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Monoterpene Alcohol Metabolism: Identification, Purification, and Characterization of Two Geraniol Dehydrogenase Isoenzymes from *Polygonum minus* Leaves

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NADP$^+$-dependent geraniol dehydrogenase (EC 1.1.1.183) is an enzyme that catalyzes the oxidation of geraniol to geranial. Stable, highly active cell-free extract was obtained from *Polygonum minus* leaves using polyvinylpolypyrrolidone, Amberlite XAD-4, glycerol, 2-mercaptoethanol, thiourea, and phenylmethylsulfonylfluoride in tricine-NaOH buffer (pH 7.5). The enzyme preparation was separated into two activity peaks, geraniol-DH I and II, by DEAE-Toyopearl 650M column chromatography at pH 7.5. Both isoenzymes were purified to homogeneity in three chromatographic steps. The geraniol-DH isoenzymes were similar in molecular mass, optimal temperature, and pH, but the isoelectric point, substrate specificity, and kinetic parameters were different. The K_m values for geraniol of geraniol-DH I and II appeared to be 0.4 mM and 0.185 mM respectively. *P. minus* geraniol-DHs are unusual among geraniol-DHs in view of their thermal stability and optimal temperatures, and also their high specificity for allylic alcohols and NADP$^+$.

Key words: monoterpene; secondary metabolite; isoenzyme; substrate specificity

Geraniol (3,7-dimethylocta-*trans*-2,6-dien-1-ol) and citral (3,7-dimethylocta-2,6-dienal) are commercially important monoterpenes that are present in the essential oils of several aromatic plants,[1,2] including *Polygonum odoratum*[3] and *P. minus* (Baharum *et al.*, unpublished results). Citral is the name given to a natural mixture of two isomeric acyclic monoterpene aldehydes: geranial (*trans*-isomer) and neral (*cis*-isomer).[4] Geraniol is one of the most important molecules in the flavor and fragrance industry.[1] A survey of consumer products revealed that geraniol and citral are present in 42% and 25% of cosmetic and household products respectively.[5] In addition, geraniol and citral also exhibit various biochemical and pharmacological properties, including antioxidant, anticancer, and antimicrobial activities.[1,6,7]

The geraniol catabolism pathway has not been well elucidated in plants. It begins with the biosynthesis of geraniol by geraniol synthase (EC 4.2.3.-) from geranyl pyrophosphate.[4] Subsequently, geraniol is oxidized by NADP$^+$-dependent geraniol dehydrogenase (geraniol-DH) (EC 1.1.1.183) to form geranial (3,7-dimethylocta-*trans*-2,6-dienal) (Fig. 1). Geraniol-DH activity has been detected in extracts of *Citrus sinensis*,[8] *Cymbopogon flexuosus* Stapf.,[9] *Zingiber officinale* Roscoe,[10] and *Ocimum basilicum*.[11] To understand the regulation of the geraniol metabolic pathway better, we investigated the enzymes that participate in this monoterpene metabolism pathway in *P. minus*. *P. minus*, which belongs to the Polygonaceae family, is an annual herb that grows by roadsides and in swamps and ditches in Asia and the Pacific Islands.[12] It has been found to possess a wide range of medicinal properties.[12–15] Here the purification and characterization of two isoenzymes of geraniol-DH isolated from *P. minus* leaves are described. The properties of the purified isoenzymes are compared with those of other geraniol-DHs.[8–11,16]

Materials and Methods

Materials. The leaves of *P. minus* used in the study were obtained from plants growing in an experimental field of the Institute of Systems Biology of Universiti Kebangsaan Malaysia (UKM). Geraniol (98%), citral (95%), borneol (97%), *p*-cumic alcohol (98%), menthol (99%), Amberlite XAD-4, polyvinylpolypyrrolidone (PVPP), protease inhibitor cocktail for plant cell and tissue extracts, and polyvinylpyrrolidone were purchased from Sigma-Aldrich (St. Louis, MO). β-Citronellol (98%), nerol (98%), linalool (98%), cinnamyl alcohol (98%), and farnesol (97%) were from Tokyo Chemical Industry (TCI) (Tokyo). Carveol (97%) was from Alfa Aesar (Ward Hill, MA). Percentages in parentheses show levels of purity for the commercially available compounds. DEAE-Toyopearl 650M, Phenyl-Toyopearl 650M, AF-Red Toyopearl 650ML were from Tosoh (Tokyo). Superdex 200 10/300 GL was from GE Healthcare (Uppsala, Sweden). Standard proteins for gel filtration were from Bio-Rad (Hercules, CA). All other reagents were commercial products of analytical grade. Water-insoluble chemicals were dissolved in absolute dimethyl sulfoxide (DMSO) or acetone, and subsequent dilutions were conducted in water. The presence of DMSO or acetone in the reaction mixture had no effect on enzyme activity.

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Abbreviations: DMSO, dimethyl sulfoxide; geraniol-DH, geraniol dehydrogenase; 2-ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonylfluoride; PVPP, polyvinylpolypyrrolidone

Table 1. Influence of Extraction Buffer Composition on *P. minus* Geraniol-DH Specific Activity

Composition of buffer	Specific activity ($U \cdot mg^{-1}$)	Relative activity (%)
Buffer A + 50% (w/w) PVPP	0	0
Buffer A + 50% (w/w) PVPP + 25% (w/w) Amberlite XAD-4	2.1×10^4	33
Buffer A + 50% (w/w) PVPP + 50% (w/w) Amberlite XAD-4	6.4×10^4	100
Buffer A + 50% (w/w) PVP-10[a] + 50% (w/w) Amberlite XAD-4	0	0
Buffer B + 50% (w/w) PVPP + 50% (w/w) Amberlite XAD-4	10.3×10^4	161
Buffer C + 50% (w/w) PVPP + 50% (w/w) Amberlite XAD-4	7.5×10^5	12

Buffer A: 50 mM tricine-NaOH buffer (pH 7.5) containing 2.5 mM 2-ME, 1 mM PMSF, 1 mM thiourea, 15% (w/v) glycerol
Buffer B: 100 mM tricine-NaOH buffer (pH 7.5) containing 2.5 mM 2-ME, 1 mM PMSF, 1 mM thiourea, 15% (w/v) glycerol
Buffer C: 50 mM tris-HCl buffer (pH 7.5) containing 2.5 mM 2-ME, 1 mM PMSF, 1 mM thiourea, 15% (w/v) glycerol
[a]PVP-10, polyvinylpyrrolidone

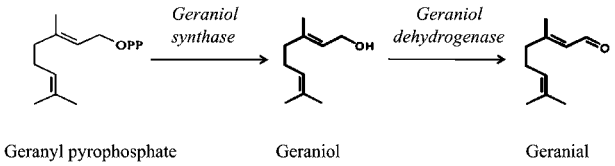

Fig. 1. Metabolic Pathway of Geraniol in Plants.

Optimization of enzyme extraction. Fresh leaves (5 g) were washed and ground using a mortar and pestle under liquid nitrogen. The fine powder was homogenized for 5 min in 20 mL of ice-cooled extraction buffer (Table 1). After filtration through four layers of cheesecloth, the crude homogenate was centrifuged at $20,000 \times g$ at 4 °C for 30 min to remove cell debris. Cell-free extracts were obtained by centrifugation.

Enzyme assay. Geraniol-DH activity was measured by observing the increase or decrease in absorbance at 340 nm at 37 °C. The standard reaction mixture (1.5 mL) contained 100 mM of glycine–NaOH buffer (pH 9.5), 2.0 mM of geraniol in DMSO, 0.2 mM of $NADP^+$, and an appropriate amount of enzyme. The reaction was started by addition of the enzyme. For the reduction reaction, the reaction mixture (1.5 mL) contained 100 mM of potassium phosphate buffer (pH 6.0), 0.2 mM of NADPH, 2.0 mM of citral, and an appropriate amount of enzyme. Enzyme activity was calculated using an extinction coefficient of $6,200\ M^{-1} \cdot cm^{-1}$ for NADPH. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of NADPH per min under the assay conditions. Specific activity was defined in units of enzyme activity per mg of protein.

Product confirmation. Identification of reaction product was done by gas chromatography-mass spectrometry (GC-MS) analysis according by the method described by Iijima *et al.* (2006), with slight modifications.[11] Geraniol (2 mM) was incubated in 100 mM of glycine–NaOH buffer (pH 9.5) containing 0.2 mM of $NADP^+$ with the cell-free extract. Two controls were used: (a) as above without the cell-free extract, and (b) as above without geraniol. After a 3 h of incubation at 37 °C, 500 µL of hexane was added, and the mixture was briefly vortexed and centrifuged at $1,500 \times g$ for 30 min to separate the phases. The hexane layer was immediately subjected to GC-MS analysis on a Clarus® 600 gas chromatograph from Perkin-Elmer (Shelton, CT), equipped with an Elite-5MS capillary column (30 m, 0.25 mm, and 0.25 µm). The sample (1 µL) was injected with a Clarus GC Autosampler. The injector and detector temperatures were set at 220 °C and 250 °C respectively. The temperature of the column was set initially at 55 °C for 2 min and then increased by 2 °C per min to a final temperature of 220 °C. Helium was used as carrier gas at a flow rate of 1 mL per min. Data acquisition and processing were performed using Turbo Mass software (Perkin-Elmer). Compounds separated on the column were identified by comparing their retention times and mass fragmentation patterns with authentic standards and library matches.

Protein measurement. Protein was measured by the Lowry method[17] using bovine serum albumin as standard, or by the absorbance at 280 nm, and an $E^{1\%}_{1cm}$ value of 10.0 was employed.

Purification of geraniol-DH. Purification of geraniol-DH was done at 4 °C. Throughout the purification procedure, 100 mM of tricine-NaOH buffer (pH 7.5) containing 2.5 mM of 2-mercaptoethanol (2-ME) was used, unless otherwise stated. Centrifugation was done at $20,000 \times g$ at 4 °C for 30 min, unless otherwise stated. Cell-free extract was prepared from 500 g of fresh leaves of *P. minus*.

The cell-free extract was put on a DEAE-Toyopearl 650M column (2.6×63 cm) equilibrated with the buffer. The proteins absorbed on column were eluted with a linear gradient of potassium chloride (0–0.8 M) in the buffer. Two peaks of geraniol-DH activity were detected, one in the wash fractions (designated geraniol-DH I), and the second in the absorbed fractions (designated geraniol-DH II) of DEAE-Toyopearl 650M column chromatography. The active fractions from each peak were pooled separately and subjected to further purification. To the active fractions of DEAE-Toyopearl, 2.0 M of ammonium sulphate solution in the buffer was added to a final concentration of 1.0 M. The resulting solutions were loaded onto a Phenyl-Toyopearl 650M column (2.6×17 cm) equilibrated with the buffer, which contained 1.0 M of ammonium sulphate. The column was washed with the same buffer, and the absorbed proteins were eluted with a linear gradient of ammonium sulphate (1.0–0 M) in the buffer. The active fractions were combined, concentrated with a Macrosep 10K Omega centrifugal device (Pall Life Sciences, Ann Arbor, MI), and dialyzed against the buffer (2.0 L) overnight with three changes. The dialyzed solution was applied to an AF-Red Toyopearl 650ML column (1.6×12 cm) equilibrated with the buffer. After washing with the same buffer, the protein was eluted with a linear gradient of potassium chloride (0–1.0 M) at a flow rate of $1.2\ mL \cdot min^{-1}$. The active fractions were pooled and then stored at −80 °C.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis (native-PAGE) was done using 12.5% gel at pH 8.8 by the Laemmli buffer system without SDS.[18] The protein was stained with silver staining and checked for enzyme activity. The reaction mixture contained 100 mM of glycine–NaOH buffer (pH 9.5), 2 mM of geraniol in DMSO, 54 µM of 1-methoxy phenazine methosulphate, 0.3 mM of nitroblue tetrazolium, and 0.2 mM of $NADP^+$. SDS polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 12.5% gel by the method of Laemmli.[18] The PageRuler™ Prestained Protein Ladder, about 10–170 kDa (SM0671) (Fermentas, St. Leon-Rot, Germany) was used as molecular marker. The gel was stained by silver staining.

Measurement of molecular mass. The molecular masses of the enzymes were estimated by gel filtration on a Superdex 200 10/300 GL column (10×300 mm) equilibrated with 0.1 M of tricine-NaOH buffer (pH 7.5) containing 2.5 mM of 2-ME. The standard proteins used were thyroglobulin, γ-globulin, ovalbumin, myoglobin, and vitamin B_{12}.

Isoelectric focusing. Isoelectric focusing was done on 18-cm ReadyStrip IPG strips at pH 3–10 (GE Healthcare Bioscience, Uppsala, Sweden). Purified geraniol-DH I and II were resuspended in isoelectric focusing buffer containing 8.0 M of urea, 4% (w/v) of CHAPS, 0.5% (v/v) of ampholines at pH 3–10, 30 mM of 2-ME, and 0.002% of bromophenol blue. Isoelectric focusing was done by the Ettan IPGphor system (Amersham Biosciences, Freiburg, Germany) following the instructions of the manufacturer. The gels were silver-stained for protein determination.

Results and Discussion

Although geraniol-DH activity has been reported for four plants,[8–11] two microorganisms, and an insect,[16] only the enzyme from the insect has been purified to homogeneity. Two cDNAs encoding $NADP^+$-dependent dehydrogenases which can use geraniol as substrate, designated *CAD1* and *GEDH1* were identified in *O. basilicum*. The enzyme encoded by *CAD1* reversibly oxidizes geraniol to produce geranial at half the efficiency of its activity with cinnamyl alcohol. The second cDNA, *GEDH1*, encodes an enzyme with sequence similarity to *CAD1*, which is capable of reversibly oxidizing geraniol and nerol with equal efficiency.[11] In addition, the enzymatic preparation of *C. flexuosus* also showed two distinct geraniol-DH activities.[9] In this study, we succeeded in purifying two types of geraniol-DH to homogeneity from *P. minus* leaves.

Optimization of geraniol-DH extraction and identification of the reaction product

Extraction protocols require optimization for plant tissues with high phenolic contents. Phenolic compounds, oil, and resins inactivate plant enzymes and are known to be a particularly severe problem as to the evergreen leaves of tannin-rich and essential-oil bearing plants.[19] It is believed that enzymes and other proteins can bind to phenolic compounds through hydrogen, hydrophobic, and ionic bonding.[20] Hence, one initial objective of the present study was to maximize the level of extractable enzyme activity in order to define reaction parameters adequately and to make possible subsequent protein purification.

Several buffer compositions were tested to identify the one most suitable for geraniol-DH extraction from *P. minus* leaves (Table 1). The best extraction composition for *P. minus* geraniol-DH was 100 mM of tricine-NaOH buffer (pH 7.5) containing a reducing agent (2.5 mM of 2-ME), an osmotic reagent (15% w/w of glycerol), a phenoloxidase inhibitor (1 mM of thiourea), a protease inhibitor (1 mM of PMSF), and a mixture of additives that adsorb phenolic compounds (half of the tissue weight of PVPP and of Amberlite XAD-4). Thiourea has been reported to be the phenol oxidase inhibitor of choice for the extraction of phenol-rich plant tissues.[21] In addition, insoluble PVPP could not be replaced with soluble polyvinylpyrrolidone. Adding sodium metabisulphite, ascorbic acid, dithiothreitol, and EDTA (data not shown) to the extraction buffer, and using Tris–HCl (pH 7.5) or potassium-phosphate (pH 7.5) in the extraction buffer (data not shown) failed to enhance enzyme activity. The addition of a protease inhibitor cocktail containing 4-(2-aminoethyl)benzene-sulfonyl fluoride, bestatin, E-64, leupeptin, pepstatin A, and phenanthroline did not significantly increase enzyme activity.

To determine whether geranial was a product of the geraniol-DH reaction, GC-MS analysis was performed. A 1:1 mixture of authentic geraniol and citral is shown in Fig. 2. Citral is a mixture of geranial (*trans*) and neral (*cis*) in an approximately 3:2 ratio.[4] The retention times of the three resulting peaks for neral (*cis*-isomer), geraniol, and geranial (*trans*-isomer) were 16.41 min, 16.84 min, and 17.42 min respectively (Fig. 2A). The enzyme reaction assay containing $NADP^+$ and geraniol was incubated in the presence and the absence of a cell-free extract of *P. minus* leaves at 37 °C for 3 h and then analyzed by GC-MS. As shown in Fig. 2B, a new peak with a retention time of 17.41 min, similar to the retention time of authentic geranial, appeared when the reaction mixture was incubated with the cell-free extract, geraniol, and $NADP^+$. This peak was not detected in the control reaction, without the cell-free extract (Fig. 2C), and it was also absent from the control with cell-free extract but without geraniol (data not shown). These results confirmed that geranial was the enzymatic product, and that geraniol-DH was the enzyme catalyzing the oxidation reaction of geraniol in *P. minus*.

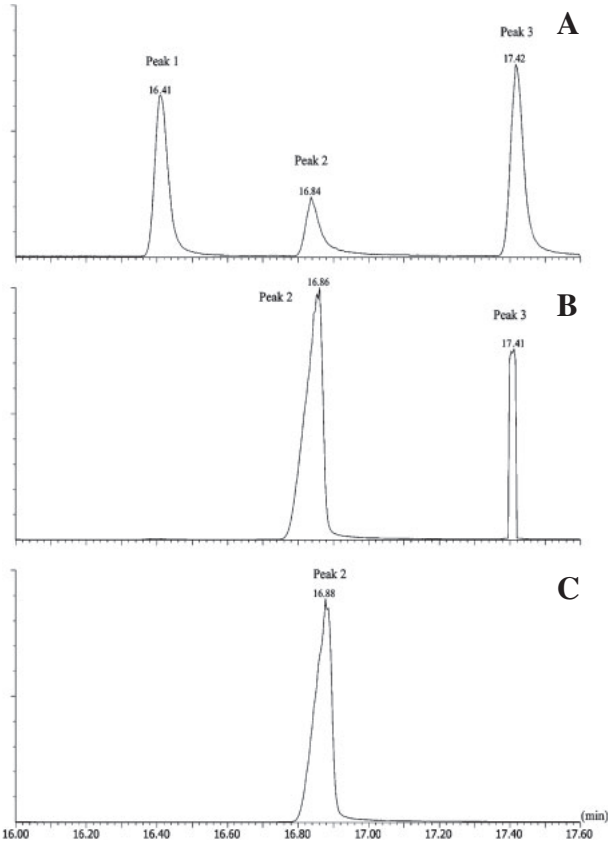

Fig. 2. Identification of Geranial as Reaction Product Following Incubation of *P. minus* Cell-Free Extract with Geraniol.

A, Separation of authentic geraniol, geranial, and neral by GC-MS. Peak 1, neral; peak 2, geraniol; peak 3, geranial. Retention times of 16.41 min, 16.84, and 17.42 min were obtained for neral, geraniol, and geranial respectively. B, GC-MS analysis following incubation of cell-free extract with geraniol and $NADP^+$. A new peak (peak 3, geranial) with a retention time of 17.41 min was seen. C, Peak 3 (geranial) was absent from control incubation without cell-free extract.

Purification of geraniol-DH

The cell-free extract prepared from 500 g of fresh *P. minus* leaves was first applied to a DEAE-Toyopearl 650M column. Ion exchange chromatography allowed the enzyme activity of the cell-free extract to be separated into two peaks, designated geraniol-DH I and II (Fig. 3). The first peak, corresponding to geraniol-DH I (fractions 10 to 32), was obtained in the wash fractions of DEAE-Toyopearl 650M column chroma-

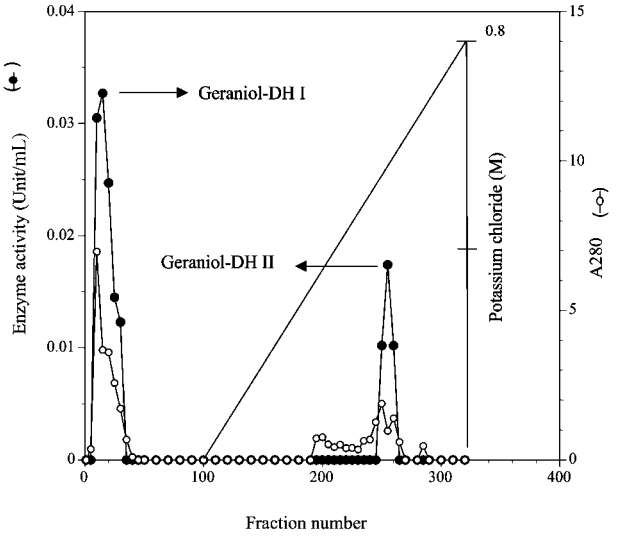

Fig. 3. Chromatographic Separation of Geraniol-DH on a DEAE-Toyopearl 650M Column.

The column was equilibrated with 100 mM of tricine-NaOH (pH 7.5) containing 2.5 mM of 2-ME, and eluted with a linear gradient of 0.8 M of potassium chloride in the buffer. The flow rate was 1.3 mL·min^{-1}. Arrows indicate protein peaks with enzyme activity. Active fractions were designated geraniol-DH I and geraniol-DH II. The straight line with hollow circles shows the peak with maximum absorbance at 280 nm. The straight line with solid circles shows the peak with geraniol-DH activity after activity measurement by the standard assay method.

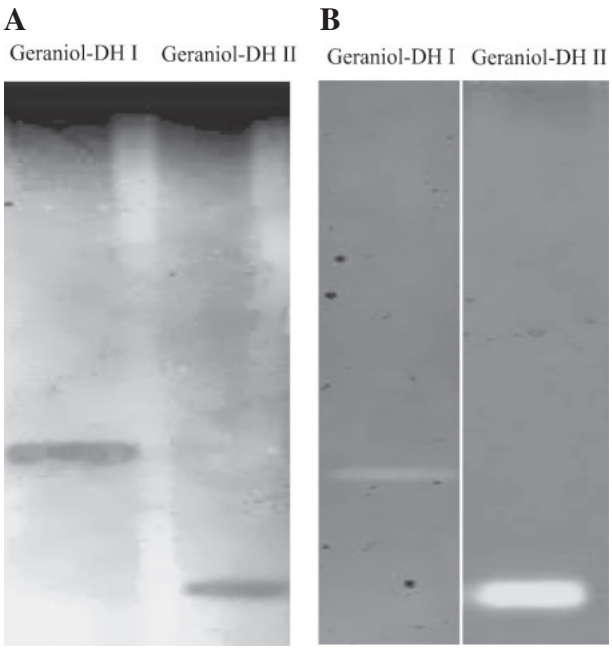

Fig. 4. Native-PAGE of Purified Geraniol-DH I and II from *P. minus* with Silver Staining (A) and Activity Staining (B).

Purified enzymes were subjected to electrophoresis in the absence of SDS with 12.5% gel at pH 8.8. The reaction mixture contained 100 mM of glycine–NaOH buffer (pH 9.5), 2 mM of geraniol in DMSO, 54 μM of 1-methoxy phenazine methosulphate, 0.3 mM of nitroblue tetrazolium, and 0.2 mM of $NADP^+$.

Table 2. Summary of Purification of Geraniol-DH Isoenzymes from *P. minus*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U·mg^{-1})	Purification (fold)	Yield (%)
Cell-free extract	13.08	8712.0	0.002	1.0	100
DEAE-Toyopearl 650M					
Geraniol-DH I	4.85	1095.0	0.004	2.9	37.0
Geraniol-DH II	0.85	61.1	0.014	9.2	6.5
Phenyl-Toyopearl 650M					
Geraniol-DH I	1.35	45.0	0.030	20.0	10.0
Geraniol-DH II	0.29	11.7	0.025	16.7	2.2
AF-Red 650ML					
Geraniol-DH I	0.20	1.10	0.175	116.7	1.5
Geraniol-DH II	0.12	0.02	0.261	173.8	0.9

tography at pH 7.5. The second peak, corresponding to geraniol-DH II (fractions 250 to 260), was obtained in the eluent fractions of the same column at pH 7.5. The two peaks were separately pooled for further purification on Phenyl-Toyopearl 650M and AF-Red Toyopearl 650ML columns. Under these column conditions, geraniol-DH I and II were eluted as single protein peaks.

The purification steps and their results are summarized in Table 2. Because sulfhydryl-reducing agents were found to protect the enzyme activity, 2-ME was included throughout the isolation procedure. Geraniol-DH I and II were purified to apparent homogeneity in three chromatographic steps. The purification procedures purified geraniol-DH I about 117-fold with about 1.5% recovery of the enzyme activity and purified geraniol-DH II about 174-fold with 0.9% recovery. Each of the purified enzymes gave single protein band on native-PAGE (Fig. 4A), and activity staining was detected at the same gel position (Fig. 4B). Geraniol-DH I and II activities were detected at different positions on activity-stained native-PAGE. In addition, when the cell-free extract was applied to CM-Toyopearl 650M column chromatography, two activity peaks were detected in the unabsorbed and eluent fractions. In view of these results, we suggest that *P. minus* contains two types of geraniol-DH isoenzymes, which may have different properties.

Protein characterization

The native molecular masses of the purified enzymes were determined by chromatography on a calibrated Superdex 200 10/300 GL column. The activities of geraniol-DH I and II were eluted with apparent molecular masses of 100 kDa and 120 kDa respectively (Fig. 5A). The molecular weights of *P. minus* geraniol-DHs have been found in general to fall within the range of 89–150 kDa as reported for other geraniol-DHs[8,9] and plant alcohol dehydrogenases.[22–27]

The subunit molecular weight was estimated by SDS–PAGE with a 12.5% polyacrylamide gel (Fig. 5B). Each

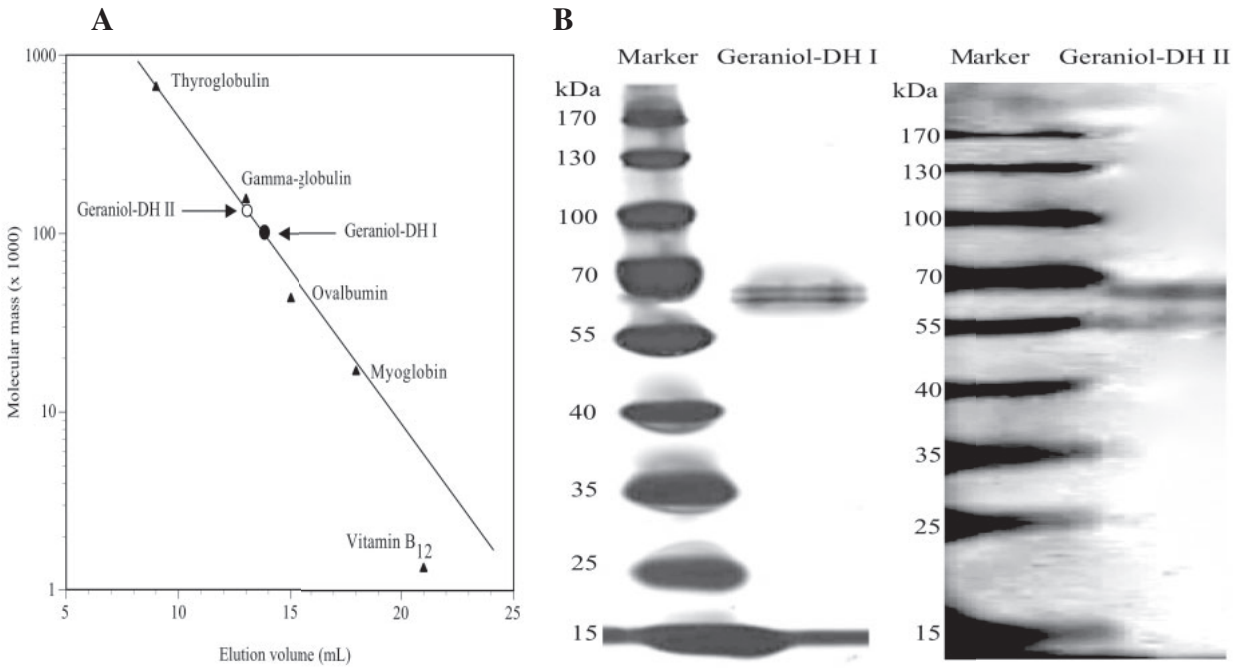

Fig. 5. Estimation of Native Molecular Masses and SDS–PAGE Analysis of the Geraniol-DH I and II from *P. minus*.

A, Estimation of native molecular masses of geraniol-DH I and II by Superdex 200 10/300 GL column. Experimental conditions are described in "Materials and Methods." Standard protein marker (solid triangle): thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1350 Da). Geraniol-DH I (solid circle). Geraniol-DH II (hollow circle). B, SDS–PAGE analysis of purified geraniol-DH I and II. Purified enzymes and standard proteins were subjected to electrophoresis in the presense of SDS with a 12.5% polyacrylamide gel. The PageRuler™ Prestained Protein Ladder, about about10–170 kDa (SM0671) (Fermentas, St. Leon-Rot, Germany) was used as molecular marker.

of the isoenzymes migrated as two bands on SDS–PAGE. The subunit molecular weights were calculated to be about 58 kDa and 62 kDa for geraniol-DH I, and the subunits of geraniol-DH II were 56 kDa and 60 kDa. The geraniol-DH I and II of *P. minus* were heterodimers with two different subunits. Cinnamyl alcohol dehydrogenase of *Eucalyptus gunni* Hook has been reported to exist as a heterodimer consisting a 42 kDa and a 44 kDa subunit.[28] In addition, *Ipomoea batatas* farnesol dehydrogenase,[27] *N. racemosa* acyclic monoterpene primary alcohol dehydrogenase,[29] and the alcohol dehydrogenases of *Triticum turgidum*,[26] *Vitis vinifera*,[30] and cultured rice cells[31] are all dimers of 37–58 kDa. On the other hand, geraniol-DH from the astigmatid mite[16] and acyclic monoterpene primary alcohol dehydrogenase from *Rauwolfia serpentina*[32] have been reported to act as monomers.

The purified enzymes were subjected to isoelectric focusing and were developed by silver staining (data not shown). Geraniol-DH I appeared to be a basic protein since an isoelectric of 8.8 was found. On the other hand, geraniol-DH II was an acidic protein with an isoelectric point of 3.5. The isoelectric points of *P. minus* geraniol-DHs were different from those of other plant alcohol dehydrogenases. The acyclic monoterpene primary alcohol dehydrogenase from *R. serpentina*[32] and the alcohol dehydrogenases of *T. turgidum*,[26] *V. vinifera*,[30] and cultured rice cells[31] were weak acidic proteins having estimated isoelectric points between 5.4–5.76.

Effects of temperature and pH

The residual activities of geraniol-DH I and II were measured after heat treatment at various temperatures for 10 min in 2.5 mM of 2-ME containing 100 mM of tricine-NaOH buffer (pH 7.5) (Fig. 6A). The decrease in the oxidation rate of geraniol-DH I was faster than geraniol-DH II. Rapid inactivation of geraniol-DH I activity occurred from 30 to 60 °C with 60% activity at 40 °C, and only 40% activity remaining at 60 °C. At 70 °C, the residual activity of geraniol-DH I was less than 20%, and geraniol-DH II retained over 80% the maximum activity after 10 min of preincubation at temperatures as low as 40 °C. From 30–50 °C, the residual acitivity of geraniol-DH II decreased slightly, but was lost rapidly at temperatures over 50 °C. Nevertheless, geraniol-DH II activity retained 60% activity at the final temperature tested, 70 °C. Both *P. minus* geraniol-DHs are more stable than previously reported geraniol-DH from *C. flexuosus*,[9] which became inactivated above 30 °C. In addition, the optimal temperature of both *P. minus* geraniol-DH isoenzymes was found to be 40 °C while the optimal temperature for *C. flexuosus*,[9] *Z. officinale*,[10] and *Carpoglyphus lactis*[16] geraniol-DHs was reported to be 25 °C.

The optimal pH values for geraniol-DH I and II were estimated by monitoring their activity between 4.0 and 10.0 pH using various buffers at a concentration of 100 mM (Fig. 6B). In the oxidation reaction of geraniol, the pH profiles of geraniol-DH I and II showed a narrow peak in the alkaline region and had less than 40% maximum activity below pH 6.5. The optimal pH values for *P. minus* geraniol-DH I and II were found to be 9.0 and 9.5 respectively. These results are comparable to the geraniol-DHs of *C. sinensis*,[8] *Z. officinale*,[10] and *O. basilicum*[11] (pH 9.0–9.5) and to the terpene alcohol dehydrogenases (pH 8.5–9.5).[22,27,29] In contrast, the optimal pH for *C. flexuosus* geraniol-DH[9] and *S. officinalis* borneol dehydrogenase[23] was 8.0.

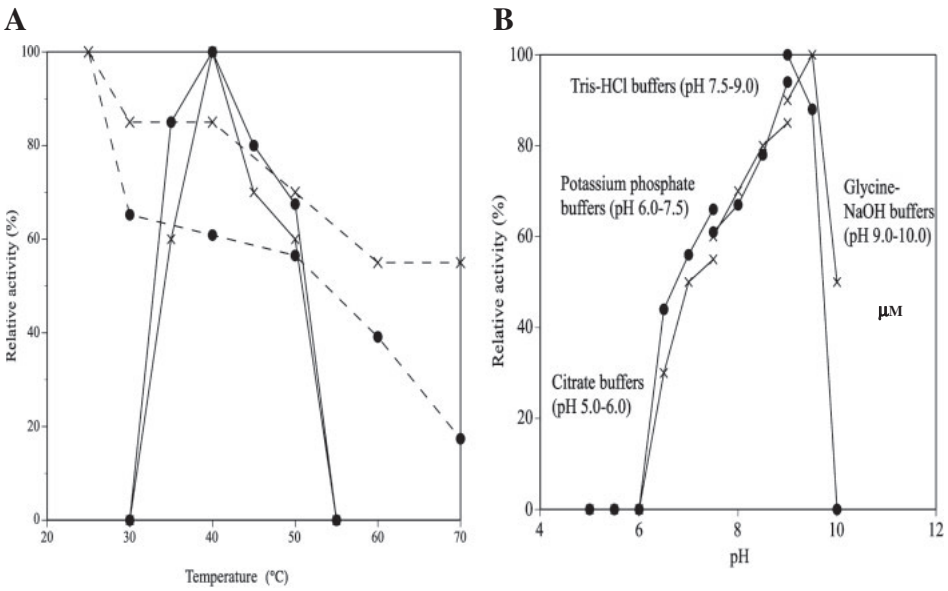

Fig. 6. Effects of Temperature and pH.

A, The effects of temperature on the enzyme activities of geraniol-DHs and the stability of the enzymes. Temperature stability was determined by incubating the purified enzymes at temperatures in a range of 25–70 °C for 10 min at pH 7.5 (100 mM of tricine-NaOH containing 2.5 mM of 2-ME). Residual geraniol-DH activity was assayed as described in "Materials and Methods." The optimal temperature was determined by performing the standard enzyme assay as described in "Materials and Methods," except that the reaction temperature was varied. Geraniol-DH I (●): thermo stability (dotted line), optimal temperature (solid line); geraniol-DH II (×): thermo stability (dotted line), optimal temperature (solid line). B, Effects of pH on enzyme activities of geraniol-DHs. Enzyme activity was assayed under the standard assay conditions, except that the following buffers were used at a final concentration of 100 mM in the incubation mixture: citrate buffers (pH 5.0–6.0), potassium phosphate buffers (pH 6.0–7.5), Tris–HCl buffers (pH 7.5–9.0), and glycine–NaOH buffers (pH 9.0–10.0). Geraniol-DH I (●); geraniol-DH II (×).

Table 3. Effects of Inhibitors on the Geraniol-DH Activity

The enzyme was preincubated for 5 min at 37 °C with various reagents before addition of the substrate. Each reagent was added at a final concentration as indicated.

Reagent	Concentration (mM)	Relative activity (%)	
		Geraniol-DH I	Geraniol-DH II
None	—	100	100
Iodoacetoamide	1.0	0	0
p-Chloromercuribenzoate	1.0	0	0
Potassium cyanide	1.0	0	0
2,2′-Dipyridyl	1.0	72	75
Sodium azide	1.0	61	90
Magnesium chloride	5.0	61	45

Effects of inhibitors

The effects of inhibitors on enzyme activity were examined. The activity of enzymes was measured after incubation of purified geraniol-DH I and II with inhibitors for 5 min at 37 °C (Table 3). At a concentration of 1.0 mM, both isoenzymes were strongly inhibited by potassium cyanide, and by sulfhydryl agents such as iodoacetoamide and *p*-chloromercuribenzoate. Chelating agents for the Fe ion, 2,2′-dipyridyl, and sodium azide caused 10–40% of inhibition in geraniol-DH I and II activities. Terpene alcohol dehydrogenase activities were greatly inhibited by sulfhydryl agents.[8,9,22,23,27] Based on these results, we suggest that the sulfhydryl group is important to enzyme activity, as for other terpene alcohol dehydrogenases.

Substrate specificity and Michaelis-Menten constants

Both geraniol-DH isoenzymes were very specific for $NADP^+$, and oxidized geraniol, nerol, β-citronellol, linalool, cinnamyl alcohol, *p*-cumic alcohol, and borneol (Table 4). Carveol was oxidized only by geraniol-DH I, and 3,7-dihydrolinalool only by geraniol-DH II. Neither *P. minus* geraniol-DHs reacted with farnesol, or with other alcohols such as benzyl alcohol, menthol, methanol, ethanol, propanol, and butanol. The kinetic parameters for geraniol-DH I and II are shown in Table 4. The K_m values for geraniol, nerol, β-citronellol, linalool, cinnamyl alcohol, and

Table 4. Substrate Specificity and K_m Values for Geraniol-DHs from *P. minus*

Substrate	Geraniol-DH I	Geraniol-DH II
Activity relative to geraniol		
Geraniol	100	100
Nerol	64	50
β-Citronellol	72	63
Tetrahydrogeraniol	0	0
Linalool	42	92
3,7-Dihydrolinalool	0	18
Tetrahydrolinalool	0	0
Cinnamyl alcohol	60	50
p-Cumic alcohol	56	46
Carveol	44	0
Menthol	0	0
Borneol	52	29
Benzyl alcohol	0	0
Farnesol	0	0
K_m value (mM)		
Geraniol	0.400	0.185
Nerol	1.212	0.714
β-Citronellol	10.000	2.222
Linalool	1.333	0.741
Cinnamyl alcohol	0.435	0.556
$NADP^+$ (μM)	21.0	58.8

$NADP^+$ were calculated from double reciprocal plots. In the reduction reactions of geraniol-DH I and II, the apparent K_m values for citral were 208 μM and 109 μM respectively.

The K_m values of $NADP^+$ determined for *P. minus* geraniol-DHs are the lowest of those measured for geraniol-DHs.[8,9] NAD^+ was ineffective as a cofactor for both *P. minus* geraniol-DHs, suggesting that both isoenzymes are $NADP^+$-dependent alcohol dehydrogenases, similarly to other plant geraniol-DHs[8–11] and plant terpene alcohol dehydrogenases.[27,29,32] On the other hand, the geraniol-DH from *Carpoglyphus lactis* had an absolute requirement of NAD^+ as cofactor.[16] Both NAD^+ and $NADP^+$ can be utilized as cofactors for *M. piperita* isopiperitenol dehydrogenase[22] and *S. officinalis* borneol dehydrogenase.[23]

The *P. minus* geraniol-DH isoenzymes oxidized nerol at 64% and 50% of the rate observed for geraniol, consistently with a previous report on *Z. officinale* geraniol-DH.[10] The geraniol-DHs of *C. flexuosus*[9] and *Carpoglyphus lactis*[16] showed no or low activity against nerol. On the other hand, nerol was a good substrate as good as geraniol for *O. basilicum* geraniol-DH.[11] The K_m values for nerol in geraniol-DH I and II respectively were approximately 3 and 4 times higher than for geraniol. This indicates that *P. minus* geraniol-DHs can recognize the geometrical isomers clearly, along with other geraniol-DHs.[9,10,16] A reduction reaction was observed in *P. minus* geraniol-DH I and II. It has been reported that enzymes from *C. sinensis*,[8] *C. flexuosus*,[9] and *Z. officinale*[10] also catalyze oxidation and reduction reactions.

The K_m values of 0.4 and 0.185 mM for geraniol in geraniol-DH I and II are higher than the K_m values for other geraniol-DHs.[8,9,11,16] A gradual increase in K_m values was observed on comparing the allylic, non-allylic, and aliphatic structures of geraniol analogs. Partial and complete saturation of the 2,3-double bond of geraniol, yielding β-citronellol (3,7-dimethyl-6-octen-1-ol) and tetrahydrogeraniol (3,7-dimethyl-1-octanol), resulted in a marked decrease in the enzyme activity of both isoenzymes. β-Citronellol exhibited K_m values 25- and 12-fold higher than geraniol in geraniol-DH I and II respectively. Furthermore, tetrahydrogeraniol was not oxidized by either isoenzyme. Similarly, linalool was a good substrate for both enzymes, its non-allylic analog, 3,7-dihydrolinalool was a poor substrate, and the aliphatic analog, tetrahydrolinalool, was not a substrate for either enzyme. *P. minus* geraniol-DH I and II also showed high affinity towards an aromatic alcohol, cinnamyl alcohol. Except for geraniol, the K_m values of cinnamyl alcohol for geraniol-DH I and II were lower than those determined for the other substrates. In addition, the K_m values for cinnamyl alcohol of *O. basilicum* geraniol-DH was more than 20-fold higher than geraniol and nerol.[11] Allylic alcohol dehydrogenase from *Pseudomonas putida* exhibited the highest catalytic specificity constants with substrates containing an allylic double bond or with an aromatic ring attached to the carbinol carbon.[33] MacKintosh and Fewson reported that the *Acinetobacter calcoaceticus* benzyl alcohol dehydrogenase oxidized not only a range of aromatic alcohols related to benzyl alcohol but also the allylic alcohol moieties in perillyl, cinnamyl, and coniferyl alcohols. They suggested that for cinnamyl and coniferyl alcohols, the alkenyl group, located between the aromatic ring and the carbinol center, may help correctly to position the alcohol in the active site.[34]

We found in this study that *P. minus* leaves contained two isoenzymes of geraniol-DH, which catalyze the oxidation of geraniol to geranial. The two isoenzymes were similar in molecular mass subunits, optimal temperature, and pH, but they differed in isoelectric point, substrate specificity, and kinetic parameters. The substrate specificities of *P. minus* geraniol-DH I and II also showed similarity to other alcohol dehydrogenases, in which saturation of the double bonds and increasing the chain length severely reduces substrate binding.[9–11,16,32,33,35] Furthermore, neither *P. minus* geraniol-DH exhibited activity against aliphatic alcohols, indicating that both of the enzymes are specific for substrates containing allylic double bonds. This suggests that *P. minus* geraniol-DHs can recognize allylic alcohols with carbon chain lengths between 9 and 10 and even a small difference in the structure of the substrate. Geraniol-DHs from *P. minus* are unique in view of their thermal stability and optimal temperatures. Furthermore, both *P. minus* geraniol-DHs have high affinity for $NADP^+$ and allylic alcohols as compared to other reported geraniol-DHs. To clarify the differences in thermal stability and affinity for allylic alcohols and coenzymes, it is necessary to determine the structure of *P. minus* geraniol-DHs, specifically their amino acid sequences. Protein structure analysis might also shed light on the question whether there exists a relationship between the stability and affinity of these enzymes. Molecular cloning of the geraniol-DH genes are in progress. Substrate analog inhibition studies and mode of inhibition will be described elsewhere.

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