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Enantioselectivity-promoting factor in enzyme-mediated asymmetric hydrolysis of enol esters

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Abstract

Enantioselectivity-promoting factor enhances enantioselectivity of protonation in lipase AP-catalyzed asymmetric hydrolysis of enol esters. The factor was partially purified by chromatography using Phenyl-TOYOPEARL 650M and Sephacryl S-200HR. The hydrolysis of 2-benzyl-1-cyclohexenyl acetate by PLE in the presence of the purified factor produced (*R*)-2-benzylcyclohexanone in 92% ee, while the reaction without the factor gave the racemate.

Keywords: Asymmetric hydrolysis; Enantioselectivity; Enol esters; Hydrolysis

1. Introduction

Optically active α-substituted ketones are important intermediates for the synthesis of natural products. Enantioselective protonation of prochiral enolates is a very simple and attractive procedure for the preparation of α-chiral ketones [1–12]. In the previous papers, we reported that enzyme-mediated enantioselective protonation was achieved by hydrolysis of enol esters with a yeast *Pichia farinosa* IAM 4682 [1–4]. Recently, we reported a novel mechanistic aspect of the reaction, i.e., enantioselectivity at protonation was promoted by an unknown factor derived from *P. farinosa* (Scheme 1) [5]. The factor also promotes the asymmetric hydrolysis of enol esters by other hydrolytic enzymes. The factor appeared to be a protein, however, its insolubility made further purifications difficult.

It has been revealed that the reaction of 2-benzyl-1-cyclohexenyl acetate (**1a**, R = Me) with lipase AP gave an optically active (*R*)-2-benzylcyclohexanone (**2**), while other conventional hydrolytic enzymes, such as lipase OF and pig liver esterase (PLE), gave only the racemate **2**. We then investigated the characteristics of the asymmetric hydrolysis with lipase AP.

2. Experimental

2.1. Materials

Ketone **2** was synthesized as previously described [3]. Enol ester **1a** was prepared by a standard method [13] from the corresponding racemic ketone **2**. The spectral data (^{1}H-NMR and IR) of **1a** were identical with the previous reported data [3]. The other substrates were pre-

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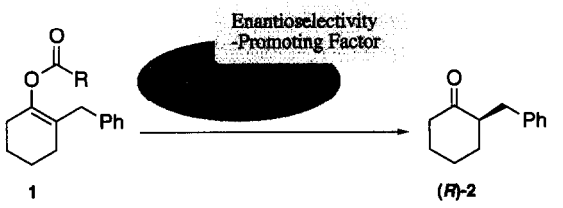

Scheme 1. Asymmetric hydrolysis of enol esters.

pared by the same procedure from the corresponding ketones. Lipase (EC 3.1.21.1) AP from *Aspergillus niger* was purchased from Amano Pharmaceutical Co., Ltd. (AP-12), lipase OF from *Candida rugosa* from Meito Sangyo Co., and PLE (EC 3.1.1.1) in $(NH_4)_2SO_4$ suspension from Sigma Chemical Co. (No. E-3128). Standard proteins (albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen A, 25 kDa) were purchased from Pharmacia–LKB Biochemistry. All other chemicals were also obtained from commercial sources.

2.2. Preparation of lipase AP solution

Commercially available lipase AP was suspended in 6 ml/g of Buffer A (pH 7.5, 50 mM sodium phosphate including 1 mM EDTA and 10 mM 2-mercaptoethanol), stirred at 0°C for 2 h, and centrifuged at 3,000 rpm for 15 min. The supernatant (Fraction I) was used in the following experiments.

All purifications were carried out at 4°C. Protein was measured by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc.) with bovine plasma γ-globulin as the standard.

2.3. Measurement of enantiomeric excess

Cyclic enol ester **1a** was chosen as the substrate for assay of enantioselectivity because the enantiomeric excess (ee) of the product **2** can be easily determined by HPLC analysis. The absolute configuration of **2** was determined by comparing sign of optical rotation with that reported, $[\alpha]_D$ +41.4° (*c* 5, MeOH), *R*-form (88% ee) [14]. A mixture (total volume, 1 ml) consisting in 10% ethanol solution of **1a** (20 μl, 9.3 mM), 380 μl of Buffer B (pH 6.5, 100 mM sodium phosphate), Buffer A, PLE (1.5 μl, 1,850 units/ml) and each fraction from chromatography was incubated at 30°C for 15 min. The products were extracted with diethyl ether, and the organic layer was passed through a short column on silica gel and dried over anhydrous Na_2SO_4. After evaporation under reduced pressure, the yield and the ee of **2** were analyzed by HPLC (CHIRALCEL OJ, Daicel Chemical Industries, Ltd.; eluent, hexane/2-propanol = 96/4; flow rate, 0.5 ml/min) [6]. Spontaneous hydrolysis of the substrate did not occur under the reaction condition.

2.4. Enzyme assay

One unit is defined as the amount of enzyme that has the hydrolytic ability of enol esters to convert 1 μmol of 2-methyl-1-cyclohexenyl acetate (**3**) to 2-methylcyclohexanone (**4**) at 37°C in 1 min. Total volume of 200 μl of Buffer B contained 10% ethanol solution of **3** (5 μl, 3.1 mM) and the enzyme solution was incubated for 10 min. After the reaction was terminated by addition of diethyl ether, the yield of **4** was determined by GLC. The conditions of GLC analysis were as follows: column, 15% butanediol succinate (GL Sciences Inc.); injection, 110°C; oven, 90 → 110°C; carrier gas, N_2; flow rate, 55 ml/min.

3. Results and discussion

3.1. Reaction using commercially available lipase AP

First, conversion of **1a** with lipase AP (Fraction I) to **2** was carried out under various conditions. Fig. 1 shows the characteristic features of the reaction. Increasing the volume of Fraction I improved not only the reactivity but also the ee of the resulting ketone (*R*)-**2** (Fig. 1a). On the other hand, heat treatment of Fraction I at more than 50°C for 3 min prior to incubation scarcely changed the reactivity, but caused a remarkable decrease in enantioselectivity (Fig. 1b). These results were similar to those previously observed for *P. fari-*

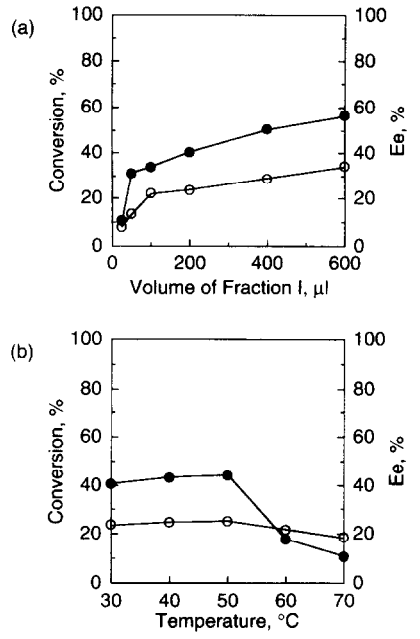

Fig. 1. (a) Enantioselective protonation of **1a** to **2** using Fraction I. (b) Thermal stability of Fraction I. The reaction was carried out using 100 μl of Fraction I. Conversion % (○); The ee of **2** (●).

nosa [1,3,5]. We assumed that the reaction mechanism was the same as that of *P. farinosa*, that is, commercially available lipase AP also included an unknown factor which promoted enantioselectivity during facially selective hydrolysis. In the following section, we called the factor as 'enantioselectivity-promoting factor' and examined the chromatographic studies to elucidate the factor in the lipase.

3.2. Purification steps

To Fraction I (from 15 g of lipase AP), $(NH_4)_2SO_4$ was added to 20% saturation and the suspension was centrifuged (10,000 rpm, 10 min). The supernatant (Fraction II) was subjected to hydrophobic interaction chromatography (Fig. 2). Fraction II was diluted with an equal volume of Buffer A containing 20% $(NH_{42}SO_4$ (Buffer C) and then loaded onto a ϕ 2.2 cm × 18 cm Phenyl-TOYOPEARL 650M column (TOSOH Co. Ltd.) equilibrated with Buffer C. The column was washed with 360 ml of Buffer C and developed with a gradient from 20 to 0% $(NH_4)_2SO_4$ in 240 ml of Buffer A, followed by eluting with the same buffer. Hydrolytic activity existed in fractions 20–35 and 43–47 but enantioselectivity appeared at fractions 37–41 (Fraction IIIa) and fractions 49–51 (Fraction IIIb), which indicate that ee peaks obviously differ from hydrolytic activity peaks.

A portion of Fraction IIIa or IIIb was further purified on a ϕ 1.5 cm × 80 cm Sephacryl S-200HR (Pharmacia–LKB Biochemistry) gel filtration column equilibrated with Buffer A containing 0.5 M NaCl. Elution of Fractions IIIa and IIIb with the same buffer gave the ee peak at fractions 15–25 (Fraction IVa) and at fractions 19–29 (Fraction IVb), respectively (Fig. 3). Although these fractions can show higher enantioselectivity in this reaction system, the use of a single fraction means that the hydrolysis of the substrate proceeds to a negligible extent (Fraction IVa, ca. 0.5 units/ml; IVb, 0 unit/ml), compared with the one with use of PLE described above. These results indicate that the enantioselectivity-promoting factor in Fractions IVa and IVb enhances enantioselectivity of hydrolysis. This is the first example of partially purified enantioselectivity-promoting factor. The characteristics of the factor are still unspecified, however the gel

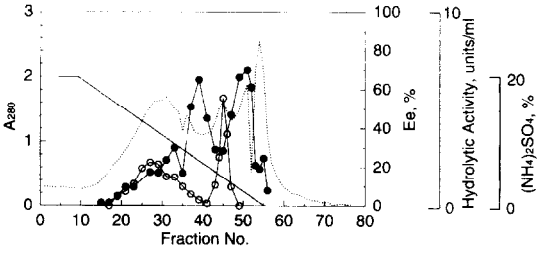

Fig. 2. Phenyl-TOYOPEARL column chromatographic profile. Abs (···); The ee of **2** (●); Hydrolytic activity (○); Conc. of $(NH_4)_2SO_4$ (—).

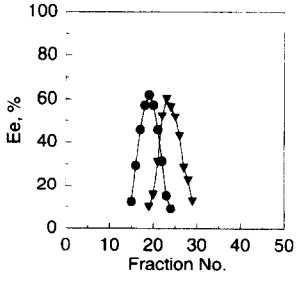

Fig. 3. Sephacryl S-200HR column profile. The ee of **2** from Fraction IIIa (●); The ee of **2** from Fraction IIIb (▼).

filtration profile shows that the factor would be macromolecular compounds (ca. 60 kDa in Fraction IVa; ca. 40 kDa in Fraction IVb).

Fraction IVb on standing at 4°C for 2 days was found to have reduced the enantioselectivity (30% ee → 9% ee) while Fraction IVa on standing under the same conditions did not. These results show that the factor in Fraction IVa is easier to handle than that in Fraction IVb.

3.3. Asymmetric reaction using PLE and the purified factor

Next, the effect of the purified factor to the enantioselectivity of PLE-catalyzed hydrolysis of **1a** was examined. The partially purified factor (Fraction V) was prepared from lipase AP by successive treatment by Phenyl-TOYOPEARL column chromatography, 80% $(NH_4)_2SO_4$ precipitation of Fraction IIIa, and dialyzation against Buffer A. Through these steps, the factor was purified from 10,200 mg protein (70 g of lipase AP) in 420 ml to 120 mg protein in 8 ml. Fraction V contained a small amount of hydrolytic enzymes which correspond to 11 units/ml. The asymmetric hydrolysis of **1a** with Fraction V (100 μl), PLE (1.5 μl) in Buffer B (total, 1 ml) at 30°C for 15 min gave the chiral ketone (*R*)-**2** in 92% ee, $[\alpha]_D^{23}$ +41.4° (*c* 0.36, MeOH) (Table 1), while the reaction without Fraction V gave the racemate **2**. The present reaction afforded enantioselectivity much better than that using the whole cells of *P. farinosa* [1,3,5]. The reaction was completed within about 12 h without racemization of the product and formation of byproducts.

Table 1
Enantioselective protonation of **1** to **2**

	R	Time min	Conv. %	ee of **2** %
1a	Me	15	33	92 [a]
1b	n-Pr	15	18	86
1c	Ph	60	17	77

[a] $[\alpha]_D^{23}$ +41.4° (*c* 0.36, MeOH).

We then examined the effect of structure of acyl group in the substrate. As shown in Table 1, changing the structure of the acyl moiety affected the reaction of **1** to **2** with Fraction V and PLE. The n-butyryl derivative (**1b**, R = n-Pr) gave lower selectivity (86% ee) than **1a** (92% ee). Displacement of acetyl moiety to benzoyl (**1c**, R = Ph) remarkably reduced the reactivity (17% conversion in 60 min) and the enantioselectivity (77% ee).

Although the details are not yet clear, the interaction between the hydrolytic enzyme and the enantioselectivity-promoting factor under the hydrolytic process must be very important in the asymmetric hydrolysis of the enol ester.

4. Conclusion

We have disclosed that during the lipase AP catalyzed asymmetric hydrolysis of enol ester **1**, the enantioselectivity-promoting factor enhances the enantioselectivity of the α-chiral ketone **2**. The hydrolysis of **1a** even with PLE increases its enantioselectivity by addition of the partially purified factor. These results shed light on the reaction mechanism of the enzyme-mediated asymmetric protonation. Further investigations are now in progress.

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