A Novel Reductase Participating in the Hydrogenation of an Exocyclic C–C Double Bond of Enones from *Nicotiana tabacum*

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A novel 74 kDa enone reductase which catalyzes the reduction of the exocyclic C–C double bond of enones was isolated from cultured cells of *Nicotiana tabacum*. The reductase was found to catalyze the enantiofacially selective reduction of the C–C double bond of 2-alkylidenecyclohexanone to give optically active (*S*)-2-alkylcyclohexanone.

Enone reductases occur widely in plants and microorganisms. Many studies on the reduction of the C–C double bond of enones by plant cell cultures and microorganisms have revealed that there are two different types of enone reductase with respect to the substrate specificity; one is responsible for reducing the endocyclic double bond and the other is for reducing the exocyclic double bond of enones.[1–8] Recently, we reported the isolation of two enone reductases participating in the reduction of the endocyclic C–C double bond of enones from cultured cells of *N. tabacum*; carvone reductase was able to reduce only a C–C double bond bearing a hydrogen atom at the β position to the carbonyl group,[9] and verbenone reductase was capable of reducing a broad range of enones.[10,11] We have now isolated a novel reductase which participates in the reduction of the exocyclic C–C double bond of enones, such as pulegone (**1**), from *N. tabacum*,[12] and here report on the details concerning the characterization of the reductase and the stereospecificity in the enzymatic reduction of enones.

Results and Discussion

A soluble cell-free extract was obtained from cultured cells of *Nicotiana tabacum*. The crude extract was subjected to chromatography on a DEAE-Toyopearl column to give three reductases of 44, 74 and 90 kDa, participating in the reduction of the C–C double bond of enones (see Fig. 1). The 90 kDa and 44 kDa reductases were identified as the verbenone reductase[11] and the carvone reductase,[9] respectively, as reported in our previous paper. However, the 74 kDa reductase was different from the known reductases. Chromatographic purification of the 74 kDa reductase resulted in a 160-fold enhancement of the reductase activity, as shown in Table 1. SDS-PAGE of the enone reductase revealed a single protein band corresponding to a molecular mass of 37 kDa, as shown in Fig. 2. The molecular mass in the native state was estimated to be 74 kDa by gel filtration chromatography on a Sephadex G-150 column using marker proteins. These observations indicate that the native enzyme is a dimeric structure of 37 kDa subunits. The enzyme reaction was found to require NADH or

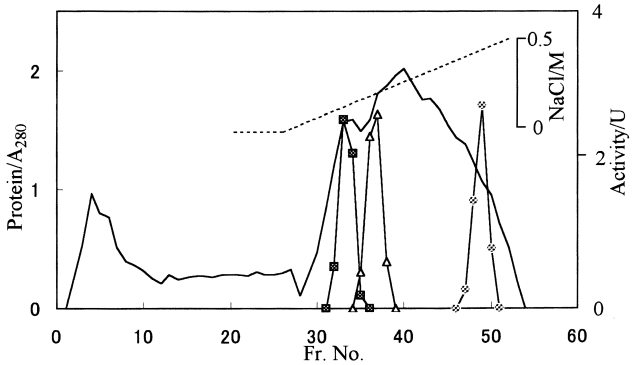

Fig. 1. Elution profiles of enone reductases on DEAE-Toyopearl column chromatography. —▩—: 74 kDa, —△—: 44 kDa, and —▨—: 90 kDa enone reductase.

Table 1. Purification of a 74 kDa Enone Reductase from Cultured Cells of *N. tabacum*

Step	Total protein	Total activity	Specific activity	Fold
	mg	U[a)]	U mg^{-1}	
Crude extract	120	12	0.1	1
DEAE-Toyopearl	8	3	0.4	4
Hydroxylapatite	0.5	1.5	3	30
Red Toyopearl	0.07	1.1	16	160

a) One unit of the reductase activity is defined as the quantity of enzyme that reduces 1 μmol of (*R*)-Pulegone (**1**) per one minute under the standard assay condition.

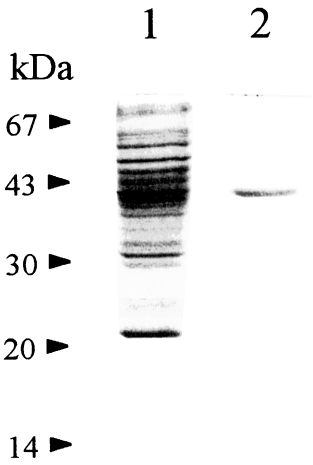

Fig. 2. SDS-gel electrophoretic analyses of a 74 kDa enone reductase. Lane 1: Crude extract; Lane 2: Red Toyopearl Fr.

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p74 red.                TXLLFDDTQKQFK
S. tuberosum-a      19  TSLLFDDTQKQFK
S. tuberosum-b      30  TSLLLDDTQKQFK
A. thaliana-a       27  SSLLFDDTQLQFK
A. thaliana-b       27  SSLLFDDTQLQFK
                        : **:**** ***
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Fig. 3. Comparison of amino acid sequences of the 74 kDa reductase (p74 red.) and the reported sequences deduced from nucleotide sequences of four genes for isovaleryl-CoA dehydrogenases: S. tuberosum-a from *Solanum tuberosum* (Gene Bank accession no. AJ278988) [17], S. tuberosum-b from *Solanum tuberosum* (AJ278987) [17], A. thaliana-a from *Arabidopsis thaliana* (AAD45605) [17], and A. thaliana-b from *Arabidopsis thaliana* (T47470) [14]. Numbers indicate positions relative to the putative initial methionine or the amino terminus.

NADPH as the coenzymes, and NADPH was a better electron donor by a factor of 3 compared with NADH. The enzyme had a pH optimum at 7.1 in Na–Pi buffer.

The partial amino acid sequences of the enone reductase were analyzed with an automated protein sequencer. However, the amino terminus of the reductase was blocked. The reductase, therefore, was digested with lysylendopeptidase directly on the gel of SDS-PAGE. The amino acid sequence for a 29 kDa peptide fragment was found to be Thr-X-Leu-Leu-Phe-Asp-Asp-Thr-Gln-Lys-Gln-Phe-Lys. A comparison of the peptide sequence against a protein database using the NCBI "blastp" program showed a significant homology with isovaleryl-CoA dehydrogenase (Fig. 3).[13–15]

In order to examine the substrate specificity and stereospecificity in the reduction of enones with the 74 kDa reductase, enzymatic reductions were carried out using several enones, **1**–**10**, as substrates (Chart 1). When monoterpene enones, such as (*R*)-pulegone (**1**), (*R*)-carvone (**2**), (*S*)-carvone (**3**), (1*S*,5*S*)-verbenone (**4**), and (1*R*,5*R*)-verbenone (**5**), were used as the substrate, the 74 kDa reductase catalyzed a stereospecific reduction of (*R*)-pulegone (**1**), which has the exocyclic C–C double bond to give (1*R*,4*R*)-isomenthone (**11**) (> 99% d.e.), but

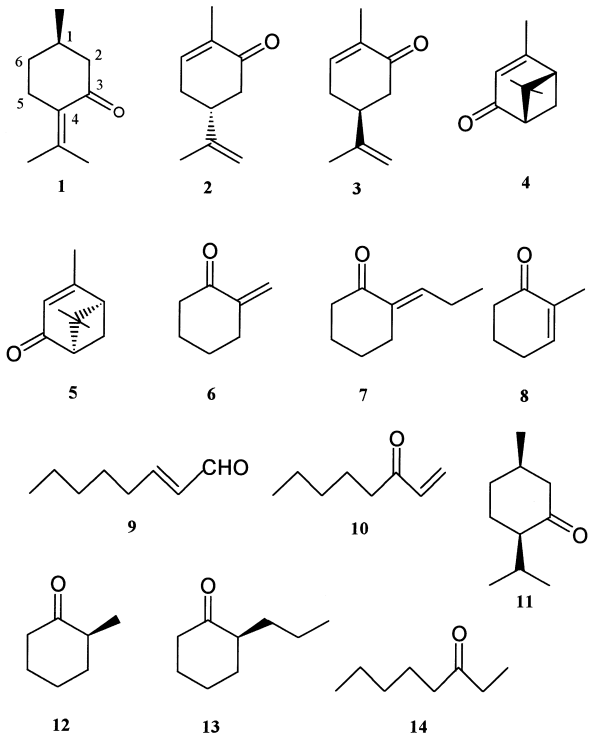

Chart 1. Formulas **1**–**14**.

Table 2. Reduction of Enones, **1**–**10**, with the p74 Reductase from Cultured Cells of *N. tabacum*

Substrate	Product	Conv.[a]/%	e.e.[b]	Config.[c]
1	**11**	> 99	> 99[d]	*R*
2	—	0	—	—
3	—	0	—	—
4	—	0	—	—
5	—	0	—	—
6	**12**	> 99	97	*S*
7	**13**	85	75	*S*
8	—	0	—	—
9	—	0	—	—
10	**14**	37	—	—

a) The conversions were expressed as the percentage of the products in the enzymatic reaction mixture. b) Enantiomeric excess on the basis of GLC on CP-cyclodextrin β 236 M-19 column. c) Configuration at the α-position to the carbonyl group of the products. d) Diastereomeric excess.

no reduction occurred in the case of the enones, **2**–**5**, which have an endocyclic C–C double bond, as shown in Table 2.

On the other hand, 2-alkylidenecyclohexanones (**6** and **7**), having an exocyclic C–C double bond, were converted to the corresponding saturated ketones (Table 2). The reduction at C-2 of the enones took place stereoselectively from the *re–re* face of the C–C double bond of **6** and the *si–re* face of that of **7** to give ketones having an *S* configuration at C-2. However, no 2-methylcyclohex-2-en-1-one (**8**) having the endocyclic C–C double bond was suitable as a substrate for the reductase. In the case of the reduction of acyclic enones, (*E*)-2-octenal (**9**) was not reduced, but 1-octen-3-one (**10**) was reduced to give

octan-3-one (**14**), as shown in Table 2.

The K_m values in the enzymatic reduction of enones **1**, **6**, **7** and **10**, were determined to be 25, 35, 150 and 83 μM, respectively. These facts suggested that the 74 kDa enone reductase is characteristic to the hydrogenation of the exocyclic C–C double bond of enones, especially pulegone (**1**).

Thus, the 74 kDa enone reductase, named pulegone reductase, was isolated from cultured cells of *N. tabacum*. We recently reported that both the carvone reductase and verbenone reductase from *N. tabacum* reduced the endocyclic C–C double bond of enones, but no reduction occurred for enones with an exocyclic C–C double bond.[12] The substrate specificity and the stereoselectivity of the pulegone reductase were quite different from those of the carvone reductase and verbenone reductase. The pulegone reductase was able to reduce enantioselectively the exocyclic C–C double bond of enones to afford optically active (*S*)-2-alkylated ketones. The substrate specificity of the reductase was similar to that of the Reductase-II (132 kDa), which was isolated from *N. tabacum* by Tang et al.[16] However, the pulegone reductase was apparently different from the Reductase-II with respect to the molecular mass and the optimum pH. On the other hand, two enone reductases catalyzing the enantioselective reductions of (*E*)- and (*Z*)-2-phenyl-2-butenal into (*R*)-2-phenylbutanal were recently characterized from *Saccharomyces cerevisiae*, but both of them were reported to hardly catalyze the reduction of pulegone (**1**).[17] Therefore, the pulegone reductase isolated here is different from the enone reductases from *S. cerevisiae* with respect to the substrate specificity. It is worth noting that the asymmetric synthesis of highly optically pure 2-alkylated ketones could be achieved by a selective use of the enone reductases isolated from cultured cells of *N. tabacum*.

Experimental

General. Analytical and prep. TLC were carried out on glass sheets (0.25 mm and 0.5 mm) coated with silica gel (Merck silica gel 60; GF_{254}). GLC analyses were carried out with FID and a glass column (3 mm×2 m) packed with 15% DEGS on Chromosorb W (AW-DMCS; 80–100 mesh) at 120 °C, or a capillary column (0.25 mm×25 m) coated with 0.25 μm CP cyclodextrin β 236M-19 (WCOT) using N_2 as a carrier gas (column temp: 100 °C, split ratio: 50, make up: 50 mL min^{-1}). GC-MS (Shimadzu) was carried out on a mass spectrometer equipped with an EI ion source (70 eV) and a gas chromatograph equipped with a capillary column (0.25 mm×25 m) coated with 0.25 μm OV-101. ^1H NMR spectra were obtained on a JEOL GSX-500 spectrometer using tetramethylsilane as an internal standard in $CDCl_3$.

Plant Cells and Substrates. Prior to their use in experiments, suspended cells of *N. tabacum*[2] were cultured on a rotary shaker (75 rpm) at 25 °C for 3 weeks in 500 mL conical flasks containing 200 mL Murashige and Skoog's medium under illumination (4000 lux).

NAD^+, $NADP^+$, NADH and NADPH were purchased from Boehringer. FAD and FMN were from Sigma. DEAE-Toyopearl, Red-Toyopearl and a TSK G3000 SW HPLC column were from Tosoh Co. Ltd. Hydroxylapatite was obtained from Wako Chemical Co. Ltd.

(*R*)-Pulegone (**1**) {GLC > 99%, $[\alpha]_D^{25}$ +22.3 (neat); lit.[4]: $[\alpha]_D^{25}$ +22.4° (neat)}, (*R*)-carvone (**2**) {99% pure on GLC, $[\alpha]_D^{25}$ −60.1° (neat); lit.[2]: $[\alpha]_D^{25}$ −59.7° (neat)} and (*S*)-carvone (**3**) {GLC > 99%, $[\alpha]_D^{25}$ +57.1° (neat); lit.[2]: $[\alpha]_D^{25}$ +60.0 (neat)} were purchased from Sigma. (1*S*,5*S*)-Verbenone (**4**) {99% pure on GLC, $[\alpha]_D^{25}$ −209.3° (neat); lit.[11]: $[\alpha]_D^{25}$ −208° (neat)} and (1*R*,5*R*)-verbenone (**5**) {99% pure on GLC, $[\alpha]_D^{25}$ +210.5° (neat); lit.[11]: $[\alpha]_D^{25}$ +210° (neat)} were prepared from (−)- and (+)-α-pinenes, respectively, by oxidation with *t*-butyl chromate.[11,18]

2-Methylidenecyclohexanone (**6**), 2-propylidenecyclohexanone (**7**) and 2-methylcyclohex-2-en-1-one (**8**) were prepared as described previously.[12] (*E*)-2-Octanal (**9**) and 1-octan-3-one (**10**) were purchased from Sigma.

Enzyme Preparation. All of the operations were carried out at 4 °C. The cultured cells (200 g) of *N. tabacum* (cultivated for 3 weeks) were ground in a Waring blender with 400 mL of 0.1 M (1 M = 1 mol dm^{-3}) Na–Pi buffer (pH 6.8) containing 10 mM 2-mercaptoethanol and 5 mM dithiothreithol. The resulting slurry was filtered through three layers of cheesecloth. The filtrate was centrifuged at 10000 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% satn 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 soln was freed 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 (**1**). The reductase fraction was further applied to a hydroxylapatite column (1×10 cm) equilibrated with buffer A. The column was washed with 100 mL of buffer A and the enzymes were eluted with 50 mL of a linear gradient of 50–500 mM Tris-HCl buffer. The active fraction was collected and concentrated by ultrafiltration and subsequently subjected to further purification on 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. The active fractions were collected and used as the purified enzyme (pulegone reductase).

SDS-PAGE was performed by a standard protocol by Laemmli[19] on a vertical slab gel. Samples were treated for 5 min at 100 °C in the presence of 6% SDS before application to the gel. After electrophoresis, the protein bands were visualized by staining with Coomassie Brilliant Blue. The molecular mass of the enzyme was calculated by using the LMW electrophoresis calibration kit (Pharmasia). Gel filtration chromatography was carried out on a Sephadex G-150 column (1×100 cm) with 0.1 M Na–Pi buffer (pH 7.0) containing 0.3 M NaCl by use of the calibration proteins with the indicated molecular mass, aldolase (158 k), bovine serum albumin (67 k), ovalbumin (43 k) and ribonuclease A (13.7 k), as references.

The pH optimum in the reduction of pulegone (**1**) with the enone reductase was determined by an enzyme reaction in a 100 mM Na–Pi buffer with the pH adjusted from 6.5 to 8.0.

Enzyme Activity Assay and Determination of Kinetic Parameters. The standard assay mixture (2 mL) was composed of the enzyme prepn, 200 μmol NADPH, 100 μmol pulegone (**1**) and 0.2 mL 1% Triton X-100 in 50 mM Tris-HCl buffer (pH 7.0). The mixture was incubated at 35 °C for 8 h and then extracted with ether (3 mL×3). The organic layer was dried over Na_2SO_4 and the

solvent was removed by evapn. The concentrated ether layer was subjected to GLC and GC-MS analyses. One unit of activity is defined as the enzyme amount of reducing 1 μmol of the enone per min. The protein concentration was determined according to the Bradford method with BSA as a standard.[20]

The influence of the substrate concentration on the initial velocity was measured using pure enzymes under standard assay conditions. The initial rates of substrate reduction were obtained for each substrate concentration, varying between 20 and 500 μM while maintaining the concentration of NADPH at 3 mM; also, the apparent Michaelis constants (K_m) were calculated from direct linear plots.

Amino Acid Sequence of the Enzyme. The purified enzyme was subjected to SDS-PAGE (12.5% gel), and then blotted onto a poly(vinylidene disulfide) membrane (Immobilon P^{SQ}, Millipore) by semi-dry blotting method. Attempts to sequence the reductase using Edman degradation with an Applied Biosystems Model 473A pulsed liquid sequencer with an online phenylthiohydantoin amino acids analyzer were unsuccessful. The reductase, therefore, was digested with lysyl endopeptidase directly in the SDS-gels to give two main peptid fragments (29 kDa and 20 kDa). The resulting peptides were sequenced. The obtained amino acid sequences were analyzed by the database screening with the NCBI "blastp" program.

Product Identification. The product in the reduction of (*R*)-pulegone (**1**) with the enone reductase was identified as (1*R*,4*R*)-isomenthone (**11**) [MS *m*/*z* 154 (M^+), ^{1}H NMR δ 0.83 (3H, d, J = 6.5 Hz, 7-Me), 0.95 (3H, d, J = 6.5 Hz, 9-Me), 0.98 (3H, d, J = 6.5 Hz, 10-Me) by direct comparison on GLC, GC-MS and ^{1}H NMR with those of the authentic sample.[4] The products in the reduction of 2-alkylidenecyclohexanones (**6** and **7**) were identified as their corresponding 2-alkylcyclohexanones (**12** and **13**). The absolute configurations and enantiomeric purities of the 2-alkylcyclohexanones obtained were determined by the circular dichroism (CD) spectra, and the peak area of the corresponding enantiomers by GLC analyses on CP cyclodextrin β 236M-19 column. The CD data of the products were as follows. **12**: 97% e.e. on GLC, $[\theta]_{288}$ +960 (*c* 0.25, MeOH) {lit.[21] $[\theta]_{288}$ −987 for *R* enantiomer} and **13** 75% e.e. on GLC, $[\theta]_{288}$ +1860 (*c* 0.25, MeOH) {lit.[22] $[\theta]_{288}$ +2480}.

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