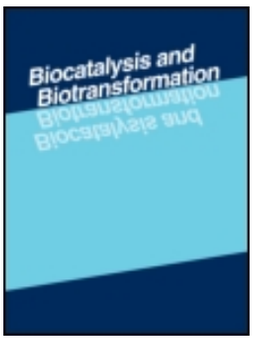

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SHORT COMMUNICATION

Substrate specificity of an esterase from the archaeon *Sulfolobus tokodaii* bearing a GGG(A)X motif

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Abstract

A GGG(A)X-type esterase (Est0071) from an archaeon catalyzes asymmetric hydrolysis of prochiral bulky malonic diesters in good enantioselectivity. The selectivity of Est0071 was for the opposite enantiomer to that previously shown for pig liver esterase, and the resulting enantiomeric excess of the products was higher. Est0071 could also catalyze the hydrolysis of various acetates of secondary alcohols, and showed moderate enantioselectivity in these reactions.

Keywords: *Esterase*, *GGG(A)X-motif*, *hydrolysis*, *malonic diester*, Sulfolobus tokodaii

Introduction

Enzymatic transformations of synthetic substrates are playing an increasingly important role in synthetic organic chemistry, especially in asymmetric synthesis. Optically active compounds are important intermediates for the synthesis of pharmaceuticals and naturally occurring compounds. Chiral carbon centers are found in many of these compounds. The attractive routes for synthesis of these compounds are hydrolytic desymmetrization of prochiral malonate diesters into their chiral monoesters or kinetic resolution of the racemic esters by hydrolytic enzymes (e.g. lipase and esterase). The optically active products may serve as chiral building blocks in the synthesis of more complex compounds of biological and pharmacological interest (Schneider et al. 1984; Toone et al. 1991; Canet et al. 1992; Breznik et al. 1997; María et al. 2005; Wallert et al. 2005; Iosub et al. 2010).

In this study, we focused on an esterase from the thermostable archaeon *Sulfolobus tokodaii* strain 7. The putative esterase gene (ST0071) was selected by *in silico* screening from the total genome of the strain and the characterized esterase expressed in *Escherichia coli* (Suzuki et al. 2004). The enzyme, called Est0071, was comprised of 303 amino acid residues and exhibited extremely thermostability with optimum activity observed at >80 °C. Recently, we have developed a thermally driven domino reaction for the synthesis of (*S*)-α-arylpropionates (profens) using Est0071 (Wada et al. 2013). However, the substrate specificity of the enzyme was not determined. Est0071 showed moderate identity with some esterases (e.g. lipase from *Candida rugosa* (CRL), *p*-nitrobenzylesterase from *Bacillus subtilis* (BsubpNBE)) which belong to a group of α/β-hydrolases containing a GGG(A)X motif, which is located in the active center of the hydrolases and is involved in the formation of the oxyanion hole. This motif has been reported to determine the activity of esterases or lipases toward bulky molecules such as tertiary alcohols (Henke et al. 2002; Kourist et al. 2007).

Pig liver esterase (PLE) is commercially obtained by extraction from animal tissue and is composed of isozymes (α, β, and γ). The γ-isoenzyme of pig liver esterase (γ-PLE) contains a GGGX motif, and cloning and functional expression of γ-isoenzymes in *Pichia pastris* and *E. coli* has been successful (Brüsehaber et al. 2007). A comparison of recombinant isozymes of PLE revealed significant differences in kinetic resolution reactions

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(Musidlowska-Persson et al. 2002). There have been many reports on pig liver esterase (PLE) catalyzed hydrolysis of prochiral bulky malonate diesters to optically active *(R)*-malonate monoesters. In contrast, there is no report of an esterase exhibiting selectivity opposite to that of PLE. Thus, we examined whether Est0071 could catalyze the hydrolysis of prochiral malonate diesters.

Methods

Preparation and enzyme assay of Est0071

The gene encoding ST0071 was cloned, over-expressed in *E. coli*, and the His-tagged fusion enzyme was purified (Suzuki et al. 2004). The esterase activity against *p*-nitrophenyl esters was determined by measuring the amount of resulting *p*-nitrophenol via enzymatic hydrolysis. One unit (U) of the esterase activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per minute. The specific activity of Est0071 was 165 U/mg protein at 30 °C.

Enzyme-catalyzed hydrolysis

The reaction of various esters with Est0071 was performed with 10 mM concentration of the substrate and 450 U of the enzyme in 200 mM HEPES buffer (pH 7) for malonic diesters or 200 mM Tris–HCl (pH8) for acetates of secondary alcohols in a total volume of 10 ml. After stirring at 30 °C for 36–264 h (malonic diesters) or 2 h (acetates), the reaction was stopped by the addition of 2 M HCl (2 ml) and the mixture was extracted with diethyl ether. After removal of the solvent in vacuo, the enantiomeric excess of the products was then analyzed with HPLC.

HPLC system

High-performance liquid chromatography (HPLC) was carried out on an LC-2010A HT system (Shimadzu, Kyoto, Japan) equipped with a chiral column OD (4.6 mm × 250 mm, Daicel Corporation, Japan). The separation of enantiomer peaks was achieved with a flow of 0.5 ml/min at 260 nm, by using a mobile phase composed of hexane–i-PrOH–trifluoroacetic acid (93:7:1.5).

Results and discussion

Est0071 catalyzed the hydrolysis of prochiral bulky diesters to give half ester with high selectivity and yield (Figure 1), and the results are summarized

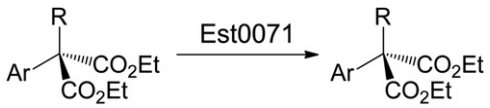

Figure 1. Est0071-catalyzed hydrolysis of diethyl malonates.

Table 1. Asymmetric hydrolysis of prochiral malonate diesters.

Run	Ar	*R*	Time (h)	Yield (%)	e.e. (%)	Config.
1	Ph	Me	48	96	96	*S*
2	Ph	Et	240	94	26	*S*
3	Ph	H	72	79	0	–
4	*p*-methylphenyl	Me	36	77	>99	*S*
5	2-naphthyl	Me	120	81	94	*S*
6	Benzyl	Me	264	99	83	*R*

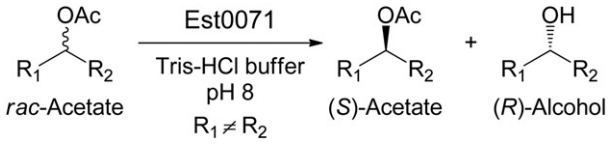

Figure 2. Enantioselective hydrolysis of various acetates.

in Table 1. The product derived from the substrate with phenyl and methyl groups (Run 1) was obtained in high yield and high excess of the *S*-enantiomer. However, when the aryl group was benzyl (Run 6), the configuration of the product was *R*. When the *R* group was changed to ethyl (Run 2) or hydrogen (Run 3), the enatiomeric excesses was low (ethyl: 26% e.e., hydrogen: 0% e.e.). This indicated that the *R* group was significant for enatioselectivity. Furthermore, the pocket, which the alkyl group fits within, in the active site of this enzyme, may not be so large. When the aryl group was *p*-methylphenyl (Run 4) or 2-naphthyl group (Run 5), the reaction rates were slower than for the other substrates, indicating that the enzyme has a large hydrophobic pocket in the active site. Furthermore, the selectivity of Est0071 was opposite that of PLE.

The results of hydrolysis of various secondary alcohol acetates with Est0071 (Figure 2) are summarized in Table 2. The enzyme showed moderate enantioselectivity toward 1-phenylethyl acetate (Table 2, Run 1, $E = 11$) in contrast to the naturally occurring PLE mixture ($E = 7$) (Hummel et al. 2007). Interestingly, the preferences of Est0071 and PLE were the same in the kinetic resolution of this compound. When the *R* group was changed to ethyl (Run 2) or propyl (Run 3), the enatioselectivity gradually decreased.

Table 2. Enantioselectivities of Est0071 in the kinetic resolution of acetate of various secondary alcohols.

Run	R_1	R_2	Time (h)	Conversion (%)	Acetate e.e. (%)	Alcohol e.e. (%)	*E* value
1	Ph	CH_3	2	40	49	74	11
2	Ph	CH_2CH_3	2	32	39	81	15
3	Ph	$(CH_2)_2CH_3$	2	8.6	5	55	3.6
4	$PhCH_2$	CH_3	2	12	7	50	3.2

Conclusions

Est0071 was shown to hydrolyze a wide range of substrates with high chemical yield. Additionally, this esterase showed high enantioselectivity compared with that of PLE. Highly enantioselective hydrolysis of diethyl 2-phenyl-2-methylmalonate (Table 1, Run 1) to the (*S*)-monoester by this esterase was successful. Moreover, Est0071 catalyzed the hydrolysis of various acetates of secondary alcohols (Table 2). Thus, Est0071 is a useful tool for the synthesis of pharmaceuticals and naturally occurring compounds with a chiral center.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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