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Journal of Molecular Catalysis B: Enzymatic 19–20 (2002) 231–235

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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A novel dihydrocoumarin hydrolase from *Acinetobacter calcoaceticus*: application to the production of useful compounds

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Received 7 September 2001; received in revised form 21 May 2002; accepted 4 June 2002

Abstract

A novel aromatic lactone hydrolyzing enzyme, dihydrocoumarin (DHC) hydrolase, was isolated from *Acinetobacter calcoaceticus* F46. Primary structure of the enzyme showed high similarity to those of the esterases of *Pseudomonas* strains and bacterial non-heme haloperoxidases. The enzyme catalyzed both hydrolysis of ester compounds such as aromatic lactones and bromination of monochlorodimedon. DHC hydrolase also could hydrolyze methyl esters of β-acetylthioisobutyrate and cetraxate. The products of these reactions were identified as D-β-acetylthioisobutyrate (>99% *ee*) and cetraxate, respectively, suggesting that the hydrolysis reactions catalyzed by DHC hydrolase are enantio- and/or regio-selective. The reaction products, cetraxate and D-β-acetylthioisobutyrate, are useful compounds as pharmaceuticals.

Keywords: Lactonase; Dihydrocoumarin hydrolase; Non-heme haloperoxidase; *Acinetobacter calcoaceticus*

1. Introduction

Lactonases, catalyzing the reversible or irreversible hydrolysis (ring-cleaving) of lactone compounds, belong to esterase-family. The lactonase from a fungus *Fusarium oxysporum* catalyzes the stereoselective hydrolysis of D-pantoyl lactone, and it has been shown to be useful for the large-scale optical resolution of racemic pantoyl lactone [1–5]. However, studies on lactonases including screening of their potential sources, their biochemistry, structures and functions, etc., have not been performed sufficiently so far.

Recently, we found *Acinetobacter calcoaceticus* F46 produced a novel aromatic lactone (dihydrocoumarin; DHC)-hydrolase [6]. The enzyme also catalyzes the bromination of monochlorodimedon in the presence of H_2O_2. Brominating activity was observed, only when the enzyme of *A. calcoaceticus* F46 was incubated in the presence of H_2O_2 and DHC (or an organic acid, such as acetic acid and *n*-butyric acid). The reaction mechanism for the halogenation reaction catalyzed by DHC hydrolase has been suggested to be similar to those of the non-heme haloperoxidases [7,8] as follows: (a) nucleophilic attack of an active serine residue at the carboxyl carbon atom of dihydrocoumarin or an organic acid, and then the formation of an acyl-enzyme; (b) hydrolysis of the acyl-enzyme through nucleophilic attack by H_2O_2, and then the formation of a peroxoacid as a reaction intermediate; (c) non-enzymatic formation of hypohalous acid from the peroxoacid and a halide ion; and (d) non-enzymatic halogenation of

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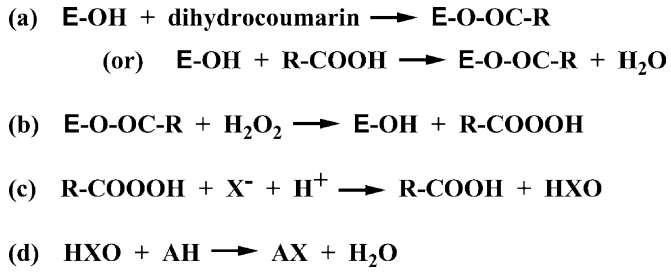
1381-1177/02/$ – see front matter
PII: S1381-1177(02)00172-8



Scheme 1. Mechanism of halogenation reaction catalyzed by DHC hydrolase of *A. calcoaceticus* F46. E, DHC hydrolase; X^-, halide ion; A, monochlorodimedon.

monochlorodimedon by the hypohalous acid [6] (Scheme 1). This is the first case of a lactonase with haloperoxidase activity.

Here, we report the application of DHC hydrolase of *A. calcoaceticus* F46 to the synthesis of useful organic compounds.

2. Materials and methods

DHC hydrolase was purified from cells of *A. calcoaceticus* F46 (AKU 724; Graduate School of Agriculture, Kyoto University, Kyoto, Japan) as described previously [6]. The enzyme activity was determined as described previously [6].

The DHC-hydrolase-overproducing strain, *E. coli* JM109 bearing pDCH21 (DHC hydrolase gene), was also used (unpublished data). Luria-Bertani (LB) medium containing 1% Bacto tryptone (Difco Laboratories, USA), 0.5% yeast extract (Oriental Yeast Co., Japan) and 1% NaCl, pH 7.0, was used. *E. coli* JM109 bearing pDCH21 was cultivated in a 2-l shaking flask containing 500 ml LB medium supplemented with 100 μg/ml ampicillin and 1 mM isopropyl β-D-thiogalactopyranoside at 37 °C with shaking for 24 h.

Washed cells of *E. coli* JM109 bearing pDCH21 (250 mg wet weight) were incubated with 10 ml of 50% (w/v) methyl DL-β-acetylthioisobutyrate or 25% (w/v) methyl cetraxate hydrochloride at 30 °C with shaking. The pH of the reaction mixture was adjusted to 7.0 by adding 6 M NaOH automatically. The concentrations of substrates and products were determined by high-performance liquid chromatography as described previously [9–11].

3. Primary structure of DHC hydrolase of *A. calcoaceticus* F46

The gene encoding DHC hydrolase of *A. calcoaceticus* F46 was cloned and sequenced (unpublished data). The deduced amino acid sequence of the enzyme exhibits significant similarity with those of the serine-esterases of *Pseudomonas* strains [12–15] and bacterial non-heme haloperoxidases [16–20], and contains a consensus motif, Gly-X-Ser-X-Gly around the active serine residue of the catalytic triad, as found in these esterases and non-heme haloperoxidases (Fig. 1). Serine-esterase inhibitors such as diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride inactivate the enzyme [6]. These facts suggest that DHC hydrolase of *A. calcoaceticus* F46 might be a protein in the serine-esterase family [21].

4. Application of DHC hydrolase to the production of useful compounds by enantio- and regio-selective hydrolysis

In addition to aromatic lactones such as DHC, 2-coumaranone and homogentisic acid lactone, the enzyme can hydrolyze the methyl esters of DL-β-acetylthioisobutyrate (K_m = 25.9 mM, V_{max} = 1440 U/mg) and cetraxate (K_m = 6.88 mM, V_{max} = 185 U/mg) among the linear esters tested. The products of these reactions were identified as D-β-acetylthioisobutyrate (>99% *ee*) and cetraxate, respectively, i.e. the hydrolysis reactions catalyzed by the enzyme are stereospecific and/or regioselective. The reaction product, D-β-acetylthioisobutyrate, has been used as a chiral building block for the synthesis of a series of

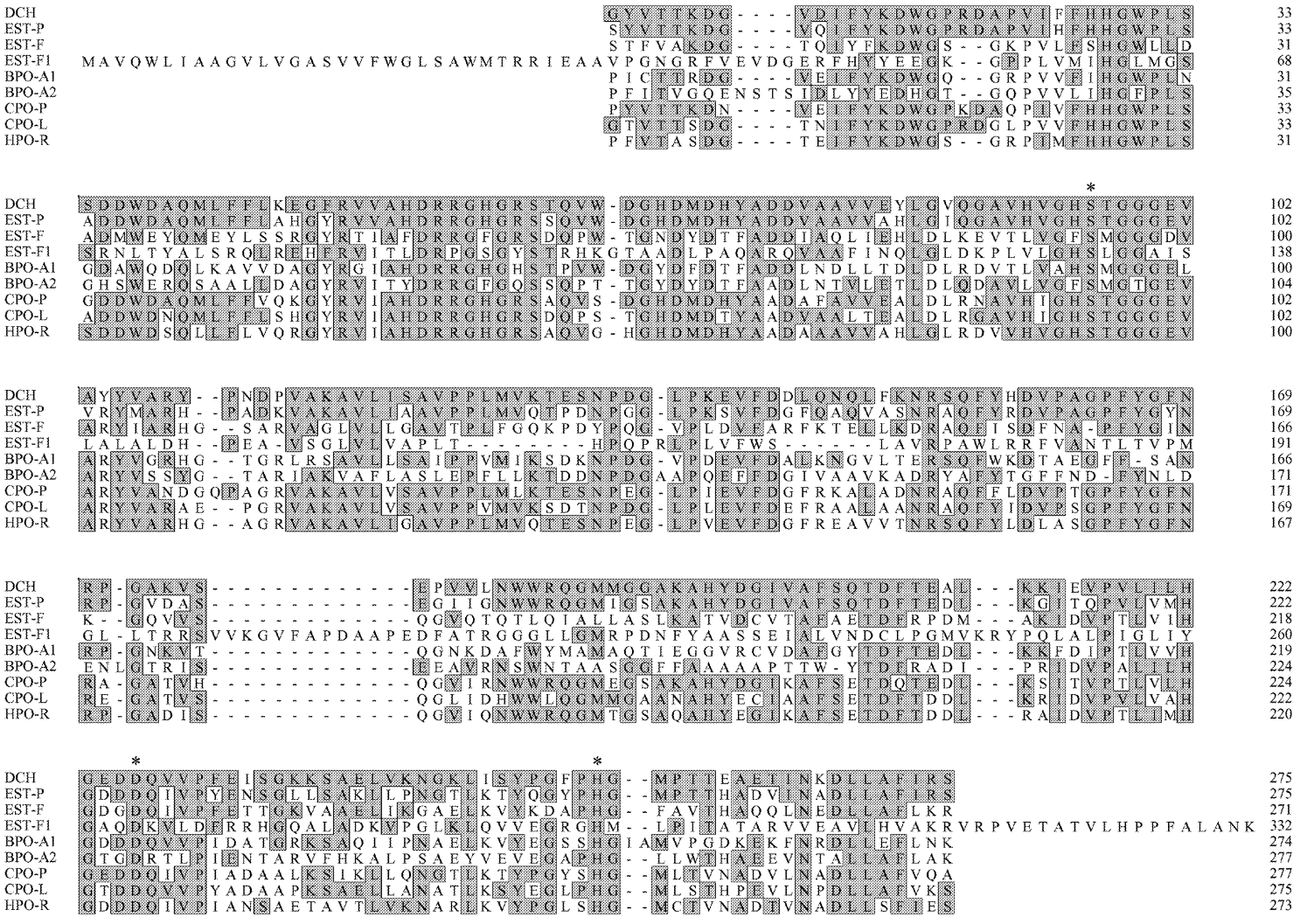


Fig. 1. Alignments of the DHC hydrolase of *A. calcoaceticus* F46 with esterases and non-heme haloperoxidases. DCH, DHC hydrolase of *A. calcoaceticus* F46; EST-P, esterase of *Pseudomonas putida* MR-2068 [12]; EST-F, esterase of *Pseudomonas fluorecens* SIK WI [13,14]; EST-F1, esterase of *Pseudomonas fluorecens* DSM 50106 [15]; BPO-A1 and BPO-A2, two bromoperoxidases of *Streptomyces aureofaciens* [16,17]; CPO-P, chloroperoxidase of *Pseudomonas pyrrocinia* [18]; CPO-L, chloroperoxidase of *Streptomyces lividans* [19]; HPO-R, non-heme haloperoxidase of *Rhodococcus erythropolis* [20]. Gaps in the aligned sequences are indicated by dashes (-). Identical amino acid residues are enclosed in boxes. Ser, Asp and His residues, which are thought to be involved in the catalytic triad, are indicated by the symbol '*'.

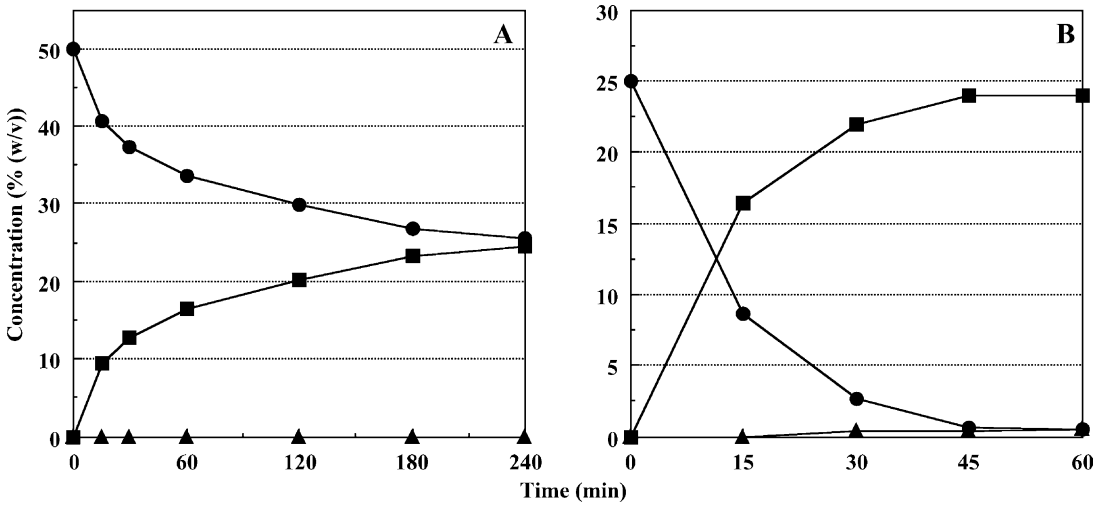


Fig. 2. Enantio- and regio-selective hydrolysis of methyl DL-β-acetylthioisobutyrate (A) and methyl cetraxate (B) catalyzed by recombinant *E. coli* cells bearing DHC hydrolase gene. (A) The reaction conditions are described in the text. Symbols: ●, methyl β-acetylthioisobutyrate; ■, D-β-acetylthioisobutyrate; ▲, L-β-acetylthioisobutyrate. (B) The reaction conditions are described in the text. Symbols: ●, methyl cetraxate hydrochloride; ■, cetraxate hydrochloride; ▲, 3-(4-hydroxyphenyl)propionate.

angiotensin-converting enzyme inhibitors [9,10]. The enzyme is also useful for the regioselective hydrolysis (specific for terminal ester bonds) of methyl cetraxate to cetraxate, which is widely used as an antiulcer agent. The hydrolysis reaction of methyl cetraxate proceeded without the formation of a by-product, i.e. hydroxyphenyl propionic acid or its methyl ester.

Thus, using *E. coli* cells expressing the DHC hydrolase gene from *A. calcoaceticus* F46, production of D-β-acetylthioisobutyrate and cetraxate through regio- and enantio-selective hydrolysis was examined. With 50% (w/v) racemic mixture of methyl β-acetylthioisobutyrate, only D-β-acetylthioisobutyrate (>99% *ee*) was formed without any hydrolysis of the L-isomer of methyl β-acetylthioisobutyrate (Fig. 2A). The regio-selective hydrolysis reaction of methyl cetraxate forming cetraxate with 25% (w/v) substrate stoichiometrically proceeded (Fig. 2B).

5. Conclusion

DHC hydrolase isolated from *A. calcoaceticus* F46 was a unique lactonase catalyzing two different reactions (i.e. ester hydrolysis and haloperoxidation). Furthermore, ester-hydrolyzing reactions catalyzed by DHC hydrolase showed high enantio- and regio-selectivities, which are useful for enzymatic production of several organic compounds. These facts suggested that lactonases are promising resources of novel biocatalysts for enzymatic production and biotransformation.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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