Journal of Molecular Catalysis B: Enzymatic 59 (2009) 158–162

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Stereospecific hydrogenation of the C=C double bond of enones by *Escherichia coli* overexpressing an enone reductase of *Nicotiana tabacum*

Toshifumi Hirata[a,*], Akihito Matsushima[b], Yuya Sato[a], Toshihiko Iwasaki[a], Hidetaka Nomura[a], Takayoshi Watanabe[a], Saki Toyoda[a], Shunsuke Izumi[a]

[a] *Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan*
[b] *Natural Science Center for Basic Research and Development, Radioisotope Center, Hiroshima University, 1-4-2 Kagamiyama, Higashi-Hiroshima 739-8526, Japan*

ARTICLE INFO

Article history:
Received 2 December 2008
Received in revised form 20 January 2009
Accepted 8 February 2009
Available online 20 February 2009

Keywords:
Biotransformation
Nicotiana tabacum
Recombinant *E. coli* expressing enone reductase
Recombinant pulegone reductase
Stereospecific hydrogenation
Bovine serum albumin

ABSTRACT

We examined the biotransformation of enantiomeric pairs of enones such as pulegone and carvone in recombinant *Escherichia coli* expressing *Nicotiana tabacum* pulegone reductase. It was found that recombinant *E. coli* cells acquired the ability for stereospecific hydrogenation of the exocyclic C=C double bond of pulegone. However, stereospecificity in hydrogenation with the recombinant *E. coli* cells was opposite to that in hydrogenation with *N. tabacum* cells. On the other hand, the isolated recombinant pulegone reductase (rPRase) from the recombinant *E. coli* cells catalyzed hydrogenation of the exocyclic C=C double bond of pulegone; the hydrogen atoms participating in the reduction at C-8 and C-4 of pulegone originate from the *pro*-4*R* hydrogen of NADPH and the medium (H_2O), respectively. Stereospecificity was lost in the hydrogenation of pulegone with the isolated rPRase, but was recovered when bovine serum albumin was added to the enzymatic reaction as an auxiliary factor.

1. Introduction

It is well known that transformation using biocatalysts allows structural modifications and generate useful chiral substances under mild and ecologically compatible conditions [1,2]. Many studies on the enantioselective reduction of the C=C double bond of enones by biocatalysts have revealed that several enone reductases can be used for the production of chiral ketones (see [3–17] for examples). We have previously reported the isolation of three different types of enone reductase involved in stereospecific hydrogenation of the C=C double bond from cultured cells of *Nicotiana tabacum*; one is responsible for reducing the exocyclic double bond of enones such as pulegone [6,7], and the others reduce the endocyclic double bond of enones such as carvone [3,6] and verbenone [4,6]. Recently, the recombinant pulegone reductases (rPRase) were prepared by genetic engineering; pulegone reductases from *Mentha piperita* [18] and *N. tabacum* [19] were expressed in *Escherichia coli*. However, stereospecificity in the hydrogenation of the C=C double bond of pulegone with these rPRases was low compared with native reductase [18,19].

To develop useful bioreactors, we further investigated the stereospecificity in the biotransformation of enones, such as pulegone and carvone, using whole cells of recombinant *E. coli* expressing *N. tabacum* pulegone reductase and the isolated rPRase. This report details the stereochemistry in the hydrogenation with the recombinant *E. coli* cells, and also the potential use of bovine serum albumin (BSA) as an auxiliary factor for stereospecific control of rPRase hydrogenation.

2. Experimental

2.1. Generals

Gas chromatography (GC) with a flame ionizing detector (FID) was performed on a capillary column (0.25 mm × 25 m) coated with 0.25 μm HiCap-CBP20 using N_2 as a carrier gas (column temperature: 130–140 °C, split ratio: 50, make up: 50 ml min^{-1}). GC–mass spectrometry (GC–MS) was performed using a MSD 5971 mass spectrometer (ion source 200 °C, 70 eV) and a gas chromatograph equipped with a DB-1 fused silica capillary column (30 m × 0.53 mm i.d.; film thickness 0.25 μm). Optical rotations were measured with a JASCO DIP-370 digital polarimeter. MS spectra were performed on a JEOL SX-102A mass spectrometer equipped with an electron spray ion source (ESI10HS). ^{1}H NMR (500 MHz)

* Corresponding author. Tel.: +81 824 24 7435 fax: +81 824 24 7435.
E-mail address: hirata-001@hiroshima-u.ac.jp (T. Hirata).

1381-1177/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2009.02.009

spectra were obtained using a JEOL LA-500 spectrometer using tetramethylsilane (TMS) as an internal standard.

2.2. Materials

(*R*)-Pulegone (**1a**) {99% pure on gas liquid chromatography [GLC], $[\alpha]_D^{25}$ +22.3 (neat); lit. [7]: $[\alpha]_D^{25}$ +22.4 (neat)}, (*S*)-pulegone (**1b**) {99% pure on GLC, $[\alpha]_D^{25}$ −22.0 (neat)}, (*S*)-carvone (**2a**) {99% pure on GLC, $[\alpha]_D^{25}$ −60.1 (neat); lit. [3]: $[\alpha]_D^{25}$ −59.7 (neat)} and (*R*)-carvone (**2b**) {99% pure on GLC, $[\alpha]_D^{25}$ +57.1 (neat)} were purchased from Sigma–Aldrich.

(4*R*)-[4-^{2}H]NADPH was synthesized as reported previously [4] with some modifications. A total of 35 mg of $NADP^+$ (2.8 mM final concentration), 1.2 ml of ethanol-d_6 (99.8% ^{2}H-enrichement) (0.5 M final concentration), and 75 units of yeast alcohol dehydrogenase were dissolved in 15 ml of 25 mM Tris buffer (pH 9.0). The reaction mixture was allowed to stand at 37 °C for 30 min. The product was purified on a diethylaminoethyl (DEAE) Toyopearl column to give (4*R*)-[4-^{2}H]NADPH (>99% ^{2}H-enrichment; 12 mg), *m*/*z* 746 ($M+H^+$), δ_H (^2H_2O) 2.66 (1H, bs, 4-H), 6.18 (2H, d, OMe) and 6.91 (1H, s, 2-H).

(4*S*)-[4-^{2}H]NADPH was synthesized as previously reported [20]. The reaction mixture contained 35 mg $NADP^+$ (9.3 mM final concentration) 12 mg of D-glucose-1-*d* (99.5% ^{2}H-enrichment) (15 mM final concentration), 2.6 ml of 0.1 M phosphate buffer, pH 8.0, 1.7 ml of dimethyl sulfoxide (DMSO) (40% final concentration), and 50 units of glucose-6-phosphate dehydrogenase. The reaction mixture was allowed to stand at room temperature for 1 h. The product was purified on a DEAE Toyopearl column to give (4*S*)-[4-^{2}H]NADPH (>99% ^{2}H-enrichment; 21 mg), *m*/*z* 746 ($M+H^+$), δ_H (^2H_2O) 2.81 (1H, bs, 4-H), 6.18 (2H, d, OMe) and 6.91 (1H, s, 2-H).

Bovine serum albumin (BSA), β- and γ-cyclodextrin and casein were purchased from Sigma–Aldrich.

2.3. Cultivation

E. coli BL21 cells were transformed by insertion of full-length *N. tabacum* pulegone reductase cDNA (*NtPRase*, Accession No. AB036735) in the multiple cloning site using pGEX expression vector (GE Healthcare Bio-Science) [19], and then stored and cultivated by general methods. Luria-Bertani (LB) medium (pH 7.9) containing 1% tryptone, 0.5% yeast extract, 1% NaCl, and 50 μg/ml of ampicillin was used for cultivation and biotransformation with recombinant *E. coli* cells. For measurement of background enzyme assay *in situ*, wild-type *E. coli* BL21 cells in LB medium were used.

N. tabacum cells have been stored for more than 20 years in our laboratory [21]. Prior to use for biotransformation experiments, the cultured cells were transferred to a 300-ml conical flask containing 100 ml of Murashige-Skoog (MS) medium (pH 7.5) with 2% sucrose and 10 mM of 2,4-dichrolophenoxyacetic acid (2,4-D), and cultured on a rotary shaker (110 rpm) for 10 days at 25 °C under illumination (4000 lx).

2.4. Reduction of enones with the whole cells of recombinant *E. coli*

A 500-μl aliquot from an overnight culture of recombinant *E. coli* was added to 50 ml of LB medium (pH 7.9) supplemented with 200 μg/ml ampicillin in a 200-ml Erlenmeyer flask. The culture was incubated at 30 °C, with shaking at 100 rpm, until it reached an OD_{600} value between 0.4 and 0.5, then isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 0.01 mM. The culture was incubated at 30 °C, with shaking at 100 rpm, for an additional 30 min, and then a solution of substrate (10 mg) in 400 μl of DMSO was added and incubated for 24 h at 30 °C.

After incubation, the *E. coli* cells were removed by centrifugation (4000 × *g*, 30 min), the supernatant was extracted with diethyl ether (3 × 20 ml), and the combined ether extracts were then dried with Na_2SO_4 and concentrated by rotary evaporation to give a crude product. The crude product was subjected to GC analyses and the yields of products were determined, as shown in Table 1. Retention time (minute) for these products in the GC analyses were as follows: HiCap-CBP20 column at 140 °C [isomenthone (4.4), menthone (4.7), menthol (5.7), isomenthol (6.5)] for the reduction products from pulegone and HiCap-CBP20 column at 130 °C [dihydrocarvone (6.7), isodihydrocarvone (7.1), neodihydrocarveol (9.4), dihydrocarveol (10.3), neoisodihydrocarveol (11.6), isodihydrocarveol (12.7)] for the reduction products from carvone. The final products were identified by comparison of GC, GC–MS, and/or ^{1}H NMR spectra with those of known samples.

2.5. Reduction of enones with cultured cells of *N. tabacum*

To the flasks containing the suspended *N. tabacum* cells (about 20 g) in MS medium (pH 7.5) (100 ml), each substrate (20 mg) in DMSO (0.2 ml) was administered, and the cultures were incubated for 7 days at 25 °C on a rotary shaker (110 rpm) under illumination (4000 lx). After incubation, the cells and media were separated by filtration with suction. The filtrated medium was extracted with diethyl ether (3 × 20 ml) and the extracts were concentrated by

Table 1
Hydrogenation of pulegones (**1a** and **1b**) and carvones (**2a** and **2b**) by *E. coli* and *N. tabacum*.

Substrates	Products	Conversion (%)		
		Recombinant *E. coli* cells	Wild type *E. coli* cells	*N. tabacum* cells
(*R*)-Pulegone (**1a**)	(1*R*,4*S*)-Menthone (**3a**)	28	0.0	3.5
	(1*R*,4*R*)-Isomenthone (**4a**)	29	0.0	22
	(1*R*,3*S*,4*S*)-Neomenthol (**5**)	0.0	0.0	1.0
	(1*S*,3*S*,4*S*)-Neoisomenthol (**6**)	0.0	0.0	0.0
(*S*)-Pulegone (**1b**)	(1*S*,4*R*)-Menthone (**3b**)	7.0	0.0	17
	(1*S*,4*S*)-Isomenthone (**4b**)	32	0.0	9.1
	(1*R*,3*S*,4*R*)-Isomenthol (**7**)	0.0	0.0	2.3
	(1*S*,3*S*,4*R*)-Menthol (**8**)	0.0	0.0	0.5
(*R*)-Carvone (**2a**)	(1*R*,4*R*)-Dihydrocarvone (**9a**)	86	77	11
	(1*S*,4*R*)-Isodihydrocarvone (**10a**)	12	9.3	0.1
	(1*R*,2*S*,4*R*)-Neodihydrocarveol (**11**)	0.0	0.0	2.1
	(1*S*,2*S*,4*R*)-Isodihydrocarveol (**12**)	0.0	0.0	0.0
(*S*)-Carvone (**2b**)	(1*S*,4*S*)-Dihydrocarvone (**9b**)	29	39	2.2
	(1*R*,4*S*)-Isodihydrocarvone (**10b**)	71	61	22
	(1*S*,2*S*,4*S*)-Dihydrocarveol (**13**)	0.0	0.0	1.5
	(1*R*,2*S*,4*S*)-Neoisodihydrocarveol (**14**)	0.0	0.0	0.3

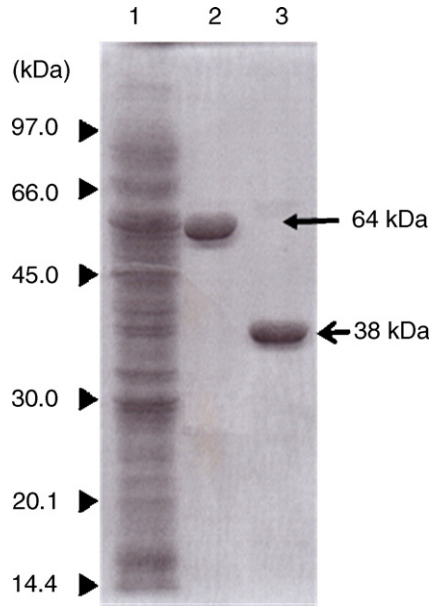

Fig. 1. SDS-PAGE in the purification of recombinant proteins, rPRase and rPRase-GST, from recombinant *E. coli* cells. Lane 1, crude soluble fraction; lane 2, purified rPRase-GST; lane 3, purified rPRase.

rotary evaporation. The concentrated ether layer was subjected to GC analyses and the yields of products were determined (Table 1). The final products were identified by GC, GC–MS, and/or ^{1}H NMR spectrometry.

2.6. Purification of recombinant pulegone reductase (rPRase) from the recombinant E. coli cells

To the recombinant *E. coli* cells in 50 ml of LB medium, IPTG was added to a final concentration of 0.01 mM and the cells were incubated at 30 °C for 16 h. After centrifugation, the *E. coli* pellet was sonicated and the soluble fraction was isolated. The glutathione *S*-transferase fusion protein (rPRase-GST) was purified using glutathione Sepharose 4B (GE Healthcare Bio-Science) affinity column chromatography, and was then digested using the PreScission protease (GE Healthcare Bio-Science) to give recombinant pulegone reductase (rPRase), as shown in Fig. 1. The molecular weights of purified rPRase and rPRase-GST were confirmed by SDS-PAGE and MALDI TOF-MS. Protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad).

2.7. Enzyme activity assay

The standard assay mixture (2 ml) was composed of enzyme (50 μg), NADPH (100 mM), substrate (50 mM), and Triton X-100 (1 mM) in 50 mM Na-phosphate buffer (pH 7.0). The mixture was incubated at 35 °C for 3 h. After incubation, the reaction mixture was extracted with diethyl ether (3 ml × 3). The ether layer was dried over Na_2SO_4 and the solvent was removed by evaporation. The concentrated ether layer was subjected to GC and GC–MS analyses. The enzyme activity was determined by measuring the amounts of products.

2.8. Incubation of enones with rPRases

Following the standard assay method, enzymatic reductions of several enones such as pulegones (**1a** and **1b**) and carvones (**2a** and **2b**) were performed. The yields of products were determined by GC analysis, as shown in Table 2. The products were identified

Table 2
Hydrogenation of (*R*)- and (*S*)-pulegones (**1a** and **1b**) with the recombinant reductases rPRase and rPRase-GST.

Substrates	Products	Conversion (%)	
		rPRase	rPRase-GST
(*R*)-Pulegone (**1a**)	(1*R*,4*S*)-Menthone (**3a**)	15	18
	(1*R*,4*R*)-Isomenthone (**4a**)	15	19
(*S*)-Pulegone (**1b**)	(1*S*,4*R*)-Menthone (**3b**)	5.0	11
	(1*S*,4*S*)-Isomenthone (**4b**)	5.1	10

by direct comparison of GC–MS and/or NMR spectra with those of known samples. Menthone (**3a**): *m*/*z* (rel. int.) 154 (M^+, 36), 139 (47), 112 (100), 97 (30), 83 (23) and 69 (58); δ_H ($CDCH_3$) 0.86 (3H, d, *J* = 6.0 Hz, 1-Me), 0.94 (3H, d, *J* = 6.0 Hz, 9-Me), 1.02 (3H, d, *J* = 6.0 Hz, 10-Me). Isomenthone (**4a**): *m*/*z* (rel. int.) 154 (M^+, 33), 139 (44), 112 (100), 97 (29), 83 (24) and 69 (70); δ_H ($CDCH_3$) 0.83 (3H, d, *J* = 6.5 Hz, 1-Me), 0.95 (3H, d, *J* = 6.5 Hz, 9-Me), 0.98 (3H, d, *J* = 6.5 Hz, 10-Me). Dihydrocarvone (**9a**): *m*/*z* (rel. int.) 152 (M^+, 20), 137 (15), 109 (29), 95 (82) and 67 (100); δ_H ($CDCH_3$) 1.02 (3H, d, *J* = 6.5 Hz, 1-Me), 1.75 (3H, s, 8-Me), 4.78 (2H, bs, C=CH_2). Isodihydrocarvone (**10a**): *m*/*z* (rel. int.) 152 (M^+, 35), 137 (16), 109 (40), 95 (89) and 67 (100); δ_H ($CDCH_3$) 1.08 (3H, d, *J* = 6.0 Hz, 1-Me), 1.76 (3H, s, 8-Me), 4.81 (2H, d, *J* = 8.0 Hz, C=CH_2).

2.9. Reduction of (R)-pulegone (*1a*) with rPRase in the presence of (4R)-[4-^{2}H]NADPH

The reaction mixture (1 ml) was composed of enzyme (50 μg), (*R*)-pulegone (**1a**) (1 mM), Triton X-100 (1 mM), and (4*R*)-[4-^{2}H]NADPH (4.4 mM) in 50 mM Na-phosphate buffer (pH 7.0). The mixture was incubated at 37 °C for 12 h. After incubation, the reaction mixture was extracted with diethyl ether (3 ml × 3). The concentrated ether layer was subjected to GC–MS analysis. Menthone (**3a**) (99% ^{2}H-enrichiment): *m*/*z* (rel. int.) 155 (23), 140 (29), 112 (100) and 69 (44). Isomenthone (**4a**) (99% ^{2}H-enrichiment): *m*/*z* (rel. int.) 155 (12), 140 (16), 112 (100) and 69 (37).

2.10. Reduction of (R)-pulegone (*1a*) with rPRase in the presence of (4S)-[4-^{2}H]NADPH

In the presence of (4*S*)-[4-^{2}H]NADPH, (*R*)-pulegone (**1a**) was converted into menthone (**3a**) (no ^{2}H-enrichment) *m*/*z* (rel. int.) 154 (38), 139 (49), 112 (100), 69 (60), and isomenthone (**4a**) (no ^{2}H-enrichment) *m*/*z* (rel. int.) 154 (31), 139 (42), 112 (100), 69 (56).

2.11. Reduction of (R)-pulegones (*1a*) with rPRase in the presence of ^2H_2O

The reaction mixture (1 ml) was composed of enzyme (50 μg), substrate (1 mM), Triton X-100 (1 mM), and NADPH (4.4 mM) in 50 mM Na-phosphate buffer (pH 7.0), prepared by use of deuterated water (^2H_2O; 99% enrichment). The mixture was incubated at 37 °C for 6 h and then extracted using diethyl ether (3 ml × 3). The products were analyzed by GC–MS analysis. Menthone (**3a**) (87% ^{2}H-enrichment): *m*/*z* (rel. int.) 155 (32), 140 (44), 113 (100) and 69 (53). Isomenthone (**3a**) (84% ^{2}H-enrichment): *m*/*z* (rel. int.) 155 (25), 140 (36), 113 (100) and 69 (52).

2.12. Reduction of pulegones (*1a* and *1b*) by rPRase with auxiliary factors

The assay mixture (1 ml) was composed of enzyme (50 μg), substrate (1 mM), NADPH (3 mM), and an auxiliary factor [Triton X-100 (1 mM), β-cyclodextrin (1 mM), γ-cyclodextrin (1 mM), casein (10 μM) or BSA (10 μM)] in 50 mM Na-phosphate buffer (pH 7.0).

Table 3
Hydrogenation of (*R*)- and (*S*)-pulegones (**1a** and **1b**) with the recombinant reductase rPRase in the presence of auxiliary factors.

Substrates	Cofactors	Conversion (%)			
		3a	**4a**	**3b**	**4b**
(*R*)-pulegone (**1a**)	None	4.0	4.0		
	Triton X-100	15	14		
	β-cyclodextrin	5.4	5.6		
	γ-cyclodextrin	14	13		
	Casein	9.4	8.4		
	BSA	6.4	18		
(*S*)-pulegone (**1b**)	None			0.6	0.6
	Triton X-100			4.9	5.1
	β-cyclodextrin			1.0	1.1
	γ-cyclodextrin			4.8	5.2
	Casein			3.8	3.4
	BSA			16	2.1

The mixture was incubated at 37 °C for 6 h. After incubation, the reaction mixture was extracted with diethyl ether (3 ml × 3). The concentrated ether layer was subjected to GC analysis and the yields of products were determined, as shown in Table 3.

On the other hand, enzymatic reductions of pulegones (**1a** and **1b**) with different concentration of BSA were done by the assay mixture (1 ml) containing enzyme (50 μg), substrate (1 mM), NADPH (3 mM), and BSA (0, 1.4, 3.6, 7.5 and 15 μM). The mixture was incubated at 37 °C for 5 h. After incubation, the reaction mixture was extracted with diethyl ether (3 ml × 3). The concentrated ether layer was subjected to GC analysis and the yields of products were determined. The relative yields of menthone and isomenthone are shown in Fig. 2.

3. Results and discussion

3.1. Biotransformation of enones with transformed *E. coli*

To determine the potentiality use of recombinant *E. coli* cells for stereospecific reduction, biotransformation of enones with whole cells of recombinant *E. coli* expressing a pulegone reductase of *N. tabacum* [19] was assessed and compared with transformations with wild-type *E. coli* BL21 cells and *N. tabacum* cells. As shown in Table 1, pulegones (**1a** and **1b**) were converted to corresponding ketones in the reaction with recombinant *E. coli* cells, although no reduction occurred in the reaction with wild-type *E. coli* cells. The reduction of (*S*)-pulegone (**1b**) with recombinant *E. coli* was stereospecific to mainly give isomenthone (**4b**) of the 4*S* configuration; however, the stereospecificity was 4*R* in the case of *N. tabacum* cells.

On the other hand, (*S*)- and (*R*)-carvones (**2a** and **2b**) were also converted to corresponding ketones by recombinant *E. coli* cells and wild-type *E. coli* cells, and the stereospecificities of both reactions were similar. This indicates that *E. coli* cells originally contain a reductase that can reduce the endocyclic C=C double bond of carvone.

Thus, it was clarified that recombinant *E. coli* cells acquired the ability for stereospecific hydrogenation of the exocyclic C=C double bond of enone, although stereospecificity in hydrogenation with the recombinant *E. coli* cells was opposite to that in hydrogenation with *N. tabacum* cells. Such different stereospecificities might mean that there are different stereospecificity-controlled factors in the *E. coli* and *N. tabacum* cells.

3.2. Stereochemistry in the hydrogenation of enones with rPRase

To determine stereospecificity in the reduction with proteins from recombinant *E. coli*, enzymatic reactions of enones, such as (*R*)- and (*S*)-pulegones (**1a** and **1b**) and (*S*)- and (*R*)-carvones (**2a** and **2b**), were examined using the recombinant proteins rPRase and rPRase-GST. Both of the isolated recombinant proteins catalyzed a reduction of pulegones (**1a** and **1b**) to give the corresponding ketones, menthones (**3a** and **3b**) and isomenthones (**4a** and **4b**), as shown in Table 2, but there was no reduction of the carvones (**2a** and **2b**). These results indicate that these recombinant proteins have the potential for reduction of enones with the exocyclic C=C double bond, but not enones with the endocyclic C=C double bond. On the other hand, the stereospecificity of hydrogenation was lost in the reduction of pulegones (**1a** and **1b**) with the recombinant proteins rPRase and rPRase-GST (Table 2). However, the reduction of (*R*)-pulegone (**1a**) with native pulegone reductase from *N. tabacum* cultured cells was highly stereospecific and mainly gave (1*R*,4*R*)-isomenthone (**4a**) [7].

To fully determine the origin of hydrogen atoms that are introduced to the C-4 and C-8 positions of pulegone in the reduction of the C=C double bond with rPRase, the following three experiments were performed. (*R*)-pulegone (**1a**) was incubated with rPRase in the presence of: (a) NADPH in ^2H_2O, (b) (4*R*)-[4-^{2}H]NADPH in H_2O and (c) (4*S*)-[4-^{2}H]NADPH in H_2O. The labeling pattern of deuterium and the deuterium contents of the resulting products, **3a** and **4a**, were determined by mass spectroscopy. Both products of incubation in the presence of NADPH in ^2H_2O [experiment (a)] showed a peak at *m*/*z* 155 (84–87% ^{2}H-enrichment) in the mass spectrum, that is, one mass unit higher than the molecular ion peak (*m*/*z* 154) of the corresponding known samples. A fragment ion peak (*m*/*z* 113) produced by expulsion of an isopropyl group from the menthone structure with McLafferty rearrangement [22] was also one mass unit higher than the fragment ion peak for known samples. This indicates that a deuterium atom is present in the cyclohexane-ring moiety of menthone, and that the hydrogen atom originating from H_2O is incorporated at the C-4 position of pulegone (**1a**). On the other hand, the mass spectra of products **3a** and **4a** that are

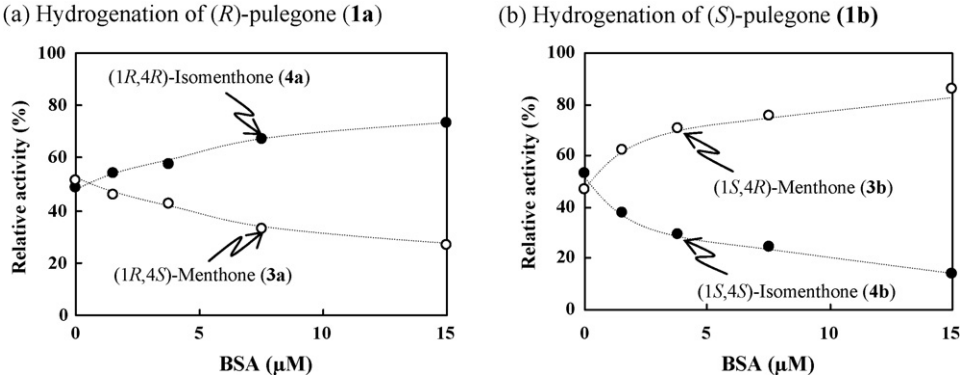

Fig. 2. Effects of BSA concentration on the stereoselectivity of the hydrogenation of (*R*)- and (*S*)-pulegones (**1a** and **1b**) with the recombinant reductase rPRase.

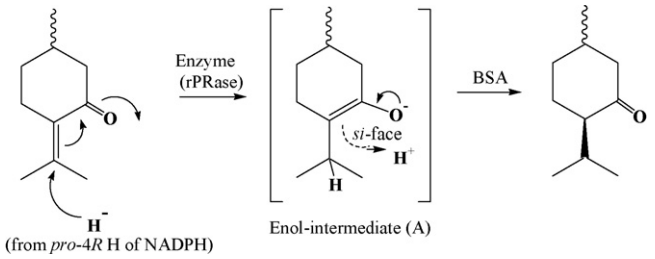

Fig. 3. Predicted mechanism of enzymatic hydrogenation of pulegones (**1a** and **1b**) with rPRase and BSA.

produced when (4*R*)-[4-^{2}H]NADPH was included in the incubation mixture [experiment (b)] showed molecular ion peaks at *m*/*z* 155 (99% ^{2}H-enrichment). A fragment ion peak due to the cyclohexane-ring moiety was seen at *m*/*z* 112, indicating that the deuterium atom originating from NADPH is not incorporated into the cyclohexane moiety but is instead incorporated into the isopropyl moiety. In the enzymatic reaction using (4*S*)-[4-^{2}H]-NADPH [experiment (c)], neither of the products were deuterated. This observation indicates that only the pro-4*R* hydrogen of NADPH is incorporated into the C=C double bond of pulegone by enzymatic reduction with rPRase.

Thus, it was determined that (a) the recombinant pulegone reductases catalyze the hydrogenation of the exocyclic C=C double bond of pulegone, (b) the hydrogen atoms that participate in the reductions of pulegone at C-8 and C-4 originate from the *pro*-4*R* hydrogen of NADPH and the medium (H_2O), respectively, and (c) the hydrogen attack at C-4 position of pulegone is not stereospecific.

3.3. Biotransformation of enones with rPRase in the presence of auxiliary factors

The lack of stereospecificity in reaction with the recombinant reductases might be due to a lack of proper factors in the enzymatic reaction system. Therefore, we investigated the enzymatic reaction with rPRase in addition to several auxiliary factors such as Triton X-100, β-cyclodextrin, γ-cyclodextrin, casein and bovine serum albumin (BSA), which might affect the solubility of substrates and/or stabilization of enzyme. As shown in Table 3, Triton X-100, γ-cyclodextrin and casein improved the reactivity of hydrogenation of pulegones, but had no effect on the stereospecificity. On the other hand, addition of BSA strongly affected both reactivity and stereospecificity of hydrogenation; (*R*)-pulegone (**1a**) was mainly transformed to (1*R*,4*R*)-isomenthone (**4a**) (diastereomeric excess: 48%), and (*S*)-pulegone (**1b**) to (1*S*,4*R*)-menthone (3b) (d.e. 77%). These effects of BSA were confirmed by the enzymatic reaction of pulegones (**1a** and **1b**) with rPRase and different concentrations of BSA. As shown in Fig. 2, the stereospecificity of hydrogenation was increased with increasing concentrations of BSA, which indicates that, in the hydrogenation reaction with BSA, a hydrogen attack at C-4 position of pulegones occurs mainly from the *si*-face of the C=C double bond to give products with the 4*R*-configuration.

Thus, it can be suggested that: (a) the C-8 position of pulegone is hydrogenated by a hydride ion from NADPH; (b) the resulting enol-intermediate (A) was bound to BSA and the *re*-face of the C=C double bond of the intermadiate (A) may be blocked from protonation; and (c) protonation occurs only from the *si*-face to give products with the 4*R*-configuration, as shown in Fig. 3. It is interesting to note that BSA can be used as an auxiliary factor for chiral-induced reactions, although further investigations are necessary for a complete understanding of the chiral-control mechanism.

References

[1] C. Wong, G.M. Whitesides, Enzymes in Synthetic Organic Chemistry, Pergamon, Oxford, 1994.
[2] J.M. Brown, in: E.N. Jacobsen, A. Pfaltz, H. Yamamoto (Eds.), Comprehensive Asymmetric Catalysis, vol. I, Springer-Verlag, New York, 1999.
[3] T. Hirata, Y.X. Tang, K. Okano, T. Suga, Phytochemistry 28 (1989) 3331–3333.
[4] K. Shimoda, D.I. Ito, S. Izumi, T. Hirata, J. Chem. Soc., Perkin Trans. 1 (1996) 355–358.
[5] P. Wanner, R. Tressl, Eur. J. Biochem. 255 (1998) 271–278.
[6] T. Hirata, K. Shimoda, T. Gondai, Chem. Lett. (2000) 850–851.
[7] K. Shimoda, S. Izumi, T. Hirata, Bull. Chem. Soc. Jpn. 75 (2002) 813–816.
[8] M. Kataoka, A. Kotaka, A. Hasegawa, M. Wada, A. Yoshizumi, S. Nakamori, S. Shimizu, Bosci. Biotechnol. Biochem. 66 (2002) 2651–2657.
[9] M. Wada, A. Yoshizumi, Y. Noda, M. Kataoka, S. Shimizu, H. Takagi, S. Nakamori, Appl. Environ. Microbiol. 69 (2003) 933–937.
[10] K. Shimoda, N. Kubota, H. Hamada, Tetrahedron: Asym. 15 (2004) 2443–2446.
[11] M.A. Swiderska, J.D. Stewart, J. Mol. Catal. B: Enzym. 42 (2006) 52–54.
[12] A. Muller, B. Hauer, B. Rosche, Biotechnol. Bioeng. 98 (2007) 22–29.
[13] J.F. Chaparro-Riggers, T.A. Rogers, E. Vazquez-Figueroa, K.M. Polizzi, A.S. Bommarius, Adv. Synth. Catal. 349 (2007) 1521–1531.
[14] M. Hall, C. Stueckler, B. Hauer, R. Stuermer, T. Friedrich, M. Breuer, W. Kroutil, K. Faber, Eur. J. Org. Chem. (2008) 1511–1516.
[15] M. Hall, C. Stueckler, H. Ehammer, E. Pointner, G. Oberdorfer, K. Gruber, B. Hauer, R. Stuermer, W. Kroutil, P. Macheroux, K. Faber, Adv. Synth. Catal. 350 (2008) 411–418.
[16] B. Kosjek, F.J. Fleitz, P.G. Dormer, J.T. Kuethe, P.N. Devine, Tetrahedron Asym. 19 (2008) 1403–1406.
[17] P. Schweiger, H. Gross, S. Wesener, U. Deppenmeier, Appl. Microbiol. Biotechnol. 80 (2008) 995–1006.
[18] K.L. Ringer, M.E. McConkey, E.M. Davis, G.W. Rushing, R. Croteau, Arch. Biochem. Biophys. 418 (2003) 80–92.
[19] A. Matsushima, Y. Sato, M. Otsuka, T. Watanabe, H. Yamamoto, T. Hirata, Bioorg. Chem. 36 (2008) 23–28.
[20] R.E. Viola, P.F. Cook P.F., W.W. Cleland, Anal. Chem. 96 (1979) 334–340.
[21] T. Hirata, H. Hamada, T. Aoki, T. Suga, Phytochemistry 21 (1982) 2209–2212.
[22] M.V. Kulkarni, E.J. Eisenbraun, M.C. Hamming, J. Org. Chem. 35 (1970) 686–689.