ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 8 (2000) 255–264

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Biotransformation of enones with biocatalysts — two enone reductases from *Astasia longa*

Kei Shimoda, Toshifumi Hirata *

Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan

Received 14 May 1999; received in revised form 7 June 1999; accepted 11 June 1999

Abstract

The stereochemistry and mechanism in the reduction of the C–C double bond of carvone by the cultured cells of *Astasia longa*, a nonchlorophyllous cell line classified in *Euglenales*, was studied. The reduction of the C–C double bond of carvone with the cultured cells involved the *anti*-addition of hydrogen atom from the *si* face at the α-position and the *re* face at the β-position of carbonyl group. Two different enone reductases were isolated from the cultured cells of *A. longa*. Both reductases catalyzed stereospecifically the *anti*-addition of hydrogen atoms from the *si* face at C-1 and the *re* face at C-6. However, one of the reductases participated in a hydrogen transfer of the *pro*-4*R* hydrogen of NADH to C-6 position of carvone and the other used the *pro*-4*S* hydrogen of NADH.

Keywords: *Astasia longa*; *Euglenales*; Stereochemistry; Enone; Carvone; Reduction of C–C double bond; Enone reductase

1. Introduction

Several studies on the reduction of enones with plant cell cultures have been reported [1–8]. In recent studies [9,10], it has been shown that *Euglena gracilis* Z reduces stereospecifically the C–C double bond of enones and possesses enone reductase of molecular mass 55 kDa which reduces the C–C double bond of carvone by *anti*-addition of hydrogen atoms from the *si* face at C-1 and the *re* face at C-6. In extension of the studies, *Astasia longa*, which is a colorless relative of *Euglena* that contains no visible proplastid-like bodies [11], was chosen as a biocatalyst for the biotransformation of enones. Although *A. longa* cells are considered to contain enone reductases similarly to that from *E. gracilis* Z [10], two different enone reductases were isolated from *A. longa* cells. We now report on the isolation of the enone reductases and stereochemistry in the reduction of the C–C double bond of enones using *A. longa* cells and the enone reductases from the cells.

* Corresponding author. Fax: +81-824-24-7435; E-mail: thirata@sci.hiroshima-u.ac.jp

1381-1177/00/$ - see front matter
PII: S1381-1177(99)00076-4

2. Experimental

2.1. Analysis

Analytical and prep. TLC (0.25 mm thick) were carried out on a silica-gel plate (Merck, Type 60, GF_{254}). GC analysis was performed by using a capillary column (0.25 mm × 25 m) coated by CP cyclodextrin β 236M-19 with N_2 as carrier gas (column temp., 100°C; split ratio of 50:1; flow late of N_2, 50 ml min^{-1}). ^1H NMR spectra were obtained on a JEOL GSX-270 spectrometer using tetramethylsilane as an internal standard in $CDCl_3$.

2.2. Cultivation

A. longa (cat. #K3-15-2725, Carolina Biol. Supply) was cultivated in the medium containing Na-acetate (1 g), polypepton (1 g), tryptone (2 g), yeast extract (2 g) and $CaCl_2$ (10 mg) in 1000 ml of water on a rotary shaker (70 rpm) at 25°C in the dark. Full growth cells (ca. 200 mg as wet wt. in 100 ml medium) cultivated for 3 weeks under continuous shaking was used in the present work.

2.3. Substrates

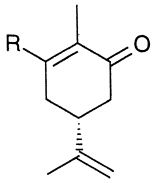
(*R*)-(−)-Carvone (**1a**), $[\alpha]_D^{25} - 60.1$ (neat) and (*S*)-(+)-carvone (**1c**), $[\alpha]_D^{25} + 57.1$ (neat) were purchased from Aldrich.

(*R*)-[6-^{2}H]Carvone (**1b**) [96% ^{2}H-enrichment; IR ν_{max} cm^{-1}: 1670 (C=O); ^1H NMR: δ 1.75 (s, 3H, 8-Me), 1.78 (s, 3H, 1-Me), 2.21–2.75 (m, 5H, 3-H_2, 4-H and 5-H_2), 4.76 (s, 1H, 9-H*cis*), and 4.80 (s, 1H, 9-H*trans*); ^2H NMR: δ 6.80 (s, 2-^{2}H); MS *m/z* (rel. int): 151 $[M]^+$ (10), 136 $[M - CH_3]^+$ (4), 109 (31), 94 (29) and 83 (100); $[\alpha]_D^{25} - 58$ (EtOH; c 1.0)] was prepared by reduction of (*S*)-(+)-carvone (**1c**) with NaB^2H_4 (99.9% atom D; Wako), isomerization of the resultant (+)-*cis*-carveol to (−)-enantiomer, and then oxidation of the enantiomer [12].

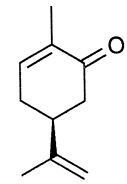
2.4. Biotransformation of (R)- and (S)-carvones (*1a* and *1c*) with the cultures of A. longa

The substrate (10 mg per flask; total 50 mg) was added to the medium (200 ml per 500 ml conical flask) containing *A. longa* cells (about 400 mg cells per flask) and the cultures were incubated at 25°C for 5 days on a rotary shaker in the dark. For time course experiments, aliquotes (100 ml) of the medium were taken from the cultured broth (1000 ml per 2000 ml conical flask) at a regular time interval. After incubation, the micro-organisms were removed by centrifugation at 600 × *g* for 10 min and the broth was extracted with diethyl ether. The total recoveries of products and substrates in the ether extracts were more than 80%. The transformation products were isolated from the ether extract by a prep. GC using a glass column (3 mm × 2 m) packed with 15% DEGS with N_2 as carrier gas (column temp, 100°C; flow rate of N_2, 50 ml min^{-1}), and identified by direct comparison of physical

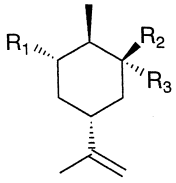
constants, TLC, GLC and spectral data with those of authentic samples [10]. Physicochemical and spectroscopic data of the products are as follows:

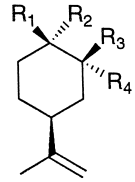


1a R = H
1b R = ^{2}H



1c





2a R_1 = H, R_2,R_3 = O
3a R_1 = H, R_2 = OH, R_3 = H
2b R_1 = ^{2}H, R_2,R_3 = O
3b R_1 = ^{2}H, R_2 = OH, R_3 = H

2c R_1 = Me, R_2 = H, R_3,R_4 = O
3c R_1 = Me, R_2 = H, R_3 = OH, R_4 = H
4 R_1 = H, R_2 = Me, R_3,R_4 = O

(1*R*,4*R*)-Dihydrocarvone (**2a**). $[\alpha]_D^{25}$ + 12.5 (EtOH; c 0.16) [Ref. [13] $[\alpha]_D^{25}$ + 13.1 (EtOH; c 6)]; IR ν_{max} cm^{-1} 1710 (C=O) and 1642 (C=C); ^{1}H NMR: δ 1.03 (d, 3H, J = 6 Hz, 1-Me), 1.75 (s, 3H, 8-Me), 4.77 (brs, 2H, > C=C*H*$_2$); MS *m*/*z* (rel. int): 152 $[M]^+$ (25), 137 $[M - Me]^+$ (19), 109 (35), 95 (81), 67 (100).

(1*R*,4*S*)-Isodihydrocarvone (**2c**). $[\alpha]_D^{25}$ − 19.0 (EtOH; c 0.1) [Ref. [14] $[\alpha]_D^{25}$ − 16.0 (EtOH; c 7.6)]; IR ν_{max} cm^{-1}: 1710 (C=O) and 1640 (C=C); ^{1}H NMR: δ 1.08 (d, 3H, J = 6 Hz, 1-Me), 1.75 (s, 3H, 8-Me), 4.81 (d, 2H, J = 8 Hz, > C=C*H*$_2$); MS *m*/*z* (rel. int): 152 $[M]^+$ (39), 137 $[M - Me]^+$ (15), 109 (40), 95 (92), 67 (100).

(1*R*,2*S*,4*R*)-Neodihydrocarveol (**3a**). $[\alpha]_D^{25}$ + 29.1 (EtOH; c 0.30) [Ref. [13] $[\alpha]_D^{25}$ + 32.3 (EtOH; c 1.5)]; IR ν_{max} cm^{-1} 3442 (OH) and 1645 (C=C); ^{1}H NMR: δ 0.98 (d, 3H, J = 6 Hz, 1-Me), 1.70 (s, 3H, 8-Me), 3.92 (brs, 1H, > C*H*–OH), 4.73 (brs, 2H, > C=C*H*$_2$); MS *m*/*z* (rel. int): 154 $[M]^+$ (9), 136 $[M - H_2O]^+$ (56), 121 (68), 107 (77), 93 (53), 79 (61), 41 (100).

(1*R*,2*S*,4*S*)-Neoisodihydrocarveol (**3c**). $[\alpha]_D^{25}$ − 20.0 (EtOH; c 0.2) [Ref. [14] $[\alpha]_D^{25}$ − 25.4 (EtOH; c 2.0)]; IR ν_{max} cm^{-1} 3374 (OH) and 1646 (C=C); ^{1}H NMR: δ 0.94 (d, 3H, J = 7 Hz, 1-Me), 1.72 (s, 3H, 8-Me), 3.75 (brs, 1H, > CH–OH), 4.70 (brs, 2H, > C=C*H*$_2$); MS *m*/*z* (rel. int): 154 $[M]^+$ (11), 136 $[M - H_2O]^+$ (56), 121 (67), 107 (75), 93 (100), 79 (55), 41 (65).

*2.5. Biotransformation of (R)-[6-^{2}H]carvone (**1b**) with the cultures of A. longa*

Incubation of (*R*)-[6-^{2}H]carvone (**1b**) (10 mg per flask; total 50 mg) with *A. longa* in the same procedure as above gave ^{2}H-labeled dihydrocarvone (**2b**) [*m*/*z* (rel. int.) 153 $[M + 1]^+$ (5), 138 (11),

109 (35), 95 (81), 67 (100); ^{1}H NMR ($CDCl_3$) δ 1.03 (d, 3H, $J = 6$ Hz, 1-Me), 1.75 (s, 3H, 8-Me), 4.77 (brs, 2H, $>C=CH_2$); ^{2}H NMR ($CHCl_3$) δ 2.13 (s, 6-^{2}H *cis*-oriented to the 1-H)] and neodihydrocarveol (**3b**) [m/z (rel. int.) 155 $[M+1]^+$ (9), 137 (75), 122 (71), 108 (100), 94 (67) and 79 (55); ^{2}H NMR ($CHCl_3$) δ 1.44 (s, 6-^{2}H *cis*-oriented to the 1-H)].

2.6. Preparation of (4R)- and (4S)-[4-^{2}H]NADH

Following the reported method [15], (4*R*)-[4-^{2}H]NADH was prepared by reduction of β-NAD^+ (120 mg) with EtOH-d_6 (99% ^{2}H-enrichment; 400 mg) and yeast alcohol dehydrogenase (27 units; 10 mg). The crude product was subjected to chromatography on a DEAE-Toyopearl column to give (4*R*)-[4-^{2}H]NADH (60 mg, 99% ^{2}H-enrichment), ^{1}H NMR (D_2O) δ 2.66 (brs, 1H, 4-H), 6.18 (d, 1H, OCH_2) and 6.90 (s, 1H, 2-H).

(4*S*)-[4-^{2}H]NADH was prepared by enzymatic reduction of [4-^{2}H]NAD^+ (120 mg) with EtOH (400 mg) and yeast alcohol dehydrogenase (27 units; 10 mg; Wako). The reaction mixture was chromatographed on a DEAE-Toyopearl column to give (4*S*)-[4-^{2}H]NADH (30 mg, 99% ^{2}H-enrichment), ^{1}H NMR (D_2O) δ 2.81 (brs, 1H, 4-H), 6.19 (d, 1H, OCH_2) and 6.92 (s, 1H, 2-H).

2.7. Isolation of reductases from A. longa

A. longa cells (5 g) harvested 3 weeks after inoculation was frozen with liquid N_2 and homogenized with 100 mM Na–Pi buffer (pH 6.8, 10 ml) containing 0.25 M sucrose, 10% glycerol, 5 mM dithiothreitol, 5 mM $Na_2S_2O_5$ and 0.5% Triton X-100 in a Waring Blendor. All purification procedures were carried out at 4°C. Protein concentrations were determined according to the Bradford method [16] with bovine serum albumin as standard. The homogenate was centrifuged ($10\,000 \times g$ for 10 min) and the supernatant was desalted on a Sephadex G-25 column using Tris buffer (pH 8.0) containing 1 mM $Na_2S_2O_5$ and 1 mM dithiothreitol. The protein material was then applied onto a DEAE-Toyopearl column (3.1×23 cm) equilibrated with the standard buffer. After elution of unadsorbed protein, a linear gradient of NaCl in the standard buffer with a slope of 0–0.5 M was started. Two enone reductases named Reductase-I and -II were obtained in different fractions. The enone reductases were separately subjected to the affinity chromatography on Blue-Toyopearl 650 ML column (1.2×9 cm) equilibrated with the standard buffer. After elution of unadsorbed protein, the enzymes were eluted with a linear NaCl gradient (0–2 M in the standard buffer). The purification schemes of the reductases are summarized in Tables 1 and 2. Effluent fractions containing the active enzyme were pooled and used for experiments.

The molecular masses of the enzymes were estimated by the gel filtration of native enzymes through a Sephadex G-150 column (1.5×75 cm) using aldolase, bovine serum albumin, ovalbumin and ribonuclease A as marker proteins and the SDS-PAGE under denaturating conditions according to

Table 1
Purification of Reductase-I from *A. longa*

	Total protein(mg)	Total reductase (units)	Specific activity (units/mg pro.)	Fold
Crude extract	98.0	0.45	0.005	1
DEAE-Toyopearl	5.4	0.32	0.059	12
Blue-Toyopearl	0.01	0.012	1.2	240

Table 2
Purification of Reductase-II from *A. longa*

	Total protein (mg)	Total reductase (units)	Specific activity (units/mg pro.)	Fold
Crude extract	98.0	0.45	0.005	1
DEAE-Toyopearl	9.1	0.41	0.045	9
Blue-Toyopearl	0.02	0.02	1.0	200

Laemmli's method [17]. The stacking gel consisted of 3% and running gel of 12.5% acrylamide gels were stained with Coomasie Blue.

For estimating the pH optimum of the enzyme reaction, the reaction mixture was composed of 100 mM 3-(*N*-morpholino)propane sulfonic acid buffer with pH adjusted from 6.0 to 8.5.

2.8. Enzyme assay

The standard assay mixture consisted of 1 ml enzyme prep. in 25 mM Na–Pi buffer with pH adjusted to 7.2, 3 μmol (*R*)-carvone (**1a**), 6 μmol NADH and 0.1 ml 1% Triton X-100. The reaction mixture was incubated for 12 h at 36°C and then extracted with diethylether. The ether layer was subjected to GLC and GC-MS analyses. The enzyme activity was expressed as unit, which is defined as the enzyme amount of producing 1 μmol of dihydrocarvone per minute.

*2.9. Incubation of (R)-carvone (**1a**) in the presence of deuterated NADH and the deuterated medium with enone reductases*

In order to gain enough of the products, incubation of (*R*)-carvone (**1a**) in the presence of deuterated NADH and the deuterated medium with enone reductases was performed in similar condition as used for the standard assay system except that the scale was 10- to 20-fold enlarged. The incubation product was extracted with diethyl ether and isolated by preparative GLC. The spectral data of the resulting dihydrocarvone under each incubation condition are listed below. The ^{2}H enrichment factor of deuterated dihydrocarvone was calculated from the molecular ion at m/z 152 and m/z 153.

(a) Dihydrocarvone obtained in the presence of (4*R*)-[4-^{2}H]NADH with Reductase-I: m/z (rel. int.) 153 $[M+1]^+$ (20), 138 (9), 110 (32), 95 (55) and 67 (100), 99% atom D; ^{2}H NMR ($CHCl_3$) δ 1.37 (s, 6-^{2}H *trans*-oriented to the 1-H).

(b) Dihydrocarvone obtained in the presence of (4*S*)-[4-^{2}H]NADH with Reductase-I: m/z (rel. int.) 152 $[M]^+$ (7), 137 (5), 109 (27), 95 (58) and 67 (100); ^{2}H NMR ($CHCl_3$) no ^{2}H-signal was detected.

(c) Dihydrocarvone obtained in the presence of ^2H_2O with Reductase-I: m/z (rel. int.) 153 $[M+1]^+$ (11), 138 (5), 110 (26), 95 (53) and 67 (100), 99% atom D; ^{2}H NMR ($CHCl_3$) δ 2.37 (s, 1-^{2}H).

(d) Dihydrocarvone obtained in the presence of (4*R*)-[4-^{2}H]NADH with Reductase-II: m/z (rel. int.) 152 $[M]^+$ (3), 137 (3), 109 (13), 95 (53) and 67 (100); ^{2}H NMR ($CHCl_3$) no ^{2}H-signal was detected.

(e) Dihydrocarvone obtained in the presence of (4*S*)-[4-^{2}H]NADH with Reductase-II: m/z (rel. int.) 153 $[M+1]^+$ (8), 138 (8), 110 (17), 95 (37) and 67 (100), 99% atom D; ^{2}H NMR ($CHCl_3$) δ 1.37 (s, 6-^{2}H *trans*-oriented to the 1-H).

(f) Dihydrocarvone obtained in the presence of ^2H_2O with Reductase-II: m/z (rel. int.) 153 $[M + 1]^+$ (12), 138 (6), 110 (12), 95 (45) and 67 (100), 99% atom D; ^2H NMR ($CHCl_3$) δ 2.37 (s, 1-^2H).

3. Results and discussion

3.1. Biotransformation of carvones by the cultures of A. longa

The time courses in the biotransformation of (*R*)- and (*S*)-carvones (**1a** and **1c**) by *A. longa* are shown in Fig. 1. In the case of the reduction of (*R*)-carvone (**1a**), the cells reduced the C–C double bond adjacent to the carbonyl group and then the carbonyl group to give (1*R*,4*R*)-dihydrocarvone (**2a**) and (1*R*,2*S*,4*R*)-neodihydrocarveol (**3a**), but the formation of (1*S*,4*R*)-isodihydrocarvone and (1*S*,2*S*,4*R*)-isodihydrocarveol was not found. On the other hand, in the case of (*S*)-carvone (**1c**), (1*R*,4*S*)-isodihydrocarvone (**2c**) and (1*R*,2*S*,4*S*)-neoisodihydrocarveol (**3c**) were specifically obtained. These observations showed that the stereospecificity in the reduction of the C–C double bond was quite high, although the stereospecificity in the reduction of C–C double bond by the cultured cells of *Nicotiana tabacum* was not strict, e.g., the cultured cells of *N. tabacum* reduced (*S*)-carvone (**1c**), yielding both of (1*R*,4*S*)-isodihydrocarvone (**2c**) and (1*S*,4*S*)-dihydrocarvone (**4**) [1].

The preferential formation of these products indicates that reduction of carvones occurred stereospecifically resulting in the products with the configuration of *R* at C-1 and *S* at C-2. These observations showed that the hydrogen attack at the conjugated C–C double bond takes place stereospecifically from the *si* face at C-1 of (*R*)- and (*S*)-carvones (**1a** and **1c**). To complete the

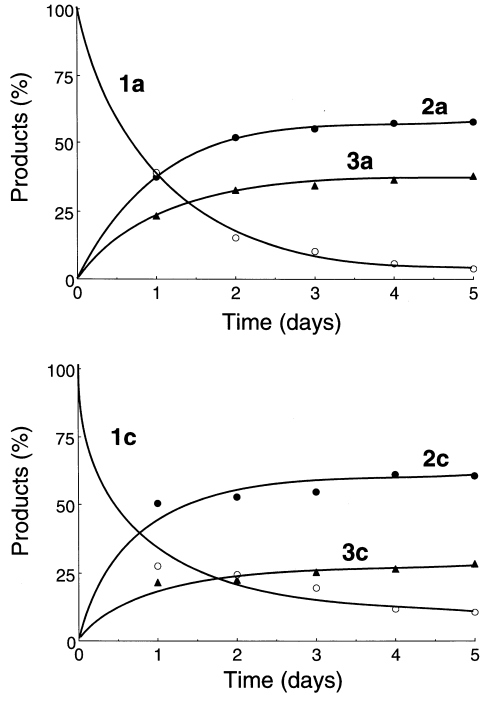


Fig. 1. Time courses for the biotransformation of (*R*)- and (*S*)-carvone (**1a** and **1c**) by *A. longa*.

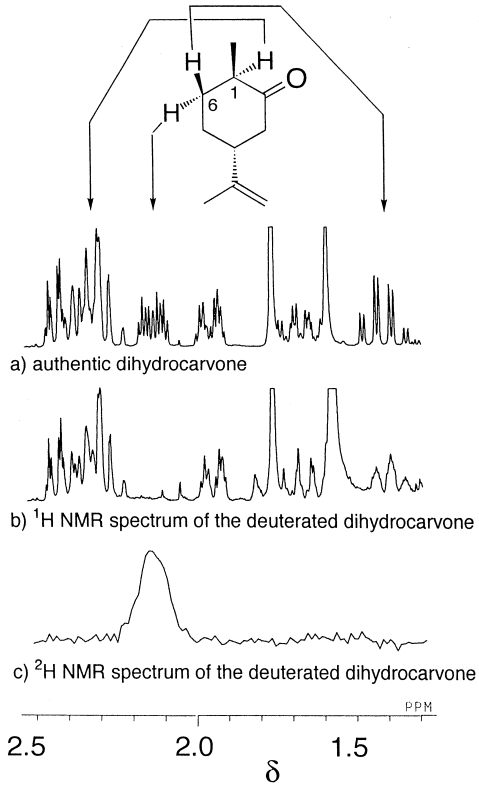


Fig. 2. Comparison of the ^{1}H and ^{2}H NMR spectra of ^{2}H-labeled product **2b** obtained by the reduction of (*R*)-[6-^{2}H]carvone (**1b**) with that of nonlabeled dihydrocarvone (**2a**).

stereochemistry in the reduction of the C–C double bond, the hydrogen addition to the 6-position of carvone was studied by examining the ^{2}H-orientation in the reduction product in the biotransformation of (*R*)-[6-^{2}H]carvone (**1b**) by *A. longa*. Incubation of **1b** (96% ^{2}H-enrichment) with *A. longa* was carried out in a manner described in Section 2 to yield deuterated dihydrocarvone (**2b**) and neodihydrocarveol (**3b**). The ^{1}H NMR spectrum of **2b** revealed the absence of the signal at δ 2.13 due to the *cis*-proton at C-6 to the hydrogen atom at C-1 of authentic dihydrocarvone [Fig. 2(b)]. The ^{2}H NMR spectrum of **2b** showed only a signal at δ 2.13 due to the ^{2}H at C-6 *cis* oriented to the hydrogen atom at C-1 [Fig. 2(c)]. Such a stereochemistry in the reduction was confirmed by the fact that the ^{2}H NMR spectrum of **3b** exhibited a signal at δ 1.44 due to the ^{2}H at C-6 *cis* oriented to the hydrogen atom at C-1. Therefore, it was demonstrated that the *pro-R* hydrogen atom at the 6-position of dihydrocarvone (**2b**) was introduced when the C–C double bond of carvone was reduced.

Thus, the stereochemistry of the hydrogen addition in the reduction of carvone with *A. longa* was established as shown in Fig. 3. The hydrogen attack to the carbocyclic double bond takes place

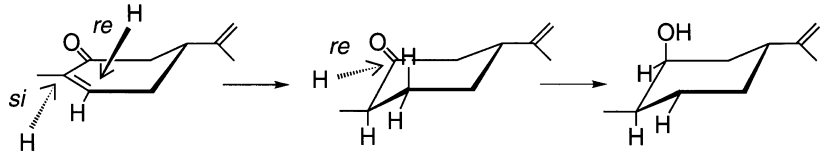


Fig. 3. Stereospecific hydrogen attack in the reduction of the C–C double bond and the carbonyl group of (*R*)-carvone (**1a**) by *A. longa*.

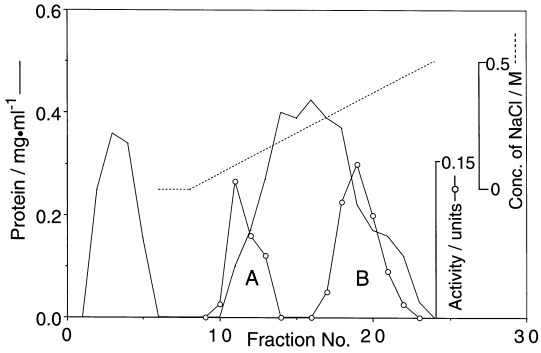


Fig. 4. Elution profiles of Reductase-I and -II by DEAE-Toyopearl column. Peak A: activity for Reductase-I; Peak B: activity for Reductase-II.

stereospecifically from the *si* face at C-1 and the *re* face at C-6 by *anti*-addition. In addition, the hydrogen attack to the carbonyl group at the 2-position takes place stereospecifically from the *re* face.

3.2. Reduction of carvones by enone reductases

In order to clarify the enzymatic reduction of carvones, enone reductases were isolated from the cultured cells of *A. longa*. The elution profiles of enone reductases in chromatography on DEAE-Toyopearl are portrayed in Fig. 4. Reductase-I and -II were isolated by further purification of a Blue-Toyopearl column.

Gel chromatography on a Sephadex G-150 column indicated that molecular masses of the native Reductase-I and -II were 35 and 36 kDa, respectively. Reductase-I showed only one protein band at 35 kDa on SDS gel electrophoresis, while Reductase-II showed the band at 36 kDa (Fig. 5). These observations indicate that both of the native Reductase-I and -II are considered to be monomer proteins. pH optimum was 7.2 for Reductase-I and 7.3 for Reductase-II. Reductase-I required NADH and NADPH as coenzymes. NADH was a better donor of hydride ion by a factor of 3 as compared to NADPH. On the other hand, Reductase-II with NADH showed almost the same activity with NADPH. FAD and FMN were ineffective for both Reductase-I and -II.

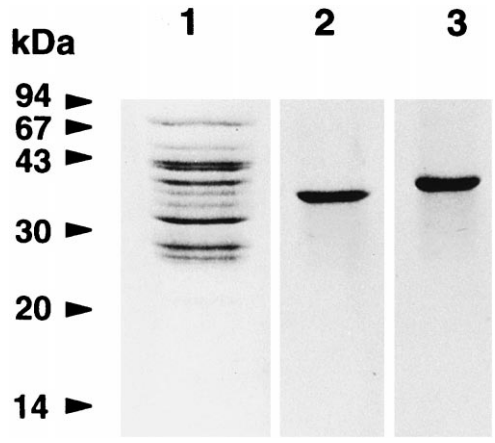


Fig. 5. SDS-gel electrophoretic analyses of the enone reductases from *A. longa* cells. 1: Crude extract; 2: Reductase-I; 3: Reductase-II.

In the reduction of carvones with the Reductase-I and -II, (*R*)-carvone (**1a**) was reduced stereospecifically to yield (1*R*,4*R*)-dihydrocarvone (**2a**) and (*S*)-carvone (**1c**) was converted to (1*R*,4*S*)-isodihydrocarvone (**2c**). In order to determine the stereochemistry of the hydrogen attack in the reduction of the C–C double bond by the enone reductases, conversion of (*R*)-carvone (**1a**) was performed in the presence of (4*R*)-[4-^{2}H]NADH, (4*S*)-[4-^{2}H]NADH or deuterated medium. In case of the reduction of **1a** by Reductase-I together with (4*R*)- and (4*S*)-[4-^{2}H]NADH, the mass spectra of resulting dihydrocarvones showed a molecular ion peak at *m/z* 153 and 152, respectively. This indicate that ^{2}H from only (4*R*)-[4-^{2}H]NADH was incorporated in the enzymatic reduction. ^{2}H NMR spectrum of dihydrocarvone obtained in the incubation with (4*R*)-[4-^{2}H]NADH showed a signal at δ 1.37 due to the ^{2}H at C-6 *trans*-oriented to the hydrogen atom at C-1. On the other hand, in the case of reduction of **1a** by Reductase-I in a deuterated medium (^2H_2O), the ^{2}H NMR spectrum of obtained dihydrocarvone showed only a signal at δ 2.37 due to the ^{2}H at C-1 (99% atom D). These observations indicate that the deuterium atoms at C-6 and C-1 of dihydrocarvone originated from (4*R*)-[4-^{2}H]NADH and ^2H_2O, respectively.

In another set of experiments with Reductase-II, significant incorporation of ^{2}H (99% atom D) was observed in the reduction of **1a** with (4*S*)-[4-^{2}H]NADH. The ^{2}H NMR spectrum of the ^{2}H-labeled product showed that ^{2}H atom was located at C-6 *trans* to the hydrogen atom at C-1. On the other hand, it was found that ^{2}H labeled site of dihydrocarvone produced in the presence of ^2H_2O was C-1 by ^{2}H NMR measurement of deuterated product. These results suggest that the deuterium atoms at C-6 and C-1 of dihydrocarvone originated from (4*S*)-[4-^{2}H]NADH and ^2H_2O, respectively.

Thus, two enone reductases, Reductase-I and Reductase-II, were isolated from *A. longa* cells. It was clarified that the reduction of the C=C bond of carvones with Reductase-I occurs stereospecifically by the *anti*-addition of hydrogen atoms from the *si* face at C-1 and the *re* face at C-6 of the C=C bond of carvone (**1a**). The hydrogen atoms participating in the reduction at C-1 and C-6 originated from the medium and the *pro*-4*R* hydrogen of NADH, respectively. Reductase-I was similar to the reductase from *E. gracilis* Z in respect to the stereochemistry of hydrogen transfer. On the other hand, the reduction of the C=C bond of **1a** with Reductase-II also involved the *anti*-addition of hydrogen atoms from the *si* face at C-1 and the *re* face at C-6. However, it was found that the *pro*-4*S* hydrogen of NADH participated in the reduction at C-6. In comparison with the enone reductase in *E. gracilis* Z cells, it is interesting that *A. longa*, a nonchlorophyllous cell line of *Euglenales*, contained two different enone reductases.

Acknowledgements

The authors thank Instrument Center for Chemical Analysis of Hiroshima University for the measurements of ^{1}H and ^{2}H NMR spectra. The authors thank Dr. Toshinobu Suzaki, Faculty of Science, University of Kobe, for supplying the cultures of *A. longa*.

References

[1] T. Hirata, H. Hamada, T. Aoki, T. Suga, Phytochemistry 21 (1982) 2209.
[2] T. Suga, H. Hamada, T. Hirata, Chem. Lett. (1987) 471.
[3] T. Suga, T. Hirata, H. Hamada, S. Murakami, Phytochemistry 27 (1988) 1041.
[4] A.J. Burbott, W.D. Loomis, Plant Physiol. 65 (1980) 96, suppl.
[5] D.V. Banthorpe, J.A. Wirz, J. Chem. Soc., Perkin Trans. 1 (1972) 1769.

[6] R. Croteau, K.V. Venkatachalam, Arch. Biochem. Biophys. 249 (1986) 306.
[7] W.B. Stephen, J.S. Melanie, W. David, M.D. Paul, J. Chem. Soc., Chem. Commun. (1982) 157.
[8] D.A. Smith, S.W. Banks, Phytochemistry 25 (1986) 979.
[9] Y. Noma, Y. Asakawa, Phytochemistry 31 (1992) 2009.
[10] K. Shimoda, T. Hirata, Y. Noma, Phytochemistry 49 (1998) 49.
[11] L.S. Shashidhara, G.S. Alison, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 63.
[12] T. Hirata, T. Higata, K. Shimoda, D.I. Ito, S. Izumi, J. Labelled Compd. Radiopharm. 39 (1996) 285.
[13] Y. Noma, C. Tatsumi, Nippon Nogeikagaku Kaishi 47 (1973) 705.
[14] Y. Noma, S. Nonomura, H. Ueda, C. Tatsumi, Agric. Biol. Chem. 38 (1974) 735.
[15] S.P. Colowick, N.O. Kaplan, Methods Enzymol. 4 (1957) 840.
[16] M. Bradford, Anal. Biochem. 72 (1976) 248.
[17] U.K. Laemmli, Nature 277 (1970) 680.