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ORIGINAL PAPER

Production of epoxide hydrolases in batch fermentations of *Botryosphaeria rhodina*

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Abstract The filamentous fungus *Botryosphaeria rhodina* (ATCC 9055) was investigated related to its ability for epoxide hydrolase (EH) production. Epoxide hydrolase activity is located at two different sites of the cells. The larger part is present in the cytosol (70%), while the smaller part is associated to membranes (30%). In media optimization experiments, an activity of 3.5 U/g_{DW} for aromatic epoxide hydrolysis of *para*-nitro-styrene oxide (*p*NSO) could be obtained. Activity increased by 30% when *p*NSO was added to the culture during exponential growth. An increase of enzyme activity up to 6 U/g_{DW} was achieved during batch-fermentations in a bioreactor with 2.7 l working volume. Evaluation of fermentations with 30 l working volume revealed a relation of oxygen uptake rate to EH expression. Oxygen limitation resulted in a decreased EH activity. Parameter estimation by the linearization method of Hanes yielded K_m values of 2.54 and 1.00 mM for the substrates S-*p*NSO and R-*p*NSO, respectively. v_{max} was 3.4 times higher when using R-*p*NSO. A protein purification strategy leading to a 47-fold increase in specific activity (940 $U/mg_{Protein}$) was developed as a first step to investigate molecular and structural characteristics of the EH.

Keywords Epoxide hydrolase · *Botryosphaeria rhodina* · Filamentous fungi · Protein purification

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Introduction

More than 100 identified or predicted epoxide hydrolases (EHs, EC 3.3.2.3) have been grouped into two major superfamilies: the microsomal EH superfamily and the cytosolic EH superfamily [6]. EHs have recently found widespread interest due to their ease of use as enzymes for syntheses without cofactor requirements. They convert epoxides into the corresponding vicinal 1,2-diols by addition of a water molecule [5, 10, 35]. In cells, EHs can play important roles such as detoxification agents for epoxide derivatives, for the biosynthesis of signal molecules and the utilization of epoxides as carbon source [2, 37]. EHs are ubiquitous in nature and have been found in bacteria [15, 16], yeasts [17, 40], filamentous fungi [21, 27], plants [1, 22], insects [33, 41], mammals [3] and human tissues [11, 18].

Besides their medical and physiological relevance [8], the utilization of these enzymes for biocatalytic production of pharmaceutical compounds has gained special interest. The preparation of enantiopure epoxides and diols by enzymatic hydrolytic resolution of racemic epoxides is a promising synthesis strategy for chemicals and pharmaceuticals. Although bacterial EHs from *Rhodococcus rhodochrous* as well as *Agrobacterium radiobacter* and fungal EHs from *Aspergillus niger* are already commercially available as versatile biocatalysts, the extension to EHs from fungi and yeast is desirable. These EHs are able

to use a wide range of substrates with high enantio- and regio-selectivity [4, 34, 38, 39].

In this report, EH production in batch fermentations of the filamentous fungus *Botryosphaeria rhodina* (synonym: *Diplodia gossypina*) is described. *Botryosphaeria rhodina* commonly appears as endophyte and pathogen on plants [28]. Research interest in this fungus was initiated when its ability to produce exopolysaccharides (EPS) was discovered [9, 31, 32]. EH from *B. rhodina* can, for example, be used for biohydrolytic formation of (1S, 2R)-indene oxide, a useful precursor for the important HIV-protease inhibitor indinavir [7, 25].

In this work, the expression of EH activity in *B. rhodina* is characterized under different culture conditions and the main influencing parameters are determined. Analytical and preparative methods for the enzyme purification steps are described. Kinetic parameters and enantioselectivity are evaluated and compared to EHs expressed in other genera.

Material and Methods

Microorganism

The fungus *B. rhodina* (ATCC 9055) was obtained from the German National Resource Centre for Biological Material (DSMZ GmbH, Braunschweig, Germany). Spores from the fungus were stored on agar plates containing fine oat flakes (30 g/l water) and 1.5% (w/v) agar at 35 °C for 7 days. After formation of thin white mycelium and black spores, plates were stored at 4 °C.

Media optimization

For media optimization experiments, growth of *B. rhodina* was observed in shake flask experiments with both complex and minimal media. All the tested media are listed in Table 1. After 5 days of shaking at 30 °C, cultures were tested for biomass formation and EH activity. If not otherwise stated, chemicals were purchased from Sigma-Aldrich, Munich, Germany.

Influence of metal ions and inducers on EH expression

Experiments regarding the influence of metal ions were carried out with 40 ml medium in 200 ml shaking flasks. For observing effects of metal ion addition, cultures were grown on a modified minimal medium [31] supplemented with CaOH, $CaCl_2$, $FeSO_4 \cdot 7\ H_2O$, $MgSO_4$ or $ZnSO_4$ (in concentrations between 0.01 and 0.5 g/l), respectively. Furthermore, the influence of supplementing the medium with 100 µl/l trace element solution consisting of EDTA (1,000 mg/l), $FeCl_3 \cdot 6H_2O$ (50 mg/l), H_3BO_3 (1 mg/ml), $MnCl_2 \cdot 4H_2O$ (15 mg/l), $ZnCl_2$ (10 mg/l) and $CoCl_2 \cdot 6H_2O$ (5 mg/l) on EH activity and biomass formation was tested.

For experiments with inducers, a volume of 400 µl containing either 100 mM phenyloxirane or 100 mM racemic *para*-nitro-styrene oxide (*p*NSO) was added to the 2.7 l scale fermentation during the late exponential growth phase, the retardation phase and the late stationary phase.

Table 1 Summary of media used for optimization experiments

Media	Composition
BM-1	10 g/l glucose, 1 g/l yeast extract, 0.5 g/l KCl, 3 g/l $NaNO_3$, 0.5 g/l $MgSO_4$.7 H_2O, 1 g/l KH_2PO_4
MM-A	10 g/l glucose, 1.4 g/l yeast extract, 0.2 g/l leucin, 0.5 g/l KCl, 6 g/l $NaNO_3$, 0.5 g/l $MgSO_4$.7 H_2O, 1.5 g/l KH_2PO_4
NM-90	30 g/l malt extract, 3 g/l soy peptone
NM-B	20 g/l sugar beet molasses, 5 g/l yeast extract
NM-D	20 g/l glucose, 10 g/l soy peptone
UMY	10 g/l glucose, 3 g/l malt extract, 3 g/l yeast extract, 5 g/l soy peptone
YM	15 g/l glucose, 20 g/l malt extract, 5 g/l yeast extract, 5 g/l soy peptone

Cultivation experiments with *B. rhodina*

Cultivation processes were performed in stirred tank bioreactors either with a reactor volume of 3.5 l (reaction volume 2.7 l) or a reactor volume of 150 l (reaction volume 30 l) (Bioengineering AG, Wald, Switzerland). The aeration rate was set to 0.3 vvm at an impeller speed of 300 rpm and the cultivation temperature was maintained at 28 °C. The pH (setpoint at 6.5) was controlled and kept constant by the addition of 2 M H_3PO_4 or 2 M NaOH. Depending on size, the reactor was inoculated by adding 200 ml/1.2 l of pre-culture which had been grown for 4 days in 1,000 ml Erlenmeyer flasks (reaction volume 200 ml) at 30 °C. The bioreactor media contained 30 g/l malt extract and 3 g/l soy peptone. The dissolved oxygen was measured with an Ingold DO-sensor (Mettler-Toledo GmbH, Giessen, Germany). Oxygen in the exhaust gas was analyzed with a Maihak Oxygor 6 N, carbon dioxide was measured with a Maihak Unor 6N (both Sick Maihak GmbH, Reute, Germany).

Determination of biomass and protein concentration

Biomass formation was determined by filtration of a constant volume of fermentation broth using a cellulose acetate

filter, pore size 0.2 μm (Sartorius AG, Göttingen, Germany) in a stainless steel vacuum filtration unit (Sartorius AG, Göttingen, Germany). Biomass was washed twice with water and dried for 24 h at 100 °C. The protein contents of solutions were determined by the Bradford staining method using Coomassie brilliant blue (Biorad Laboratories Inc., Munich, Germany) as dye.

Preparation of crude extracts

Optimal cell disruption was experimentally determined after 15 min of homogenization at 4 °C using a bead-mill (Willy A. Bachofen AG, Basel, Switzerland) with a loading of glass beads of 50% (v/v). After this time the enzyme was released from the cell compartments into the solution. Cell fragments, whole cells and glass beads were removed from the protein solution by filtration (single use cellulose folded filter, 15–19 μm, Whatman, Dassel, Germany). The filtrate was centrifuged at 15,000*g* and 4 °C for 15 min, the supernatant was collected and the pellet including cell walls was resuspended in working buffer which consisted of 0.1 M Tris/HCl-buffer, 1 mM EDTA, 1 mM cysteine, and 0.5 mM phenylmethansulfonylfluorid (PMSF) adjusted to pH 7.5.

Determination of enzyme activity with whole cells and cell free extract

EH activity was assayed using racemic phenyloxirane and pNSO. All substrates were dissolved in dimethylsulfoxide (DMSO) to a concentration of 100 mM (stock solutions). EH activities during cultivation of *B. rhodina* were assayed using racemic pNSO as substrate. About 1 ml of cultivation broth was centrifuged at 25,000*g* and 4 °C. After discarding the supernatant, the same volume of working buffer was added to resuspend the biomass. To 0.5 ml of pre-incubated biomass suspension (2 min at 30 °C), 20 μl of stock solution were added to reach a final concentration of 4 mM racemic pNSO. Incubation was performed for 20 min at 30 °C and 700 rpm in a thermo-mixer (Eppendorf GmbH, Hamburg, Germany). After 20 min, 0.5 ml MeOH was added to stop the reaction. Samples were centrifuged at 25,000*g* and 4 °C. After filtration using a 0.2 μm PET-Chromafil single use filter (Macherey-Nagel GmbH & Co KG, Düren, Germany), the quantification of the product was carried out with an HPLC System (Waters 600 Delivery System and Waters 717 Autosampler (Waters GmbH, Eschborn, Germany), UVIS 204 Detector (Linear Instruments, Reno, NV, USA), ER-3612 Degasser Unit (Erma Inc., Tokyo, Japan), Kontron 450 Data System Integrator (Kontron AG, Eching, Germany) reverse phase column ODS-Hypersil; 250 × 4.6 mm, 5 μm (M&W Chromatographietechnik GmbH, Berlin, Germany), mobile phase 40/60% (v/v) acetonitrile/water at a flow rate of 1.0 ml/min).

Assays using cell free extracts were carried out in 1 ml vials containing 400 μl of enzyme solution. The reaction was started by addition of 15 μl of *p*NSO stock solution under the same incubation conditions as stated above.

To determine the parameters of the Michaelis–Menten equation (K_M and maximum rate of epoxide conversion v_{max}) using R-*p*NSO, S-*p*NSO or the racemic mixture of *p*NSO (50% R-*p*NSO, 50% S-*p*NSO) as substrates, solutions of various concentrations (0.02, 0.05, 0.1, 0.3, 0.5, 1, 2, 5 and 8 mM) were prepared in Tris–HCl buffer (100 mM, pH 7.5 including 1 mM EDTA, 1 mM L-cysteine, 0.5 mM PMSF). To 500 μl of pre-incubated crude-extract including the soluble epoxide hydrolase, a defined volume of *p*NSO solution was added and the reaction was started at 30 °C and 500 min^{-1} for 20 min in the thermo-mixer. After 20 min, the *p*NSO conversion was stopped by adding 500 μl cold (−20 °C) MeOH for the inactivation of EH activity. The diol formed was quantified by HPLC as described above.

One unit (U) is defined as the amount of enzyme, which hydrolyzes 1 μmol pNSO per minute at pH 7.5 and 30 °C.

Preparation of cell free extract and membrane free extract

After cultivation, biomass was harvested by centrifugation. The wet mycelium was suspended in 0.1 M Tris/HCl adjusted to pH 7.5, containing 1 mM EDTA, 1 mM cysteine and 0.5 mM PMSF. The vessel (total volume 120 ml) of a pre-cooled bead mill Dyno-Mill (Glenn Mills Inc., Clifton, NJ, USA) loaded with 60 ml glass beads of 1.0 mm diameter was filled with 60 ml of biomass suspension. Cell disruption was performed for 15 min at 4 °C. Unbroken cells and debris were removed by centrifugation for 15 min at 8,000*g* and 4 °C. The supernatant was regarded as cell free extract and used for further experiments.

For localization experiments, a pellet from debris of broken cells was resuspended in working buffer and centrifuged again. After a second resuspension in working buffer, the pellet was investigated for EH activity. The cell free extract was collected and centrifuged at 65,000*g* by a Sorvall CombiPlus ultracentrifuge (Thermo Electron Inc., Langenselbold, Germany). The EH activity determinations in membrane free extracts were performed as described above.

Purification of EH

Cell-free extract was centrifuged at 70,000*g* for 1.5 h at 4 °C to remove remaining cell membranes. Membrane free

supernatant was treated with 60% (w/v) $(NH_4)_2SO_4$ followed by centrifugation. Precipitated proteins were dissolved in working buffer supplemented with 20% (w/v) $(NH_4)_2SO_4$. The concentrated protein solution was applied onto an octyl-sepharose column 1.5 × 10 cm (Biorad Laboratories Inc.) equilibrated with working buffer supplemented with 30% (w/v) $(NH_4)_2SO_4$ at a flow rate of 1 ml/min. Fractions containing EH activity were pooled after elution with working buffer (0.3 ml/min) containing ethylene glycol with a linear time gradient in concentrations from 0 up to 50% (v/v). Concentrated fractions with EH activity were applied onto a DEAE-cellulose 52 column (Whatman International Ltd, Maidstone, UK) equilibrated with working buffer (pH 7.5) and eluted with a linear gradient (0.4 ml/min, 0–1 M NaCl in 450 min). Active fractions were pooled and concentrated by an Amicon ultra-filtration unit (Millipore GmbH, Schwalbach, Germany) using a 10 kD MWCO ultrafiltration membrane. Concentrated protein solution was applied onto a Sephacryl-S 300 column 3 × 60 cm equilibrated with working buffer adjusted to pH 7.5 containing 10 mM NaCl. The separation of active fractions was performed at a flow rate of 0.3 ml/min. The active fractions were pooled and concentrated by ultrafiltration as described above. Samples of each purification step except gel filtration were used for SDS—polyacrylamide gel preparation.

SDS–PAGE

SDS–PAGE was conducted on 1 mm thick slab gels consisting of 10% acrylamide resolving gel and 4% acrylamide stacking gel (Biorad Laboratories Inc.) adjusted to pH 8.8 in the presence of 0.05% (w/v) SDS. Samples and the protein standard were prepared by adding one volume of sample buffer containing 4 × Tris/HCl/SDS pH 6.8, 20% (v/v) glycerol, 2% (v/v) 2-mercapto-ethanol, 0.001% (w/v) bromophenol blue and 4% (w/v) SDS. The mixture was incubated for 10 min at 95 °C and 350 rpm. Proteins were stained over night with 0.1% (v/v) colloidal coomassie brilliant blue (Biorad Laboratories Inc.). The gel was decolorized in a solution of 30% (v/v) acetic acid and 10% (v/v) methanol for 3 h.

Results

Production of epoxide hydrolase in *B. rhodina*

Localization of epoxide hydrolase activity

The total protein concentration of the cell free extract was 0.81 g/l, while after ultracentrifugation the total protein content decreased to 0.56 g/l. This finding is attributed to the removal of membrane-associated proteins. Comparison of total EH activity in the supernatant after cell disruption (cell free extract) and after ultracentrifugation (membrane-free extract) indicated a 30% higher total amount of EH activity in the former samples, which agrees with 3.5 ± 0.3 units. The decrease down to 2.5 units of total EH activity in samples using membrane free extract might be due to the loss of proteins of about 0.25 g/l owing to membrane association. Hence, the specific activity (U/$g_{protein}$) remained at the same level (2.7 U/$g_{protein}$) in both samples, that is the cell-free as well as the membrane-free extracts.

Due to the membrane association, it was expected that the loss compared to total EH activity of 1 unit would be recovered completely in the membrane part. However, only 0.048 (4.8%) units were determined in the membrane compartment, which could be due to the poor resuspension characteristics of the centrifuged membrane and the instability of the membrane associated EH.

Media optimization

Influence of medium composition and trace elements The highest EH activity was measured when using BM-1 medium supplemented with 0.1 g/l trace elements solution. On this medium, the specific EH activity was 3.5 U/g_{DW} being more than twice the activity compared to cultures growing on BM-1 without trace element solution (1.5 U/g_{DW}). EH activities in cultures grown on MM-A medium without and with trace element solution changed from 2.4 to 3.0 U/g_{DW}, respectively. This difference is small compared to the effect when using BM-1 medium. All other tested media (NM-B, UMY, YM, NM-D, and NM-90) did not lead to higher specific activities than 3 U/g_{DW}.

Influence of ions Experiments were carried out using medium BM-1. Regarding the metal ion content, the medium consists of 0.5 g/l KCl, 3 g/l $NaNO_3$, 1 g/l KH_2PO_4 and 0.5 g/l $MgSO_4$.

When supplementing 0.01 g/l of Fe^{2+}, Zn^{2+} or Ca^{2+} ions to BM-1 without trace element solution, the specific EH production was negatively influenced at concentrations between 0.01 and 0.5 g/l. The specific activities decreased from 1.7 U/g_{DW} to 1.2, 1.1 and 0.7 U/g_{DW} for Fe^{2+}, Zn^{2+} or Ca^{2+} ions, respectively.

The presence of 0.01 g/l Mg^{2+}ions revealed an increase in the specific activity of 25% (from 1.1 to 1.4 U/g_{DW} ± 0.05). However, the specific activity remained constant at about 1.8 U/g_{DW} when 0.25 g/l Mg^{2+}or more was supplemented to the medium. In cultivation media supplemented

with Mg^{2+}ions in a range from 0 to 0.01 g/l $MgSO_4$, the biomass yield was unaffected at 0.3 g biomass per g glucose consumed ($g_{DW}/g_{glucose}$). Hence, a better yield was obtained due to increased biomass production, when media were supplemented with 0.25 up to 2 g/l $MgSO_4$ (0.49 $g_{DW}/g_{glucose}$).

Influence of inducers An increase of specific enzyme activity of 30% compared to the control with 1.1 U/g_{DW} was observed when 0.1 g/l *p*NSO was supplemented to the culture during the exponential growth phase. The specific EH activity was decreased down to 0.55 U/g_{DW} when phenyloxirane was supplemented to the same phase of the culture.

With the addition of phenyloxirane to the late stationary phase, the specific activity initially decreased about 20%. After 20 h, an increase of specific EH activity of over 20% compared to the control was observed. A decrease of activity in the following 24 h occurred during the transition into the lytic phase.

Batch-fermentation

2.7 l batch fermentation In 2.7 l bioreactor experiments with *B. rhodina* using medium NM-90 including supplements (0.5 g/l $MgSO_4$ and 0.1 g/l trace element solution), the specific activity could be increased from 1.5 to 6 U/g_{DW} compared to fermentations using the same medium without supplements. An exponential growth phase was observed between 15 and 30 h of cultivation time. A maximal specific growth rate of $\mu_{max} = 0.13\ h^{-1}$ could be determined. The volumetric EH activity increased from 2 to 16 U/l. The maximum of volumetric activity was achieved in the late stationary phase after 75 h of cultivation. The end of the stationary phase and transition into the lytic phase was observed after 90 h of fermentation time (Fig. 1).

30 l batch fermentation A scale-up of the process using the media NM-90 including supplements as stated above was conducted in batch fermentation with a working volume of 30 l. In these fermentations, a maximum specific activity of 2.5 U/g_{DW} was measured. The maximal specific growth rate could be determined to be $\mu_{max} = 0.11\ h^{-1}$. The volumetric activity obtained was slightly decreased to 15 U/ml. Values of parameters at the onset of the smaller and larger scaled fermentations are shown in Table 2 to point out differences in conditions. The stirrer tip velocity was allowed to be higher in the larger scale fermentation

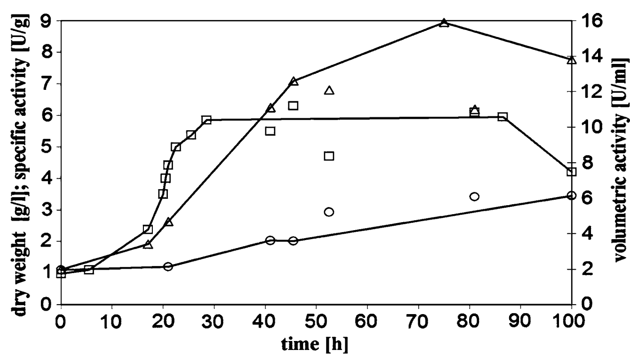

Fig. 1 Biomass concentration (*open square*) in the 2.7 l batch fermentation experiment. Volumetric (*open triangle*) and specific (*open circle*) epoxide hydrolase activity were determined using racemic pNSO as substrate

Table 2 Parameters of *B. rhodina* batch cultivation experiments

	Batch fermentation working volume	
	2.7 l	30 l
Stirrer tip velocity	0.48 m/s	0.76 m/s
Superficial gas velocity	3.16 m/h	3.32 m/h
Reynolds number $Re = \frac{nd^2\rho}{\eta}$	8.036	39.918
Dimensionless gas flow $Q = \frac{\dot{V}_G}{nd_r^3}$	0.042	0.029

yet avoiding damage to mycelia caused by high shear forces.

The volumetric oxygen transfer coefficient (k_La) in the 30 l fermentation was determined following the quasi-steady state method [29]. The results are shown in Fig. 2. To compare conditions in all fermentations, k_La was also predicted using the empirical equation formulated by Henzler with the extension made by Hoecker et al. [13, 14]. The power input P was calculated from the equation by Michel and Miller [20] which provided a ratio of $P_{2.7l}/P_{30l} = 0.02$. Based on this, the k_La ratio could be derived to $k_La_{2.7l}/k_La_{30l} = 0.33$. The predicted k_La, therefore, was larger in the large scaled fermentation.

In order to find dependencies between fermentation conditions and EH activity A_{EH}, various correlations were tested to describe the measured time course of activity. A correlation was found to the slope of the oxygen uptake rate OUR. The behavior of the volumetric activity could therefore be represented by:

$$\frac{dA_{EH}}{dt} = k_1 \frac{dOUR}{dt} \quad (1)$$

k_1 could be determined to be 1.83 (U h)/($mmol_{O2}$ g_{DW}), the mean standard error to be 0.02. The model-derived volumetric activity is shown in Fig. 2.

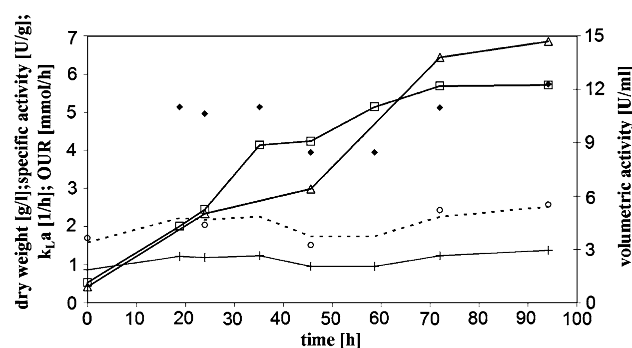

Fig. 2 Biomass concentration (*open square*), k_La (*filled diamond*) and oxygen uptake rate (OUR) (+) in the 30 l batch fermentation experiment. Volumetric (*open triangle*) and specific (*open circle*) epoxide hydrolase activity were determined using racemic pNSO as substrate. The specific activity derived by the model including the OUR is indicated by the *dashed line*

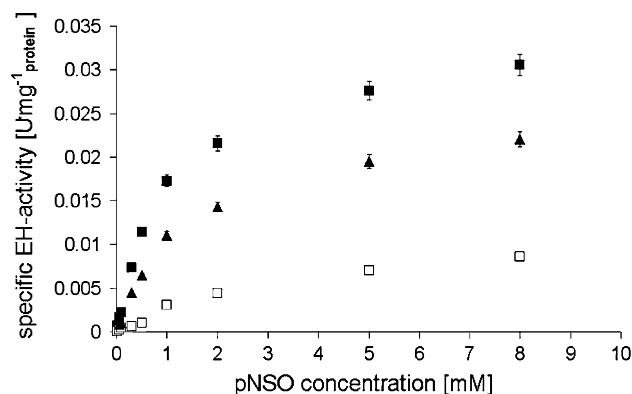

Fig. 4 EH activity depending on the substrate concentration for R-pNSO, S-pNSO and the racemic pNSO

Studies of epoxide hydrolase in *B. rhodina*

Enzyme properties

Influence of temperature The influence of temperature on EH activity is shown in Fig. 3. Activity measured at 4 °C is decreased by 50% with respect to the maximum specific activity measured at 30 °C. Temperatures above 30 °C led to a diminished activity with an almost complete loss of activity at 60 °C. Evaluation between 4 and 30 °C in an Arrhenius plot yields a rather low activation energy of 17 kJ/mol. In this range, the temperature influence on the enzyme-catalyzed reaction can be considered as being low.

Enantioselectivity and substrate concentration effects Enzyme kinetic measurements were performed for the soluble epoxide hydrolase allowing a comparison of the conversion kinetics using R-*p*NSO and S-*p*NSO. After cell disruption, the crude extract had a protein concentration of 1,295 ± 60 µg/ml.

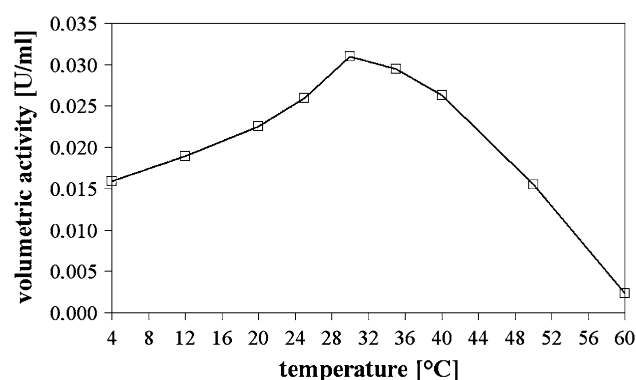

Fig. 3 Effect of temperature on volumetric epoxide hydrolase activity

The characteristics of the EH activity depending on the concentration of R-*p*NSO, S-*p*NSO and racemic *p*NSO are shown in Fig. 4, exhibiting Michaelis–Menten type kinetics. The R-*p*NSO had both a lower K_M and a higher v_{max} compared to S-*p*NSO (Table 3), which were determined by a Hanes linearization (Fig. 5). It should be noted, that v_{max} is related to the protein concentration of the crude extract and not to purified epoxide hydrolase. An apparent enantioselectivity of the kinetic resolution could be determined for epoxide hydrolase with a clear preference for R-*p*NSO. The maximal velocity of R-*p*NSO conversion in the crude extract was 3.4-fold higher than for the S-*p*NSO.

For the determination of substrate concentration effects on the EH-activity, studies with concentrations of the racemic epoxide up to the maximal solubility (50 mM) were carried out where the EH showed no inhibition effects due to high epoxide concentrations. A maximum reaction rate was observed at substrate concentrations above 20 mM *p*NSO (data not shown).

Effect of product concentration After 140 min of reaction time, product concentration was rising to a final maximum value of 2.5 mM *para*-nitro-styrene diol (*p*NSD) from racemic *p*NSO and 5.5 mM *p*NSD from R-*p*NSO, respectively (Fig. 6). While the product concentration was rising, the specific EH activity decreased down to 5% compared to

Table 3 Kinetic parameters of soluble EH using R-*p*NSO, S-*p*NSO and racemic *p*NSO

	R-*p*NSO	S-*p*NSO	rac. *p*NSO
K_M (mM)	1.0	2.54	1.46
v_{max} ($U/g_{protein}$)	0.034	0.01	0.026

Parameters were estimated from a Hanes linearization (Fig. 4)

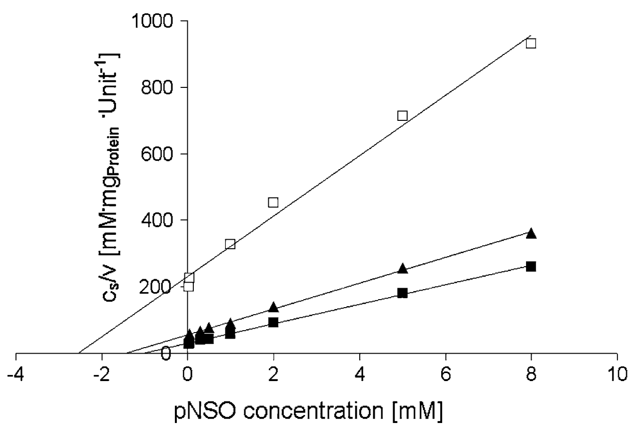

Fig. 5 Plot of EH activities by the linearization method by Hanes for estimation of kinetic parameters (K_M, v_{max})

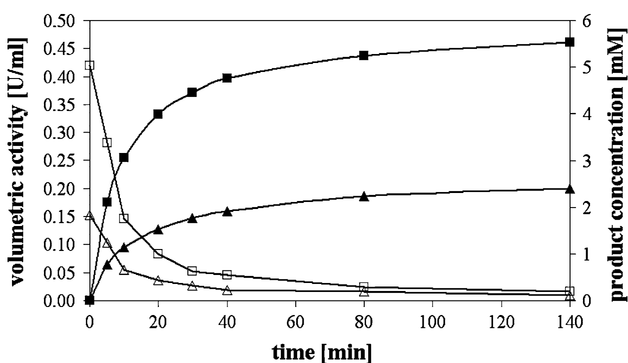

Fig. 6 Product inhibition from conversion of racemic pNSO (*Filled triangle*) and R-pNSO (*Filled square*). Specific activities decrease in the presence of the product pNSD for racemic pNSO (*open triangle*) and for R-pNSO (*open square*)

the initial value. Without supplementation of substrate, 90% of initial EH activity were retained after 24 h at 24 °C. More than 50% of the detected product had been formed within the first 5 min after the reaction had started. From these observations, a product inhibition of the enzymatic reaction can be assumed.

Enzyme purification

Results of EH purification are summarized in Table 4. Evaluating the complete purification procedure, a final increase of specific activity by a factor of 47 with a total yield of 10% of the cytosolic EH was achieved. Preliminary experiments revealed that phenyl-sepharose is an appropriate matrix for binding and eluting the target protein. In batch binding experiments followed by an elution with 50% (v/v) ethylene glycol, nearly 90% of the enzymatic activity could be recovered. In contrast to this, only 60% of enzyme activity could be recovered after

Table 4 Summary of the purification steps of epoxide hydrolase from *B. rhodina*

Procedure	Total proteine (mg)	Vol. activity (U/L)	Spec. activity (U/$g_{protein}$)	Yield (%)	Purity
Ultracentrifugation	49.8	4.5	20	100	1
Precipitation with 30% (w/w) $(NH_4)_2SO_4$	31.2	3	25	67	1.25
Precipitation with 60% (w/w) $(NH_4)_2SO_4$	20	2.7	35	60	1.8
Octyl-Sepharose	0.5	0.6	570	13	28
DEAE-anion-exchange/ ultrafiltration	0.06	0.45	940	10	47

hydrophobic interchange chromatographie (HIC) using octyl-sepharose. After three passages of cell disruption by bead mill and additional ultra-centrifugation, a volumetric activity of 5 U/l was detected in the membrane free supernatant. After dialysis of the dissolved protein pellets, a loss of 15% of volumetric enzymatic activity was observed. In the following treatment by HIC on an octyl-sepharose column, nearly 70% of the hydrolase could be recovered by elution using ethylene glycol. Fractions containing most of the detectable enzyme activity were used for further investigation. They were applied onto a DEAE cellulose-based column and eluted by a linear NaCl gradient. Nearly 85% of active hydrolase were recovered when 30% of the final NaCl concentration was attained. From this final fraction, five single bands could be detected in the SDS gel. The molecular weights of these protein bands ranged from 28 to 40 kDa (Fig. 7).

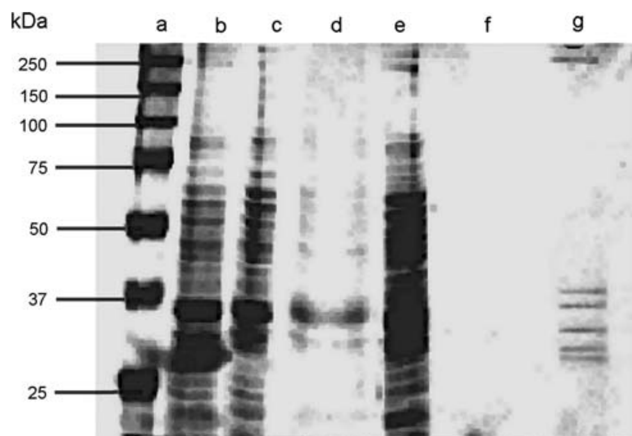

Fig. 7 SDS/PAGE including 30 µl molecular weight marker (**a**), 30 µl of crude extract after cell disruption (**b**), and ultra-centrifugation (**c**), proteins after precipitation by salt treatment with 30% (**d**), and 60% of $(NH_4)_2SO_4$ (**e**), and active fractions after HIC (**f**) and DEAE/ultra-filtration (**g**)

Discussion

The volumetric and specific activities measured in this study are similar to activities measured in experiments with *A. niger* [24]. Specific enzyme activities detected in shaking flasks were fourfold lower than in bioreactor experiments. Bioreactor experiments were carried out under pH control, in contrast to the cultivation using shaking flasks.

After supplementation of epoxide derivatives to the culture and incubation for more than 5 h in the stationary phase, an increase in EH activity could be observed. This increase of more than 20% indicates a potential induction effect in the presence of epoxide derivatives, in particular phenyloxirane. This can be related to a cellular protection mechanism, controlling intracellular epoxides by hydrolase and transferase interactions [26]. The solubility of these toxic compounds will be increased by formation of a vicinal diol allowing the removal from the cell. The underlying control of EH expression in fungi was described previously for *A. niger* and *Beauveria bassiana* [12, 23, 24].

It could be shown that EH expression in *B. rhodina* is related to the OUR of the microorganism. A positive influence of oxygen on EH expression was reported in the insect cell baculovirus expression system [36]. From calculations using empirical equations, it can be predicted that $k_{L}a$ values in the small-scale fermentation are lower than in the large-scale fermentation. Yet observation of specific and volumetric enzyme activities yields an opposite result to the expected behavior. An explanation is the change of viscosity of the culture broth by growth, leading to zones with poor oxygen supply. In the larger vessel, poorly mixed regions are larger, leading to increased diffusional limitation. Therefore larger parts of the reactor volume could have been present where cells were not supported with oxygen, leading to lower EH expression.

The major part of the EH from *B. rhodina* is located in the cytosol whereas the smaller part is associated to the membrane, consistent with other fungal EH belonging to the cytosolic EH superfamily [6]. It was not clarified if both cytosolic and membrane bound EHs are similar to their kinetic resolution. The cytosolic EH shows a K_m value, which is similar to results of EH isolated from *A. niger* [19, 21]. The determined reaction rate is 3.4 times higher for R-*p*NSO compared to S-*p*NSO, an indication for enantiospecific catalysis by the EH from *B. rhodina.*

A first purification strategy to isolate the cytosolic EH has been established resulting in a 47-fold increase in activity. The molecular weights of the five single bands detected at the SDS/PAGE are similar to results for EHs from various other organisms [1, 16, 21, 30]. In these cases, molecular weights for the epoxide hydrolases from soy bean, *Rhodutorula glutinis*, *Nocardia* sp., *A. niger* and *Agrobacterium radiobacter* were determined to be 33, 45, 34, 45 and 34 kDa, respectively. Further investigations of isolated samples of these bands to elucidate the biochemical structure and characteristics of the EH from *B. rhodina* will allow the determination of the sequence similarity and properties of this promising EH for the preparation of e-nantiopure epoxides and their corresponding diols.

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