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A 38 kDa allylic alcohol dehydrogenase from the cultured cells of *Nicotiana tabacum*

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Abstract

An $NADP^+$-dependent alcohol dehydrogenase (allyl-ADH) was isolated from the cultured cells of *Nicotiana tabacum*. The allyl-ADH was found to be efficient for the dehydrogenation of secondary allylic alcohols rather than saturated secondary alcohols and it was specific for the *S*-stereoisomer of the alcohols. The enzyme catalyzed the reversible reaction whereby the carbonyl group of enones is reduced to the corresponding allylic alcohol or vice versa. Two possible primary structures of the allyl-ADH were deduced by the sequence analyses of full-length cDNAs (*ally-ADH1* and *ally-ADH2*), which were cloned by the PCR method. These analyses indicated that the allyl-ADHs are composed of 343 amino acids having the molecular weights 38 083 and 37 994, respectively, and they showed approximately 70% homology to the $NADP^+$-dependent oxidoreductases belonging to a plant ζ-crystallin family.

Keywords: *Nicotiana tabacum*; Solonaceae; Allylic alcohol dehydrogenase; Allylic alcohol; Enone; Carveol; Carvone; ζ-crystallin family

1. Introduction

Alcohol dehydrogenases (ADHs) acting on primary alcohols (Dalziel and Dickinson, 1966; Dickinson and Dalziel, 1967; Lange et al., 1976; Tassin and Vandecasteele, 1972) and secondary alcohols (Coleman and Perry, 1985; Barrett et al., 1980; Bryant et al., 1988) are well characterized. However, little attention has been paid to those participating in the oxidation of allylic alcohols. It has been shown that cultured cells of *Nicotiana tabacum* transform monoterpene allylic alcohols, such as carveol and verbenol, into the corresponding enones (Hamada, 1988). In connection with studies on the biotransformation of enones with cultured plant cells as biocatalysts (Hirata et al., 1982), we isolated from the cultured suspension cells of *N. tabacum* an allylic alcohol dehydrogenase (allyl-ADH) having a dehydrogenase activity specific for the (*S*)-alcohol. The primary structure of the allyl-ADH was deduced by analyzing the nucleotide sequence of the *allyl*-ADH cDNA in the cultured cells of *N. tabacum*.

2. Results and discussion

2.1. Isolation and characterization of the allyl-ADH

The allylic alcohol dehydrogenase was purified from a cell free extract of the cultured cells of *N. tabacum* by DEAE-Toyopearl anion exchange column chromatography, followed by Red-Toyopearl affinity column chromatography. The purification steps of the enzyme are summarized in Table 1. SDS–PAGE of the purified enzyme fraction yielded a single protein band at 37 kDa. Gel filtration chromatography on a TSK G3000SW HPLC column revealed the molecular mass of the enzyme to be approximately 74 kDa. These results suggested that the native enzyme is a dimer composed of two identical subunits. The enzyme had a pH optima of 8.0 for the dehydrogenation of (2*S*, 4*S*)-carveol (**1a**) and at 7.4 for the reduction of the carbonyl group of (*S*)-

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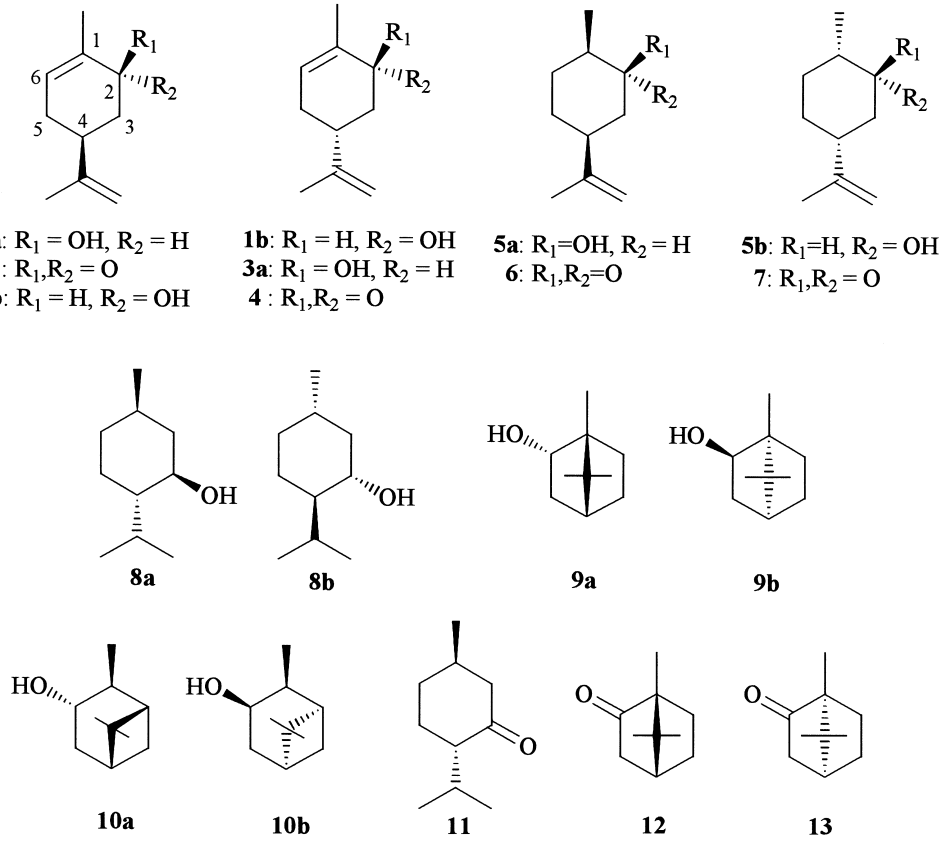

Table 1
Purification of the allyl-ADH from *N. tabacum*

Step	Total protein (mg)	Total activity (units[a])	Specific activity (units/mg protein)	Fold
Crude extract	552	0.29	0.53×10^{-3}	1
DEAE-Toyopearl	61	0.28	4.6×10^{-3}	9
Red-Toyopearl	0.09	0.01	0.11	210

[a] One unit of enzyme activity is defined as the amount of enzyme that catalyzed the oxidation of 1.0 μmol of (2*S*, 4*S*)-carveol (**1a**) per min.

carvone (**2**). The utilization of $NADP^+$ by the enzyme was four times greater than for NAD^+.

The substrate specificities in the dehydrogenation of each enantiomer of the alcohols, **1**, **3**, **5**, and **8–10**, with the allyl-ADH were examined, and are shown in Table 2. Carveols, **1** and **3**, were converted into carvones, **2** and **4**, respectively, whereas saturated alcohols, **5** and **8–10**, were not oxidized. This indicated that the allyl-ADH catalyzed specifically the dehydrogenation of the secondary alcohols adjacent to the C−C double bond, rather than the saturated secondary alcohols. In order to complete the characterization of the substrate specificity of the enzyme, the Michaelis parameter (K_m) for the enzymatic dehydrogenation was measured: 2.5×10^{-3} M for **1a**, 3.8×10^{-2} M for **1b**, 1.9×10^{-3} M for **3a**, 5.2×10^{-2} M for **3b**, and 5.9×10^{-1} M for **5a**. The K_m values for the (*S*)-alcohols were one order of magnitude lower than those for the (*R*)-alcohols, indicating that the allyl-ADH has a higher affinity for the *S*-configuration than for the *R*-configuration. It is interesting to note that the affinity for the allylic alcohol was much higher than that for the saturated secondary alcohol according to the differences of K_m values by up to two orders of magnitude. These showed that the allyl-ADH favored allylic alcohols having the *S*-configuration.

The possibility that allyl-ADH catalyzes a reversible reaction for the dehydrogenation of alcohols was examined. The yields in the enzymatic reactions of ketones, **2**, **4**, **6**, **7**, and **11–13**, as substrates are summarized in Table 3. Carvones, **2** and **4**, having a conjugated C−C double bond were transformed into the corresponding allylic alcohols, whereas the saturated ketones, **6**, **7**, and **11–13** were not, suggesting that the allyl-ADH is specific for the reduction of a ketone adjacent to the C−C double bond, in agreement with the results described above. It is of interest that the allyl-ADH from *N. tabacum* hardly reduced saturated ketones. In the reduction of carvones, (*S*)-allyl alcohols were preferentially obtained rather than (*R*)-stereoisomers, showing that the stereospecificity of the reduction by the allyl-ADH corresponds to the Prelog rule (Prelog, 1964) for reduction the of ketone, resulting in the predominant formation of a *S*-alcohol by *re*-face

Table 2
Dehydrogenation of alcohols with the allyl-ADH from *N. tabacum*

Substrate	Product	$K_{cat}(min^{-1})$[a]
(2*S*,4*S*)-Carveol (**1a**)	(*S*)-Carvone (**2**)	4.6
(2*R*,4*R*)-Carveol (**1b**)	(*R*)-Carvone (**4**)	1.2
(2*S*,4*R*)-Carveol (**3a**)	(*R*)-Carvone (**4**)	5.8
(2*R*,4*S*)-Carveol (**3b**)	(*S*)-Carvone (**2**)	1.5
(1*R*,2*S*, 4*S*)-Neoisodihydrocarveol (**5a**)	(1*R*,4*S*)-Isodihydrocarvone (**6**)	0.2
(1*S*,2*R*,4*R*)-Neoisodihydrocarveol (**5b**)	(1*S*,4*R*)-Isodihydrocarvone (**7**)	0
(1*R*,3*R*,4*S*)-Menthol (**8a**)	–	0
(1*S*,3*S*,4*R*)-Menthol (**8b**)	–	0
(1*R*,2*S*,4*R*)-Borneol (**9a**)	–	0
(1*S*,2*R*,4*S*)-Borneol (**9b**)	–	0
(1*S*,2*S*,3*S*,5*R*)-Isopinocampheol (**10a**)	–	0
(1*R*,2*R*,3*R*,5*S*)-Isopinocampheol (**10b**)	–	0

[a] K_{cat} are expressed as turnover number in the enzymatic formation of the product.

Table 3
Hydrogenation of ketones with the allyl-ADH from *N. tabacum*

Substrate	Product	$K_{cat}(min^{-1})$[a]
(*S*)-Carvone (**2**)	(2*S*, 4*S*)-Carveol (**1a**)	1.5
	(2*R*,4*S*)-Carveol (**3b**)	0.6
(*R*)-Carvone (**4**)	(2*R*,4*R*)-Carveol (**1b**)	0.7
	(2*S*,4*R*)-Carveol (**3a**)	1.9
(1*R*, 4*S*)-Isodihydrocarvone (**6**)	(1*R*,2*S*, 4*S*)-Neoisodihydrocarveol (**5a**)	0.2
(1*S*, 4*R*)-Isodihydrocarvone (**7**)	–	0
(1*R*, 4*S*)-Menthone (**11**)	–	0
(1*R*, 4*R*)-Camphor (**12**)	–	0
(1*S*, 4*S*)-Camphor (**13**)	–	0

[a] K_{cat} are expressed as turnover number in the enzymatic formation of the product.

attack of hydrogen. These results show that the enzymatic reaction with the allyl-ADH is a reversible oxidoreduction and the reaction is enantioselective.

2.2. Cloning and sequencing of a cDNA encoding the allyl-ADH

The N-terminal amino acid of the allyl-ADH was blocked and consequently the partial amino acid sequences were determined for two peptide fragments prepared by digestion of the allyl-ADH with lysyl endopeptidase. The sequences obtained were KGETV FVSAASGAVGQLVGQFAKMLG and VLESGDPX FQKGDLVXG.

The complete amino acid sequence of the allyl-ADH was determined from the cDNA clone, which was obtained by reverse transcriptase–polymerase chain reaction (RT-PCR), 3′- and 5′- rapid amplification of cDNA ends (RACE) method. The first RT-PCR was performed by using primers determined from the partial amino acid sequence given above. These PCR experiments produced two full-length cDNA clones (*allyl-ADH1* and *allyl-ADH2*) with 1243-base pairs (bp). These nucleotide sequences contained a 1029-base open reading frame (ORF), as shown in Fig. 1. The *allyl-ADH1* and *allyl-ADH2* were different in two bp at the 5′-untranslated regions and four bp at the ORF. The *allyl-ADH1* and *allyl-ADH2* encoded polypeptides of 343 amino acids with calculated molecular weights of 38 083 and 37 994, respectively, and the amino acid sequences of the deduced allyl-ADHs were different in four amino acid residues. The sequence variation of *allyl-ADH* cDNA and deduced polypeptide from the sequence in Fig. 1 is thus; nucleotide a20g, g27a, a80g, t120c, t765c, and g864a, polypeptide T17A, V30A, L345P, and R278H. The estimated molecular weight almost coincided with the values for the allyl-ADH observed by SDS–PAGE, and the partial amino acid sequences completely agreed with the sequence deduced from the cDNA clones (Fig. 1; underlined).

The deduced amino acid sequence from two allyl-ADH cDNA clones showed about 70% sequence homology to NADPH-dependent oxidoreductase/ζ-crystallin family from *Arabidopsis thaliana* (GenBank accession no. Z49768 and Z49268; Babiychuk et al., 1995). The fragment 160–178 of the amino acid sequence had significant homology to $NADP^+$-dependent leukotriene B4 12-hydroxydehydrogenase (67–86% homology) and also to the ζ-crystallin family/quinone reductase (58–72% homology) containing the putative NAD^+/$NADP^+$ binding domain of the other short chain

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   1 cacattatta ttaacagaaa agaagcgaaa aatggcagaa gaagtgagca acaaacaggt
   1                                 M  A  E   E  V  S  N   K  Q  V
  61 cattcttaaa aactatgtca caggttaccc taaggaatcc gacatggaaa tcaagaatgt
  11  I  L  K   N  Y  V  T   G  Y  P   K  E  S   D  M  E  I   K  N  V
 121 caccattaaa ctcaaagttc cagaaggttc taatgatgtg gttgtgaaga atctttactt
  31  T  I  K   L  K  V  P   E  G  S   N  D  V   V  V  K  N   L  Y  L
 181 gtcttgtgac ccttatatgc gtagccgcat gaggaaaatt gagggtagct atgttgaatc
  51  S  C  D   P  Y  M  R   S  R  M   R  K  I   E  G  S  Y   V  E  S
 241 cttcgctcct ggctccccta tcacgggata tggagtggct aaagttttgg agtctggtga
  71  F  A  P   G  S  P  I   T  G  Y   G  V  A   K  V  L  E   S  G  D
 301 tccaaaattc caaaaaggtg acttagtttg gggaatgact ggatgggaag agtatagtat
  91  P  K  F   Q  K  G  D   L  V  W   G  M  T   G  W  E  E   Y  S  I
 361 tataacacct actcagactc tctttaaaat tcatgacaag gatgtgcctc tttcctacta
 111  I  T  P   T  Q  T  L   F  K  I   H  D  K   D  V  P  L   S  Y  Y
 421 cacaggaatc ctcgggatgc ctgggatgac agcttatgct ggttttcatg aggtttgctc
 131  T  G  I   L  G  M  P   G  M  T   A  Y  A   G  F  H  E   V  C  S
 481 ccccaagaag ggggaaactg tctttgtttc agctgcatct ggagcagttg gtcagctcgt
 151  P  K  K   G  E  T  V   F  V  S   A  A  S   G  A  V  G   Q  L  V
 541 tgggcaattt gcaaagatgc tgggttgcta cgttgttggt agtgctggaa gcaaagaaaa
 171  G  Q  F   A  K  M  L   G  C  Y   V  V  G   S  A  G  S   K  E  K
 601 ggttgatctg ttgaagagca aatttgggtt tgacgaagct tttaactata aagaggagca
 191  V  D  L   L  K  S  K   F  G  F   D  E  A   F  N  Y  K   E  E  Q
 661 ggatttaagt gcagctttga agaggtactt ccctgatgga attgacatct actttgagaa
 211  D  L  S   A  A  L  K   R  Y  F   P  D  G   I  D  I  Y   F  E  N
 721 tgtgggaggg aagatgcttg atgcagttct tgtgaacatg aaactctatg gccgtattgc
 231  V  G  G   K  M  L  D   A  V  L   V  N  M   K  L  Y  G   R  I  A
 781 tgtgtgtggg atgatttcgc aatacaacct tgagcagact gaaggagtgc acaacttgtt
 251  V  C  G   M  I  S  Q   Y  N  L   E  Q  T   E  G  V  H   N  L  F
 841 ttgcctcatc acaaaacgaa tccgcatgga aggatttctt gtttttgatt actatcatct
 271  C  L  I   T  K  R  I   R  M  E   G  F  L   V  F  D  Y   Y  H  L
 901 ttaccccaaa tatttggaaa tggtcattcc tcaaataaag gcaggcaagg ttgtttatgt
 291  Y  P  K   Y  L  E  M   V  I  P   Q  I  K   A  G  K  V   V  Y  V
 961 ggaagatgtt gcccatggcc ttgaaagtgc tcccactgct ctagttggtc tcttctctgg
 311  E  D  V   A  H  G  L   E  S  A   P  T  A   L  V  G  L   F  S  G
1021 tcgcaatatt ggaaagcaag tcgtgatggt ttcgcgtgaa tgaatgtcac caatggatat
 331  R  N  I   G  K  Q  V   V  M  V   S  R  E   *
1081 cttgccgtga tatttgtgtt taataaactt tccctttgt agtagagctg tttgtgttta
1141 cgcgctttga attatcattg gttttgttta attgcattgt gtatctgctt cagggaaaaa
1201 gaacgaacta gatcaataat ttctggccaa aaaaaaaaaa aaa
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Fig. 1. Nucleotide sequences and deduced amino acid sequence of the *allyl-ADH1* clone. Two peptide sequences of the purified allyl-ADH are underlined. The bold letters show the putative $NADP^+/NAD^+$ binding motif.

alcohol dehydrogenases (Scrutton et al., 1990; Baker et al., 1992; Persson et al., 1994), as shown in Fig. 2. It was reported that the nucleotide-binding domain with the consensus amino acid sequences, AXXGXXG or GXXGXXG, preferentially bind, $NADP^+$ as a co-factor (Persson et al., 1994; Thorn et al., 1995). Since the allyl-ADH had the sequence AXXGXXG, its co-factor specificity was predicted to be a $NADP^+$ rather than NAD^+. It has been suggested that three glycines at 152, 155, and 166 of leukotriene B4 12-hydroxydehydrogenase from human and pig have a crucial role for the enzyme activity by producing an $NADP^+$ binding

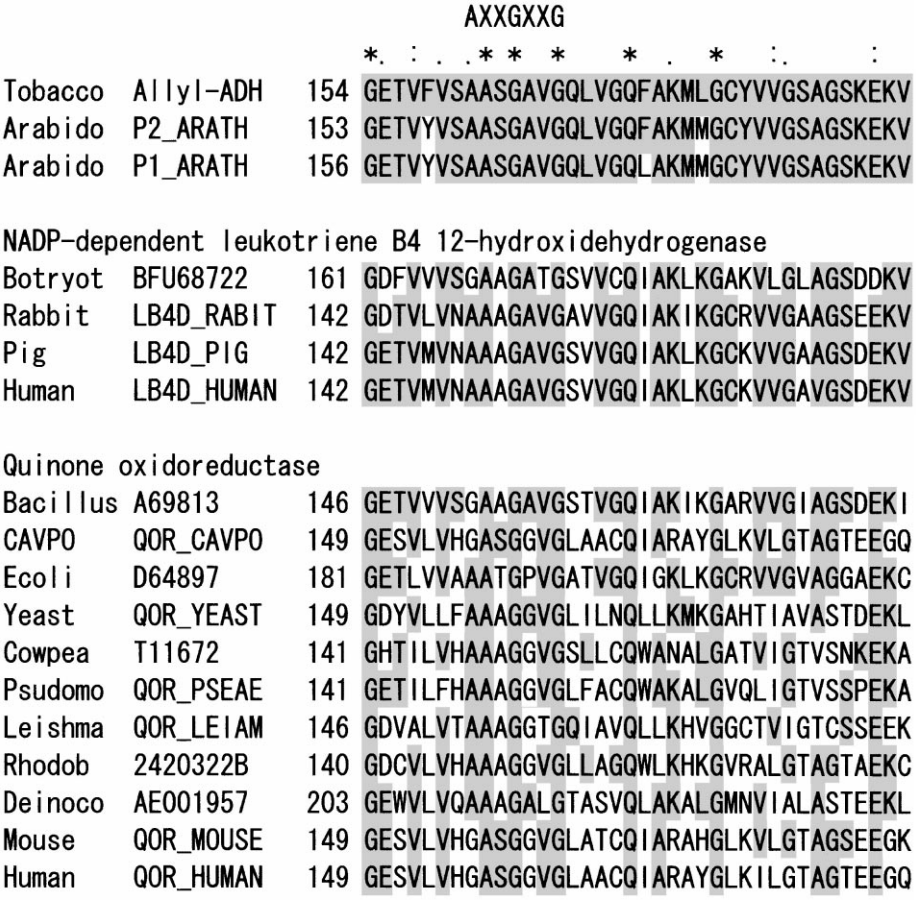

Fig. 2. Amino acid alignment of NAD^+/$NADP^+$-binding domains of allyl-ADH and other homologous proteins. Amino acid sequence alignments were generated using the CLUSTAL W multiple sequence alignment program (Thompson et al., 1994). Identical amino acid residues with allyl-ADH are indicated by a black background. Upper-column indicates the NADPH-dependent oxidoreductases: *Arabidopsis thaliana* P1_ARATH (GenBank accession no. Q39172), and *Arabidopsis thaliana* P2_ARATH (Q39173). Center-column indicates the NADP-dependent leukotriene B4 12-hydroxidehydrogenase: *Botryotinia fuckeliana* BFU68722 (U68722), rabbit LB4D_RABIT (Q28719), pig LB4D_PIG (Q29073), and human LB4D_HUMAN (Q14914). Bottom-column indicates the quinone oxidoreductases: *Bacillus subtilis* A69813 (PIR), *Cavia porcellus* QOR_CAVPO (P11415), *Escherichia coli* D64897 (PIR), *Saccharomyces cerevisiae* QOR_YEAST (P38230), cowpea T11672 (PIR), *Pseudomonas aeruginosa* QOR_PSEAE (P43903), *Leishmania amazonensis* QOR_LEIAM (P42865), *Rhodobacter capsulatus* 2420322B (PRF), *Deinococcus radiodurans* AE001957 (AAF10634), mouse QOR_MOUSE (P47199), and human QOR_HUMAN (Q08257).

pocket (Yokomizo et al., 1996). A similar pattern for the positioning of three glycines was found in all dehydrogenases cited in Fig. 2.

Thus, the primary structure of the allyl-ADH from *N. tabacum* was demonstrated to be similar to the NADPH-dependent oxidoreductase belonging to the plant ζ-crystallin family. Further biochemical studies and molecular analyses will be required to determine why the pocket of allyl-ADH from *N. tabacum* is specific for allylic alcohols.

3. Experimental

3.1. General experimental procedures

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-236 M-19 (WCOT), using N_2 as carrier gas (column temp: 110°C, split ratio: 50, make up: 50 ml min^{-1}). GC–MS was carried out with a capillary column (0.25 mm×25 m) coated with 0.25 μm of OV-101.

3.2. Substrate

(*S*)-Carvone (**2**) {$[\alpha]_D^{25}$ +57.1 (neat)}, (*R*)-Carvone (**4**) {$[\alpha]_D^{25}$ −60.1 (neat)}, (1*R*,3*R*,4*S*)-menthol (**8a**) {$[\alpha]_D^{25}$ −49.1 (*c* 1.5, EtOH)}, (1*S*,3*S*,4*R*)-menthol (**8b**) {$[\alpha]_D^{25}$ +48.9 (*c* 1.5, EtOH)}, (1*R*,2*S*,4*R*)-borneol (**9a**) {$[\alpha]_D^{25}$ +37.5 (neat)}, (1*S*,2*R*,4*S*)-borneol (**9b**) {$[\alpha]_D^{25}$ −37.3 (neat)}, (1*S*,2*S*,3*S*,5*R*)-isopinocampheol (**10a**) {$[\alpha]_D^{25}$ +30.7 (*c* 2.0, EtOH)}, (1*R*,2*R*,3*R*,5*S*)-isopinocampheol (**10b**) {$[\alpha]_D^{25}$ −31.7 (*c* 2.0, EtOH)}, (1*R*,4*S*)-menthone (**11**)

$\{[\alpha]_D^{25}$ −27.3 (*c* 1.0, EtOH)}, (1*R*,4*R*)-camphor (**12**) $\{[\alpha]_D^{25}$ +42.9 (*c* 1.5, EtOH)} and (1*S*,4*S*)-camphor (**13**) $\{[\alpha]_D^{25}$ −42.6 (*c* 1.5, EtOH)} were purchased from Aldrich. (2*S*, 4*S*)- and (2*R*,4*S*)-carveols (**1a** and **3b**) and (2*R*,4*S*)- and (2*S*,4*R*)-carveols (**1b** and **3a**) were prepared from (*S*)- and (*R*)-carvones (**2** and **4**), respectively, by reduction with $NaBH_4$. (1*R*,2*S*, 4*S*)- and (1 *S*, 2*R*,4*R*)-Neoisodihydrocarveols (**5a** and **5b**) were given by the $NaBH_4$ reduction of (1*R*,4*S*)- and (1*S*,4*R*)-isodihydrocarvones (**6** and **7**), which were prepared from (*S*)- and (*R*)-carvones (**2** and **4**), respectively (Hirata et al., 1982).

3.3. Purification of the enzyme

Cultured cells of *N. tabacum* (Hirata et al., 1982) grown for 4 weeks in Murashige and Skoog's medium were used in the present work. The fresh cells (150 g) were frozen with liquid N_2, ground in a chilled mortar, and homogenized with 500 ml of 50 mM Na–Pi buffer (pH 6.8). The resulting suspension was filtered through four layers of cheesecloth. The crude extract was centrifuged at 10 000 ***g*** for 30 min and then the supernatant was treated with 40–80% sat. of ammonium sulfate to give a crude enzyme preparation. The preparation was suspended in 25 mM Tris–HCl buffer at pH 8.0 (basic buffer) and dialyzed overnight against the basic buffer. The dialyzate was applied to a Sephadex G-25 column equilibrated with the basic buffer. The desalted protein material was subsequently loaded onto a 6×35 cm column packed with DEAE-Toyopearl (Tosoh Co. Ltd.) equilibrated with the basic buffer. After non-adsorbed proteins were eluted, adsorbed proteins were eluted with the basic buffer containing a 0–1.0 M linear gradient of NaCl. The active enzyme fraction for the dehydrogenation of (2*S*, 4*S*)-carveol (**1a**) was collected. The enzyme fraction was further purified on a Red-Toyopearl affinity column (Tosoh Co. Ltd.) (2×20 cm) equilibrated with the basic buffer. After washing with the buffer solution, the proteins were eluted with a 0–1.0 M linear gradient of NaCl in the basic buffer. The active enzyme fractions were pooled and used for subsequent experiments.

The purified enzyme was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) on a 12.5% gel according to the standard protocol of Laemmli (Laemmli, 1970). The molecular mass of the denatured enzyme was determined by SDS–PAGE with the LMW electrophoresis calibration kit (Pharmacia). The molecular mass of the native enzyme was estimated by gel filtration through a TSK G3000SW HPLC column using aldolase, bovine serum albumin, ovalbumin and ribonuclease A, respectively as marker proteins.

The pH optimum of the enzyme reaction was estimated by reaction of substrate **1a** in a buffer solution of 100 mM 3-(*N*-morpholino)propane sulfonic acid with pH adjusted from 6.0 to 8.5.

3.4. Enzyme assay

The standard assay mixture was composed of 2 ml enzyme preparation (in 25 mM Tris–HCl buffer at pH 8.0), 10 μmol $NADP^+$, and 5 μmol (2*S*, 4*S*)-carveol (**1a**) solubilized by Triton X-100 (0.3% final concentration). The mixture was incubated for 24 h at 35°C and then extracted with diethyl ether. The organic layer was subjected to GLC and GC–MS analyses. The enzyme activity was determined as the amount of (*S*)-carvone (**2**) produced. One unit of enzyme activity is defined as the amount of enzyme that catalyzed the oxidation of 1.0 μmol of (2*S*, 4*S*)-carveol (**1a**) per min.

In the activity assay for the oxidation of alcohols, the assay mixture was composed of purified enzyme (10 μg) in 2 ml of 25 mM Tris–HCl buffer at pH 8.0, 1.0 μmol $NADP^+$, and 1.0 μmol substrate solubilized by Triton X-100 (0.3% final concentration). The mixture was incubated for 3 h at 35°C and the amount of product was estimated by GLC analyses. In the activity assay for the reduction of ketones, the reactions were carried out under the same conditions containing 2 ml enzyme solution (10 μg) in 25 mM Na–Pi buffer at pH 7.4, 1.0 μmol NADPH, and 1.0 μmol substrate solubilized with 0.3% Triton X-100.

3.5. Partial amino acid sequence of allyl-ADH

Purified protein on a 12.5% SDS–PAGE gel was degraded partially by lysyl endopeptidase and the peptide fragment on the SDS–PAGE gel was blotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-PSQ, Millipore). The Coomassie stained peptide band was excised. Sequence analysis was performed with an online phenylthiohydantoin amino acids analyzer (Applied Biosystems Model 473A pulsed liquid sequencer).

3.6. Cloning a cDNA encoding allyl-ADH

Total cellular RNA was isolated from cultured cells (9.3 g) of *N. tabacum* with guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). Using dT20-M4 primer and M-MLV reverse transcriptase (Sawady Technology Co. Ltd.), single-stranded cDNA was synthesized from the total RNA. The single-stranded cDNA was used as template for 1st PCR amplification with EX *Taq* DNA polymerase (Takara Shuzo Co., Ltd.) and the primers ADH1 (5′-aaa gtr yta gaa tct ggr gat cca-3′) and ADH2 (5′-ctt gtg gga caa ttt gct aag atg-3′). The amplification was carried out at 94°C for 1 min for denaturation, 56°C for 1 min for annealing, and 72°C for 1 min for

synthesis, followed by 30 cycles. The first PCR solution was used as template for second PCR using the primers ADH3 (5′-gag aga cag ttt ttg tgt cag ctg-3′) and ADH4 (5′-ttc cag gta ggw gay tta gtc-3′). 3′-RACE-PCR was performed with the following primers; nucleotides 448–473, which are derived from the RT-PCR clone, for upstream primer, and 3′-RACE1 (5′-tgg aag aat tcg cgg-3′) and M13M4 (5′-gtt ttc cca gtc acg ac-3′) for downstream primer. 5′-RACE-PCR was performed with the following primers; nucleotides 336–382, which are derived from the RT-PCR clone, for downstream primer, and 5′-RACE1 (5′-tgg aag aat tcg cgg ccg ctt aag ggg ggg ggg ggg-3′) and 5′-RACE2 (5′-cgc ggc cgc tta-3′) for upstream primer. The amplified cDNA fragment was excised from an agarose gel, purified, and ligated to the pPCR-Script Amp vector (Stratagene). DNA sequence analysis was performed with an ABI PRISM310 Genetic Analyzer with BigDye terminator cycle sequencing kit (PE Biosystems). Two full-length cDNA (*ally-ADH1* and *ally-ADH2*) of a 1243-bp were sequenced (DDBJ/EMBL/GenBank accession no. AB036735). The nucleotide sequence of the cDNA contained a 1029-base ORF (Fig. 1).

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