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Authors: Kazunori Yamamoto, Yuko Oku, Atsutoshi Ina, Atsushi Izumi, Masaharu Doya, Syuji Ebata, and Yasuhisa Asano

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FULL PAPER

Purification and characterization of a novel enone reductase from *Sporidiobolus salmonicolor* TPU 2001 reacting with large monocyclic enones

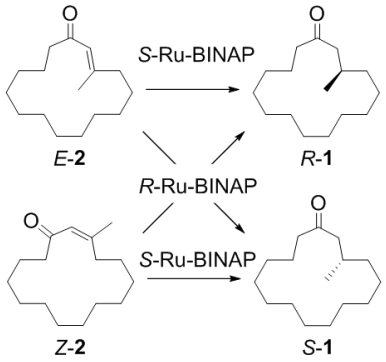
Kazunori Yamamoto,[a,b] Yuko Oku,[a,b] Atsutoshi Ina,[a,b] Atsushi Izumi,[a,b] Masaharu Doya,[c] Syuji Ebata,[c] and Yasuhisa Asano*[a,b]

We discovered a novel enone reductase from *Sporidiobolus salmonicolor* TPU 2001 (*Ss*ERD) and expressed the gene in *Escherichia coli*. The enzyme catalyzed the reduction of (*E*)-3-methylcyclopentadec-2-en-1-one (*E*-**2**), cyclopentadec-2-en-1-one (**3**), and cyclododec-2-en-1-one (**4**) to (*S*)-muscone (*S*-**1**), cyclopentadecan-1-one (**5**), and cyclododecan-1-one (**6**), respectively. The apparent K_m and V_{max} values for *E*-**2** were estimated to be 4.9 ± 0.4 μM and 100 ± 1.4 nmol min^{-1} mg^{-1}, respectively. The enzyme was specific to NADPH, and cysteine residue strongly affected the enzyme activity. The enzyme exhibited the highest activity at pH 8.0 and high stability in the pH range from 4.5 to 11.0. Using 10 mU of the enzyme, *S*-**1** was synthesized from 0.1 mM *E*-**2** with 94.8% yield and 100% enantiomeric excess by incubation at pH 7.0 and 30 °C for 60 min. We further successfully constructed enone reductase with high specific activity by mutation of *Ss*ERD. The Y67A variant from *Ss*ERD exhibited 4.5 times higher specific activity and 3 times higher catalytic efficiency toward *E*-**2**. This is the first report of the enzyme catalyzing reduction of carbon-carbon double bond of large monocyclic enones.

Introduction

The enzymes catalyzing asymmetric reduction of activated carbon-carbon double bonds such as α,β-unsaturated aldehydes, ketones, and esters, have been widely studied for application in production of chiral building blocks in the synthesis of bioactive compounds.[1] For example, enzymes that react with small ring enones, such as 5- or 6-membered ring enones, have been reported. Old yellow enzyme (OYE) (EC 1.6.99.1) catalyzes reduction of 2- or 3-alkyl-substituted cyclohexen-1-ones to the corresponding ketones.[2] 12-Oxophytodienoate reductases (EC 1.3.1.42) are available for asymmetric reduction of 2- and 3-methylcyclopenten-1-one.[3] Enzymes catalyzing reduction of carbon-carbon double bond of polycyclic enones have been also reported. Progesterone 5β-reductase (EC 1.3.99.6) catalyzes reduction of progesterone to 5β-pregnane-3,20-dione.[4] Morphinone reductase (EC 1.3.1.42) catalyzes reduction of morphinone to hydromorphone.[5] However, enzymes catalyzing reduction of large monocyclic enones have not been studied in detail, although certain chemical methods have been reported.

(*R*)- or (*S*)-muscone, (*R*)- or (*S*)-3-methylcyclopentadecan-1-one,[6] are produced using enantioselective chemical methods (Scheme 1).[7,8] (*R*)-Muscone (*R*-**1**), which is well known as a natural perfume, is produced by the enantioselective hydrogenation of (*E*)-3-methylcyclopentadec-2-en-1-one (*E*-**2**) using $Ru_2Cl_4[(S)$-*p*-tolyl-binap$]_2NEt_3$ (*S*-Ru-BINAP) or of (*Z*)-3-methylcyclopentadec-2-en-1-one (*Z*-**2**) using $Ru_2Cl_4[(R)$-*p*-tolyl-binap$]_2NEt_3$ (*R*-Ru-BINAP) with 100% yield and 98% enantiomeric excess (ee).[8c] (*S*)-Muscone (*S*-**1**) is also produced by the same method from *E*-**2** using *R*-Ru-BINAP or *Z*-**2** using *S*-Ru-BINAP with 100% yield and 98% ee. However, these methods require expensive Ru-BINAP catalyst and rigorous reaction conditions.



S-Ru-BINAP: $Ru_2Cl_4[(S)$-*p*-tolyl-binap$]_2NEt_3$
R-Ru-BINAP: $Ru_2Cl_4[(R)$-*p*-tolyl-binap$]_2NEt_3$

Scheme 1. Chemical syntheses of (*R*)-muscone (*R*-**1**) and (*S*)-muscone (*S*-**1**) by asymmetric hydrogenation using Ru-BINAP catalyst. The structure of *Z*-**2** is rotated counterclockwise 45 degrees relative to other three compounds.

In this study, we investigated development of biochemical methods for production of muscone. First, we screened enzymes catalyzing the reduction of large monocyclic enone *E/Z*-**2**, and isolated the enzyme catalyzing this reduction from *Sporidiobolus salmonicolor* TPU 2001. The enzyme responsible for the reduction of large monocyclic enones and asymmetrically synthesized *S*-**1**, but not *R*-**1**. Although *S*-**1** is not useful for perfumery, the enzymes catalyzing the reduction of large monocyclic enones are important as catalysts to elucidate the

[a] *Dr. K. Yamamoto, Y. Oku, Dr. A. Ina, Dr. A. Izumi, Prof. Dr. Y. Asano*
Biotechnology Research Center and Department of Biotechnology
Toyama Prefectural University
5180 Kurokawa, Imizu, Toyama (Japan)
E-mail: asano@pu-toyama.ac.jp
[b] *Dr. K. Yamamoto, Y. Oku, Dr. A. Ina, Dr. A. Izumi, Prof. Dr. Y. Asano*
Asano Active Enzyme Molecule Project, ERATO, JST
5180 Kurokawa, Imizu, Toyama (Japan)
[c] *Dr. M. Doya, Dr. S. Ebata*
Toho Earthtec, Inc.
1450 Kurotori, Nishi-ku, Niigata, Niigata (Japan)

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reaction mechanism of enzymatic reduction of large monocyclic enones. The present paper describes purification and characterization of the enzyme from *S. salmonicolor* TPU 2001. The optimal conditions for asymmetric synthesis of *S*-**1** are also described.

Results

Screening and purification of enone reductase reacting with large monocyclic enones

In preliminary experiments, we incubated *E*/*Z*-**2** with several OYEs from strains of the genus *Saccharomyces*, which were able to reduce 6-membered ring enones. However, those enzymes did not exhibit the reductase activity toward *E*/*Z*-**2** (data not shown). Therefore, enzymes catalyzing reduction of the carbon-carbon double bond in large monocyclic enones were screened using *E*/*Z*-**2** with about 300 yeast and 400 bacterial strains. All bacterial strains including *Pseudomonas putida* with morphinone reductase[5] and *Bacillus subtilis* having OYE family enzyme, YqjM[9], did not reduce *E*/*Z*-**2**. We found that three yeast strains, belonging to the genus *Sporidiobolus*, exhibited high reductase activity toward *E*/*Z*-**2**. Enzyme from the strain *Sporidiobolus salmonicolor* TPU 2001 exhibited the highest reductase activity (0.14 mU mg^{-1} of protein) in the crude enzyme solution. The reductase, *Ss*ERD, was next purified with an overall yield of 12.6% from the strain using four column chromatography experiments (Figure S1, Table 1). The purified enzyme exhibited a specific activity of 98.2 mU mg^{-1} of protein with NADPH, but no activity with NADH (data not shown).

Molecular mass and amino acid sequence

Molecular mass of *Ss*ERD was estimated to be 80 kDa (data not shown), which was composed of two identical subunits of 37 kDa each. The N-terminal amino acid sequence was determined to be APITNKKTIF, and those of six internal peptides were as follows: 1) TIFAEVPSGVPEPGK, 2) TLSLSLDPYLR, 3) SYVPPFELGQPIANFGTGEVLK, 4) VEVAFNYK, 5) NIFQVVSK, and 6) GLDNGESFNDLFTGANFGK (Figure S2). The BLAST search using these amino acid sequences showed that the identical sequences were present in an uncharacterized protein from *S. salmonicolor* CBS 6832[10] (accession number: CEQ42599).

Table 1. Purification of enone reductase from *S. salmonicolor* TPU 2001.

Step	Activity (mU)	Protein (mg)	Specific activity (mU mg^{-1})	Yield (%)
Cell-free extract	855	6104	0.14	100
Ammonium sulfate	803	3210	0.25	93.9
Toyopearl Butyl	340	250	1.36	39.8
Q Sepharose	328	30.2	10.9	38.4
Toyopearl AF-Blue	203	5.2	39.0	23.7
MonoQ 5/50 GL	108	1.1	98.2	12.6

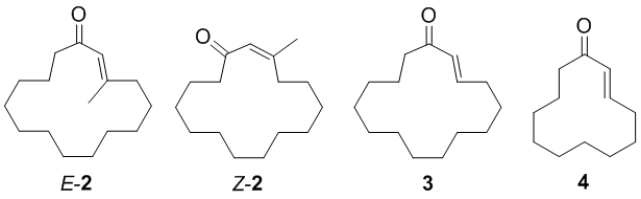
Cloning and expression of the *Ss*ERD gene, and purification of recombinant enzyme

It was difficult to obtain a large amount of the enzyme from the selected strain (only 0.4 mU of enzyme in 1 mL of the culture). Therefore, we investigated cloning and expression of the *Ss*ERD gene as follows: The *Ss*ERD gene was cloned from total cDNA using primers corresponding to N- and C-terminal amino acid sequence of CEQ42599, as described in the Experimental section. The amino acid sequence deduced from the cloned *Ss*ERD gene showed 92% identity to that of CEQ42599. In addition, above amino acid sequences of the N-terminal and six internal peptides were contained in the deduced amino acid sequence (Figure S2). The nucleotide sequence of *Ss*ERD was registered in DDBJ/EMBL/GenBank (accession number: LC209205). The *Ss*ERD gene was next ligated with the pET28a vector, and *Escherichia coli* BL21 (DE3) was transformed by the plasmid harboring the *Ss*ERD gene. In this expression experiment, the strain harboring the expression vector produced 10.2 mU of enzyme per 1 mL of the culture, indicating that the enzyme productivity was increased 25 times using the recombinant method. The recombinant enzyme purified using the same procedure as that used for the native enzyme (Table S1) exhibited similar specific activity (101 mU mg^{-1} of protein) as that of the native enzyme. Therefore, we used the purified recombinant enzyme in following studies.

Substrate specificity and its kinetic analysis of recombinant *Ss*ERD

The substrate specificity of *Ss*ERD was investigated using *E*-**2**, *Z*-**2**, cyclopentadec-2-en-1-one (**3**), cyclododec-2-en-1-one (**4**), and various 5- and 6-membered ring compounds, such as 3-methylcyclohex-2-en-1-one[2, 9, 11], cyclohex-2-en-1-one[12], ketoisophorone[3, 12], (*R*)-(-)-carvone[13], (+)-pulegone[13], 2-

Table 2. Substrate specificity and kinetic parameters of recombinant enone reductase.



E-**2** *Z*-**2** **3** **4**

Substrate	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
E-**2**	4.9 ± 0.4	100 ± 1.4	3.7 ± 0.1	0.8 ± 0.07
Z-**2**	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
3	4.3 ± 0.2	47 ± 0.6	1.7 ± 0.0	0.4 ± 0.02
4	1.9 ± 0.3	160 ± 3.2	5.9 ± 0.1	3.1 ± 0.3

Each experiment was performed using 3.9 μM–125 μM of substrate under standard assay condition. Data represent mean values ± SD of three replicates. *Ss*ERD did not react with 3-methylcyclohex-2-en-1-one, cyclohex-2-en-1-one, ketoisophorone, (*R*)-(-)-carvone, (+)-pulegone, 2-methylcyclopent-2-en-1-one, 3-methylcyclopent-2-en-1-one, *N*-ethylmaleimide, and *N*-phenylmaleimide. [a] Not detected.

methylcyclopent-2-en-1-one[3, 11], 3-methylcyclopent-2-en-1-one[3, 9, 11], *N*-ethylmaleimide[12, 14], and *N*-phenylmaleimide[3, 14] (Table 2). The enzyme exhibited reductase activity toward *E*-**2**, **3**, and **4**, but not toward *Z*-**2** and all small ring compounds. Thus, the enzyme reacted with 15-membered ring enone with methyl group and 15- and 12-membered ring enone without methyl group, but not with 5- and 6-membered ring compounds, which were widely utilized as the substrates for activity measurements in other ERDs. The results also indicated that the enzyme was specific to the *E*-isomer in 15-membered ring enone bearing a methyl group in the β-position. The K_m and V_{max} values of *Ss*ERD for *E*-**2** were estimated to be 4.9 ± 0.4 μM and 100 ± 1.4 nmol min^{-1} mg^{-1}, respectively. The V_{max} value for 15-membered ring enone **3** (47 ± 0.6 nmol min^{-1} mg^{-1}) was 2 times lower than that of *E*-**2**, whereas the K_m value (4.3 ± 0.2 μM) was similar to that of *E*-**2**. *Ss*ERD exhibited the highest V_{max} (160 ± 3.2 nmol min^{-1} mg^{-1}) and affinity (K_m :1.9 ± 0.3 μM) for 12-membered ring enone **4** among the three reactive compounds.

Identification of reaction products

To identify the reaction products of three reactive enones *E*-**2**, **3**, and **4**, each reaction was performed by incubation with the recombinant *Ss*ERD at pH 7.0 at 30 °C. When 0.1 mM of *E*-**2**, **3**, or **4** was incubated with 0.2 mM NADPH and 10 mU of *Ss*ERD for 60 min in 1 mL of 20 mM potassium phosphate buffer (KPB), they were converted into *S*-**1**, cyclopentadecan-1-one (**5**), and cyclododecan-1-one (**6**) with 98.4%, 97.5%, and 98.8% yields, respectively, and the ee of *S*-**1** was 100% (Figure 1).

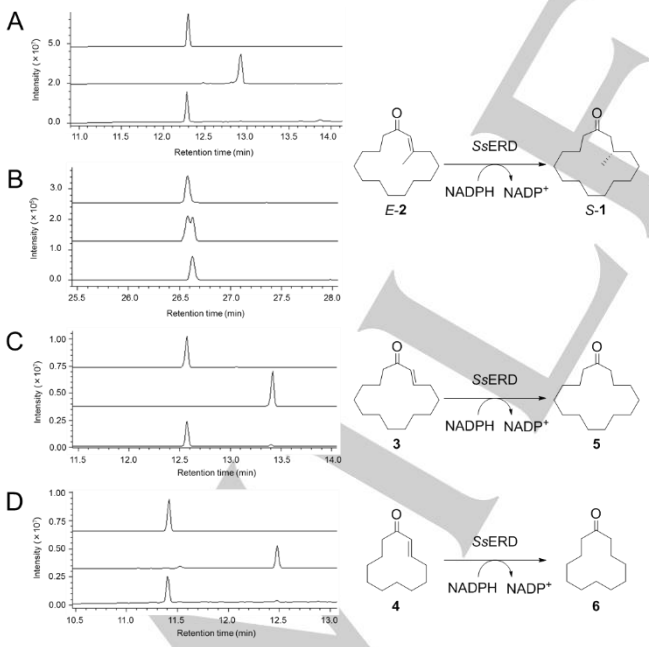


Figure 1. Total ion chromatograms of the reaction products. A) Detection of muscone. Authentic *rac*-**1** (above), authentic *E*-**2** (middle), and reaction product (below). B) Determination of stereochemistry. Authentic *R*-**1** (above), authentic *rac*-**1** (middle), and reaction product (below). C) Detection of cyclopentadecan-1-one **5**. Authentic **5** (above), authentic **3** (middle), and reaction product (below). D) Detection of cyclododecan-1-one **6**. Authentic **6** (above), authentic **4** (middle), and reaction product (below).

Effects of pH and temperature on enone reductase activity and stability

When reductase activity of the recombinant *Ss*ERD toward *E*-2 was analyzed in the pH range of 3.0–10.5, the enzyme exhibited the ERD activity from pH 4.5 to 10.0, and the highest activity was recorded at pH 8.0 (Figure 2A). The enzyme exhibited optimal temperature at 45 °C, and the ERD activity drastically decreased over 50 °C (Figure 2B). To determine its stability, the enzyme was incubated under varying pH and temperature conditions of 2.5–13.0 at 30 °C and 10 °C–60 °C at pH 7.0 for 30 min, respectively. The enzyme was stable in a broad pH range of pH 4.5 to 11.0 (Figure 2C), and up to a temperature of 30 °C, but unstable over 50 °C at pH 7.0 (Figure 2D).

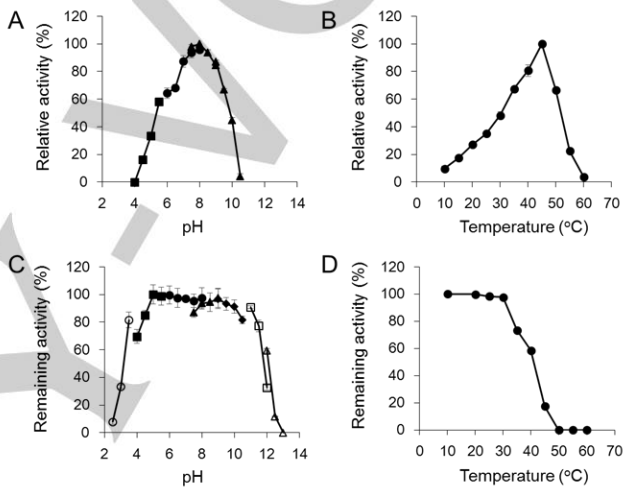


Figure 2. Effects of pH and temperature on enzyme activity and stability. A) optimal pH, B) optimal temperature, C) pH stability, D) temperature stability. Error bars indicate the standard deviations of three replicates. ○: KCl-HCl, pH 2.5–3.5, ■: Sodium acetate, pH 4.0–5.5, ●: KPB, pH 6.0–8.0, ▲: Tris-HCl, pH 7.5–9.0, ♦: Gly-NaOH, pH 9.0–10.5, □: Na_2HPO_4-NaOH, pH 11.0–12.0, Δ: KCl-NaOH, pH12.0–13.0

Effects of chemicals and metals on enone reductase activity

One millimolar each of the chemicals and metals was added into the reaction mixture, and the ERD activity was analyzed under standard assay conditions using recombinant *Ss*ERD. The enzyme activity was strongly inhibited by mercaptide-forming reagents such as $AgNO_3$, $HgCl_2$, $CuSO_4$, and *p*-chloromercuribenzoic acid, but the activities were recovered by addition of cysteine into the reaction mixture (Table 3). The ERD activity was not inhibited by chelating reagents and carbonyl reagents, and was increased silightly on adding reducing reagents such as dithiothreitol and cysteine

Effects of site-directed mutagenesis on enzyme activity

A structure model of *Ss*ERD was constructed based on the crystal structure of a double bond reductase from *Zingiber officinale* (PDB ID: 4WGG)[15], which showed the highest identity (39.3%) among enzymes with elucidated crystal structure, and four amino acid residues, Y67, M139, C251, and I281 were selected from its

putative active site (Figure S3). Then, these residues were substituted with alanine, and the ERD activity toward *E*-**2** was analyzed. The Y67A mutant exhibited higher activity than the recombinant *Ss*ERD, whereas M139A and I281A mutants showed lower activity. C251A mutant did not exhibit the ERD activity. Then, the kinetic parameters of four mutants for *E*-**2** and NADPH were analyzed. The Y67A mutant enzyme showed an apparent K_m value of 6.6 ± 0.6 μM and V_{max} value of 454 ± 0.0 nmol min^{-1} mg^{-1} for *E*-**2**, in which the V_{max} value was 4.5 times higher than that of the recombinant *Ss*ERD, while the K_m value was similar to that of the recombinant *Ss*ERD (Table4). However, the apparent V_{max} values of M139A and I281A mutants for *E*-**2** were lower than that of the recombinant *Ss*ERD, while K_m values for *E*-**2** were similar to or lower than that of the recombinant *Ss*ERD. Since M139A and I281A mutants showed higher K_m values for NADPH (120 ± 7.4 μM and 167 ± 17 μM, respectively), the low V_{max} values of both mutants for *E*-**2** might be affected by the low affinity to NADPH. In the C251A mutant, ERD activity was completely lost, indicating that cysteine residue might strongly affect the *Ss*ERD activity.

Discussion

ERDs catalyze reduction of carbon-carbon double bond of α, β-unsaturated ketones,[1] and the enzymes catalyzing the reduction of small ring enones such as 5- or 6-membered ring enones[2, 3, 9, 11-14, 16, 17] or polycyclic enones such as progesterone[4] and morphinone[5] have been widely studied. For example, OYEs from *Saccharomyces carlsbergensis*[2] and *Candida macedoniensis*[12] catalyze the reduction of 6-membered ring enones, such as cyclohexen-2-en-1-one, 3-methylcyclohexen-2-en-1-one, ketoisophorone, and (*R*)-(-)-carvone. 12-Oxophytodienoate reductases from *Lycopersicon esculentum*,[3] enone reductases from *Nicotiana tabacum*,[11] and YqjM from *Bacillus subtilis*[9] catalyze the reduction of 6-membered ring enones and 5-membered ring enones, such as 2-methylcyclopent-2-en-1-one and 3-methylcyclopent-2-en-1-one. It was also reported that some of the above enzymes and *N*-ethylmaleimide reductase from *Yarrowia lipolytica*[14] react with *N*-ethylmaleimide and *N*-phenylmaleimide. However, there are no reports of enzymes catalyzing the reduction of large monocyclic enones, such as 12- or 15-membered ring enones. Therefore, we screened for enzymes catalyzing the reduction of large monocyclic enones and discovered a new enzyme from *S. salmonicolor* TPU 2001, *Ss*ERD, which catalyzes the reduction of carbon-carbon double bond of *E*-**2**.

Table 3. Effects of chemicals and metals on enone reductase activity.

Compounds	Relative activity (%)
None	100
EDTA	97
o-Phenanthroline	101
NH_2OH	101
Semicarbazide	93
Iodoacetic acid	76
$AgNO_3$	0
$HgCl_2$	0
$CuSO_4$	28
$PbCl_2$	97
N-Ethylmaleimide	97
p-Chloromercuribenzoic acid	42
KCN	100
Cysteine	110
Dithiothreitol	132
$AgNO_3$ + Cysteine [a]	90
$HgCl_2$ + Cysteine [a]	74
$CuSO_4$ + Cysteine [a]	113
p-Chloromercuribenzoic acid + Cysteine [a]	117

Each experiment was performed using 1 mM of chemicals or metals under standard assay condition.
[a] After addition of 1 mM of chemicals or metals, 2 mM of cysteine was added to the reaction mixtures, and enzyme activity was assayed under standard condition.

The *Ss*ERD catalyzes the reduction of carbon-carbon double bond of not only (*E*)-3-methylcyclopentadec-2-en-1-one (*E*-**2** in Table 2) but also cyclopentadec-2-en-1-one (**3** in Table 2) and cyclododec-2-en-1-one (**4** in Table 2). However, the enzyme did not catalyze the reduction of (*Z*)-3-methylcyclopentadec-2-en-1-one (*Z*-**2** in Table 2), and small ring enones. Thus, *Ss*ERD is the first enzyme catalyzing the reduction of large monocyclic enones. In addition, the enzyme could recognize the structural difference between *E*-**2** and Z-**2**. Since *Ss*ERD showed unique substrate specificity, its other characteristics were further compared in detail with ERDs catalyzing the reduction of small ring enones.

ERDs can be classified into two groups based on their cofactor specificity: one contains a flavin and NAD(P)H dependent enzymes such as enzymes of the OYE family,[2, 3, 9, 12, 14] and another contains NAD(P)H dependent enzymes such as ERDs from *N. tabacum* (*Nt*DBR),[13] guinea-pig leukotriene B4 12-hydroxydehydrogenase/15-oxo-prostaglandin 13-reductase (LTB_4 12-HD/PGR),[16] double bond reductase from *Arabidopsis thaliana* (At5g16970),[17] and double bond reductase from *Zingiber officinale* (*Zo*DBR).[15] Therefore, we searched for binding site of *Ss*ERD using BLAST program, and obtained the result that a similar NAD(P) binding site was conserved as in LTB_4 12-HD/PGR (PDB ID: 1V3V)[16] and human 15-ketoprostaglandin delta-13-reductase (PDB ID: 2ZB4),[18] which were registered in PDB as the complexes with NADP. This result indicates that *Ss*ERD is an NADPH dependent enzyme, although the total amino acid sequence is not so much similar to that of the above NAD(P)H dependent ERDs (amino acid identity is 35% of *Nt*DBR, 33% of LTB_4 12-HD/PGR, 38% of At5g16970, and 39% of *Zo*DBR).

The inhibitor specificity and site-directed mutagenesis suggested that the interaction with C251 residue and NADPH was important for enzyme activity of *Ss*ERD. The enzyme activity of *Ss*ERD was remarkably inhibited by incubation with mercaptide-forming reagents and recovered on addition of cysteine (Table 3). The enzyme activity of *Ss*ERD was also completely lost with the mutation of C251 (Table 4). It was reported that *Zo*DBR (PDB ID: 4WGG), LTB_4 12-HD/PGR (PDB ID: 1V3T), and At5g16970 (PDB

Table 4. Kinetic parameters for *E*-**2** and NADPH of mutant enzymes.

Mutant	*E*-**2**[a]				NADPH[b]			
	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
Recombinant *Ss*ERD	4.9 ± 0.4	100 ± 1.4	3.7 ± 0.1	0.8 ± 0.07	8.6 ± 0.7	106 ± 0.0	3.9 ± 0.0	0.5 ± 0.0
Y67A	6.6 ± 0.6	454 ± 0.0	17 ± 0.0	2.6 ± 0.3	10 ± 0.6	454 ± 0.0	17 ± 0.0	1.6 ± 0.1
M139A	5.3 ± 0.2	35 ± 0.2	1.3 ± 0.0	0.2 ± 0.0	120 ± 7.4	47 ± 0.5	1.7 ± 0.0	0.014 ± 0.001
C251A	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]
I281A	2.7 ± 0.2	5.8 ± 0.2	0.2 ± 0.0	0.08 ± 0.003	167 ± 17	6.8 ± 0.4	0.25 ± 0.01	0.002 ± 0.001

[a] Kinetic parameters for *E*-**2** were analyzed using 3.9 μM–125 μM of *E*-**2** and 200 μM NADPH under standard assay condition.
[b] Kinetic parameters for NADPH were analyzed using 6.3 μM–200 μM of NADPH and 100 μM *E*-**2** under standard assay condition.
[c] Not detected.
Data represent mean values ± SD of three replicates.

ID: 2J3I) have cysteine residues in their active sites and interact with NADPH, although the cysteine residue is not always conserved in all ERDs.

The site-directed mutagenesis of Y67, M139, and I281, which were present in the putative active sites in the homology model, provided additional information regarding to the substrate specificity and relationship between NADPH and enzyme activity. Y67A mutant exhibited 4.5 times higher specific activity toward *E*-**2**, but lower specific activity toward compounds **3** and **4**, as compared with those of the recombinant *Ss*ERD (Table S2). The kinetic analysis of Y67A mutant indicated that the K_m values for *E*-**2** and NADPH were similar to those of the recombinant *Ss*ERD. On the other hand, mutant enzymes of M139A and I281A exhibited higher K_m values for NADPH compared with that of the recombinant enzyme. Thus, we revealed that M139 and I281 residues might play an important role in the interaction with NADPH, and affected to the enzyme activity.

We revealed that *Ss*ERD can catalyze the synthesis of large monocyclic ketones from the corresponding enones. Large monocyclic ketone derivatives are applicable as synthetic precursors of bicyclic or polycyclic compounds.[19] Here, we reported for the first time, an enzyme that catalyzes the reduction of large monocyclic ketones, and successfully developed new biochemical method for synthesis of large monocyclic ketones.

Conclusions

In this study, we found a new enone reductase catalyzing the reduction of carbon-carbon double bond of large monocyclic enones. Thus, the enzyme can produce (*S*)-muscone by asymmetric reduction of β-methyl substituted 15-membered ring enone. The enzyme also catalyzes the synthesis of cyclododecan-1-one and cyclopentadecan-1-one from 12- and 15-membered ring enones, which do not possess methyl groups. Large monocyclic ketone derivatives are also important intermediates in the synthesis of bicyclic or polycyclic compounds. The enzyme may be useful for development of new environmental friendly methods for biochemical synthesis of various large monocyclic ketones.

Experimental Section

Chemicals

E/Z-**2** was synthesized by Toho Earthtech (Niigata, Japan). *E*- and *Z*-isomers of **2** were separated using the protocol given by Yamamoto et al.[8c] *R*-**1** was prepared using the protocol of Takabe et al.[20] Compunds **3** and **4** were prepared from **5** and **6**, respectively (Figure S4 and S5). All other chemicals used were of analytical grade and commercially available.

Screening for *E/Z*-2 reducing enzymes

Screening for *E/Z*-**2** reducing enzymes was carried out using yeast strains of our laboratory at Toyama Prefectural University, Toyama, Japan (TPU). The strains were cultivated in 10 mL of YPD medium (1% yeast extract, 2% Polypepton, and 2% glucose, pH 7.0) at 30 °C for 24 h, and the cells were harvested by centrifugation (9,000 × *g*, 5 min, 4 °C). The cells were then disrupted using a Multi-beads Shocker instrument (Yasui Kikai, Osaka, Japan) (2,500 rpm, 60 s on time, 60 s off time, 6 cycles), and cell debris was discarded after centrifugation (20,000 × *g*, 20 min, 4 °C). The supernatant was dialyzed against 20 mM KPB, pH 7.0, and the dialyzed enzyme solution was used following ERD activity measurement. The dialyzed enzyme solution containing 1 mg of proteins was incubated with 1 mM *E/Z*-**2** and 2 mM NADPH in 20 mM KPB (1 mL), pH 7.0, at 30 °C. After 1 h of incubation, the reaction mixture was vigorously mixed with ethyl acetate (1 mL), and the products of organic layer were analyzed by GC-MS to confirm the production of muscone.

Enzyme assay

GC-MS method: The reaction mixture (1 mL) contained 0.1 mM *E*-**2**, 0.2 mM NADPH, an appropriate amount of enzyme, and 20 mM KPB, pH 7.0. The reaction was initiated by adding 2 μL of 50 mM *E*-**2** solution in DMSO, and incubated at 30 °C for 1 h. The reaction was terminated by adding ethyl acetate (1 mL), and muscone in organic layer was analyzed by GC-MS equipped with TC-70 column (φ 0.25 μm, 60 m × 0.25 mm, GL-Science, Japan). The amount of muscone was quantified using a standard curve of the authentic muscone. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of muscone per min.

UV method: The reaction mixture (total volume of 1 mL) containing 0.1 mM *E*-**2**, an appropriate amount of enzyme, and 20 mM KPB, pH 7.0, was incubated at 30 °C for 3 min, and the reaction was initiated by the addition of 0.2 mM NADPH. The reaction was monitored at 30 °C for 3 min by measuring absorbance at 340 nm. One unit of enzyme activity was defined

as the amount of enzyme catalyzing the dehydrogenation of 1 μmol of NADPH per min.

Analysis of muscone

Muscone was analyzed using the GC-MS system (GC-2010/GCMS-QP2010 Plus, Shimadzu, Japan) equipped with a TC-70 capillary column. The column was held at 80 °C for 3 min after injection, and followed by 25.0 °C min^{-1} increase to 290 °C. The carrier gas used was helium at a flow rate of 28 mL min^{-1} and a column head pressure of 194 kPa was maintained. The ion source temperature was set at 200 °C in electron ionization (EI). Muscone and substrate were identified by the *m/z* value, and the retention times were compared with authentic standards.

The stereochemistry of muscone was analyzed using the same apparatus equipped with cyclodextrin-3P column (φ 0.25 μm, 0.25 mm × 25 m, Chiral-separations com., Germany). The column was held at 80 °C for 5 min after injection, and followed by 5 °C min^{-1} increase to 230 °C. The carrier gas used was helium at a flow rate of 15.2 mL min^{-1} and a column head pressure of 150 kPa was maintained. The ion source temperature was set at 230 °C in EI. Stereochemistry of muscone was identified by comparison of retention time with authentic standards.

Cultivation of *S. salmonicolor* TPU 2001

S. salmonicolor TPU 2001 was cultivated in YPD medium (10 mL) at 30 °C for 24 h. This culture was then inoculated into 500 mL of the YPD medium and further cultivated at 30 °C for 24 h. The cells were harvested by centrifugation (8,000 × *g*, 10 min), washed with 20 mM KPB, pH 7.0, and stored at -80 °C until use.

Purification of enone reductase from *S. salmonicolor* TPU 2001

All procedures were performed at 4 °C using KPB, pH 7.0, unless otherwise stated.

Cells (85 g of wet cell weight from 2 L of culture) were suspended with 320 mL of 20 mM buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted using a Multi-beads Shocker (2,500 rpm, 60 s on time, 60 s off time, 6 cycles). The supernatant (330 mL) was collected after centrifugation (20,000 × *g*, 20 min), and 69 g of solid ammonium sulfate was added into the enzyme solution up to 35% saturation. The resulting precipitate was removed after centrifugation at 20,000 × *g* for 20 min, and 92 g of solid ammonium sulfate was further added to the supernatant up to 75% saturation. The precipitate formed was collected by centrifugation at 20,000 × *g* for 20 min and dissolved with 20 mM buffer containing 1 mM PMSF.

Solid ammonium sulfate was added to the enzyme solution to 20% saturation, and the enzyme solution was applied to a Toyopearl Butyl-650 column (5.5 × 6.0 cm) equilibrated with 20 mM buffer containing 20% saturated ammonium sulfate. After the column was washed with a buffer containing 15% saturated ammonium sulfate, the adsorbed enzyme was eluted with the buffer containing 10% saturated ammonium sulfate. The active fractions were combined and dialyzed against 20 mM buffer.

The dialyzed enzyme solution was then applied to a Q Sepharose Fast Flow column (2.5 × 8.0 cm) equilibrated with 20 mM buffer, and unadsorbed enzymes were collected.

The enzyme solution was applied to a Toyopearl AF-Blue HC-650 column (1.5 × 2.5 cm) equilibrated with 20 mM buffer, and the column was washed with the same buffer. The adsorbed enzyme was eluted with the buffer containing 10 mM NADPH, and the active fractions were concentrated to 3 mL using Amicon Ultra-15 (Merck Millipore, MA, USA).

The concentrated enzyme solution was applied to a MonoQ 5/50 GL (GE Healthcare, IL, USA) equilibrated with 20 mM buffer and unadsorbed active fractions were combined and used for further studies.

Amino acid sequence analysis

The N-terminal amino acid sequence was analyzed by Nippi Incorporated (Tokyo, Japan). The internal sequence was determined by LC-MS/MS analysis. The enzyme on SDS-PAGE was destained with 30% acetonitrile containing 25 mM ammonium hydrogen carbonate, and reductive-alkylated with iodoacetamide. The trypsin digestion was carried out at 37 °C for 16 h, and the digested peptides were analyzed using a nanoLC-nanoESI-QTOF (Waters, MA, USA) equipped with a nanoACQUITY symmetry C18 (φ 5 μm, 180 μm × 20 mm, Waters) as a trap column and nanoACQUITY CSH 130 C18 (φ 1.7 μm, 75 μm × 200 mm, Waters) as an analysis column maintained at 35 °C. Elution was carried out with H_2O/acetonitrile containing 0.1% formic acid, 1% (0–1 min), 1–50% (1–50 min), 50–95% (50–55 min), 95% (55–75 min), 95–99% (75–78 min) acetonitrile, at a flow rate of 0.3 μL min^{-1}. LC-MS/MS conditions were as follows: electron ionization, nanoESI; cone voltage, 3 kV; fragmentation, collision-induced dissociation. The data was acquired by repeating the collision conditions (10–50 eV per second) and low energy conditions. The homologous sequences of the internal amino acid sequences obtained from de novo peptide sequencing were searched by BLASTP.

Cloning and expression of the *Ss*ERD gene, and purification of the recombinant enzyme

Total RNA was prepared from the cultivated cells using TRIzol (Thermo Fisher Scientific, MA, USA) reagent. cDNA was synthesized using PrimeScript™ RT-PCR Kit (Takara Bio, Otsu, Japan). The coding region of the enzyme was amplified by polymerase chain reaction (PCR) using forward (5'-GGAGATATACCATGGCGCCCATCACCAACAAGAAGACC-3') and reverse (5'-TGCGGCCGCAAGCTTACTCGAGGGAGATGACGG-CCTTGCC-3') primers, which were based on the sequence in accession number CENE01000030 in the GenBank, with start and stop codons. The PCR was performed by using 0.23 pmol of forward primer, 0.23 pmol of reverse primer, 42 ng of gDNA template, and PrimeSTAR MAX premix (Takara Bio) in a final volume of 25 μL. PCR conditions were as follows: 35 cycles at 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 8 s. The amplified PCR products were separated by agarose-gel electrophoresis, purified by a Wizard SV PCR and Gel Clean-Up System (Promega, WI, USA), and ligated into pET28a vector using In-Fusion® HD Cloning Kit (Takara Bio). The DNA sequence was determined using a 3500 Genetic Analyzer (Applied Biosystems, CA, USA).

The plasmid harboring the enone reductase gene was transformed into *E. coli* strain BL21 (DE3). The recombinant *E. coli* was cultivated in 5 mL of Luria-Bertani (LB) broth containing 20 μg kanamycin/mL for 16 h at 37 °C. An aliquot (1%) of the grown cells was added into 500 mL of LB medium containing the same concentration of kanamycin, and incubated for 4 h at 37 °C. In order to induce the expression, 0.5 mM IPTG was added into the culture, and the culture was further cultivated for 16 h at 16 °C. The recombinant enzyme was purified from 30 g of wet cells from 4 L of culture, using the same procedures as those used for the native enzyme.

Site-directed mutagenesis of *Ss*ERD

Site-directed mutagenesis of the enone reductase gene was performed using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, CA, USA), according to manufacturer's instruction. Primers were designed as shown in Table S3, and pET28a vector carrying enone reductase was used as a template for the mutagenesis reactions. PCR conditions were as follows: 95 °C for 2 min, 18 cycles at 95 °C for 20 sec, 60 °C for 10 sec, and 68 °C for 3.5 min, and 68 °C for 5 min.

Effects of pH and temperature on enzyme activity and stability

The optimal pH was assayed by the UV method in the pH range of 4.0–10.5 using sodium acetate, pH 4.0–5.5, KPB, pH 6.0–8.0, Tris-HCl, pH 7.5–9.0, and glycine-NaOH, pH 9.0–10.5. The optimal temperature was analyzed at pH 7.0 by varying the temperature between 5 °C and 60 °C. The pH stability was investigated by incubation at 30 °C for 30 min without enzyme using KCl-HCl, pH 2.5–3.5, sodium acetate, pH 4.0–5.5, KPB, pH 6.0–8.0, Tris-HCl, pH 7.5–9.0, glycine-NaOH, pH 9.0–10.5, Na_2HPO_4-NaOH, pH 11.0–12.0, and KCl-NaOH, pH 12.0–13.0. The temperature

stability was analyzed after incubating at 20–65 °C for 30 min in KPB, pH 7.0. After the incubation, the remaining activity was analyzed under standard assay conditions.

Other analytical methods

Protein concentration was determined by the method of Bradford using the dye reagent supplied by Nacalai tesque (Kyoto, Japan). Bovine serum albumin was used as standard.

The molecular mass of native enzyme was estimated by gel filtration on a Superdex 200 10/300 GL (GE Healthcare) equilibrated with 20 mM KPB, pH 7.0, containing 150 mM NaCl. Standard proteins were glutamate dehydrogenase (290,000 Da), lactate dehydrogenase (142,000 Da), enolase (67,000 Da), myokinase (32,000 Da), and cytochrome c (12,400 Da).

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Keywords: asymmetric synthesis • enone reductase • large monocyclic enone • muscone • mutagenesis

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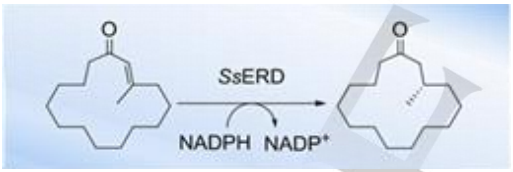

Substituting the Ru-BINAP catalyst: This is the first report of enone reductase reducing carbon-carbon double bond of large monocyclic enones. The enone reductase is available for asymmetric synthesis of (*S*)-muscone from (*E*)-3-methylcyclopentadec-2-en-1-one, which has so far only been reported by chemical reduction using Ru-BINAP catalyst.

*Kazunori Yamamoto, Yuko Oku, Atsutoshi Ina, Atsushi Izumi, Masaharu Doya, Syuji Ebata, and Yasuhisa Asano**

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Purification and characterization of a novel enone reductase from *Sporidiobolus salmonicolor* TPU 2001 reacting with large monocyclic enones