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Biohydrolysis of substituted styrene oxides by *Beauveria densa* CMC 3240

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Abstract

Resting whole cell suspensions of the fungus *Beauveria densa* CMC 3240 containing an epoxide hydrolase have been used to resolve a series of *para*-substituted styrene oxides, with stereoinversion of the hydrolysed epoxide enantiomer. The regio- and hence, enantioselectivity of hydrolysis is compromised where *ortho*- and *para*-methyl- and chlorostyrene oxides are substrates. Negligible activity was observed with *para*-nitrostyrene oxide as substrate. The results appear to confirm a general mechanism of enzyme-catalysed acid hydrolysis for *Beauveria* spp. acting on styrene oxides.

Keywords: Epoxide hydrolase; Chiral epoxide ; Chiral diol ; *Beauveria* spp.

1. Introduction

Microbial epoxide hydrolases (EHs) from both bacteria and fungi, for a recent review see [1], are assuming increasing potential as valuable catalysts for the production of natural products via chiral epoxide or chiral diol intermediates [2–4]. Whilst bacterial epoxide hydrolases are now isolable from many sources [5–7], literature on the fungal equivalents is still rare and indeed to our knowledge no isolation of a fungal EH has been reported, although these activities appear to be well distributed, especially among dematiaceous fungi [8–10]. The fungal hydrolysis of styrene oxide derivatives has proved especially interesting, in that two distinct mechanisms have been described whereby either retention or inversion of the stereochemistry of the hydrolysed enantiomer is observed depending on the fungus employed [11]. Chiral styrene oxides are, of course, valuable synthons leading to the production of many bioactive compounds such as fluoxetine [12]. Whole cell preparations of *Aspergillus niger* LCP 521 yielded (*S*)-epoxide and (*R*)-glycol, whereas hydrolyses by suspensions of *Beauveria sulfurescens* ATCC 7159 resulted in (*R*)-epoxide and (*R*)-glycol. A screen of an industrial collection revealed that another fungus of the latter genus, *Beauveria densa* CMC 3240, also possessed considerable activity toward these aromatic epoxides, with the same enantioselectivity for unsubstituted styrene oxide as reported for *Beauveria sulfurescens* ATCC 7159. In this pa-

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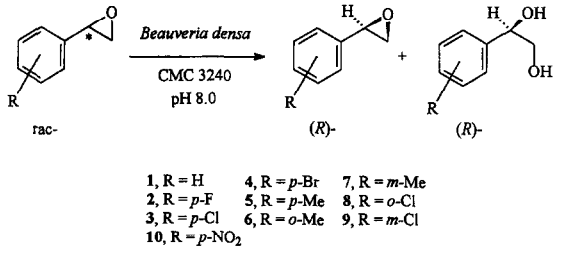

Fig. 1. Biohydrolysis of substituted styrene oxides by *Beauveria densa* CMC 3240.

per the substrate specificity and enantioselectivity of this enzyme expressed in resting cells are studied with a series of styrene oxides substituted in the aromatic ring (Fig. 1).

2. Experimental

2.1. Maintenance and growth of microorganisms

Beauveria densa CMC 3240 was obtained from Chiroscience, Cambridge, UK. Mycelia were maintained on malt extract agar slopes at 25°C. A loopful of fungus was used to inoculate 60 ml medium containing 10 g l^{-1} glucose and 7.5 g l^{-1} corn steep liquor in a 250 ml Erlenmeyer flask. This was grown for 3 d at 200 rpm in an orbital shaker at 25°C. The mycelium was then transferred to 600 ml of the same medium in a 2 l Erlenmeyer flask which was grown under the same conditions for 2 d. The fungus was harvested by centrifugation at 4000 rpm for 20 min after which it was washed with an equal volume of 50 mM phosphate buffer pH 8.0 and then harvested by Buchner filtration to obtain a 'dry' cake.

2.2. Chemicals

Styrene oxides **2–9** were prepared by oxidation of the corresponding commercially available styrenes by oxidation with *meta*-chloroperbenzoic acid according to the method of Imuta and Ziffer [13]. *Para*-nitrostyrene oxide was synthesised according to the method of Westkaemper and Hanzlik [14] All glycols and acetonides thereof were prepared according to the method of Pedragosa-Moreau et al. [15]. All other reagents and chemicals were purchased from Aldrich Chemical Company, Poole, Dorset, UK.

2.3. Epoxide hydrolase assays

Epoxide hydrolase activity was assayed by extracting 500 μl reaction mixture into 500 μl diethyl ether with subsequent analysis on a capillary BP 10 GC column. Hexadecane was employed as internal standard. For comparative epoxide hydrolysis assays, mycelium from one growth flask was evenly suspended at a concentration of 50 mg ml^{-1}, divided into aliquots and challenged with 5 mM of the appropriate substrate in ethanol to 1% final reaction volume.

2.4. Preparative whole cell biotransformations

To 500 ml 50 mM phosphate buffer pH 8.0 in a 2 l Erlenmeyer flask was added 10 g cake of *Beauveria densa* CMC 3240. This was agitated at 200 rpm on a rotary shaker for 1 h to ensure equilibration and even dispersal of mycelium. Substrate was then added to the mixture at a concentration of 15 mM in 1% final reaction volume of ethanol (apart from **10**, *para*-nitrostyrene oxide, see Section 3). Reactions were monitored as above. At the appropriate time the mycelium was removed by Buchner filtration and the liquor extracted with 3×150 ml diethyl ether. The combined organic fractions were dried over anhydrous magnesium sulphate and after filtration, the solvent was removed in vacuo. Residual epoxides and product diols were purified by flash silica chromatography using petroleum ether/diethyl ether as solvent.

2.5. Determination of enantiomeric excesses

Enantiomeric excesses of epoxides and diols were determined using data from capillary chi-

ral GC chromatograms on either Lipodex E or Chirasil-Dex CB columns. Diols were derivatised to the corresponding acetonides prior to injection. It is important to note that 'absolute configurations' have only been assigned tentatively according to elution order with respect to standards of enantiopure (*R*)-styrene oxide and the acetonide of enantiopure (*R*)-styrene glycol.

3. Results and discussion

As the expression of fungal epoxide hydrolase activity has been shown to vary markedly throughout the growth curve of some fungi [8], it was considered important to determine the stage of optimal epoxide hydrolase titre for harvesting mycelia for biotransformation purposes. Epoxide hydrolase activity is apparently expressed without induction throughout the growth of the organism on glucose/corn steep liquor (Fig. 2). The expression of activity prior to stationary phase is in sharp contrast to the epoxide hydrolase activity expressed by dematiaceous hyphomycetes. Although maximum specific activity is observed at the onset of stationary phase, activity is still present after eight days growth. Routinely, mycelia was harvested after 2 d growth in 600 ml culture.

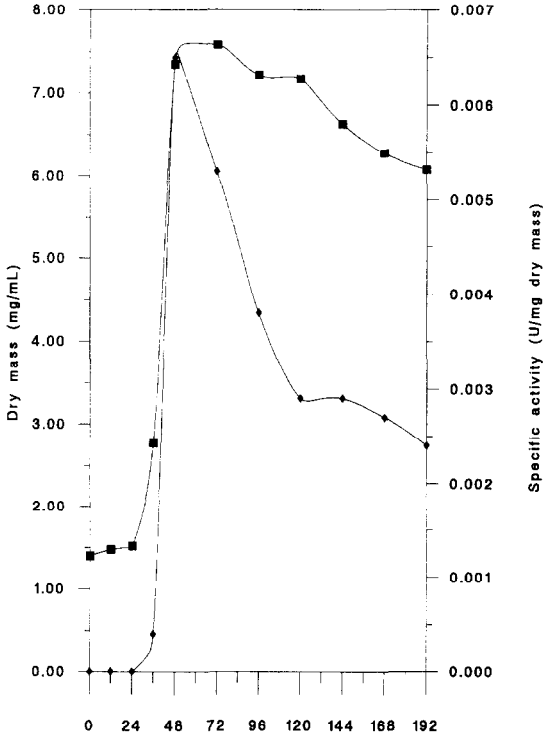

Fig. 2. Growth/EH activity profile for *Beauveria densa* CMC 3240. Biomass from 60 ml culture was harvested at intervals for dry mass (■) and specific activity (◆) determinations. Cells were assayed for EH activity using 10 mM *para*-chlorostyrene as substrate.

Table 1
Comparative specific activities of identical amounts of homogeneous culture of *Beauveria densa* CMC 3240 toward substituted styrene oxides

Substrate	Specific activity (U/mg $\times 10^{-3}$)
1	0.12
2	0.54
3	0.19
4	0.08
5	0.41
6	0.03
7	0.07
8	0.01
9	0.00

Using identical amounts of mycelium from the same culture, the relative activity of the *Beauveria* epoxide hydrolase with respect to a series of aromatic epoxides was studied (Table 1). Although the kinetics of styrene oxide metabolism by *Beauveria sulfurescens* ATCC 7159, a related fungus, have proved difficult to characterise [16], these results offer an empirical indication of the effect of ring substitution on hydrolytic activity by *Beauveria densa* CMC 3240. The rate of enzymatic hydrolysis of the *para*-halogenated styrene oxides, taking into account the rate of background reaction, was observed to be fastest for those bearing more electronegative substituents i.e. *p*-F > *p*-Cl > *p*-Br. The rate of hydrolysis of *para*-methylstyrene oxide was also rapid, even when corrected for the considerable amount of background hydrolysis observed for this substrate. Reaction rate was greatly affected by substituent position, decreasing in the order *para* > *meta* > *ortho* for both chlorinated and methylated series of substrates.

Table 2
Enantioselectivity of biohydrolyses of substituted styrene oxides by *Beauveria densa* CMC 3240

Substrate	Time (h)	Yield *E* (%)	ee *E* (%)	Yield *D* (%)	ee *D* (%)
1	2–2.5	18	> 95 (*R*)	34	78 (*R*)-
2	2	18	> 95 '*R*'	36	77'*R*'
3	4	17	> 95 '*R*'	36	82 '*R*'
4	5	19	> 95 '*R*'	41	87 '*R*'
5	0.5–1	21	> 95 '*R*'	48	61 '*R*'
6	20	24	12 '*R*'	36	5 '*R*'
7	20	19	50 '*R*'	44	67 '*R*'
8	20	77	0	0	0
9	20	60	0	10	21 '*R*'

These results are embellished by the observed enantioselectivities of these reactions (Table 2). The hydrolysis of unsubstituted styrene oxide is accomplished with similar enantioselectivity to that observed with *Beauveria sulfurescens* ATCC 7159. This suggests an epoxide hydrolase activity unique and distinct to this genus. When the substrate range is broadened to accommodate *para*-halo- and *para*-methyl styrene oxide, this enantioselectivity is conserved, resulting in resolutions of the '*R*' enantiomers of these epoxides. Using $H_2{}^{18}O$ incorporation studies, stereoinversion of hydrolysed styrene oxide enantiomers has been proven to occur both in hydronium ion mediated solvolysis via a benzylic carbocation after epoxide protonation or in 'spontaneous' chemical hydrolysis with nucleophilic attack of water in a concerted mechanism [17]. Such stereoinversion has also been proven in the hydrolysis of styrene oxide by *Beauveria sulfurescens* ATCC 7159 [16]. That the corrected rate of *para*-methylstyrene oxide hydrolysis by *Beauveria densa* CMC 3240 is greater than the other epoxides may suggest extra stabilisation of an acid-mechanism induced carbocation and point to an enzyme-catalysed hydronium-ion dependent hydrolysis. This would also appear to be confirmed by the failure of this fungus to hydrolyse *para*-nitrostyrene oxide even at low concentrations (2–5 mM) aided by higher concentrations of ethanol necessary for even partial substrate solubilisation. The stability of the acid generated carbocation would be greatly reduced by the presence of this electron withdrawing substituent. This is in contrast to the state of affairs observed with the epoxide hydrolase from *Aspergillus niger* LCP 521, which has been shown to open styrene oxide at the terminal carbon and hence accepts *para*-nitrostyrene oxide readily [18], electron withdrawing groups having been shown to promote hydroxyl attack in a basic environment at this site [17].

Benzylic carbocation stability should not be greatly affected when the methyl substituent is *meta*-substituted. However, hydrolysis of **7** is much slower than that of **5** by *B. densa*, with reduced enantioselectivity, presumably because of reduced regioselectivity. We must implicate other factors in this phenomenon, therefore, which may include restricted access of nucleophilic hydroxyl ion to the benzylic carbocation. This effect is indeed more pronounced for *ortho*-methylstyrene oxide **6**, which is a very poor substrate for both steric and electronic factors (the stabilisation effect is removed as the arrangements are no longer planar). *Ortho*- and *meta*-substituted chlorostyrene oxides **8** and **9** are poorer substrates for the fungus than their methylated counterparts. This may be due to the combined effects of greater steric hindrance and even less residual benzylic carbocation stabilisation than may occur with **6** and **7**. Indeed, negligible hydrolysis of **9** was recorded.

The hydrolysis of *meta*-chlorostyrene oxide is apparent however but the residual epoxide is racemic, whereas the diol produced exhibits a small enantiomeric excess of 20% '*R*'. This asymmetrisation may suggest a mixed benzylic/terminal carbon attack mechanism whereby both '*R*' and '*S*' enantiomers are converted predominantly to the '*R*' diol in an enantioconvergent process. This may be a less pronounced example of the same phenomenon observed when *cis*-1-methyl, 2-phenylethylene oxide is hydrolysed by *Beauveria sulfurescens* ATCC 7159 [15], although mechanistically, this result is difficult to rationalise. Conclusions re-

garding the effects of different phenyl substituents on hydrolyses by *B. densa* would be greatly aided by $H_2{}^{18}O$ incorporation experiments which have proved useful in delineating the precise regiochemistry of microbial epoxide hydrolase reactions previously [16,19].

The unique enantioselectivity of epoxide hydrolases expressed in *Beauveria* spp. may be due to the cellular location of the enzyme. Whereas highly active soluble epoxide hydrolase activities are readily obtained from *Ulocladium*, *Zopfiella* and *Aspergillus* spp., all apparently catalysing terminal carbon attack when challenged with terminal oxiranes, it has proved very difficult to isolate a soluble cell fraction of the corresponding enzyme from *Beauveria densa* CMC 3240. It may be that this activity is closely associated with the membrane bound hydroxylating systems found in both *Beauveria densa* CMC 3240 [20] and *Beauveria sulfurescens* [21]. Alternatively, the activities may be isolated in discrete acidic environments in membrane vesicles, which may help to explain their distinct mechanism. Research is currently underway in our laboratory to investigate these possibilities, and to incorporate epoxide hydrolase methodologies into chemoenzymatic syntheses.

Acknowledgements

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