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Asymmetric transformation of enol acetates with esterases from *Marchantia polymorpha*

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Abstract

Two esterases catalyzing the asymmetric hydrolysis of enol acetates to give optically active α-alkylated ketones were isolated from cultured cells of *Marchantia polymorpha* by a three-step procedure: hydrophobic chromatography, anion exchange chromatography and gel-filtration chromatography. These esterases had opposite stereoselectivities on protonation of the enol intermediate in the hydrolysis and one of them obviously reversed the stereoselectivity when the chain length and the bulkiness of substituents at the β-position to the acetoxyl group were increased. The internal amino acid sequences of peptide fragments obtained by the proteolysis of the esterases with lysyl endopeptidase had no similarity to those of other hydrolytic enzymes.

Keywords: *Marchantia polymorpha*; Esterase; Enantioface-differentiating hydrolysis; Enol acetate; Optically active ketone

1. Introduction

Optically active carbonyl compounds bearing a stereogenic center α to the carbonyl group are important reaction intermediates or synthons for the asymmetric synthesis [1–3]. Enantioselective protonation of prochiral enol derivatives is a very simple and attractive route for the preparation of optically active α-substituted ketones and a number of examples on the protonation of metal enolates by chiral proton sources have been reported [4–11]. Recently, a new type of enzymatic hydrolysis has been disclosed, e.g., the hydrolysis of enol esters with yeast and plant cell cultures of liverwort *Marchantia polymorpha* to afford optically active α-substituted ketones [12–17]. It was also found that the hydrolytic enzymes from yeast and commercially available lipases from microorganisms could exhibit enantioselectivity in the hydrolysis of enol esters only in the presence of an enantioselectivity-promoting factor partially purified from yeast [18,19]. However, there is no information on the stereoselectivity of hydrolytic enzymes participating in the hydrolysis of enol esters from plant cells. We have isolated two esterases which participate in the asymmetric hydrolysis of enol acetates from *M. polymorpha* [20], and here report the characterization and the partial amino acid sequencing of the esterases in detail.

2. Experimental

2.1. Materials

Cyclohexanone enol acetates, **1**–**7**, were prepared by treatment of their corresponding ketones with perchloric acid and acetic anhydride [21]. The following chromatography media were used: Butyl-Toyopearl, DEAE-Toyopearl (both from TOSO Co. Ltd.), Sephadex G-75 (Pharmacia). For the estimation of molecular mass, protein standards (Pharmacia) were used. All other chemicals were purchased from commercial sources as reagent grade.

2.2. Analysis

Analytical and prep. TLC was carried out on glass sheets (0.25 and 0.5 mm) coated with silica gel (Merck silica gel

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60; GF_{254}). GLC analyses were carried out with FID and a capillary column (0.25 mm × 25 m) coated with 0.25 μm CP cyclodextrin β 236M-19 (WCOT) using N_2 as carrier gas (column temperature: 100 °C, split ratio: 50, make up: 50 ml min^{-1}); GC-MS was carried out with a capillary column (0.25 mm × 25 m) coated with 0.25 μm OV-101.

2.3. Purification of esterases from cultured cells of M. polymorpha

Suspension cells of *M. polymorpha* [22] were cultivated in MSK-II medium on a rotary shaker (75 rpm) at 25 °C for 3 weeks prior to use for enzyme preparation. All purification steps were carried out at 4 °C. Cultured cells of *M. polymorpha* (500 g, fr. wt) were frozen with liquid nitrogen and added to 1000 ml potassium phosphate buffer (100 mM, pH 7.0). The mixture was homogenated in a Waring Blender and the homogenate was then filtered through four layers of cheesecloth. The filtrate was centrifuged (10 000 × *g* for 30 min) to give crude enzyme. The crude enzyme fraction was subjected to $(NH_4)_2SO_4$ precipitation. The precipitated protein between 40 and 80% saturation was collected after centrifugation (10 000 × *g* for 30 min) and solubilized in 100 ml potassium phosphate buffer (50 mM, pH 7.0) containing 30% $(NH_4)_2SO_4$ (buffer A). The protein solution was added to a Butyl-Toyopearl column (2 cm × 30 cm) which was pre-equilibrated with buffer A. After washing the column with buffer A of 200 ml, the enzymes were eluted with a linear $(NH_4)_2SO_4$ gradient (30–0%). Two esterases named Est I and II were obtained in different fractions. Fractions containing these esterase activities were individually combined and dialyzed overnight against 3000 ml Tris–HCl buffer (25 mM, pH 8.0; buffer B). The protein solution was then applied to a DEAE-Toyopearl column (1.5 cm × 20 cm). After washing the column with 100 ml of buffer B, proteins were eluted with a linear gradient of NaCl (0–1 M). Fractions of 5 ml were collected and assayed for esterase activity. The active fractions were combined and concentrated by ultra-filtration. The enzyme solution was chromatographed on a 1.5 cm × 100 cm column containing Sephadex G-75, which had been equilibrated earlier with potassium phosphate buffer (25 mM, pH 7.0; buffer C). The column was eluted with buffer C and the fractions exhibiting esterase activity were combined and used for experiments.

The molecular masses of esterases were estimated by gel-filtration chromatography on a Sephadex G-75 column and calibrated with the following protein standards: bovine serum albumin (67k), ovalbumin (43k), chymotrypsinogen A (25k) and ribonuclease A (13.7k). SDS-PAGE was performed on a vertical slab gel according to the standard protocol by Laemmli [23]. After electrophoresis, the protein bands were visualized by staining with Coomassie Brilliant Blue. The molecular masses of esterase subunits were calculated by using the LMW electrophoresis calibration kit (Pharmacia).

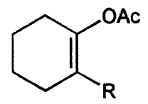
2.4. Determination of esterase activity

The standard assay mixture consisted of 2 ml of the sodium phosphate buffer containing enzyme (pH 7.0) and 2-methylcyclohexanone enol acetate **1** (5 mg). The reaction was carried out at 35 °C for 5 min. The reaction mixture was extracted with *n*-pentane and the product was identified by direct comparison with the authentic sample by GLC and GC-MS analyses. It was confirmed that neither non-enzymatic hydrolysis nor racemization of the product occurred under the incubation conditions. One unit of enzyme activity is defined as the quantity of enzyme that hydrolyses 1 μmol of **1** per 1 min under the conditions given above. Protein concentrations were determined by the Bradford dye-binding assay using BSA as a standard [24].

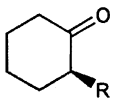
The influence of the substrate concentration on the initial velocity was measured using esterases, which were concentrated by ultra-filtration (150 μg/ml), under standard conditions. Initial rates of substrate hydrolysis were obtained for each substrate concentration (0.05–5 mM), and apparent Michaelis constants (K_m) were calculated from direct linear plots.

2.5. Product identification

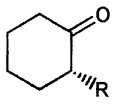
In order to obtain the products adequate for identification, incubation of substrates with esterases was performed in a similar condition to the standard assay system except that the scale was 50-fold enlarged and the reaction mixture was incubated for 4 h. The absolute configuration and optical purity of the resulting ketone in the enzymatic hydrolysis of (**1**–**7**) were determined by circular dichroism (CD) spectra and the peak area of the corresponding enantiomers by GLC analyses on CP cyclodextrin β 236M-19. Retention times for the products in the GLC were as follows: **8** and **15**, 11.8 and 12.8 min; **9** and **16**, 12.7 and 12.9 min; **10** and **17**, 23.8 and 24.0 min; **11** and **18**, 18.1 and 18.4 min; **12** and **19**, 27.7 and 27.9 min; **13** and **20**, 72.1 and 72.8 min; **14** and **21**, 60.1 and 61.2 min. The CD data of the products obtained in the hydrolysis with Est I were the following—**8**: $[\theta]_{288}$ + 990 (c 0.25, MeOH) (lit. [25]: $[\theta]_{288}$ − 987 for *R* enantiomer (**15**)); **9**: $[\theta]_{288}$ + 351 (c 0.25, MeOH) (lit. [26]: $[\theta]_{288}$ + 2200); **10**: $[\theta]_{288}$ + 361 (c 0.12, MeOH) (lit. [27]: $[\theta]_{288}$ + 2126); **18**: $[\theta]_{288}$ − 86 (c 0.15, MeOH) (lit. [28]: $[\theta]_{288}$ + 1690 for *R* enantiomer (**11**)); **19**: $[\theta]_{288}$ − 2485 (c 0.25, MeOH) (lit. [26]: $[\theta]_{288}$ + 2480 for *S* enantiomer (**12**)); **20**: $[\theta]_{288}$ − 669 (c 0.14, MeOH); **21**: $[\theta]_{288}$ − 1990 (c 0.15, MeOH) (lit. [26]: $[\theta]_{288}$ + 1750 for *R* enantiomer (**14**)). The CD data of the products obtained in the hydrolysis with Est II were the following—**12**: $[\theta]_{288}$ + 99 (c 0.09, MeOH); **13**: $[\theta]_{288}$ + 173 (c 0.17, MeOH); **14**: $[\theta]_{288}$ + 278 (c 0.15, MeOH). The CD data of the products (**11**, **15**–**17**) could not be obtained due to the low yield and the lack of the products.



1: R=Me **5**: R=*n*-Pr
2: R=Et **6**: R=*n*-Pnt
3: R=*i*-Pr **7**: R=Bnz
4: R=*t*-Bu



8: R=Me **12**: R=*n*-Pr
9: R=Et **13**: R=*n*-Pnt
10: R=*i*-Pr **14**: R=Bnz
11: R=*t*-Bu



15: R=Me **19**: R=*n*-Pr
16: R=Et **20**: R=*n*-Pnt
17: R=*i*-Pr **21**: R=Bnz
18: R=*t*-Bu

2.6. Sequencing of esterases

The Est I and II were subjected to SDS-PAGE (12.5% gel), respectively, and then blotted onto a polyvinylidene disulfide membrane (Immobilon P^{SQ}, Millipore) by semi dry blotting method. Attempts to sequence the esterases using the Edman degradation with an Applied Biosystems Model 473A pulsed liquid sequencer with an online phenylthiohydantoin amino acids analyzer were unsuccessful. The esterases, therefore, were digested with lysyl endopeptidase directly in the SDS-gels. The resulting peptides (18k peptide No. 1 from Est I and 20k peptide No. 2 from Est II) were sequenced.

The obtained peptide sequences were used for screening the SwissProt protein sequences library by the sequence analysis program BLAST.

3. Results and discussion

A cell free extract was obtained from cultured cells of *M. polymorpha*. The crude extract was subjected to chromatography on a Butyl-Toyopearl column to give two esterases (Est I and II) (Fig. 1). The results of a typical purification procedure of Est I and II are shown in Tables 1 and 2, respectively. The molecular mass in the native state of Est I was estimated to be 54 kDa by gel-filtration chromatography on a Sephadex G-75 column using marker proteins.

Table 1
Purification of the Est I from cultured cells of *M. polymorpha*

Step	Total protein (mg)	Total activity (U[a])	Specific activity (U/mg)	Fold
Crude extract	35	22	0.63	1
Butyl-Toyopearl	1.8	13	7.2	11
DEAE-Toyopearl	0.5	12	24	38
Sephadex G-75	0.08	10	125	198

[a] One unit of esterase activity is defined as the quantity of enzyme that hydrolyses 1 μmol of **1** per 1 min under the standard assay condition.

Table 2
Purification of the Est II from cultured cells of *M. polymorpha*

Step	Total protein (mg)	Total activity (U[a])	Specific activity (U/mg)	Fold
Crude extract	35	22	0.63	1
Butyl-Toyopearl	0.6	1.2	2.0	3
DEAE-Toyopearl	0.2	1.1	5.5	9
Sephadex G-75	0.03	0.9	30	48

[a] One unit of esterase activity is defined as the quantity of enzyme that hydrolyses 1 μmol of **1** per 1 min under the standard assay condition.

Electrophoresis on SDS-PAGE showed a single protein band with estimated molecular mass of 27 kDa (Fig. 2). The comparison of the molecular masses obtained by these methods reveals that Est I holoenzyme is a 54 kDa dimer with two 27 kDa subunits. Est II was determined to be a 45 kDa dimer structure composed of two 22.5 kDa subunits by the same methods. The N-termini of both Est I and Est II were blocked. The internal sequences of esterases were analyzed after digestion with a peptidase as described in Section 2 (Table 3). Comparisons of these peptide sequences against a protein database using the NCBI "blastp" program showed no significant homology with protein which has a function relating to hydrolase activity.

Enzymatic hydrolyses of several cyclohexanone enol acetates (**1**–**7**) with these esterases were carried out to clarify

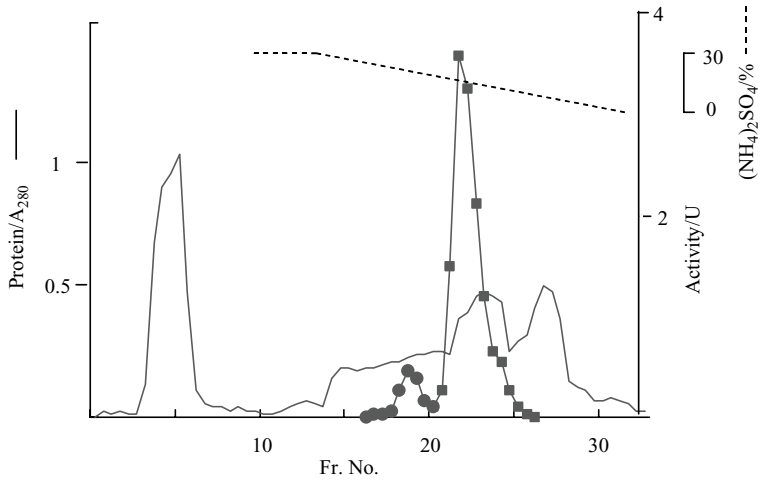


Fig. 1. Elution profiles of esterases on Butyl-Toyopearl column chromatography: (■) Est I; (●) Est II.

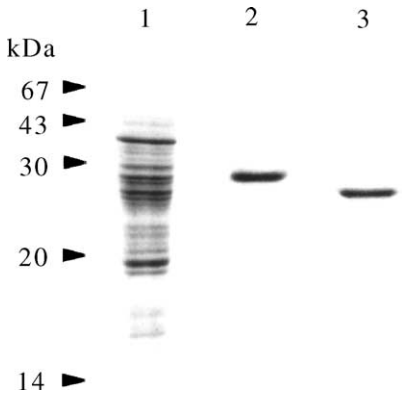


Fig. 2. SDS-PAGE analyses of esterases: lane 1, crude extract; lane 2, Est I; lane 3, Est II.

Table 3
Sequences of esterase peptide fragments after digestion with lysyl endopeptidase

No. of peptides	Amino acid sequence
No. 1 (Est I)	Leu–Leu–Glu–Thr–Gly–Val–Arg–Val–Thr–Trp
No. 2 (Est II)	Ile–Gln–Ala–Ile–Ser–Leu–Trp–Ala–Met–Ala–Glu

the effect of various substituents at the β-position to the acetoxyl group on the enantiomeric ratio and the catalytic activity of enzymes. The conversion yield and enantiomeric excess in the hydrolysis with Est I are summarized in Table 4. Hydrolysis of **1**–**3** by Est I gave the corresponding optically active ketones (**8**–**10**). The protonation of the enol intermediates from **1**–**3** occurred preferentially from the same enantiotopic face of the C=C bond, judged by the fact that the CD curves of the products (**8**–**10**) were positive. However, when **4**–**7** were used as substrates, the CD curves of the corresponding products (**18**–**21**) were negative. This result demonstrates that the stereoselectivity of Est I in the protonation of these enol intermediates is reversed by long chain ($C \geq 3$) and bulky substituent at the β-position to the acetoxyl group. Surprisingly, Est I converted **7** into optically active **21** in high optical yield (>99% e.e.) although the hydrolysis of **7** with hydrolytic enzymes from yeast gave racemic ketone [18,19]. On the other hand, the conversion yield and enantiomeric purity in the hydrolysis of **1**–**7** by Est II were low in comparison to the case of Est I (Table 5). Nevertheless, the stereoselectivity of Est II in the protonation of the enol intermediate might be opposite to that of Est I.

Table 4
Enantioselectivity on the hydrolysis of enol acetates by Est I

Substrate	Product	Conversion (%)	e.e. (%)	Configuration[a]
1	**8**	>99	>99	*S*
2	**9**	>99	14	*S*
3	**10**	10	17	*R*
4	**18**	21	5	*S*
5	**19**	>99	>99	*R*
6	**20**	20	26	*R*
7	**21**	15	>99	*S*

[a] Preferred configuration at the α-position to the carbonyl group of the products.

Table 5
Enantioselectivity on the hydrolysis of enol acetates by Est II

Substrate	Product	Conversion (%)	e.e. (%)	Configuration[a]
1	**15**	4	4	*R*[b]
2	**16**	3	2	*R*[b]
3	**17**	3	3	*S*[b]
4	**11**	20	2	*R*[b]
5	**12**	5	4	*S*[b]
6	**13**	16	7	*S*[b]
7	**14**	11	14	*R*[b]

[a] Preferred configuration at the α-position to the carbonyl group of the products.
[b] Hard to deduce.

Table 6
Kinetic parameters for the Est I-catalyzed hydrolysis of enol acetates

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
1	0.09	77	855
2	0.11	63	572
3	1.87	14	7
4	2.54	7	3
5	0.54	79	146
6	0.72	42	58
7	0.97	20	21

In order to clarify the substrate specificity of Est I, the kinetic constants for several enol acetates were measured (Table 6). The K_m value for the acetate **1** was the smallest among these substrates. The K_m value increased when long substituents were introduced at the β-position to the acetoxyl group; the K_m value for **6** having *n*-pentyl group as the β-substituent was eight times larger than that for **1**. When *i*-propyl and *t*-butyl groups were introduced as the β-substituent (**3** and **4**), the K_m values drastically increased. These facts indicate that Est I prefers acetate with short β-substituent rather than that with long and bulky β-substituent. On the other hand, the specificity of Est II was slightly different from that of Est I (Table 7). In the case of the substrates **1**–**6**, Est II showed the similar tendency to Est I; the K_m value increased when long and bulky substituents were introduced at the β-position to the acetoxyl group. Interestingly, the K_m value for **7** was the smallest among the substrates tested, indicating that Est II was specific to the substrate having phenyl group as the β-substituent.

Table 7
Kinetic parameters for the Est II-catalyzed hydrolysis of enol acetates

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
1	0.07	76	1086
2	0.15	52	347
3	0.81	11	14
4	2.01	17	8
5	0.20	19	95
6	0.30	26	87
7	0.06	33	550

Thus, two esterases with opposite stereoselectivities in the enantioselective hydrolysis of enol esters were isolated from cultured cells of *M. polymorpha*. It was shown that the enantioselectivity of Est I reversed in the hydrolysis of substrate with long side chain, bulky *t*-butyl group or benzyl group at β-position to the acetoxyl group, contrasted with the substrate having short side chain. It is postulated that the turn over of the substrate in the active site of the enzyme due to steric hindrance by the β-substituent may cause the inversion of the enontioselectivity in the protonation of enol intermediate. Recently, Matsumoto et al. reported that the enantioselectivity-promoting factor from yeast operated the enantioselectivity of hydrolytic enzymes from yeast and commercially available lipases from microorganisms [18,19]. The esterases from *M. polymorpha* were able to differentiate the enantiotopic face of the C=C bond of the enol intermediate in the hydrolysis without enantioselectivity-promoting factor, suggesting that they are apparently different from the hydrolytic enzymes from microorganisms. Analysis of the whole sequences using molecular biological techniques based on the sequences obtained here seems to be necessary in order to understand the molecular bases of their properties.

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