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New *Pseudomonas* esterases by genetic engineering

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Abstract

The two new esterases, EP6 and EP10, from *Pseudomonas marginata* were studied with respect to their substrate specificities and selectivities. Esters of aliphatic and cyclic secondary alcohols were shown to be substrates for EP10, which could be accepted stereoselectively in hydrolysis and transesterification reactions. EP6 showed no selectivity in hydrolysis of the studied substrates.

Keywords: *Pseudomonas marginata*; Esterase; Hydrolysis; Stereoselectivity; Secondary alcohols

1. Introduction

Recent years have seen an exponential growth of chemo-enzymatic methods for obtaining enantiomerically pure chemicals. Enzymes are particularly important for the separation of enantiomers and hydrolysis is still the most common enzymatically catalyzed reaction [1]. Esterases represent a group of hydrolytic enzymes of broad natural diversity concerning substrate and reaction type. Due to their diverse substrate specificities which differ from that of lipases, esterases may provide a substantial broadening of the currently available tool kit for biocatalytic applications in organic syntheses [2–7].

In contrast to conventional screening methods, where hundreds of different organisms are screened for the production of a stereoselective enzyme, we try to exploit the potential of a specific organism. Working with a library of genes of such an organism allowing the expression in *E. coli* strains exhibiting low endogenous esterase activities offers several advantages over traditional screening programs. A major advantage of this strategy is to get access to essentially all esterases that are encoded in the genetic program of this organism as expression is made independent of the natural regulatory circuits. This also includes the immediate separation of a single enzyme from the background of other esterase activities expressed in that particular donor organism and thus allows rapid identification of distinct specificities and selectivities. Fast direct evolution using sequential generations of random mutagenesis is possible [8]. In addition, heterologous overexpression to high levels facilitates the purification of the

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desired enzyme or even makes this unnecessary and allows rapid up-scaling of the enzyme production process to a large scale.

Here, we report on the comparison of two new cloned *Pseudomonas* esterases with respect to their specificities and selectivities.

2. Experimental

2.1. Enzyme preparation

Pseudomonas marginata (= *Burkholderia gladioli* pathovar *gladioli*) was obtained from the 'National Collection of Plant Pathogenic Bacteria', UK (NCPPB1891). The cloned genes of the two esterases EP10 and EP6 were overexpressed in *E. coli* using an IPTG inducible *tac* promoter expression system. Whole-cell extracts were prepared by ultrasonic disruption. In the case of EP10 a solubilization step by treatment with Triton X-100 was performed prior to purification. FPLC (Pharmacia) purification of the proteins was done by hydrophobic interaction and ion exchange chromatography to a purity > 90% as judged by denaturing SDS–PAGE [9,10] Enzymatic activity was determined by a photometric assay at 420 nm using a 4 mM solution of *o*-nitrophenyl butyrate in 0.1 M Tris/HCl, pH 7.0. One unit corresponds to the release of one micromole of *o*-nitrophenol per minute.

2.2. Syntheses of substrates

All substrates used are summarized in Fig. 1. Substrates **1** and **2** were synthesized according to literature procedures [11,12]. Substrate **3** was prepared from commercially available (D,L)-2-octanol by standard chemical procedures.

2.3. Enzymatic hydrolysis

Hydrolysis experiments were carried out in 50 ml 10 mM sodium phosphate buffer (pH = 7.0) containing 200 units of the esterase and 1

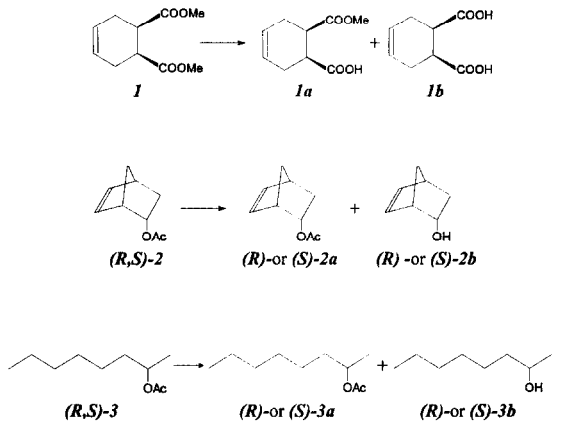

Fig. 1. Hydrolysis of dimethyl-*cis*-cyclohex-4-en-1,2-dicarboxylate (**1**), (±)-*endo*-bicyclo[2.2.1]hept-5-en-2-yl acetate (**2**) and (±)-2-octyl acetate (**3**).

mmol of substrate. The reaction was stirred vigorously and the pH was maintained at 7.0 throughout the reaction by addition of 0.1 M sodium hydroxide solution using an autotitrator [13]. After the sodium hydroxide consumption had indicated a conversion of 30–50%, the aqueous buffer solution was extracted two times with 25 ml CH_2Cl_2. The combined organic phases were dried over anhydrous sodium sulfate and the solvent was evaporated at room temperature under reduced pressure.

2.4. Determination of enantiomers by chiral gas chromatography

Gas chromatography was performed on a Shimadzu GC-14A model equipped with a flame ionisation detector. The separation of enantiomeric acetates **2a**, **3a** and alcohols **3b** was accomplished on a 25 m × 0.32 mm Chirasil-Dex-CB fused silica capillary column (0.25 μm; carrier gas: hydrogen).

In the case of **2b** the separation of the enantiomers was achieved after derivatisation to the diastereoisomeric menthyl carbonates (menthyl chloroformiate, pyridine/CH_2Cl_2 at room temperature for 60 min.) using a 25 m × 0.25 mm DB-1701 fused silica capillary column (0.25 μm; carrier gas: nitrogen)

3. Results and discussion

Esters of the prochiral cyclic dicarboxylic acid (substrate **1**) and of two chiral secondary alcohols (substrates **2**, **3**) were used as model compounds for the identification of biocatalytic specificities and differences between the two recombinant esterases EP6 and EP10 (Table 1). Substrate **1** could neither be hydrolyzed by EP6 nor by EP10 under the studied conditions. Substrates **2** and **3** were hydrolyzed by EP6 without preference of any of the two enantiomers. EP10 showed moderate selectivity towards substrates **2** and **3**. This indicates that esterase EP10 might be an enzyme, that selectively hydrolyzes esters of secondary alcohols and that enantiomers of secondary alcohols might be selectively recognized by that enzyme.

Based on these findings from the hydrolysis experiments and on the high stability of the enzyme [9] the transesterification of (D,L)-menthol with isopropenyl acetate by EP10 in supercritical CO_2 was studied. Michor et al. [7] found that EP10 showed some advantages in this reaction when compared to catalysis by several commercial lipases. Esterase EP10 not only showed a high initial velocity but also high stereoselectivity in the transesterification of this secondary alcohol. Although EP6 did not show stereoselectivity in hydrolysis of the substrates examined in this study, this enzyme seems to have other interesting features. Hickel et al. (submitted for publication) found that this enzyme was able to convert mandelonitrile to benzaldehyde reflecting hydroxynitrile lyase activity. To our knowledge this is the first time that this kind of enzymatic conversion was reported for a bacterial enzyme. As the primary structure of EP 6 shows significant homologies to several carboxylesterases and high activities in hydrolysis of esters of short chain carboxylic acids, we believe that EP6 is a typical esterase.

Further studies are in progress to characterize the substrate range applicable to EP6.

Table 1
Hydrolysis of dimethyl-*cis*-cyclohex-4-en-1,2-dicarboxylate (**1**), (±)-*endo*-bicyclo[2.2.1]hept-5-en-2-yl acetate (**2**) and (±)-2-octyl acetate (**3**) by *Pseudomonas marginata* esterases EP6 and EP10

Enzyme	Substrate	Conversion (%, GC)	$ee_{alcohol}$ (%)
EP6	1	0	0
	2	55.7	0
	3	44.8	0
EP10	1	0	0
	2	50	45
	3	28	45

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