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Enantioselectivity of a recombinant esterase from *Pseudomonas fluorescens*

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Abstract

A recombinant esterase from *Pseudomonas fluorescens* (PFE) was produced from *E. coli* cultures and purified to homogeneity resulting in a specific activity of 120 U/mg (*p*-nitrophenylacetate assay). PFE is stable in a wide range of pH (6–9) and active from 30–70°C, but rather unstable at temperatures > 50°C. PFE hydrolyzes a wide range of aliphatic and aromatic esters, but no long chain fatty acid esters. The enzyme showed high rate and enantioselectivity in the resolution of α-phenylethanol ($E > 100$) and its acetate ($E = 58$), while the closely related α-phenylpropanol was converted at very low rate and enantioselectivity. 3-Phenylbutyric acid methylester was hydrolyzed at acceptable rate, but low enantioselectivity ($E = 3.4$–3.7), whereas 2-phenylbutyric acid ethylester was not a substrate for PFE.

Keywords: Enantioselectivity; Esterase; *Pseudomonas fluorescens*; Purification

1. Introduction

Esterases belong to the group of hydrolases [Carboxylester hydrolases, E.C. 3.1.1.1]. They catalyze the formation or cleavage of ester bonds and are also active in organic solvents [1]. Some microbial esterases have been cloned [2,3], but only a few of them have been used in the resolution of chiral compounds [4,5].

In this paper, we have characterized a recently cloned and expressed recombinant esterase from *Pseudomonas fluorescens* and investigated the enantioselectivity of this enzyme towards some chiral compounds.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Fluka, Buchs, Switzerland and Sigma, Steinheim, Germany, at the highest purity available.

2.2. Production of esterase

E. coli JM109 harboring the rhamnose inducible plasmid 2792.1 [6] encoding for PFE

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was grown at 37°C in 250 ml of Luria–Bertani broth supplemented with ampicillin (100 μg/ml) until the early exponential phase (OD_{600} 0.5, approx. 3 h). Gene expression was then induced by adding rhamnose (final concentration 0.2% (w/v)) to the culture, followed by further incubation for 5.5 h at 37°C. Cells were collected by centrifugation (5000 rpm, 10 min, 4°C) and were washed twice with sodium phosphate buffer (50 mM, pH 7.5, 4°C). After resuspension in the same buffer, cells were disrupted by sonication and cell debris were removed by centrifugation (5000 rpm, 15 min, 4°C). Protein content was determined using the BioRad assay according to Bradford [7]. The crude extract was directly used in hydrolysis experiments or lyophilized and immobilized on Celite 545 (see below) for biotransformations in organic solvent. The enzyme was purified to homogeneity with a specific activity of 120 U/mg (pNPA-assay, see below) as described elsewhere [8].

2.3. Esterase activity

Esterase activity during cultivation, of the crude extract, and of the lyophilized enzyme was determined photometrically in sodium phosphate buffer (50 mM, pH 7.5) using *p*-nitrophenylacetate (pNPA, 10 mM dissolved in DMSO) as substrate and the amount of liberated *p*-nitrophenol was determined at 410 nm ($\varepsilon = 15 \times 10^3$ M^{-1} cm^{-1}) and 25°C. One unit (U) of activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per min under assay conditions. Esterase activity for biotransformations was based on a pH-stat assay: To 20 ml of an emulsion containing 5% (w/v) of ethylacetate and 2% (w/v) of gum arabic, a known amount of esterase was added at 37°C. Liberated acetic acid was titrated automatically in a pH-stat (Metrohm, Herisau, Switzerland) with 0.01 N NaOH in order to maintain the pH constant at pH 7.5. One unit of esterase activity was defined as the amount of enzyme, which liberates 1 μmol acetic acid per min under assay conditions. Similarly, temperature and pH-profiles were determined. Stability was determined after incubation of PFE for 17 h at the temperature given in Fig. 1 followed by the standard pH-stat assay at 37°C and pH 7.5. All values are corrected for autohydrolysis of ethylacetate.

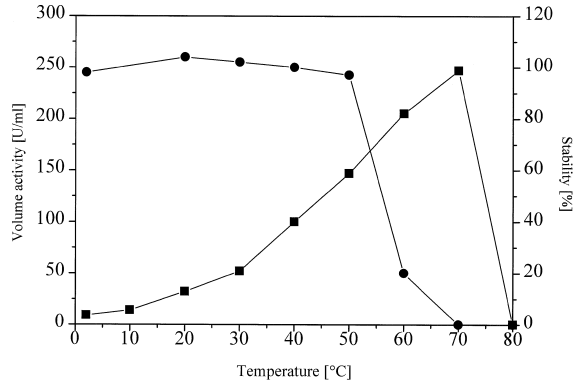

Fig. 1. Volume activity (■) and stability (●) of PFE as a function of temperature.

2.4. Immobilization on Celite 545

A total of 100 mg of lyophilized culture supernatant was dissolved in 1.5 ml of sodium phosphate buffer (50 mM, pH 7.5) and mixed for 20 min with 1 g of Celite 545. The slurry was filtrated through a Büchner funnel and washed with 20 ml of chilled acetone followed by drying at room temperature. Esterase activity was then determined by pH-stat as described above.

2.5. Chemical synthesis

The synthesis of α-phenylethylacetate, α-phenylpropylacetate, 3-phenylbutyric acid methylester and 2-phenylbutyric acid ethylester was performed using standard procedures and has been described elsewhere [9].

2.6. Biotransformations

(a) Hydrolyses of racemic α-phenylethylacetate and α-phenylpropylacetate (0.5 mmol)

Table 1
Results of esterase-catalyzed biotransformations of chiral compounds

Compound	Enantiomeric excess		Conversion[a]	E[a]
	[% ee_S]	[% ee_P]	[%]	
α-Phenylethanol	99 (S)	99 (R)	50	> 100
α-Phenylethylacetate	60 (R)	94 (S)	39	58
α-Phenylpropanol	4 (S)	74 (R)	5	7
α-Phenylpropylacetate	< 1 (R)	27 (S)	< 1	2
3-Phenylbutyric acid methylester	51 (S)	34 (R)	60[b]	3.4
3-Phenylbutyric acid methylester	31 (S)	47 (R)	40[b]	3.7

[a]Calculated from the enantiomeric excess as described by Chen et al. [10].
[b]As determined from NaOH consumption.

were performed in 3 ml of sodium phosphate buffer (50 mM, pH 7.5) and 2 ml of toluene at 40°C using 200 U (based on pH-stat assay) crude extract of PFE. Samples from the reaction mixture were centrifugated, the organic phase was analyzed by gas chromatography (FS-Cyclodex β-I/P CS-Fused Silica capillary column, CS-Chromatographie Service, Langerwehe, Germany) and enantiomeric excess, conversion, and enantioselectivity were calculated as described by Chen et al. [10]. Absolute configurations were assigned by comparison with enantiopure standards. Hydrolysis of 3-phenylbutyric acid methylester was performed in a pH-stat system by adding 1 mmol of ester to 20 ml of water (pH 7.5) at 40°C and with stirring at 500 rpm followed by addition of 400 U lyophilized PFE. After the consumption of 0.1 N NaOH corresponding to the desired conversion, the mixture was extracted two times with chloroform. The organic phase was dried over anhydrous Na_2SO_4 followed by evaporation of solvent. Substrate and product were separated by silica gel column chromatography (petrol ether:diethyl ether = 2:1) and the optical rotation of the methylester was determined to $[\alpha]_D^{20}$: 15.4° ($c = 1$, $CHCl_3$) at 60% conversion and 9.4° ($c = 1$, $CHCl_3$) at 40% conversion. Enantiomeric excess and enantioselectivity given in Table 1 were calculated from the ratio of the optical rotation of the sample to the literature value of the pure compound, because separation of enantiomers by GC or HPLC was not possible. Hydrolysis of 2-phenylbutyric acid ethylester gave almost no conversion after 24 h and substrate and product were not isolated.

(b) For acylation, 0.5 mmol of α-phenylethanol or α-phenylpropanol were dissolved in 3 ml of toluene at 40°C (dried over molecular sieve) containing 1 mmol of vinylacetate and 200 U Celite-esterase were added. Samples from the reaction mixture were centrifuged and the supernatant was analyzed by GC.

3. Results and discussion

3.1. Cultivation and isolation of P. fluorescens esterase

The esterase was produced by cultivation of *E. coli* harboring the esterase gene in 250 ml of LB-media. Highest productivity (100 U/ml) was found after 5.5 h induction (total cultivation time 8.5 h) corresponding to a total wet cell weight of 2.1 g. Isolation as described in Section 2 resulted in a specific activity of 100 U/mg protein (pNPA-assay) and 28 U/mg protein (ethylacetate) for lyophilized PFE, and 650 U/g after immobilization on Celite 545. Crude PFE was purified by immobilized zinc ion affinity chromatography yielding a homogeneous esterase in a single step with a specific activity of 120 U/mg (pNPA). Crude and pure (lyophilized) PFE are active in a wide range of pH (6–9) with an optimum between pH 7.5 to 8

(data not shown). A pH-stat assay at different temperatures revealed that PFE is active up to 70°C (Fig. 1), but is rather unstable at temperatures above 50°C. The enzyme can be stored at temperatures up to 50°C without significant loss of activity. Crude and purified PFE exhibited similar activities and stabilities [8].

3.2. Enantioselectivity

The most important applications for hydrolases is their use in the synthesis of enantiomerically pure compounds. PFE exhibited very high enantioselectivity in the resolution of α-phenylethanol ($E > 100$) and hydrolysis of its acetate ($E = 58$), thus, allowing the preparation of both enantiomers in very high optical purity. The hydrolysis reaction was faster and after 5.5 h 39% conversion and 94% ee for the (*S*)-product were achieved. In striking contrast, the closely related propanol derivatives were converted at extremely low reaction rates (reaction times > 200 h) and with poor enantioselectivity ($E = 2$ to 7) (Table 1). An aromatic ring near to a carboxylic group significantly influenced the rate of PFE-catalyzed hydrolysis: whereas 3-phenylbutyric acid methylester was hydrolyzed at acceptable rates ($E = 3.4$–3.7), almost no reaction was observed with the 2-phenylbutryric acid ester. Thus, it seems that the binding pocket of PFE is rather small, because rate as well as enantioselectivity vary significantly with variations in the substrate structure.

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