomer inhibits the conversion of the otherwise faster reacting (*S*)-enantiomer. To investigate these results in more detail, we attempted to determine the kinetic parameters of the conversion of the respective enantiomers by DhaA (Table 2). In case of the (*S*)-enantiomer, the V_{max} could not be determined because the K_m value was higher than the solubility of the substrate, which was around 35 mM. The rate at 5 mM was 3.70 μmol $min^{-1}.mg^{-1}$ from which a $k_{cat}{}^S/K_m{}^S$ of 0.42 mM^{-1} s^{-1} can be calculated. In the case of the (*R*)-enantiomer the K_m was determined by using the V_{max} (0.84 μmol min^{-1} mg^{-1}) and equals 0.48 s^{-1}. Using the experimentally determined *E* value of 5, which is equal to: $(k_{cat}{}^R/K_m{}^R)/(k_{cat}{}^S/K_m{}^S)$, it follows that the $K_m{}^R$ equals 0.23 mM. In short, despite the vastly different K_m values the *E*-value is still only 5, i.e. the difference is largely compensated by large differences in k_{cat}.

Similar conclusions are reached from fitting the data of a progress curve (Fig. 1A) to a Michaelis–Menten equation.[7] This method could not be used to obtain unique solutions for all of the kinetic parameters but it did suggest a ratio of the K_m values of (*S*)- and (*R*)-enantiomers of higher than 100. The resulting *E*-value was independent of the absolute K_m value for each enantiomer and led to an *E*-value of 5, consistent with the previous determination. A similar analysis of the ethyl derivative (entry 11, Table 1), is shown in Fig. 1B. The close resemblance of the progress curve to the one in Fig. 1A indicates the likelihood of overall similar kinetic features. Fitting the data of the progress curve resulted in an *E*-value of 9 and vastly different K_m values for the enantiomers.

Table 1. Dehalogenation of haloalkanes, monitored by gas chromatography on a chiral column; reaction conditions: substrate conc. 5–10 mM, tris-sulfate buffer (50 mM), with up to 20% DMSO to enhance solubility (entries 4 and 12, 14, 16), pH 8.2, room temperature. (n.d. = not-determined)

entry	Racemic substrates	E-value DhlA	E-value DhaA	entry	Racemic substrates	E-value DhaA	entry	Meso substrates	%ee prod. DhlA	%ee prod DhaA
1		3	1.3	7		5	13		47	18
2		4	n.d.	8		4	14		13	47
3		2	n.d.	9		1.5	15		10	10
4		2	2	10		3	16		46	29
5		2	n.d.	11		9				
6		3	n.d.	12		3				

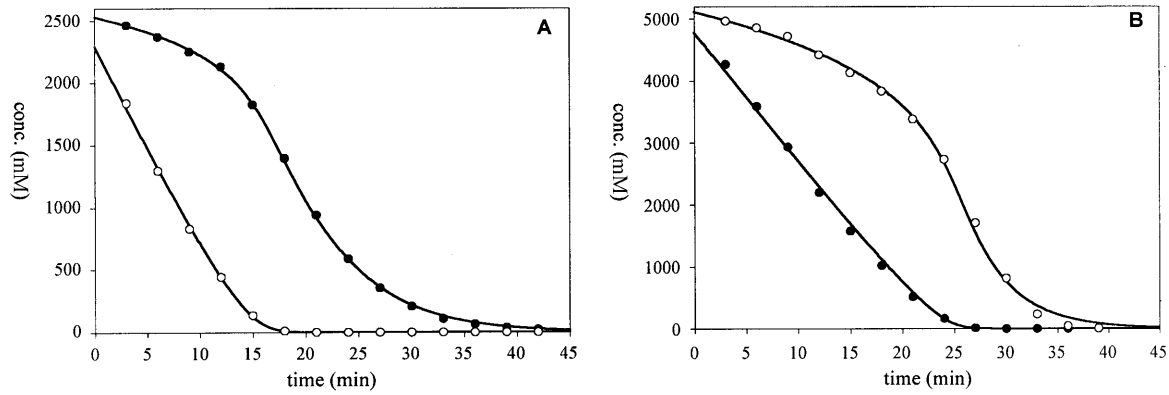


Figure 1. (A) Progress curve of the DhaA catalyzed kinetic resolution of methyl-3-bromo-2-methyl propionate (●: *S*-enantiomer, ○: *R*-enantiomer). (B) kinetic resolution of ethyl-3-bromo-2-ethyl propionate. Conditions: tris-sulfate buffer (50 mM, pH 8.2), room temperature

Table 2. Kinetic parameters of the conversion of methyl-3-bromo-methyl propionate by DhaA[a]

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
(*R*)-enantiomer	0.48	0.23	2.1
(*S*)-enantiomer	>17[b]	>30	0.42

[a] Calculated using $E = 5$.
[b] The exact kinetic parameters could not be determined due to the low solubility (35 mM) of the substrate.

In summary, we have shown the first examples of chiral recognition of haloalkane dehalogenases. The magnitude of the chiral recognition was low, but an *E*-value of 9 could be reached after some structural optimization of the substrate and indicated that the enantiodiscrimination is determined by very subtle steric factors. Remarkably, for a substrate with an *E*-value of only 5, differences of the K_m values of over a hundred were determined, which are largely compensated by opposing differences in k_{cat}.

Recently, we demonstrated that the enantioselectivity of an epoxide hydrolase from *Agrobacterium radiobacter* AD1, which has a similar reaction mechanism, could be improved by modifying the rate of the first reaction step.[8] These results make a similar or directed evolution approach towards improved enzyme variants an attractive one and activities in that direction are currently underway.[9]

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5. The rate of disappearance of the substrate enantiomers is monitored using a GC equipped with a chiral column (G-TA or B-TA, Astec).
6. The *E*-values were determined by using the extent of the conversion of the reaction and the e.e. of the substrate. In entries 7 and 11, *E*-values were also determined by using the e.e. of the substrate and the e.e. of the product. For a detailed analysis, see: Straathof, A. J. J.; Jongejan, J. A. *Enzyme Microb. Technol.* **1997**, *21*, 559.
7. For details of such an analysis, see: Lutje Spelberg, J. H.; Rink, R.; Kellogg, R. M.; Janssen, D. B. *Tetrahedron: Asymmetry* **1998**, *9*, 459
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