Available online at www.sciencedirect.com
ScienceDirect
Bioorganic Chemistry 36 (2008) 23–28
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

An enone reductase from *Nicotiana tabacum*: cDNA cloning, expression in *Escherichia coli*, and reduction of enones with the recombinant proteins

Akihito Matsushima [a], Yuya Sato [b], Miki Otsuka [b], Takayoshi Watanabe [b], Hiroaki Yamamoto [c], Toshifumi Hirata [b,*]

[a] *Natural Science Center for Basic Research and Development, Radioisotope Center, Hiroshima University, 1-4-2 Kagamiyama, Higashi-Hiroshima 739-8526, Japan*
[b] *Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan*
[c] *R&D Management Corporate Development Center, Daicel Chemical Industries, Ltd., Himeji Research Center, 1239 Shinzaike, Aboshi-ku, Himeji, Hyogo 671-1283, Japan*

Received 26 July 2007
Available online 22 October 2007

Abstract

In the course of the purification of enone reductase participating to the reduction of pulegone, two reductases (NtRed-1 and NtRed-2) were isolated from cultured cells of *Nicotiana tabacum*. The partial amino acid sequences of the reductases revealed that NtRed-1 was allyl-alcohol dehydrogenase (Accession No. BAA89423) and NtRed-2 was malate dehydrogenase (Accession No. CAC12826). cDNA cloning and expression of these reductases in *Escherichia coli* were performed. Reduction with recombinant proteins was examined with cyclic α,β-unsaturated ketones, such as pulegone, carvone and verbenone, as substrates. It was found that the recombinant NtRed-1 catalyses the hydrogenation of the exocyclic C–C double bond of pulegone.

Keywords: *Nicotiana tabacum*; Enone reductase; Allyl-alcohol dehydrogenase; Malate dehydrogenase; cDNA cloning; Recombinant proteins; Reduction of pulegone; Reduction of enones

1. Introduction

Asymmetric hydrogenation of C–C double bonds is one of the most important reactions for production of chiral compounds from alkenes [1,2]. Many studies on the enantioselective reduction of the enone C–C double bond by biocatalysts such as microorganisms and plant cell cultures have revealed that enone reductases are useful for the production of chiral ketones [3–11].

Previously, we reported the isolation of two types of enone reductase that differed with respect to the substrate specificity from cultured cells of *Nicotiana tabacum* [12]. One is responsible for reducing the exocyclic double bond of enones, such as pulegone (**1**) [13], and the other reduces the endocyclic double bond of enones, such as carvone (**2**) [14] and verbenone (**3**) [15]. Recently, many researchers have reported on enone reductases from plant cells in the research of secondary metabolism [16–18]. Ringer et al. reported [16] the characterization of pulegone reductase from *Mentha piperita* (PulR; Accession No. AAQ75423) by genetic engineering; the multiple alignment of PulR was referred to allyl-alcohol dehydrogenase (allyl-ADH; Accession No. BAA89423) from *N. tabacum* [19]. This report suggested that allyl-ADH catalyzed the reduction of the C–C double bond of enones.

We have now investigated the structure and function of the enone reductase that is involved in the reduction of the

* Corresponding author. Fax: +81 82 424 7435.
E-mail address: thirata@sci.hiroshima-u.ac.jp (T. Hirata).

0045-2068/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bioorg.2007.08.005

C–C double bond of pulegone (**1**) in *N. tabacum* cells, by cDNA cloning, expression of recombinant reductase in *Escherichia coli*, and biotransformation of enones with the recombinant reductase.

2. Material and methods

2.1. General

Analytical and preparative thin-layer chromatography (TLC) analyses were performed on glass sheets (0.25 and 0.5 mm) coated with silica gel (Merck silica gel 60; GF_{254}). Optical rotations were measured with a JASCO DIP-370 digital polarimeter. Gas chromatography (GC) analyses with flame ionization detector (FID) were performed on a capillary column (0.25 mm × 25 m) coated with 0.25-μm Rt-βDEXse (Shimadzu) using N_2 as a carrier gas (column temp: 140 °C, split ratio: 50, make up: 50 ml min^{-1}). Gas chromatography–mass spectrometry (GC–MS) was performed using a JEOL SX-102A mass spectrometer equipped with an electron ionization (EI) ion source (70 eV) and a gas chromatograph equipped with a capillary column (0.25 mm × 25 m) coated with 0.25-μm ZB-5 (Zebron). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was performed using an Ultraflex Time-of-Flight Instrument (Bruker Daltonics) equipped with a nitrogen laser operating at 337 nm. ^1H NMR spectra were obtained using a JEOL LA-500 (500 MHz) spectrometer, using tetramethylsilane (TMS) as an internal standard in $CDCl_3$. DNA sequences were determined using an Applied Biosystems Model 373A Automated Sequencer. Luria–Bertani (LB) medium containing 1% tryptone, 0.5% yeast extract and 1% NaCl was used for cultivation of *E. coli* cells. Protein concentrations were determined using a Bio-Rad Protein Assay kit (Bio-Rad laboratories) and bovine serum albumin was used as the standard protein solution.

2.2. Substrates

(*R*)-Pulegone (**1a**) {>99% pure on GLC, $[\alpha]_D^{25}$ + 22.3 (neat); lit. [13]: $[\alpha]_D^{25}$ + 22.4 (neat)}, (*S*)-pulegone (**1b**) {>99% on GLC, $[\alpha]_D^{25}$ − 22.0 (neat)}, (*S*)-carvone (**2a**) {99% on GLC, $[\alpha]_D^{25}$ − 60.1 (neat); lit. [14]: $[\alpha]_D^{25}$ − 59.7 (neat)}, (*R*)-carvone (**2b**) {>99% on GLC, $[\alpha]_D^{25}$ + 57.1 (neat)}, (1*S*,5*S*)-verbenone (**3a**) {99% on GLC, $[\alpha]_D^{25}$ − 209.3 (neat); lit. [15]: $[\alpha]_D^{25}$ − 208 (neat)}, and (1*R*,5*R*)-verbenone (**3b**) {99% on GLC, $[\alpha]_D^{25}$ + 210.5 (neat)} were purchased from Sigma–Aldrich. Oxalacetic acid (**4**) was obtained from Wako Pure Chemical Industries. (2*S*,4*S*)-Carveol (**5**) (99% pure on GLC) was prepared from (*S*)-carvone (**2a**) by $NaBH_4$ reduction [19] (Fig. 1).

2.3. Purification of pulegone reductase from N. tabacum cells

Native NtRed-1 was purified according to the procedure described previously [13]. All operations were performed at 4 °C. The cultured cells (200 g) of *N. tabacum* (cultivated for 3 weeks) [4] were ground in a Waring blender with 400 ml of 0.1 M Na–Pi buffer (pH 6.8) containing 10 mM 2-mercaptoethanol and 5 mM dithiothreitol. The resulting slurry was filtered through three layers of cheesecloth. The filtrate was centrifuged at 10,000*g* for 15 min. The supernatant was fractionated stepwise by the addition of $(NH_4)_2SO_4$, and the fraction obtained between 40% and 60% saturation was collected by centrifugation. The pellet was dissolved in 30 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM 2-mercaptoethanol and 1 mM dithiothreithol (buffer A) and the crude enzyme solution was separated from $(NH_4)_2SO_4$ by passing through a Sephadex G-25 column (3 × 40 cm) equilibrated with buffer A. The desalted proteins were applied to a DEAE–Toyopearl column (2 × 20 cm) equilibrated with buffer A. The enzymes were eluted with 200 ml of buffer A containing a 0–0.5 M linear gradient of NaCl to give an enone reductase fraction, which catalyzed the reduction of the C–C double bond of pulegone (**1a**). The reductase fraction was further applied to a Red–Toyopearl column (1 × 10 cm) equilibrated with buffer A. After non-adsorbed proteins were eluted, adsorbed proteins were eluted with buffer A containing a 0–1.0 M linear gradient of NaCl to yield pulegone reductase.

The purified reductase fraction was separated by SDS–PAGE (12.5% gel); this showed two bands, one at 36.3 kDa (NtRed-1) and one at 36.8 kDa (NtRed-2). These bands were excised from the SDS–PAGE gel, and each gel

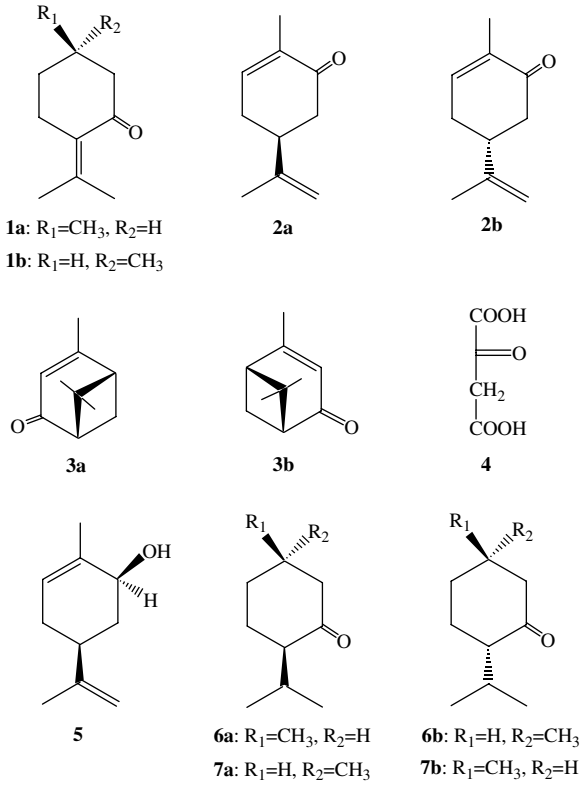

Fig. 1. Formulas **1a**–**7b**.

piece was subjected to digestion with trypsin directly to give a crude peptide fraction. The peptide fraction was subjected to preparative HPLC on a 3.5-μm C18 column (Waters; 1.0 × 150 mm). The resulting peptide fragments were sequenced, and the amino acid sequences were identified as Lys-Ile-Glu-Gly-X-Tyr-Val-Glu-X-Phe-Ala-Pro-Gly for NtRed-1 and Val-Leu-Val-Val-Ala-Asn-Pro-Ala-Asn-Thr-Asn-Ala for NtRed-2. The obtained amino acid sequences were analyzed by the NCBI 'blastp' program. Furthermore, the crude peptide fractions were subjected to MALDI-TOF MS analysis. The peptide fragments were identified by analysis with the peptide MS finger-print method using a software MASCOT (http://www.matrixscience.com).

2.4. Cloning of the NtRed-1 cDNA for expression vector

Total cellular RNA was purified from cultured cells of *N. tabacum* using ISOGEN (Nippon Gene Co.). Single-stranded cDNA synthesis of the purified RNA was then performed using oligo(dT)20-M13M4 adapter primer. Two oligonucleotides, NtRed-1-1 (5′ GGGATCCATGGCAGAA GAAGTGAGCAAC 3′ +31 to +55) and NtRed-1–2 (5′ CACTCGAGACATTCATTCACGCGAAAC 3′ +1048 to +1067), which were designed from the nucleotide sequence of allyl-ADH (Accession No. AB036735), were used as primers for polymerase chain reaction (PCR) amplification to produce *NtRed-1* cDNA from single-stranded cDNA. Both primers contain BamHI and XhoI restriction sequences. The amplification involved 30 cycles of 94 °C for 1 min for denaturation, 68 °C for 1 min for annealing, and 72 °C for 1 min for synthesis. The final PCR product was 1051 bp, and this was ligated to the pPCR-Script SK(−) vector (Stratagene), which was inserted into *E. coli* DH5α competent cells. Double-stranded DNA sequencing by the dideoxynucleotide chain termination was performed using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences) and an ABI 373 DNA sequencer (Applied Biosystems). The cDNA clone of *NtRed-1* was then digested with BamHI (TOYOBO) and XhoI (TOYOBO), and ligated to the pGEX-6P-1 expression vector (Amersham Bioscience) to construct pGEX-6P-1(*NtRed-1*). Finally, pGEX-6P-1(*NtRed-1*) was transformed into *E. coli* BL21 competent cells. The DNA sequence of cloned *NtRed-1* cDNA was also confirmed by DNA sequencing.

2.5. Expression of recombinant NtRed-1 in E. coli

The expression and purification of NtRed-1 were performed according to the manufacturer's instructions. The *E. coli* cells transformed with pGEX-6P-1(*NtRed-1*) in 50 ml of LB medium were cultivated at 30 °C, and 0.1 mM of isopropyl-β-D-thiogalactopyranoside was then added to the cultures and incubated at 16 °C for 16 h. After centrifugation, the *E. coli* pellet was sonicated. The glutathione *S*-transferase (GST) fusion proteins ware purified using glutathione Sepharose 4B (Pharmacia Biotech) affinity-column chromatography and digested by PreScission protease to release the recombinant NtRed-1 (rNtRed-1), as shown in Fig. 2. The molecular weight of purified rNtRed-1 was determined by SDS–PAGE and MALDI-TOF MS. The molecular mass of the rNtRed-1 in a state of non-denaturation was estimated by gel filtration chromatography on a Sephadex G-100 column using aldolase, bovine serum albumin, ovalubumin and ribonuclease as marker proteins.

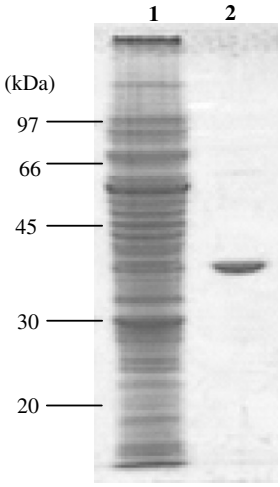

Fig. 2. Expression and purification of recombinant NtRed-1. Lane 1, crude soluble fraction; lane 2, purified recombinant NtRed-1.

2.6. Cloning and expression of recombinant NtRed-2 in E. coli

All the procedures used for the cloning and expression of recombinant NtRed-2 (r NtRed-2) in *E. coli* were similar to those for the cloning and expression of rNtRed-1 described above, except for the PCR conditions. Two oligonucleotides, MDHF-1+BHI (5′ GGATCCATGGCTAAAGATCCA GTTCGCGTT 3′ +55 to +78) and MDHR-1+XhoI (5′ CTCGAGTCACATACACTCCCCATGAGTTGA 3′ +1159 to +1178), which were designed from the nucleotide sequence of malate dehydrogenase (Accession No. CAC12826), were used as PCR primers to generate *NtMDH* cDNA from single-stranded cDNA. Both primers contained BamHI and XhoI restriction sequences. PCR amplification involved 30 cycles of 94 °C for 1 min for denaturation, 60 °C for 1 min for annealing, and 72 °C for 1 min for synthesis. The final PCR product was 1136 bp. The DNA sequence of cloned *NtRed-2* cDNA was also confirmed by DNA sequencing. After DNA sequencing, *NtRed-2* was ligated to the pGEX-6P-1 expression vector (Amersham Bioscience) to construct pGEX-6P-1(*NtRed-2*). rNtRed-2 was expressed in the *E. coli* BL21 strain according to the procedure described above. The expressed rNtRed-2 was also purified using glutathione Sepharose 4B (Pharmacia Biotech) affinity-column chromatography and digested by PreScission proteases to release the rNtRed-2 from the GST-fusion protein. The molecular weight of purified

rNtRed-2 was confirmed by SDS–PAGE and MALDI-TOF MS analyses.

2.7. Enzyme activity assay

The standard assay mixture (2 ml) was composed of the recombinant enzyme preparation (50 μg), 200 μmol NADPH, 100 μmol substrate, 1 mM dithiothreitol, 1 mM mercaptoethanol, and 0.2 ml 1% Triton X-100 in 50 mM Na-phosphate buffer (pH 7.0). The mixture was incubated at 35 °C for 3 h and then extracted with 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 analyses and the product yields were determined by the results of the GC analyses. The final products were identified by GC, GC–MS, and/or ^{1}H NMR spectrometry.

To determine the kinetic parameters for the recombinant reductase, the assay mixture (2 ml) was composed of the enzyme preparation (10 μg), 3 mM NADPH, 1 mM dithiothreitol, 0.2 ml of 1% Triton X-100, 0.1–3.0 mM of the substrate in 50 mM Na-phosphate buffer (pH 7.0). The mixture was incubated at 35 °C and then extracted with ether (3 ml × 3). The ether layer was subjected to GC analyses.

3. Results and discussion

3.1. Purification and structure determination of reductases

A soluble cell-free extract was obtained from cultured cells of *N. tabacum*. The crude extract was subjected to chromatography on a DEAE–Toyopearl column to give a crude enzyme fraction that was involved in the reduction of the C–C double bond of pulegone [12]. Further purification of the pulegone reductase on a Red–Toyopearl column resulted in about 160-fold increased reductase activity. However, SDS–PAGE of the purified reductase revealed two protein bands corresponding to molecular masses of 36.3 (NtRed-1) and 36.8 kDa (NtRed-2).

The partial amino acid sequences of two protein bands were found to be Lys-Ile-Glu-Gly-X-Tyr-Val-Glu-X-Phe-Ala-Pro-Gly for NtRed-1 and Val-Leu-Val-Val-Ala-Asn-Pro-Ala-Asn-Thr-Asn-Ala for NtRed-2. A comparison of the peptide sequence against a protein database using the NCBI 'blastp' program showed a significant homology with allyl-ADH from *N. tabacum* (Accession No. BAA89423) [19] for NtRed-1 and malate dehydrogenase from *N. tabacum* (NtMDH; Accession No. CAC12826) for NtRed-2. The identity of NtRed-1 to allyl-ADH was confirmed by the peptide mass finger-print method in the mass spectroscopic analysis of NtRed-1. However, the amino acid sequence of allyl-ADH does not contain the previously reported partial sequence for the p74 reductase catalyzing the hydrogenation of exocyclic C–C double bond of enones [13]. This may indicate that there are two different enzymes participating to the reduction of exocyclic C–C double bond of enones in *N. tabacum*.

The deduced amino acid sequence of NtRed-1 shared high identity with 2-alkenal dehydrogenase from *Arabidopsis thaliana* (AtDBR1; Accession No. NP-197199), zeta-crystallin homologue from *A. thaliana* (Accession No. CAA89262), (+)-pulegone reductase from *M. piperita* (PulR; Accession No. AAQ75423) and phenylpropenal double-bond reductase from *Pinus taeda* (PtPPDBR; Accession No. ABG91753) (Fig. 3). All of these enzymes are known to catalyze the reduction of C–C double bond of α,β-unsaturated ketones or aldehydes [16–18,20]. The *AXXGXXG* motif (Fig. 3), which is known to participate in binding of the phosphate group of $NAD(P)^+$ or NAD(P)H [21], is conserved in all these reductases. The *GXXS* motif (Fig. 3), which stabilize both adenine and nicotinamide moieties of the cofactor in NADPH-bound form of quinine oxidoreductase [22], is also conserved in all these reductases. Crystallographic analyses of AtDBR1 indicate that the deduced amino acid sequence of NtRed-1 may comprise two domains: a substrate-binding domain (residues 1–135 and 304–343) and a nucleotide-binding domain (residues 136–303) [18]. The amino acid residue that is expected to stabilize the enol form of the transition state was also conserved at Tyr-258 of NtRed-1 (indicated by an asterisk in Fig. 3).

3.2. Expression of the recombinant reductases in E. coli

For cloning of NtRed-1, the primers for amplification of *NtRed-1* cDNA were designed from the nucleotide sequence of allyl-ADH (Accession No. AB036735). The cloned nucleotide sequence of NtRed-1 has 100% identity with allyl-ADH [19] and 78% identity with pulegone reductase from *M. piperita* (Accession No. AY300163) [16]. The MALDI-TOF MS spectra showed that molecular mass of recombinant NtRed-1 was 38482 ± 10 Da (theoretical value: 38486.6 Da). The molecular mass in a state of non-denaturation was estimated to be 74 kDa by gel filtration chromatography. These may indicate that the rNtRed-1 is a dimeric structure of 38 kDa subunits in a state of active enzyme.

On the other hand, for cloning of NtRed-2, the primers used for amplification of *NtRed-2* cDNA by PCR were designed from the nucleotide sequence of NtRed-2 (Accession No. CAC12826). The cloned nucleotide sequence of NtRed-2 shows 100% homology with malate dehydrogenase, as shown by the peptide mass finger-print mass spectroscopy method. The MALDI-TOF MS spectrum showed that molecular mass of recombinant NtRed-2 was 35812 ± 10 Da (theoretical value: 35805.9 Da).

3.3. Reduction of enones with the recombinant reductases

To determine the enzymatic activity of the recombinant proteins rNtRed-1 and rNtRed-2, enzymatic reactions were investigated with the following substrates: (*R*)-pulegone (**1a**), (*S*)-pulegone (**1b**), (*S*)-carvone (**2a**), (*R*)-carvone (**2b**), (1*S*,5*S*)-verbenone (**3a**), and (1*R*,5*R*)-verbenone (**3b**),

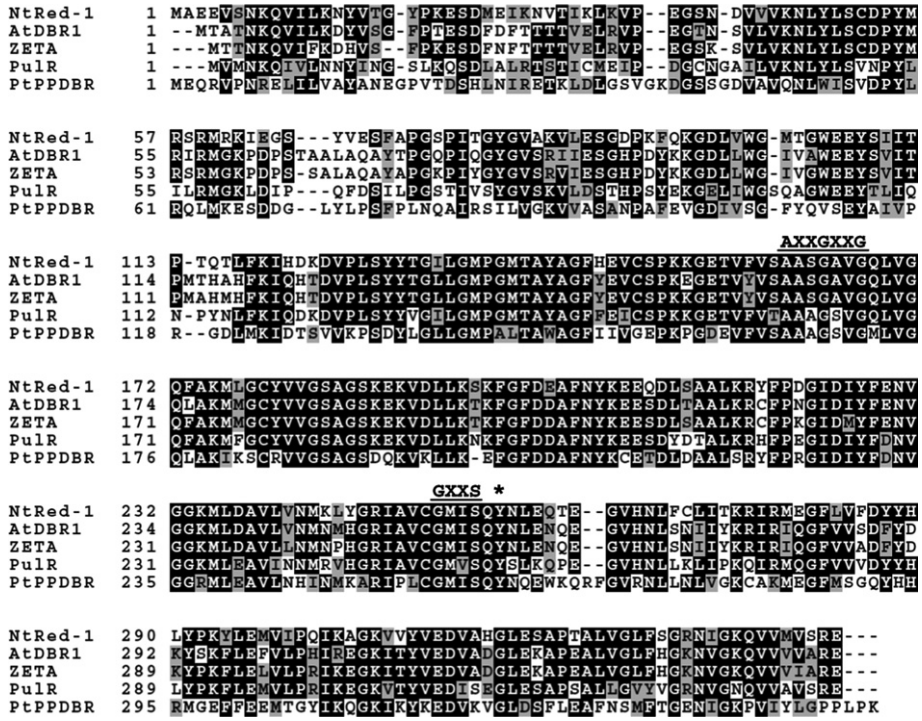

Fig. 3. Alignment of amino acid sequences deduced from the cDNA encoding pulegone reductase from *N. tabacum* (NtRed-1) and other reductases. The amino acid residue depicted indicated by an asterisk (*) functions as a general acid/base for hydride transfer. Identical and similar amino acid residues are shown in black and gray, respectively. AtDBR1, 2-alkenal dehydrogenase from *A. thaliana* (Accession No. NP-197199); ZETA, zeta-crystallin homologue from *A. thaliana* (Accession No. CAA89262); NtADH, allyl-alcohol dehydrogenase from *N. tabacum* (Accession No. BAA89423); PulR, (+)-pulegone reductase from *M. piperita* (Accession No. AAQ75423); PtPPDBR, phenylpropenal double-bond reductase from *P. taeda* (Accession No. ABG91753).

oxalacetic acid (**4**) and (2*S*,4*S*)-carveol (**5**). It was found that rNtRed-1 catalyzed a reduction of pulegones (**1a** and **1b**) with an exocyclic C–C double bond to give the corresponding ketones, isomenthone (**6**) and menthone (**7**); however, there was no reduction of the enones, **2a**, **2b**, **3a** and **3b**, with an endocyclic C–C double bond, as shown in Table 1. The kinetic parameters, K_m and K_{cat}, in the reduction of pulegone with rNtRed-1 were summarized in Table 2. (*R*)-pulegone (**1a**) was a preferred substrate over that of its (*S*)-enantiomer (**1b**). However, the substrate specificity of rNtRed-1 for (*R*)-pulegone (**1a**) (K_m 1.4 mM) was quite low, as compared with that of previously reported native reductase (K_m 25 μM) [13].

By contrast, rNtRed-2 catalyzed the reduction of the carbonyl group of oxalacetic acid (**4**), but no other substrates, especially pulegone, were reduced. This may show that malate dehydrogenase did not participate with the reduction of enones, though the malate dehydrogenase coincidently co-purified with the enone reductase.

These results show that the enzyme involved in the reduction of the C–C double bond of enones is NtRed-1. However, recombinant NtRed-1 did not catalyze a dehydrogenation of (*2S,4S*)-carveol (**5**). This indicates that the recombinant NtRed-1 lacked the catalytic function as allyl-alcohol dehydrogenase [19]; although the recombinant NtRed-1 was designed from the nucleotide sequence of allyl-ADH (Accession No. AB036735).

Table 1
Substrate specificity in the reduction of enones with the recombinant NtRed-1

Entry (reaction time, h)	Substrates	Products	Conversion (%)
1 (3)	(*R*)-Pulegone (**1a**)	(1*R*,4*R*)-Isomenthone (**6a**)	4.4
		(1*R*,4*S*)-Menthone (**7b**)	6.8
2 (12)	(*R*)-Pulegone (**1a**)	(1*R*,4*R*)-Isomenthone (**6a**)	14.3
		(1*R*,4*S*)-Menthone (**7b**)	15.7
3 (3)	(*S*)-Pulegone (**1b**)	(1*S*,4*S*)-Isomenthone (**6b**)	0.3
		(1*S*,4*R*)-Menthone (**7a**)	0.5
4 (12)	(*S*)-Pulegone (**1b**)	(1*S*,4*S*)-Isomenthone (**6b**)	1.6
		(1*S*,4*R*)-Menthone (**7a**)	2.1
5 (12)	(*R*)-Carvone (**2a**)	—	N.d.[a]
6 (12)	(*S*)-Carvone (**2b**)	—	N.d.
7 (12)	(1*S*,5*S*)-Verbenone (**3a**)	—	N.d.
8 (12)	(1*R*,5*R*)-Verbenone (**3b**)	—	N.d.
9 (12)	Oxalacetic acid (**4**)	—	N.d.
10 (12)	(2*S*,4*S*)-Carveol (**5**)	—	N.d.

[a] N.d. denotes not detected.

Table 2
Kinetic parameters for the recombinant NtRed-1

Substrates	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})
(*R*)-Pulegone (**1a**)	1.4	3.3	2.4
(*S*)-Pulegone (**1b**)	8.3	2.8	0.34

In the reduction of enones with rNtRed-1, enones with the exocyclic C–C double bond were suitable substrates and enones with the endocyclic C–C double bond were not. However, the stereospecificity of hydrogenation was lost with rNtRed-1 reduction, although the reduction of (*R*)-pulegone (**1a**) with native enzyme was highly stereospecific to give (1*R*,4*R*)-isomenthone (**6a**). The lack of stereospecificity in rNtRed-1 might mean that there are two different enzymes participating to the hydrogenation of exocyclic C–C double bond of enones in *N. tabacum*, or this is caused by a lack of cofactors in the reaction with rNtRed-1 or to changes in its quaternary structure during the folding process of the recombinant protein.

Youn et al. [18] reported that the substrate versatility of 2-alkenal dehydrogenase (AtDBR1) from *A. thaliana*, which is closely similar to NtRed-1, was fairly limited. The substrate selectivity of enone reductases is due to variability in the amino acid sequence of the substrate-binding domain. Although the amino acid sequence homologies of the nucleotide-binding domains of these proteins (Fig. 3) are high (59–80%), the substrate-binding domains are variable (residue 1–135, 32–58%; residue 136–303, 32–72%). This variability may explain the substrate selectivity of these reductases. Further investigations are necessary for a complete understanding of the enone reductases.

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] A. Kergomard, M.F. Renard, H. Veshambre, J. Org. Chem. 47 (1982) 792–798.
[4] T. Hirata, H. Hamada, T. Aoki, T. Suga, Phytochemistry 21 (1982) 2209–2212.
[5] T. Suga, H. Hirata, H. Hamada, S. Murakami, Phytochemistry 27 (1988) 1041–1044.
[6] A. Kergomard, M.F. Renard, H. Vashambre, D. Courtois, V. Petiard, Phytochemistry 27 (1988) 407–409.
[7] M. Kataoka, A. Kotaka, A. Hasegawa, M. Wada, A. Yoshizumi, S. Nakamori, S. Shimizu, Biosci. Biotechnol. Biochem. 66 (2002) 2651–2657.
[8] K. Matsumoto, Y. Kawabata, J. Takahashi, Y. Fujita, M. Hatanaka, Chem. Lett. (1998) 283–284.
[9] P. Wanner, R. Tressl, Eur. J. Biochem. 255 (1998) 271–278.
[10] M.A. Swiderska, J.D. Stewart, J. Mol. Catal. B: Enzym. 42 (2006) 52–54.
[11] A. Muller, B. Hauer, B. Rosche, J. Mol. Catal. B: Enzym. 38 (2006) 126–130.
[12] T. Hirata, K. Shimoda, T. Gondai, Chem. Lett. (2000) 850–851.
[13] K. Shimoda, S. Izumi, T. Hirata, Bull. Chem. Soc. Jpn. 75 (2002) 813–816.
[14] T. Hirata, Y.X. Tang, K. Okano, T. Suga, Phytochemistry 28 (1989) 3331–3333.
[15] K. Shimoda, D.I. Ito, S. Izumi, T. Hirata, J. Chem. Soc., Perkin Trans. 1 (1996) 355–358.
[16] K.L. Ringer, M.E. McConkey, E.M. Davis, G.W. Rushing, R. Croteau, Arch. Biochem. Biophys. 418 (2003) 80–92.
[17] H. Kasahara, Y. Jiao, D.L. Bedgar, S.-J. Kim, A.M. Pattern, Z.-Q. Xia, L.B. Davin, N.G. Lewis, Phytochemistry 67 (2006) 1765–1780.
[18] B. Youn, S.-J. Kim, S.G.A. Moinuddin, C. Lee, D.L. Bedgar, A.R. Harper, L.B. Davin, N.G. Lewis, C. Kang, J. Biol. Chem. 281 (2006) 40076–40088.
[19] T. Hirata, Y. Tamura, N. Yokobatake, K. Shimoda, T. Ashida, Phytochemistry 55 (2000) 297–303.
[20] E. Babichuk, S. Kushnir, E. Belles-Boix, M.V. Montagu, D. Inze, J. Biol. Chem. 270 (1995) 26224–26231.
[21] P.J.B. Pereira, S. Macedo-Ribeiro, A. Parrage, R. Perez-Luque, O. Cunningham, K. Darcy, T.J. Mantle, M. Coll, Nat. Struct. Biol. 8 (2001) 215–220.
[22] I. Levin, R. Schwarzenbacher, D. McMullan, P. Abdubek, E. Ambing, T. Biorac, J. Cambell, J.M. Canaves, H.-J. Chiu, X. Dai, A.M. Deacon, M. DiDonato, M.-A. Elsliger, A. Godzik, C. Grittini, S.K. Grzechnik, E. Hampton, L. Jaroszewski, C. Karlak, H.E. Klock, E. Koesema, A. Kreusch, P. Kuhn, S.A. Lesley, T.M. McPhillips, M.D. Miller, A. Morse, K. Moy, J. Ouyang, R. Page, K. Quijano, R. Reyes, A. Robb, E. Sims, G. Spraggon, R.C. Stevens, H. van den Bedem, J. Velasquez, J. Vincent, F. von Delft, X. Wang, B. West, G. Wolf, Q. Xu, K.O. Hodgson, J. Wooley, I.A. Wilson, Proteins 56 (2004) 629–633.