Functional Analyses and Application of Microbial Lactonohydrolases

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Abstract Microbial lactonohydrolases (intramolecular ester bond-hydrolyzing enzymes) with unique properties were found. The lactonohydrolase from *Fusarium oxysporum* catalyzes enantioselective hydrolysis of aldonate lactones and D-pantoyl lactone (D -PL). This enzyme is useful for the large-scale optical resolution of racemic PL. The *Agrobacterium tumefaciens* enzyme catalyzes asymmetric hydrolysis of PL, but the stereospecificity is opposite to that of the *Fusarium* enzyme. Dihydrocoumarin hydrolase (DHase) from *Acinetobacter calcoaceticus* is a bifunctional enzyme, which catalyzes not only hydrolysis of aromatic lactones but also bromination of monochlorodimedon in the presence of H_2O_2 and dihydrocoumarin. DHase also hydrolyzes several linear esters, and is useful for enantioselective hydrolysis of methyl DL-β-acetylthioisobutyrate and regioselective hydrolysis of methyl cetraxate.

Keywords: lactonohydrolase, lactone, stereospecific hydrolysis, resioselective hydrolysis

INTRODUCTION

Lactone compounds, *i.e.* cyclic compounds possessing intramolecular ester bonds, are widely distributed in nature as various biologically active substrates, metabolic intermediates, etc. Lactonohydrolases, *i.e.* enzymes catalyzing the reversible or irreversible hydrolysis of lactone compounds, comprise some of the enzymes involved in the synthesis and degradation of these compounds. For example, gluconolactonase has been suggested to participate in the formation of L-gulono-γ-lactone from L-gulonate in L-ascorbate biosynthesis [1]. Such kind of aldonate lactone-hydro-lyzing enzymes, so-called "aldonolactonase", are involved in the oxidative degradation of aldoses [2,3]. Thus, a number of lactonohydrolases from various organisms have already been reported [4-12], but detailed investigation of them lays behind that of other common ester-hydrolyzing enzymes, *e.g.*, esterases and lipases.

Making use of their high stereoselectivity and reactivity under moderate conditions, many kinds of enzymes have been applied to the industrial production of organic compounds. Ester-hydrolyzing enzymes, such as lipases, also play important roles in practical organic syntheses, *e.g.*, optical resolution, transesterification, *etc.* [13-15]. The reactions catalyzed by lactonohydrolases, as well as those by lipases and other ester-hydrolyzing enzymes, are sometimes stereospecific and/or regioselective, and might be applicable to the synthesis of useful compounds, such as optically active compounds, structured lipids, *etc.*

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These facts promoted us to attempt to find novel lactonohydrolases. We screened various microorganisms for lactone-hydrolyzing activity and found several lactonohydrolases with unique properties. Here we report the characterization of these enzymes and their application to the synthesis of organic compounds.

LACTONOHYDROLASES ACTING ON PANTOYL LACTONE

Diversity of Lactonohydrolases Catalyzing the Stereospecific Hydrolysis of Pantoyl Lacone

Cells of various kinds of microorganisms were screened for stereospecific lactonohydrolase activity with either the D- or L-enantiomer of pantoyl lactone (PL) as the substrate [16-18]. PL-hydrolyzing activity was found to be widely distributed in various microorganisms (Table 1). While bacterial strains tended to hydrolyze the L-enantiomer of PL, fungal strains preferentially hydrolyzed the D-enantiomer. Strains of *Fusarium*, *Gibberella*, *Penicillium* and *Schizophyllum* showed relatively high activity toward D-PL. On the other hand, L-PL hydrolyzing activity was largely found in *Agrobacterium* and *Bacillus* strains.

Lactonohydrolase from *Fusarium oxysporum*

A D-PL hydrolyzing lactonohydrolase was isolated from *Fusarium oxysporum* AKU3702, which showed the highest activity and enantioselectivity toward D-PL among the screened microorganisms, and the enzyme was characterized in some detail [19] (Fig. 1(A) and Table 2). The relative molecular mass of the enzyme is 125

Table 1. Stereoselective hydrolysis of PL by microorganisms

Strain	Degree of hydrolysis (%)	Optical purity of PA formed (% *ee*)
D-PL-specific		
Fusarium oxysporum AKU3702	20.0	91.7
Fusarium anguioides AKU3703	19.5	85.0
Fusarium solani AKU3710	14.9	81.1
Gibberella fujikuroi AKU3802	10.0	68.0
Gibberella fujikuroi AKU3805	11.9	72.0
Serratia plymuthicum AKU62	9.85	13.8
Penicillium notatum AKU3406	7.22	58.3
Penicillium chrysogenum AKU3407	6.89	51.4
Penicillium lilacinum AKU3414	8.98	67.0
Caldariomyces fumago AKU3910	8.08	51.6
Schizophyllum commune AKU5035	8.26	63.8
Schizophyllum sp. AKU5041	6.80	56.8
Schizophyllum commune AKU5044	13.3	85.8
L-PL-specific		
Achromobacter polymorph AKU122	2.57	16.4
Bacillus thuringiensis AKU238	2.96	14.3
Bacillus brevis AKU248	2.77	13.8
Agrobacterium radiobacter AKU302	6.12	39.9
Agrobacterium tumefaciens AKU305	9.72	56.2
Agrobacterium radiobacter AKU306	9.23	57.0
Agrobacterium radiobacter AKU308	14.4	77.6
Agrobacterium tumefaciens AKU314	7.32	20.1
Agrobacterium tumefaciens AKU315	4.68	48.3
Agrobacterium tumefaciens AKU316	14.6	75.8

See references [16-18] for details.

kDa, and its subunit molecular mass is 60 kDa. The enzyme thus appears to be a dimer consisting of identical subunits. About 1 mol of calcium per subunit and 15.4% (w/w) glucose equivalent of carbohydrate are included in the enzyme. Calcium is necessary for both enzyme activity and stability. The enzyme carries three kinds of asparagine-linked high-mannose-type sugar chains at the 28th, 106th, 179th and 277th asparagine residues of the enzyme polypeptide. The carbohydrate moiety is an essential element for the stabilization of the enzyme. The enzyme hydrolyzes aldonate lactones, such as D-galactono-γ-lactone, L-mannono-γ-lactone and D-gulono-γ-lactone, stereospecifically. Not only are the corresponding enantiomers not hydrolyzed by the enzyme, but they competitively inhibit the enzyme activity. All the substrate lactones including D-PL have a downward hydroxy group at the 2-position, when the lactone rings are drawn according to Haworth's projection. For every substrate, the reverse reaction, *i.e.* the lactonization of aldonic acids and D-pantoic acid (D-PA), takes place under acidic pH conditions. Aromatic lactones, such as dihydrocoumarin, homogentisic acid lactone, 2-coumaranone and 3-isocoumaranone, are also effective substrates, but lactonization does not take place with these aromatic substrates.

Genomic DNA and cDNA encoding the enzyme were cloned and sequenced [20]. The lactonohydrolase genomic gene consists of six exons separated by five short introns. The deduced amino acid sequence of the lactonohydrolase shows significant similarity to those of gluconolactonase from *Zymomonas mobilis* [21], and paraoxonases from man and rabbit [22]. These results suggested that these enzymes might form a unique superfamily of C-O cleaving and P-O cleaving hydrolases, and recently it was revealed that some of these paraoxonases possess lactone hydrolyzing activity [23, 24].

Lactonohydrolase from *Agrobacterium tumefaciens*

An L-PL-hydrolyzing lactonohydrolase was isolated from *Agrobacterium tumefaciens* AKU316 [18] (Fig. 1(B) and Table 2). The relative molecular mass of the native enzyme is 62 kDa, and its subunit molecular mass is 26.5 kDa. This enzyme only hydrolyzes L-PL, *i.e.* D-PL is not accepted as a substrate. Various kinds of aromatic lactones, such as dihydrocoumarin, homogentisic-acid lactone, *etc.*, are also hydrolyzed, but aldonate lactones, which are good substrates for the *Fusarium* lactonohydrolase, are not hydrolyzed. Because of its high hydrolysis activity and stereospecificity toward aldonate lactones, the *Fusarium* enzyme is thought to participate in the metabolic pathway for aldose *in vivo*. On the other hand, the *Agrobacterium* lactonohydrolase is quite differ-

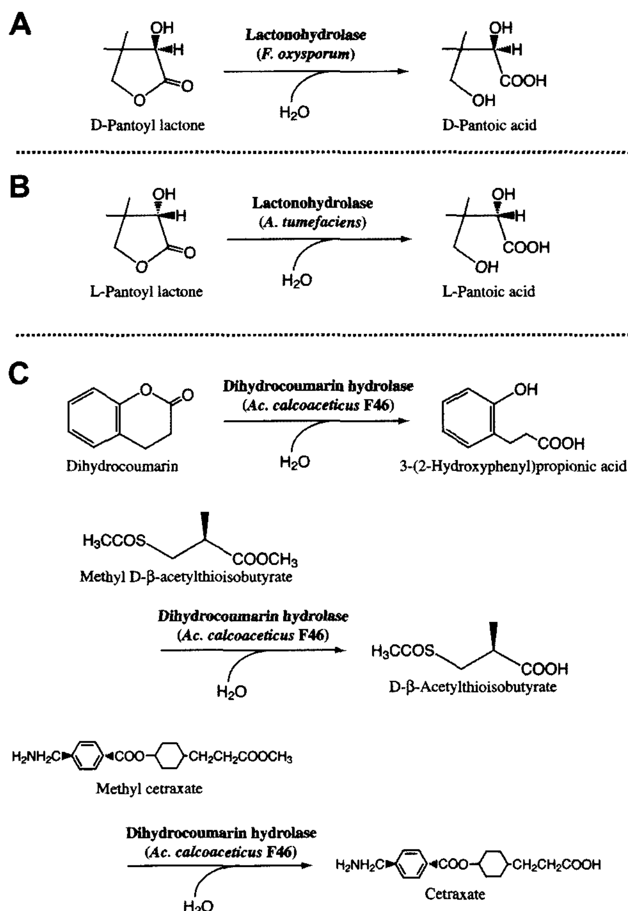

Fig. 1. The stereo- and regio-selective hydrolysis reactions catalyzed by microbial lactonohydrolases.

ent from the *Fusarium* one in many aspects other than substrate specificity. The deduced amino acid sequence of the *Agrobacterium* enzyme exhibits significant similarity to those of the polyketide cyclases of *Streptomyces peucetius* [25] and *Streptomyces argillaceus* [26]. These cyclases have been suggested to function in the biosynthesis of antitumor polyketides in these microorganisms. Although the lactonohydrolase reaction might be involved in the cyclization reaction in the biosynthetic pathway for polyketides, such secondary metabolites have not been found in *A. tumefaciens*.

Optical Resolution of Racemic Pantoyl Lactone with *Fusarium* Lactonohydrolase

D-Pantothenic acid and its derivatives are used as additives for animal feeds and as pharmaceutical products [27,28]. The commercial production of D-panto-thenate has been dependent exclusively on chemical synthesis including the optical resolution of racemic PL. A drawback of this chemical process is the troublesome resolution of racemic PL, which requires the use of an expensive alkaloid or chiral amine as a resolving reagent. We considered that the enantioselective lactonohydrolase reaction might be applicable to this optical reso lution step. For the practical purpose, the hydrolysis of the D-enantiomer is more advantageous than that of the L-enantiomer. In the case of a L-PL-specific lactonohydrolase, the optical purity of the remaining D-PL might be low, except when the hydrolysis of L-PL is complete. On the other hand, using a D-PL specific lactonohydrolase, D-PA, which can be easily converted to D-PL by heating under acidic conditions, of high optical purity could be constantly obtained regardless of the hydrolysis

Table 2. Properties of microbial lactonohydrolases

	F. oxysporum	*A. tumefaciens*	*A. calcoaceticus* F46
Native M_r	125,000	62,000	55,000
Subunit M_r (SDS-PAGE)	63,000	26,500	30,000
Number of subunits	2	2	2
Substrate specificity [K_m (mM) / V_{max} (μmol/min/mg)]			
Hydrolysis			
D-Pantoyl lactone	123 / 653	No activity	No activity
L-Pantoyl lactone	No activity	3.59 / 13.7	No activity
D-Galactono-γ-lactone	23 / 1,300	No activity	No activity
L-Mannono-γ-lactone	3.6 / 1,440	No activity	No activity
Dihydrocoumarin	6.3 / 2,800	2,560 / 7.74 × 10^4	0.801 / 4,760
Homogentisic-acid lactone	2.5 / 257	4.35 / 1.83 × 10^4	0.560 / 0.963
2-Coumaranone	8.7 / 701	455 / 1.52 × 10^4	0.761 / 8.04
3-Isochromanone	4.4/6.88	1.43/2.37	No activity
Lactonization			
D-Pantoic acid	9.1 / 103	No activity	No activity
D-Galactonic acid	52.6 / 216	No activity	No activity
Optimum pH (hydrolysis)	7-7.5	8.5	7.0
Optimum temperature (hydrolysis)	50°C	45°C	30°C
pH stability (30°C, 30 min)	5.0-10	> 5.0	> 5.0
Thermal stability [a]	< 50°C (pH 7.4, 20 min)	< 50°C (pH 7.4, 20 min)	< 75°C (pH 7.0, 30 min)
Inhibitors	Chelating reagents, L-pantoyl lactone, Mn^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}	Chelating reagents, Fe^{2+}, Pb^{2+}, Sn^{2+}, Al^{3+}, Fe^{3+}	Diisopropylphosphofluoridate, phenylmethanesulfonyl fluoride, Hg^{2+}, Fe^{3+}

[a] Enzymes retained 100% activity under the conditions with the indicated pHs or temperatures.

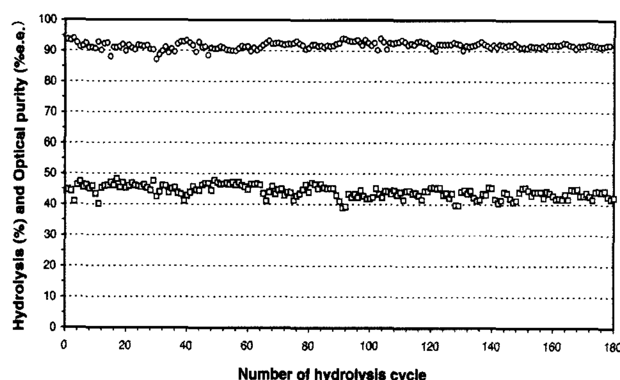

Fig. 2. Stereospecific hydrolysis of racemic PL by immobilized *F. oxysporum* cells. Here, 280 L of immobilized mycelia (comprising 15.2 kg of wet cells) was incubated with 350 L of an aqueous DL-PL solution (350 g/L) at 30°C for 21 h. The pH of the reaction mixture was automatically adjusted to 6.8-7.2 with 15 M NH_4OH. All the immobilized mycelia removed from the reaction mixture were used for subsequent reactions. The hydrolysis reaction was carried out 180 times. Symbols: O, optical purity of D-PL; □, hydrolysis rate.

yield. Thus, the *Fusarium* lactonohydrolase specific for the D-enantiomer of PL was used for the enzymatic resolution of racemic PL [16,17,29]. Under the optimal conditions, D-PL in a racemic mixture (700 mg/mL) was stereospecifically hydrolyzed to D-PA by *F. oxysporum* mycelia, with automatic control of the pH of the reaction mixture at 7.0. After 24 h, the amount of PL hydrolyzed in the reaction mixture reached 322 mg/ mL, with an optical purity of 96% *ee* for D-PA [17]. However, the mycelia recovered after the repeated reaction for several times had lost their hydrolytic activity and were inadequate for the practical use. The cutting of the mycelia by the stirring and the resulting leakage of the enzyme from the mycelia into the reaction mixture were suggested for this problem. In order to overcome this problem, the fungal mycelia were immobilized in calcium alginate gels. Using the resulting immobilized mycelia, practical hydrolysis of D-PL in a racemic mixture was possible. When the gels were incubated in 350 g/L DL-PL for 21 h at 30°C with automatic pH control (pH 6.8-7.2), 90-95% of the D-PL was hydrolyzed to D-PA of high optical purity (90-97% *ee*). After repeated batch-wise reactions for 180 cycles (*i.e.* 180 days), the immobilized mycelia retained 60% of the initial activity (Fig. 2). The improvement of the stability of the enzyme activity is thought to be partly due to calcium, as a stabilizer of the enzyme, contained in the gel. This enzymatic resolution has been commercially operated since 1999 by Daiichi Fine Chemicals, Japan, through which it has been shown that the new process is highly satisfactory not only from an economic aspect but also an environmental one (water –49%, CO_2 –30%, and BOD –62%, compared with the former chemical resolution method) (Fig. 3).

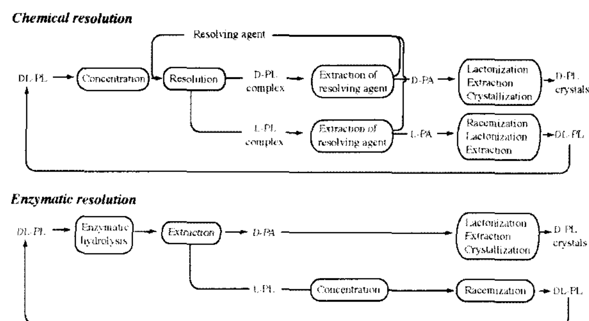

Fig. 3. Comparison of the enzymatic (lower) and conventional chemical (upper) resolution processes for DL-PL.

Since lactonohydrolases act on the intramolecular ester bonds of lactone compounds, modification of the starting substrates for the production of useful compounds, such as the esterification of carboxylic acids or acylation of alcohols, is not required, and there is no formation of by-products, *i.e.* alcohols or carboxylic acids. For example, the optical resolution of racemic PL, with *O*-acetyl- or *O*-formyl-PL, and lipases as the starting material and catalysts, respectively [30,31], requires the acylation of PL and produces acetate or formate as a by-product, while the resolution with lactonohydrolase is free from these unconvenient problems.

LACTONOHYDROLASE (DIHYDROCOUMARIN HYDROLASE) WITH HALOPEROXIDASE ACTIVITY FROM *ACINETOBACTER CALCOACETICUS*

Structure and Function Analysis of Dihydrocoumarin Hydrolase

Dihydrocoumarin hydrolase (DHase) from *Acinetobacter calcoaceticus* F46 [32] is one of the most unique lactonohydrolases, and a good example of the diversity of lactonohydrolases (Fig. 1(C) and Table 2). The enzyme was found through the screening for aromatic lactone-hydrolyzing enzymes, and specifically hydrolyzes dihydrocoumarin. Aromatic lactones other than dihydrocoumarin, such as 2-coumaranone and homogentisic acid lactone, also serve as substrates, but their susceptibilities relative to that of dihydrocoumarin are quite low. Aldonate lactones, which are good substrates for the *Fusarium* lactonohydrolase, do not serve as substrates. With regard to the reverse reactions, no detectable amounts of lactone compounds were formed. The molecular mass of the native enzyme is 52 kDa, and its subunit molecular mass is 30 kDa.

The gene encoding DHase was cloned and sequenced. The deduced amino acid sequence of DHase exhibits high similarity to those of serine-esterases of *Pseudomonas* strains [33-35] and bacterial haloperoxidases [36-40]. All of these enzymes including DHase contain a con-

sensus motif, Gly-Xaa-Ser-Xaa-Gly, around the active serine residue of the catalytic triad. Serine-enzyme inhibitors such as diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride inactivate the enzyme. These facts suggest that DHase might be a member of the serine-enzyme family [41].

Haloperoxidases are enzymes that catalyze the halogenation of various organic compounds in the presence of H_2O_2 and halide ions, and can be roughly divided into two groups. One is either heme- or vanadium-dependent and the other does not contain a prosthetic group or any metal ions [42]. All bacterial haloperoxidases are classified into the latter group. The catalytic triads of serine-esterases are conserved in the amino acid sequences of bacterial haloperoxidases, and some of them show not only halogenation activity but also ester-hydrolyzing activity [43]. DHase also catalyzes the bromination of monochlorodimedon on incubation with H_2O_2 and dihydrocoumarin (or an organic acid, such as acetic or *n*-butyric acid) [32]. The reaction mechanism for the halogenation reaction catalyzed by DHase (Fig. 4A) has been suggested to be similar to those of non-heme haloperoxidases [44,45] (Fig. 4B), 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 monochlorodimedon by the hypohalous acid. In brief, this halogenation reaction is caused by the peroxidation of organic acids catalyzed by DHase.

However, it seems improbable that DHase *in vivo* catalyzes the formation of peroxiacids, which is very toxic for cells. So, we predicted that DHase catalyzes the reverse reaction, *i.e.* hydrolytic degradation of peroxoacids, *in vivo*. Actually, the purified DHase could catalyze the hydrolysis of peracetic acid to acetic acid and H_2O_2, and a DHase-defective mutant derived from *A. calcoaceticus* F46 was more sensitive to growth inhibition by peracetic acid than the parent strain. On the other hand, recombinant *E. coli* cells expressing the DHase gene were more resistant to peracetic acid. In addition, the putative catalase gene was found downstream of the DHase coding gene, and Northern blot hybridization analysis revealed that both of them were transcript as part of a polycistronic mRNA [Honda, Kataoka, Shimizu, unpublished observation]. These suggest that DHase detoxifies peroxoacids in conjuction with the catalase, *i.e.* at first, peroxoacids are hydrolyzed to the corresponding organic acids and H_2O_2 by DHase, and then the resulting H_2O_2 is degraded by the catalase.

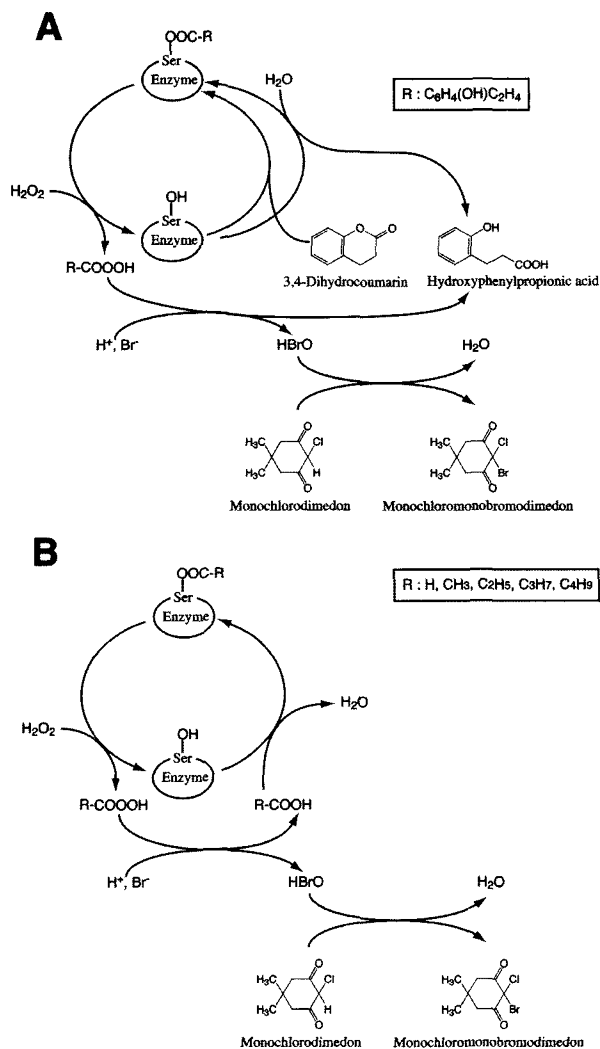

Fig. 4. Predicted reaction mechanisms of the bromination and hydrolysis reactions with DHase. The bromination and hydrolysis reactions with dihydrocoumarin catalyzed by DHase (A), and the bromination reaction with organic acids (B) are shown.

Application of Dihydrocoumarin Hydrolase to Enantio- and Regio-selective Hydrolysis Reactions

The DHase of *A. calcoaceticus* F46 catalyzes the hydrolysis of several linear esters other than the aromatic lactones described in the previous section. The substrate specificity of DHase toward linear esters is very characteristic, *i.e.* a) the enzyme is specific toward methyl esters, b) it recognizes the configuration at the 2-position, and c) it hydrolyzes diesters to monoesters. Taking advantage of these properties, we attempted the stereoselective hydrolysis of methyl β-acetylthioisobutyrate, which has a chiral center at its 2-position, and regioselective hydrolysis of cetraxate methyl ester, which has two ester bonds in its molecule, using DHase as the catalyst (Fig. 1C). The products of these enzymatic hydrolysis reactions were identified as D-β-acetylthioisobutyrate (>99% *ee*) and cetraxate, respectively.

D-β-acetylthioisobutyrate is an important intermedi-

ate for the synthesis of a series of angiotensin converting enzyme inhibitors [34,46-48]. Concerning the preparation of D-β-acetylthioisobutyrate, several processes have been reported, such as optical resolution of DL-β-acetylthioisobutyrate using various resolving agents and chemical synthesis involving D-β-hydroxyisobu-tyrate as a starting material [49]. However, these processes are quite complicated, and require large amounts of energy and expensive agents. To overcome these problems, the asymmetric hydrolysis of methyl DL-β-acetylthioisobutyrate with DHase was attempted [50]. Recombinant *E. coli* cells were used as the catalyst. Under the optimal conditions, *i.e.* using 2.5% (w/v) cells and 50% (w/v) methyl DL-β-acetylthioisobutyrate in the reaction mixture, and stirring at 30°C with pH adjustment to 7.0 with 6 M NaOH, the D-form of the racemic substrate was completely hydrolyzed to the corresponding acid in 4 h. The formation of L-β-acetylthioisobutyrate was barely detected. D-β-Acetyl-thioisobutyrate and the remaining methyl L-β-acetyl-thioisobutyrate in the reaction mixture could be separately extracted by changing the pH of the reaction mixture with a solvent such as ethyl acetate or methyl isobutyl ketone.

Cetraxate hydrochloride, which is widely used as an antiulcer medicine, has been industrially produced by means of chemical deprotection of the terminal ester of the esterified intermediate, benzyl cetraxate [51,52]. The regioselective deblocking of the terminal ester of benzyl cetraxate is quite difficult because of the coexisting phenyl ester in the molecule. Kuroda *et al.* reported the regioselective hydrolysis of the terminal ester bond of benzyl cetraxate by *Aspergillus niger* cellulase [51]. The *Aspergillus* enzyme however, scarcely acts on the lower alkyl esters of cetraxate. From the industrial viewpoint, the hydrolysis of lower alkyl esters of cetraxate is more advantageous than that of benzyl ester, because of the troublesome synthesis of benzyl ester substrate and the considerable decrease in the molecular weight after the deprotection. We have developed a method of regioselective hydrolysis of methyl esters of cetraxate with DHase [50]. The recombinant *E. coli* cells expressing the DHase gene could hydrolyze 25% (w/v) of methyl cetraxate to cetraxate, quantitatively. After a 1 h reaction, all of the substrate was converted to cetraxate, without any formation of by-products, such as hydroxyphenyl propionic acid.

Thus, DHase, which exhibits significant hydrolysis activity, with stereo and/or regioselectivity, toward methyl esters of β-acetylthioisobutyrate and cetraxate, might be applicable to the enzymatic synthesis of useful compounds, similarl to *Fusarium* lactonohydrolase.

PROSPECT

The use of "ester-hydrolyzing enzymes" in industrial production processes for useful compounds should increase in the future. Previous studies on ester-hydrolyzing enzymes as industrial catalysts were concentrated on well-known enzymes such as lipases and esterases. As shown here, lactonohydrolases are expected as alternative ester-hydrolyzing enzymes.

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