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Enantioselective hydrolysis of epoxides: the employment of the soluble fraction from *Vicia sativa* seedlings

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Abstract

Biocatalytic hydrolysis of meso and racemic aryl- and alkyl-oxiranes was accomplished by employing the epoxide hydrolase activity of the soluble fraction of *Vicia sativa* seedlings. Whereas meso epoxides were not hydrolyzed by this fraction, racemic compounds were transformed into the corresponding diols by formal anti-stereoselective water attack. Both substrate and product enantioselectivity were strongly influenced by the chains length and the presence of a hydroxyl group. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Epoxide hydrolase; Plant; Asymmetric hydrolysis

1. Introduction

Enantiomerically pure epoxides and chiral diols are important building blocks in organic synthesis and can be used as key intermediates in the synthesis of more complex enantiopure compounds. Several chemical and biological production methods have been reported [1–3]. Among several types of enzymes employed, epoxide hydrolases (EHs) are particularly attractive being very efficient biocatalysts for achieving the resolution of racemic epoxides. Chiral epoxides and vicinal diols can be obtained by formal water addition onto the epoxide ring with good to excellent enantiomeric purity. Since these enzymes are essentially involved in human detoxification processes, EHs of mammalians origin have been mostly studied up to the recent years [4–6]. In the last years, however, EH activities have been found in organism as diverse as bacteria [7], fungi [8], yeasts [9], insects [10], and plants [11] showing that these enzymes are probably ubiquitous in nature. The possibility to have EHs from different sources, which may be characterized by a different substrate and/or product enantioselectivity have much increased the interest in this kind of biocatalyst. Thus, the study of the properties of the different EHs, i.e. their molecular mechanism, catalytic behavior, substrate and product stereoselectivity is now a topic of relevant importance. EHs exist predominantly in two different forms: a microsomal (mEH) and a cytosolic or soluble (sEH). Both forms hydrolyze a large number of different substrates, in agreement with the detoxifying function. A large number of studies have been carried to characterize these enzymes in mammals, but only few data have been reported on plant EHs. A soluble EH from soybean has been purified and characterized using 9,10-epoxystearic acid as a substrate [12], more recently, soluble EHs were cloned from potato (*Solanum tuberosus*) [13] and *Arabidopsis thaliana* [14] and epoxide hydrolase activity has been found in the microsomal and soluble fraction from *Vicia sativa* seedlings using

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once again 9,10-epoxystearic acid as a substrate [11].

In this paper, we report our results about the stereochemical behavior of the hydrolysis of a number of racemic and meso epoxides by the soluble fraction from *Vicia sativa* seedlings showing that soluble plant EHs may be efficient biocatalysts for the enzymatic resolution of some racemic epoxides.

2. Experimental

2.1. Materials

(±)-1,2-Epoxyhexane (**1a**), (±)-3,3-dimethyl-1,2-epoxybutane (**1b**), (±)-1,2-hexanediol (**5a**), (±)-3,3-dimethyl-1,2-butanediol (**5b**) were commercial products and were used without further purification. (±)-2-methyl-1,2-epoxyhexane (**2**), *cis*-(±)-2,3-epoxyheptane (**3a**), *cis*-(±)-3,4-epoxyheptane (**3b**), *cis*-(±)-3,4-epoxynonan-1-ol (**3c**), *cis*-(±)-11,12-epoxyhexadecan-1-ol (**3d**), *cis*-(±)-5,6-epoxyhexadecane (**3e**), *cis*-9,10-epoxyoctadecane (**3f**), *cis*-stilbene oxide (**3g**) and cyclopentane oxide (**4**) were prepared as previously reported [5]. (±)-2-Methylhexane-1,2-diol (**6**), *threo*-(±)-heptane-2,3-diol (**7a**), *threo*-(±)-heptane-3,4-diol (**7b**), *threo*-(±)-nonane-1,3,4-triol (**7c**), *threo*-(±)-decane-1,11,12-triol (**7d**), *threo*-(±)-hexadecane-5,6-diol (**7e**), *threo*-(±)-octadecane-9,10-diol (**7f**), *threo*-(±)-1,2-diphenylethanediol (**7g**) and *trans*-cyclopentanediol (**8**) were prepared by acid catalyzed hydrolysis ($HClO_4$) of the corresponding epoxides, as previously reported [5].

2.2. Product analysis

Optical rotations were measured with a Perkin-Elmer 241 polarimeter. The ^1H and ^{13}C NMR spectra were registered in $CDCl_3$ with a Bruker AC 200 instrument using TMS as the internal reference. The e.e. of the unreacted epoxides (**1–4**) and of the formed diols (**5–8**), were determined by GLC analysis using a Carlo Erba HRGC 5300 instrument equipped with a 20 m Chiraldex G-TA (ASTEC) column, evaporator and detector 245°C, helium flow 1 ml/min, at the following temperatures: (**1a**) 40°C; (**1b**) 65°C; (**2**) 65°C; (**3a**) 70°C; (**3b**) 80°C; (**3c**) 110°C; (**5a**) 80°C for 15 min at 5°C/min and 125°C for 10 min; (**5b**) 40°C for 12 min at 5°C/min and 80°C for 15 min; (**6**) 65°C for 12 min at 5°C/min and 100°C for 20 min; (**7a**) as trifluoroacetyl derivative, 70°C; (**7b**) 80°C; (**7c**) as trifluoroacetyl derivative, 120°C. The yields were determined by GLC analysis, under the same conditions used for the determination of the e.e., by adding cyclohexanol as an internal standard. The e.e. of diol (**7d**) was determined by HPLC, after transformation into the MTPA ester, under the following conditions; nitrile S5 column, using hexane/2-propanol (99.8:0.2) as the eluent.

2.3. Preparation of plant subcellular fractions

Vicia sativa seedlings, obtained by direct sowing and allowed to grow for 4 days, were homogenized in three volumes of 50 mM Tris–HCl buffer (pH 7.4) containing KCl (1.15% w/v) and the resulting suspension was centrifuged at 10 000 × *g* for 30 min. The supernatant was further centrifuged at 30 000 × *g* for 1.5 h, leading to the soluble and microsomal fractions. Both fractions were used directly or stored at −80°C. Protein concentration of the soluble fraction was estimated using the method of Lowry [15].

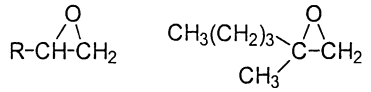
2.4. Enzymatic biotransformation

The enzymatic incubations were performed by adding to 5–10 ml of soluble fraction, containing 3-4 mg of protein/ml, solutions of epoxides (**1–4**) in ethanol (4 M, 25–50 μl) to a final epoxide amount of 20 μmol/ml. The mixtures were incubated with shaking at 27°C for the time necessary to obtain the appropriate conversion (24–48 h), then stopped by extraction with ethyl acetate (3 × 10 ml). Generally, the organic phases were diluted to an exactly known volume (50 ml) by dilution with ethyl acetate and a proper amount of the standard was added to an aliquot (5 ml) of these extracts in order to verify the conversion and the e.e. of the residual epoxides by GLC. The remaining part of the organic phases were evaporated in vacuo and the residues were analyzed by NMR, then derivatized and analyzed by GLC or HPLC to determine the e.e. of the formed diols. In the case of epoxides (**3d–g**) the conversions were determined by NMR on the basis of the ^1H NMR signals of epoxides and corresponding diols. Experiments carried out with epoxides (**1–4**) under identical

conditions, but using either a preparation deactivated by boiling, or Tris–HCl buffer alone, showed that no relevant nonenzymatic hydrolysis occured under the incubation conditions.

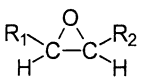
3. Results and discussion

The racemic and meso epoxides (**1–4**) were submitted to biohydrolysis with a crude *Vicia sativa* seedlings soluble fraction.



1
a: R = $(CH_2)_3CH_3$
b: R = $C(CH_3)_3$

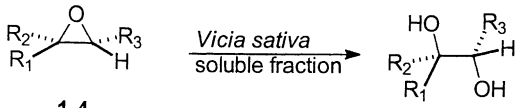
2



3
a: $R_1 = CH_3$, $R_2 = (CH_2)_3CH_3$
b: $R_1 = CH_2CH_3$, $R_2 = (CH_2)_2CH_3$
c: $R_1 = CH_2CH_2OH$, $R_2 = (CH_2)_4CH_3$
d: $R_1 = (CH_2)_9CH_2OH$, $R_2 = (CH_2)_3CH_3$
e: $R_1 = (CH_2)_9CH_3$, $R_2 = (CH_2)_3CH_3$
f: $R_1 = R_2 = (CH_2)_7CH_3$
g: $R_1 = R_2 = Ph$

4

Incubations were carried out at 27°C, pH 7.4 using a preparations containing 3–4 mg of protein/ml and a substrate amount of 20 μmol/ml. The reactions were stopped by extraction with ethyl acetate. Generally, the organic phases were analyzed by GLC, after addition of an approppriate standard, to determine the amount and the e.e. of the residual epoxides, and after derivatization to determine the e.e. of the formed diols. In the case of epoxides (**3d–g**) the conversions were determined by NMR on the basis of the ^1H NMR signals of epoxides and corresponding diols. The e.e. of diol (**7d**) was established after transformation into the corresponding tris(MTPA) ester by HPLC.



1-4 **5-8**

Due to the moderate hydrolysis rate, in order to achieve an appropriate biotransformation of the epoxides, the incubations were protracted for 24–48 h. The results are reported in Table 1, which also includes the absolute configurations of the residual epoxides and of the formed diols, which were determined by comparison of their GLC or HPLC retention times with those of optically active samples [5,16–19]. The data reported in Table 1 may be used to determine the *E*-value related to these reactions. However, the conversions determined on the extracts by GLC have not the accuracy which should be necessary to apply the related equations. Furthermore, we have not determined the regioselectivities implied in these reactions, therefore, the *E*-values have not been included in Table 1.

The substrate and product enantioselectivity is highly dependent on the substituents at the oxirane ring. Monosubstituted oxiranes (**1a**, **b**) are hydrolyzed to the corresponding diols (**5a**, **b**) with a product and substrate enantioselectivity highly dependent on substituents. The *n*-alkyl group (*n*-butyl, **1a**) cause reactions showing very low selectivity, while the branched *tert*-butyl groups (**1b**) induced a high substrate enantioselection in favor of the (R) enantiomer. Furthermore, the absolute configuration and the e.e. of the formed diols and residue epoxides were in agreement with a regioselective attack of the nucleophile on the less hindered oxirane carbon. Both these results show a striking analogy with those found for the rabbit liver mEH and sEH catalyzed hydrolysis of the same racemic substrates [16,20]. In agreement with the stereochemical behavior observed using EHs arising from other sources [17,21], the introduction of a methyl group on the oxirane carbon bearing the alkyl substituent, epoxide (**2**), determines an inversion of the substrate and product enantioselection favoring the formation of the (S) diol. Also in this case, however, the absolute configuration of the starting epoxide and formed diol and the relative e.e. are consistent with a regioselective oxirane ring opening on the less substituted carbon.

The hydrolysis of the *cis*-disubstituted epoxides (**3a–g**) shows a strongly dependent substituent stereochemical behavior, too. It is noteworthy that at variance with mammalian EHs [22,23] the meso epoxides (**3f**, **g**) and (**4**) are not substrate for this enzyme,

Table 1
Enantiomeric excesses and absolute configurations of residual epoxides and formed diols obtained by partial hydrolysis of epoxides (**1–4**) using the soluble fraction of *Vicia sativa* seedlings[a]

Substrate	Incubation time (h)	Hydrolysis (%)	Unreacted epoxide		Formed diol	
			e.e.[b]	Absolute configurations	e.e.[b]	Absolute configurations
1a	15	48	20	S	26	R
	48	100			0	
1b	48	10	8	S	>98	R
2	48	25	8	R	30	S
3a	24	40	30	(2S,3R)	38	(2R,3R)
3b	24	30	N.D.	N.D.	80	(3R,4R)
	40	50	N.D.	N.D.	80	(3R,4R)
3c	24	20	8	(3S,4R)	20	(3R,4R)
3d	24	100	–		90	(11R,12R)
3e	48	0				
3f	48	0				
3g	48	0				
4	48	0				

[a] N.D.: not determined. Reactions carried using a substrate amount of 20 μmol/ml with soluble preparations containing 3–4 mg of protein/ml.
[b] Average error of three determinations: ±2%.

independently of the aromatic or aliphatic (cyclic or not) nature of the substituents. Racemic *cis*-dialkyl substituted oxiranes give instead selectively the corresponding *threo*-diols by formal water anti-addition. The product enantioselectivity is generally in favor of the (R,R) diol (**7**) and the enantiomeric purity depends on the nature and relative size of the two substituents. The hydrolysis of epoxide (**3a**) proceeds with a moderate enantioselection while a good product enantioselection has been observed in the oxirane ring opening of the isomer epoxide (**3b**). An increase in the product enantioselectivity on going from (**3a**) to (**3b**) was observed also in the hydrolysis of the same substrates using the rabbit liver soluble fraction [24]. However, at variance with the mammalian sEH catalyzed hydrolysis the reaction of epoxide (**3c**) bearing a hydroxyl group on the ethyl substituent, with *Vicia sativa* soluble fraction shows only a moderate product enantioselection. Finally, it must be remarked that the introduction of an hydroxyl group on the longer alkyl chain (compare hydrolyses of epoxides **3d**, **e**) transforms practically a non-substrate for the hydrolytic enzyme of the soluble fraction of *Vicia sativa* seedlings in a substrate able to be hydrolyzed with a high product enantioselection and in a stereoconvergent way, in agreement with a stereoselective attack at the S carbons of each enantiomer. Thus, the presence of a hydroxyl group affects the stereoselectivy of the reaction, but this effect probably depends on the position of the hydroxyl group with respect to the oxirane ring (compare hydrolyses of epoxides **3c**, **d**).

In conclusion, although more data are desirable, these results show that plants EHs are able to hydrolyze epoxides different from 9,10-epoxystearic acid and derivatives, in agreement with a possible involvement of these enzymes in plants detoxifying reactions. Furthermore, the stereochemical behavior observed in the hydrolysis of mono- and disubstituted oxiranes, and the comparison with the behavior observed using other EH sources, indicates that, although the hydrolysis catalyzed by different EHs present similar features, the substrate and product enantioselection for a determined epoxide may be different using different EH sources. The possibility to have more EHs, arising from different sources, is therefore important in order to increase the possibility of synthetic application of these biocatalysts.

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