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Selectivity-enhancement in enantioselective hydrolysis of *sec*-alkyl sulfates by an alkylsulfatase from *Rhodococcus ruber* DSM 44541

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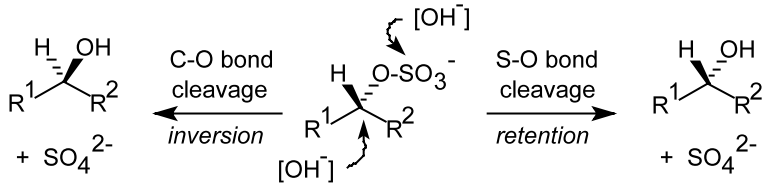
Abstract—The Enantioselectivity of the biohydrolysis of *sec*-alkyl sulfate esters using a bacterial alkylsulfatase from *Rhodococcus ruber* DSM 44541 was dramatically enhanced in presence of additives ('enhancers') such as carbohydrates, polyethylene glycol, detergents, metal ions and through enzyme immobilization. In presence of iron, the *E* value for the kinetic resolution of (±)-3- and (±)-4-octyl sulfate was improved from $E=3.9$ to ≥ 200 and $E=1.1$ to 10, respectively.

1. Introduction

In contrast to the majority of hydrolytic enzymes, such as esterases, lipases and proteases,[1] which do not affect any stereogenic center located within the substrate during catalysis, alkylsulfatases[2] [EC 3.1.6.X] are flexible concerning the stereochemistry during catalysis (Scheme 1). Depending on the nature of the enzyme and its mechanism of action, hydrolysis of a *sec*-alkyl sulfate ester may proceed either by cleavage of the S–O bond or the C–O bond, which results in *retention* or *inversion* of configuration at the stereogenic carbon.[3,4] This stereochemical flexibility makes them particularly attractive for the design of so-called deracemization processes, which allow the transformation of a racemate into a single stereoisomeric product in 100% theoretical yield.[5,6] Most recently, we have isolated an alkylsulfatase from *Rhodococcus ruber* DSM 44541 (termed RS2), which acts through absolute stereoinversion of the stereogenic *sec*-carbon atom of the substrate being attacked.[7] Thus, from a *rac*-*sec*-alkyl sulfate, (*R*)-enantiomers were preferentially hydrolyzed with *inversion* of configuration to furnish the corresponding (*S*)-*sec*-alcohol and remaining non-converted (*S*)-alkyl sulfate. As a result, a homochiral product mixture was obtained.[8]

The first enantioselective hydrolysis of *sec*-alkyl sulfates by an alkylsulfatase was discovered by the chance observation that several *rac*-alkyl sulfates were rapidly hydrolyzed until 50% conversion, whereas the remainder took place at a very much reduced reaction rate or not at all.[9,10] This observation—together with additional experiments performed with enantiopure substrates—led to the conclusion that alkylsulfatase CS1 from *Comamonas terrigena* (NCIMB 8193) and S2 from *Pseudomonas* C12B (NCIMB 11753 = ATCC 43648) exhibited a certain enantioselectivity for (*R*)-configured *sec*-alkyl sulfates.[10] An opposite enantiopreference was found for the CS2- and S1-enzyme from the above-



Scheme 1. Retention or inversion during enzymatic sulfate ester hydrolysis.

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mentioned strains. In spite of these biochemical data, which indicate the possibility of kinetic resolution, no data on the stereo- and enantioselectivities of alkylsulfatases were reported to date.[7,8]

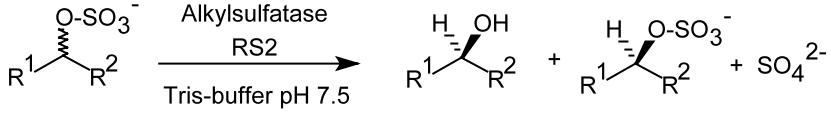
During our first study on the stereo- and enantioselective hydrolysis of *sec*-alkyl sulfates,[7] it was found that alkylsulfatase RS2 exhibited absolute stereoselectivity with respect to *inversion* of configuration during catalysis. In contrast, the corresponding enantioselectivities were less than satisfactory as they ranged from poor to acceptable and an E value of 21 for (±)-2-octyl sulfate was obtained at most. In order to broaden the applicability of this useful enzyme for the deracemization of various *sec*-alcohols through stereo- and enantioselective hydrolysis of their corresponding *rac*-sulfate esters, we studied the possibility to enhance enantioselectivities by addition of various components which are known to act as mediators for the chiral recognition process of enzymes.[11,12]

2. Results and discussion

It is a commonly observed phenomenon that the relative size of substituents attached to a stereogenic center dominates the spatial recognition process during asymmetric (bio)catalysis and thus it is the prime factor in chiral discrimination,[13] in particular for substrates possessing low polarity. This phenomenon was a suitable explanation for the fact that the enantioselectivity of enzyme RS2—expressed as the enantiomeric ratio (E value)[14]—was optimal for substrate *rac*-2-octyl sulfate (**1a**) ($E=21$) where the relative size of substituents R^1 and R^2 differs significantly (Scheme 2). In contrast, enantioselectivities markedly dropped when the stereogenic sulfate ester group was moved towards the center of the molecule, with R^1 and R^2 becoming similar in size to give 3- (**2a**, $E=3.9$) and 4-octyl sulfate (**3a**, $E=1.1$). This fact prompted us to focus our study on (±)-**2a** and (±)-**3a** which were transformed with insufficient enantioselectivities. Furthermore, whereas several methods for the preparation of nonracemic 2-octanol are available,[15] the synthesis of 3- and 4-octanol in high e.e. is difficult.[16]

2.1. Selectivity-enhancement of 3-octyl sulfate

rac-3-Octyl sulfate **2a** was resolved with low enantioselectivity in the absence of additives ($E=3.9$). The addition of carbohydrate derivatives and polyethylene glycol (PEG) showed some positive, but overall still insufficient effects on the enantioselectivity, with DEAE–dextran being best ($E=9.5$) (Table 1). These



rac-**1a**-**3a** (*S*)-**1b**-**3b** (*S*)-**1a**-**3a**

Compound	R^1	R^2
1a, **b**	*n*-C_6H_{13}	CH_3
2a, **b**	*n*-C_5H_{11}	C_2H_5
3a, **b**	*n*-C_4H_9	*n*-C_3H_7

Scheme 2. Stereo- and enantioselective hydrolysis of *sec*-alkyl sulfates by alkylsulfatase RS2.

Table 1. Enhancement of enantioselectivity for (±)-**2a** in presence of carbohydrates, PEG, enzyme immobilization and detergents (see Scheme 2).

Additive	Concentration (w/v)	Conversion (%)	E.e.$_p$ (%)	Enantioselectivity E
None[a]	–	41	48	3.9
Trehalose	5	38	50	4.0
PEG 6000	5	41	60	6.0
Dextran MW 41 kDa	5	34	64	6.3
DEAE–Dextran	5	33	74	9.5
DEAE–Sephadex A-25[b]	–	32	42	3.0[c]
TEAE–Cellulose[b]	–	26	66	6.1[c]
Ecteola–Cellulose[b]	–	26	86	17.8
Sodium dodecyl sulfate (SDS)	0.2	<1	–	–
1-Octanesulfonic acid	0.2	36	56	4.8
Triton X-100	2.0	36	60	5.5
Cetyl trimethylammonium bromide	0.2	25	90	25.5

[a] Under standard conditions using native enzyme RS2 in the absence of additive.
[b] Enzyme RS2 immobilized onto this carrier.
[c] Some of the enzyme activity was not bound.

additives are believed to exert their selectivity-enhancing effects through hydrogen bonding on the enzyme surface. Positive effects of PEG on the catalytic performance of lipases were reported recently.[12] Furthermore, DEAE–dextran combines the properties of a polysaccharide with those of an immobilization matrix due to its positively charged DEAE residues, which can bind proteins. It is assumed that this combination of properties makes it effective in comparison to 'normal' dextrans (MW 188 kDa) or DEAE–cellulose, both of which alone had no effect on the enantioselectivity.

Due to the fact that DEAE–dextran showed the most promising results, changes in enantioselectivity upon enzyme immobilization was investigated. Successful binding onto macroscopic materials was only feasible onto anionic carriers. In contrast, adsorptive binding onto various types of hydrophobic materials of Accurel-type, as well as covalent attachment though epoxy-groups (VA-Epoxy-Biosynth) or activated Sepharose (thiopropyl-activated Sepharose 6B and activated CH-Sepharose 6B) failed. Improved enantioselectivities were only detected when the enzyme was bound on Ecteola-Cellulose, a carrier possessing epichlorohydrin–triethanolamin moieties. In this case, the E value was close to preparative utility ($E=17.8$).

The most pronounced effects of detergents on the catalytic performance of enzymes were reported for lipases and the effects were largely attributed to interfacial activation.[17] Depending on the nature of the detergent, its interaction with the enzyme is predominantly based on ionic and/or hydrophobic parameters.[18] Recalling the detergent-like structure of alkyl sulfates, strong effects were anticipated. Most interestingly, substrate-like anionic detergents, such as sodium dodecyl sulfate and di-*n*-octylsulfosuccinate resulted in a complete loss of activity even at low concentration (0.2% w/v), whereas 1-octanesulfonic acid had no effect. Similar results were obtained with non-ionic detergents, such as Triton X-100 and Tween 80. In contrast, a remarkable improvement was obtained with a cationic detergent—cetyl trimethylammonium bromide—which resulted in an E value of preparative utility ($E>25$).

Due to the fact that the addition of EDTA did not abolish enzymatic activity,[19] we concluded that enzyme RS2 was not dependent on any metal ions as cofactors for its mechanism of action. This property and the fact that the substrate represents a negatively charged entity at neutral pH, led us to the assumption that metal ions might exert certain selectivity-enhancing effects (Table 2, Scheme 2). Several metals, such as Ca^{2+}, Li^{+}, Mg^{2+}, are known to improve activity and/or selectivity of lipases.[12,20] To our disappointment, a range of metals often tested for their effects on enzyme activity, such as Mg^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+} showed no effect within the limits of detectability. However, the use of iron had a dramatic effect, which was strongly concentration-dependent: Addition of increasing amounts of Fe^{2+} led to a decrease of reaction rate going in hand with an improvement of the E value (up to $E\sim80$). The same, but even more pronounced effect was observed with Fe^{3+}. In this case, virtually absolute enantioselectivity ($E\geq200$) was achieved at the price of a reduced reaction rate. The limits of this method are reached at elevated Fe^{3+}-concentration, which led to enzyme deactivation. Whereas the exact mechanism of action of iron on the alkylsulfatase remains unclear, any redox-phenomena due to spontaneous oxidation of $Fe^{2+}\rightarrow Fe^{3+}$ can be excluded since the addition of mercaptoethanol and dithiothreitol had no effect on activity and selectivity of the enzyme.[19] It appears clear, however, that the ionic strength of the metal ion seems to be an important factor.

2.2. Selectivity-enhancement of 4-octyl sulfate

Since both alkyl chains in *rac*-4-octyl sulfate differ only by a single methylene group, it is a quasi-symmetric substrate, which makes chiral recognition a very difficult task.[16d] As a consequence, no notable enantioselectivity was observed under standard conditions in the absence of enhancers ($E=1.1$). Since (poly)saccharides, PEG and detergents had little or limited effects on the enantioselectivity of substrate (±)-**2a**, they were excluded from further studies and our attention was focused on the effects of iron (Table 3, Scheme 2).

Table 2. Enhancement of enantioselectivity for (±)-**2a** in presence of metal ions (see Scheme 2)

Additive	Concentration (mM)	Conversion (%)	E.e.$_p$ (%)	Enantioselectivity E
None[a]	—	41	48	3.9
$MgCl_2$	10	41	40	3.0
$CaCl_2$	10	41	46	3.7
$MnCl_2$	10	39	38	2.8
$CoCl_2$	10	37	50	4.0
$FeCl_2$	2	37	66	7.1
$FeCl_2$	5	35	88	25
$FeCl_2$	10	18	97	81
$FeCl_3$	2	27	50	3.6
$FeCl_3$	5	9	99	≥200
$FeCl_3$	10	<1	–	–

[a] Under standard conditions using native enzyme RS2 in the absence of additive.

Table 3. Enhancement of enantioselectivity for (±)-**3a** in presence of iron (see Scheme 2)

Additive	Concentration (mM)	Conversion (%)	E.e.$_p$ (%)	Enantioselectivity E
None[a]	–	54	4	1.1
$FeCl_2$	2	50	14	1.5
$FeCl_2$	5	32	62	5.6
$FeCl_2$	10	17	80	10
$FeCl_3$	2	48	10	1.3
$FeCl_3$	5	19	78	9.7
$FeCl_3$	10	<1	—	—

[a] Under standard conditions using native enzyme RS2 in the absence of additive.

We were pleased to observe the same effects, i.e. a concentration-dependent enhancement of enantioselectivity in general, and a positive influence of the ionic strength of Fe^{2+} versus Fe^{3+}. Again, complete deactivation took place at elevated concentrations of Fe^{3+}. Overall, the addition of 10 mM Fe^{2+} improved the initial selectivity from nil to a remarkable value of $E=10$.

The use of various components, such as carbohydrates, PEG, detergents, and metal ions has been proven to be a largely empirical, but powerful method for the tuning of enzyme (enantio)selectivity for several types of biocatalyzed reactions. In general, allosteric effects denoted as 'enantioselective inhibition' have been quoted.[21] However, since their mode of interaction with the protein is still poorly understood on a molecular basis, rationales for the explanation of these effects are still highly speculative.

The data from this study allows the following points to be addressed: It has been proposed that the docking of the alkyl sulfate within the active site of the enzyme predominantly involves (strong) ionic binding via the (negatively charged) sulfate ester group, whereas chiral discrimination occurs by (weak) Van der Waal's interactions involving the positioning of the alkyl groups into two hydrophobic pockets. As a consequence, chiral recognition largely depends on discrimination by size.[10,22] It is therefore unsurprising that (among several types of additives) metal ions had the most pronounced effect on the enantioselectivity, because they are most likely to interfere with the ionic substrate binding through formation of ion pairs with the charged substrate.

3. Experimental

3.1. General remarks

All GC analyses were carried out on a HP 6890 gas chromatograph, equipped with FID and a HP-1 capillary column (30m×0.53 mm×0.5 μm, N_2). Enantiomeric purities were analyzed on a chiral column Chrompack CP7500 (25 m×0.25 mm×25 μm) using H_2 as carrier gas.

Alcohols were purchased from Aldrich (**1b**) and Fluka (**2b**,**3b**); sulfation of *rac*-**1b**–**3b** was completed as described in the preceding paper (±)-**1a**–**3a**. Additives were obtained from Sigma (immobilization carriers, detergents), Merck and Fluka (salts). For the determination of conversion, e.e.$_p$ as well as the elucidation of absolute configuration of products see the experimental section of the preceding paper.

3.2. Biocatalytic procedures

Rhodococcus ruber DSM 44541 was grown under aerobic conditions in baffled Erlenmeyer flasks at 30°C and 130 rpm using the following medium: 10 g/l glucose, 10 g/l peptone, 10 g/l yeast extract, 2 g/l NaCl, 1.5 g $MgSO_4 \cdot 7\ H_2O$, 1.3 g/l NaH_2PO_4 and K_2HPO_4. Cell growth was monitored by measurement of the optical density via the absorption at 546 nm. Biotransformations were carried out using a partially purified enzyme.

A crude enzyme preparation was obtained as follows: Cells of *R. ruber* DSM 44541 from the late exponential growth phase were disintegrated using a bead mill (Vibrogen Zellmühle, bead diameter 0.35 mm). After centrifugation of cell debris (38 000×*g*, 2 h), the supernatant was subjected to hydrophobic chromatography using a Phenyl Sepharose column (Pharmacia). Active fractions were collected, dialyzed against 10 mM Tris/HCl buffer pH 7.5 and finally lyophilized. The activity of the crude enzyme preparation was 1.2 U/ml; 1 U of sulfatase liberates 1 μmol SO_4^{2-} min^{-1}.

Immobilization of alkylsulfatase: An aliquot of partially purified alkylsulfatase preparation (1 ml, 11 mg protein/ml) in Tris/HCl buffer (0.1 M, pH 7.5) was added to the immobilization carrier (100 mg), which was pre-washed with the same buffer. The mixture was gently mixed for 5 min and allowed to stand for another 60 min at 4°C. After centrifugation and separation of the supernatant, the carrier was repeatedly washed with the same buffer and tested for alkylsulfatase activity. The relative activities in relation to the initial activity (set to 100%) were as follows: TEAE–Cellulose, ~100% bound and <1% nonbound activity; Ecteola–cellulose, 22% bound and 34% nonbound activity.

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