Isolation and Characterization of Isopiperitenol Dehydrogenase from Piperitenone-Type Perilla

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Studying the biosynthesis of oil compounds in *Perilla* will help to elucidate regulatory systems for secondary metabolites and reaction mechanisms for natural product synthesis. In this study, two types of alcohol dehydrogenases, isopiperitenol dehydrogenases 1 and 2 (ISPD1 and ISPD2), which are thought to participate the oxidation of isopiperitenol in the biosynthesis of perilla, were isolated from three pure lines of perilla. Both ISPD1 and ISPD2 oxidized isopiperitenol into isopiperitenone with an oxidized form of nicotinamide adenine dinucleotide (NAD^+) cofactor. ISPD1 used both isopiperitenol diastereomers, whereas ISPD2 used *cis*-isomer as a substrate. However, only ISPD2 was isolated from piperitenone-type perilla. These results suggests that in perilla, ISPD2 is related to the biosynthesis of piperitenone, which was formed *via* (−)-*cis*-isopiperitenol.

Key words isopiperitenol dehydrogenase; *Perilla*; molecular cloning; essential oil; biosynthetic pathway; (−)-*cis*-isopiperitenol

Perilla frutescens is an annual Asian herbaceous plant used as a fresh kitchen herb. Its red-leaf strains are used in a traditional medicine known as *soyo* (蘇葉). The mericarps of *P. frutescens* are also used in a remedy called *soshi* (蘇子). The Japanese Pharmacopoeia defines *soyo* as containing not less than 0.08% perillaldehyde (PA), calculated based on dry leaves.[1] PA is a monoterpene unique to perilla.

Monoterpenes constitute a chemically diverse group of C10 compounds formed from two isoprene units. They are generally volatile and fragrant, and are often found in plant essential oils. Monoterpenes secreted by plants play important roles in defense against microbes and insect pathogens and in the attraction of pollinators.[2–4] Although many monoterpene compounds have antibacterial or antitumor properties,[5,6] they are usually used in perfumery and for flavoring food and medicine. Natural plant essential oils are rich in terpenoids and are valuable raw materials for phytochemical industries. However, terpene biosynthetic pathways are not well understood. Our group has been studying the biosynthesis of oil compounds in *Perilla* for more than 25 years to understand the regulatory systems of secondary metabolites and elucidate reaction mechanisms for natural product synthesis.

Perilla oil can be classified into more than ten types according to its principal constituents.[7–9] Figure 1 shows the putative biosynthetic pathways of principal compounds found in the PA, perillaketone (PK), and piperitenone (PT) oils. The synthesis of the constituent compounds in each of these oils is genetically controlled in perilla, and the regulatory steps in the biosynthetic pathways have been investigated by crossing experiments using pure strains developed from repeated self-pollination.[9,10] The function of each gene can be determined by cloning the enzyme that catalyzes the relevant reaction step in the biosynthetic pathway. The initial reaction step in the putative pathways for the monoterpenes in perilla oil is the dephosphorylation of geranyl diphosphate (GDP) catalyzed by either geraniol or (−)-limonene synthase. Our previous studies revealed that geraniol synthases from *P. citriodora*, *P. frutescens*, *P. hirtella*, and *P. setoyensis* and (−)-limonene synthases from *P. citriodora* and *P. frutescens*[11–14] share very similar amino acid sequences. An enzyme in the cytochrome P450 family is also reported to function as a limonene hydroxylase (limonene 7′-hydroxylation).[15] Geraniol dehydrogenases, which oxidize primary monoterpene alcohols, such as perilla alcohol and geraniol, have recently been isolated from *Perilla* (GenBank accession nos. JX629451-JX629453 and JX855836-JX855838, submitted). Enzymes relevant to monoterpene biosynthesis in perilla are being cloned individually, and are not yet finished.

PT-type perilla was collected in northern Thailand and in Laos. Its essential oil consists of 36% PT, 23.7% limonene, and other monoterpene compounds.[9] PT in plant essential oil is thought to be synthesized from GDP *via* limonene, isopiperitenol, and then isopiperitenone[9,16] (Fig. 1). Oxidation of isopiperitenol to isopiperitenone is probably catalyzed by alcohol dehydrogenases, and (−)-isopiperitenol/(−)-carveol dehydrogenase (GenBank accession no. AY641428) has been isolated from peppermint (*Mentha×piperita*).[17] However, this enzyme has not yet been isolated from perilla. Geraniol dehydrogenases recently isolated from perilla did not transform isopiperitenol; therefore, there is another alcohol dehydrogenase in perilla that oxidizes isopiperitenol to isopiperitenone. Here, we report the cloning and characterization of two types of alcohol dehydrogenase relevant to the oxidation of isopiperitenol in PT-type perilla.

MATERIALS AND METHODS

Plant Materials All perilla plants used in this study were grown in the Experimental Station for Medicinal Plant Research, Graduate School of Pharmaceutical Sciences, Kyoto University. The pure perilla strains were bred and maintained according to a previously reported method.[11] The species name, strain number, and oil type of the perilla strains used in this study were *P. citriodora* [strain No. 5601, PT type; strain No. 5712, PK type] and *P. frutescens* [strain No. 5598, PT type].

The authors declare no conflict of interest.

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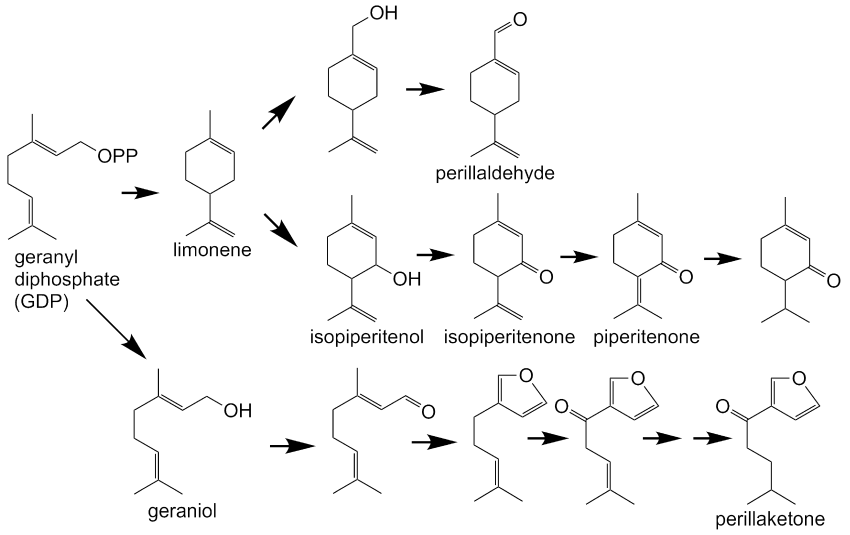

Fig. 1. Putative Biosynthetic Pathways of Oil Constituents (PA-Type, PT-Type, and PK-Type) in Perilla

Preparation of Substrates (−)-Isopiperitenone was prepared as follows. Commercially available (−)-limonene (1.5 g, 11 mmol) was oxidized as reported,[18] except that the chromium trioxide–pyridine complex, prepared from chromium trioxide (15.0 g, 150 mmol), was not isolated as it is in the literature.[19] The crude product (1.76 g) contained (−)-isopiperitenone and (−)-carvone in a 56 : 44 ratio, which were separated by column chromatography on silica gel by elution with 9% acetone in hexane to give (−)-isopiperitenone as a pale yellow oil (0.58 g, 39%). The ^{1}H-NMR spectrum agreed with the reported spectrum.[18] (−)-Isopiperitenol was prepared as a 40 : 60 mixture of *cis*- and *trans*-isomers from (−)-isopiperitenone according to a literature procedure in anhydrous diethyl ether.[20] This isomeric mixture was used in the enzymatic assays.

Construction of Expressed Sequence Tag Database and Cloning of Isopiperitenol Dehydrogenases from Perilla Kazusa DNA Research Institute constructed cDNA libraries and sequenced cDNA to build an expressed sequence tag (EST) database. Total RNA was extracted from young perilla leaves using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, U.S.A.), and was processed according to the mRNA-Seq protocol (Illumina, San Diego, CA, U.S.A.). The cDNA library was sequenced using a Genome Analyzer IIx sequencer (Illumina) with 100 bp paired-end (PE) reads. The Illumina reads were assembled using CLC Genomics Workbench version 4.7.2 (CLC Bio, Cambridge, MA, U.S.A.). Assembled contigs were used as queries against the non-redundant protein database using the BLASTX algorithm.

BLAST searches revealed two types of alcohol dehydrogenases (*ISPD1* and *ISPD2*), which showed high sequence similarity with (−)-isopiperitenol/(−)-carveol dehydrogenase from peppermint.[17] We expected that these enzymes would be relevant to the oxidation of isopiperitenol in perilla. Two pairs of primers were designed for homology-based polymerase chain reaction (PCR): 3976-f (5′-ATG GCA TCC TTT TCA ACT CTT TCT GTT TGTC-3′) as the forward primer and 3976-r (5′-AGA TTC TGG CTC CTT AAA CAC TTG CAT TGC-3′) as the reverse primer for cloning *PcPK-ISPD1*; 3204-f1 (5′-ATG GCA TAC TTT TCA ACT CTT TCT TCT CTC-3′) as the forward primer with 3204-r (5′-GGC AGA ATC TTG TGG ATT CTT GAA GAC-3′) as the reverse primer for cloning *PcPK-ISPD2*, *PfPT-ISPD2*, and *PcPT-ISPD2*. Total RNA was isolated from fresh young leaves of perilla. *PfPT-ISPD2* was isolated from strain No. 5598, *PcPT-ISPD2* was isolated from strain No. 5601, and *PcPK-ISPD1* and *PcPK-ISPD2* were isolated from strain No. 5712. The RNA was reverse transcribed to cDNA by ReverTra Ace (Toyobo, Osaka, Japan). PCR for determining cDNA sequences was performed in a reaction mixture containing KOD Dash (0.375 units, Toyobo), 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 0.3 μM forward and reverse primer, 1.5% DMSO, and cDNA (0.1 μL) with a temperature program starting at 94°C for 100 s, followed by 33 cycles of 94°C for 30 s, 50°C for 5 s, 74°C for 70 s, and a final elongation at 74°C for 70 s. Amplified *ISPD1* and *ISPD2* sequences were cloned into pTrcHis2-TOPO (Invitrogen, Carlsbad, CA, U.S.A.), sequences were confirmed by FASMAC Co., Ltd. (Kanagawa, Japan), and each clone was expressed in an *E. coli* expression system, as described below.

Heterologous Expression of cDNAs in *E. coli* and His-Tagged Protein Purification Each plasmid harboring *PcPK-ISPD1* and *PcPT-ISPD2* was transformed into TOP10F′ (Invitrogen) competent cells. Cultures initiated from single colonies were incubated in Lysogeny broth (LB) medium and cultured at 37°C for 12 h with shaking at 200 rpm. LB medium (200 mL) containing 0.1 mg/mL ampicillin was inoculated with the culture. After 0.5 h incubation at 37°C with shaking at 160 rpm, isopropylthio-β-garactoside was added to the culture to a final concentration of 1 mM. The cultures were then incubated for 37 h at 18°C with shaking at 220 rpm. Isolation and purification of His-tagged proteins were performed as described in a previous study.[21]

Enzymatic Assays and GC-MS Analysis All enzymatic reactions were performed in a 4 mL glass vial sealed with a polytetrafluoroethylene-coated silicone rubber septum. Reaction mixtures (250 μL) consisted of 50 mM Tris buffer (pH 7.0), 10% (v/w) glycerol, 1 mM dithiothreitol, 2 mM cofactor (either oxidized form of nicotinamide adenine dinucleotide (NAD^+) or reduced nicotinamide adenine dinucleotide (NADH)), 0.25 mM substrate, and the purified recombinant protein. Mixtures without cofactors were prepared as control solutions. The reaction mixtures were incubated at 30°C for 14 h. After the reaction, a solid-phase microextraction (SPME) fiber (100 μm

polydimethylsiloxane; Supelco, Bellefonte, PA, U.S.A.) was inserted into the headspace of the vial at room temperature for 30 min and was then transferred to the injector of a GC-MS (6850GC/5975MSD; Agilent Tech., Santa Clara, CA, U.S.A.). The SPME fiber was heated for 10 min at 160°C to desorb the compounds. The compounds were separated on a DB-WAX column (60 m×0.25 mm, 0.25 μm film thickness; Agilent Tech.). The temperature program began at 50°C for 5 min, increased at 5°C min^{-1} to 200°C, and remained at 200°C for 5 min. Helium was used as carrier gas, and the column flow was 1.0 mL/min. The eluted compounds were identified by comparing their ion spectra and relative retention times with authentic standards or the MS data library (NIST11; National Institute of Standards and Technology).

RESULTS AND DISCUSSION

Isolation of Two Types of Isopiperitenol Dehydrogenase from Perilla In the putative biosynthetic pathway of PT in PT-type perilla, the initial step is the dephosphorylation and cyclization of GDP by limonene synthase to form limonene. This is followed by the 3′-hydroxylation of limonene to produce isopiperitenol and the final step is expected to be the oxidation of isopiperitenol to isopiperitenone (Fig. 1). This pathway from GDP to isopiperitenone is based on the biosynthesis of peppermint oil, which has (−)-menthol stereoisomers as its principal components.[17,22] Limonene synthases have been cloned both from perilla (GenBank accession no. AAG31435, from strain no. 5601)[14] and peppermint (GenBank accession no. ABW86881),[22] and they share 64% amino acid sequence identity. Because isopiperitenol is oxidized by (−)-isopiperitenol/(−)-carveol dehydrogenase in peppermint (ISPD),[17] it is possible that the perilla dehydrogenase with a similar amino acid sequence to that of the peppermint dehydrogenase catalyzes the oxidation of isopiperitenol. Two cDNAs, contig nos. 3204 and 3976, were retrieved from the perilla EST library by searching for sequences showing homology with peppermint ISPD. Amino acid sequences of contig nos. 3204 and 3976 shared 40.4% and 41.5% identities with that of ISPD from peppermint, respectively. These identities were the highest identities with ISPD among contigs of EST library from perilla. Contig no. 3204 showed the highest amino acid sequence similarity to NAD(P)-binding Rossmann fold superfamily protein from *Theobroma cacao* (GenBank accession no. EOY08388), whereas the sequence of contig no. 3976 was very similar to that of momilactone A synthase-like isoform 1 from *Vitis vinifera* (GenBank accession No. XP_002281462). Contig Nos. 3204 and 3976 appeared to belong to the short-chain dehydrogenase/reductase (SDR) superfamily.

Based on these sequences, two sets of primers for amplifying contig Nos. 3204 and 3976 were designed to clone these genes from *P. frutescens* [strain No. 5598, PT type] and *P. citriodora* [strain No. 5601, PT type; strain No. 5712, PK type]. Consequently, *PcPK-ISPD2* and *PcPK-ISPD1* (GenBank accession Nos. KF766527, KF766526), which had very similar sequences to contig Nos. 3204 and 3976, respectively, were isolated from strain No. 5712 (Fig. 2). *PfPT-ISPD2* and *PcPT-ISPD2* (GenBank accession Nos. KF766528 and KF766529) were isolated as analogs of contig No. 3204 from strain Nos. 5598 and 5601, respectively (Fig. 2), although an analogous sequence to contig No. 3976 was not obtained from these two strains.

Sequence Analysis of *ISPD1* and *ISPD2* *PcPK-ISPD1* consisted of 825 nucleotides encoding a protein of 275 amino acids, and *PcPK-ISPD2*, *PfPT-ISPD2*, and *PcPT-ISPD2* consisted of 831 nucleotides encoding a protein of 277 amino acids (Fig. 2). In synthases cloned from perilla, the amino acid sequence identities were very high for enzymes with the

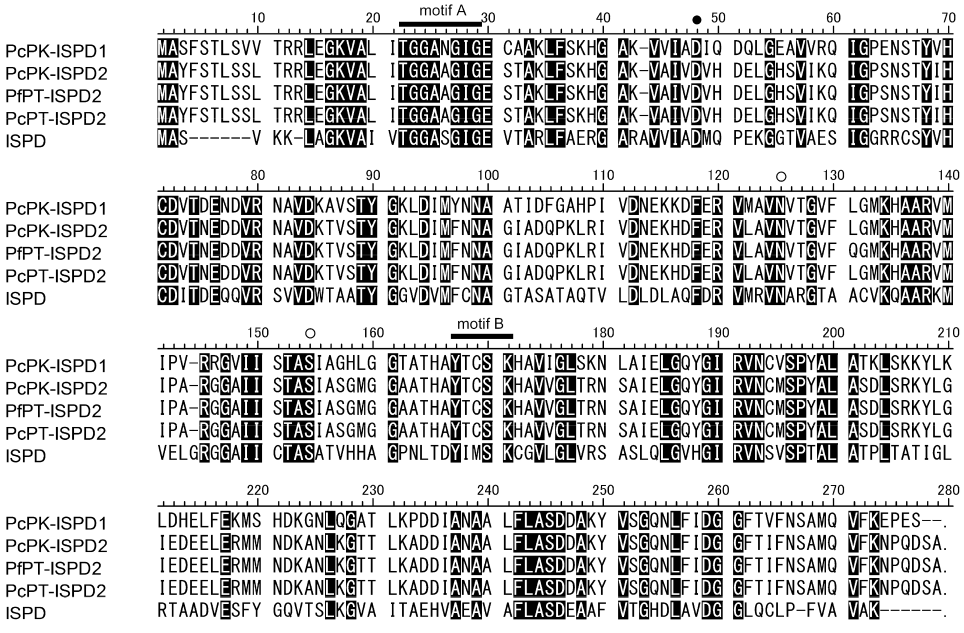

Fig. 2. Alignment of the Amino Acid Sequences of Isopiperitenol Dehydrogenases Described in This Study

PcPK-ISPD1: isopiperitenol dehydrogenase 1 from *P. citriodora* (strain No. 5712, PK-type; GenBank accession No. KF766526); PcPK-ISPD2: isopiperitenol dehydrogenase 2 from *P. citriodora* (strain No. 5712, PK-type; GenBank accession No. KF766527); PfPT-ISPD2: isopiperitenol dehydrogenase 2 from *P. frutescens* (strain No. 5598, PT-type; GenBank accession No. KF766528); PcPT-ISPD2: isopiperitenol dehydrogenase 2 from *P. citriodora* (strain No. 5601, PT-type; GenBank accession No. KF766529). ISPD: (−)-isopiperitenol/(−)-carveol dehydrogenase from *Mentha×piperita* (GenBank accession No. AY641428). Thick black bars indicate the TGXXXGXG motif, which is the conserved coenzyme binding motif (motif A), and the active site YXXXK motif (motif B). Closed circle denotes the conserved Asp47 residue indicative of NAD(H) preference. Open circles denote the residues that form a catalytic tetrad with Tyr and Lys of the YXXXK motif.

same functions, such as geraniol and linalool synthases[11,12] or primary alcohol dehydrogenases involved in monoterpene synthetic pathways (submitted). Three *ISPD2*s cloned in this study also shared more than 99.6% sequence identity. The amino acid identity between ISPD1 and ISPD2 isolated from strain No. 5712 was 76.5%.

Sequence motifs that are conserved in SDR enzymes were also found in the sequences of the four clones isolated from perilla, such as the TGXXXGXG coenzyme-binding motif[23,24] (motif A in Fig. 2) and the conserved Asp47 residue that indicates a preference for NAD(H) over NADP(H).[24] ISPD1 and ISPD2 contain the SDR YXXXK motif (motif B in Fig. 2) as an active site sequence with two residues, Tyr and Lys, which form a catalytic tetrad with Ser152 and Asn124.[17,23]

Functional Analysis of PcPK-ISPD1 PcPK-ISPD1 transformed both (−)-*cis*- and (−)-*trans*-isopiperitenol into (−)-isopiperitenone with NAD^+ as a cofactor (Fig. 3B). Because ISPD1 and ISPD2 isolated in this study contained the conserved Asp47 residue[24] indicative of NAD(H) specificity, they were expected to have an affinity for the NAD(H) cofactor. Furthermore, larger quantities of reaction products were detected in the reactions with NAD^+ than those with $NADP^+$ (data not shown). Consequently, NAD^+ was used as the cofactor for all enzymatic oxidative reductions in this study. The control solutions without cofactors produced no reaction products (data not shown).

Some SDR enzymes relevant to plant oil syntheses catalyze

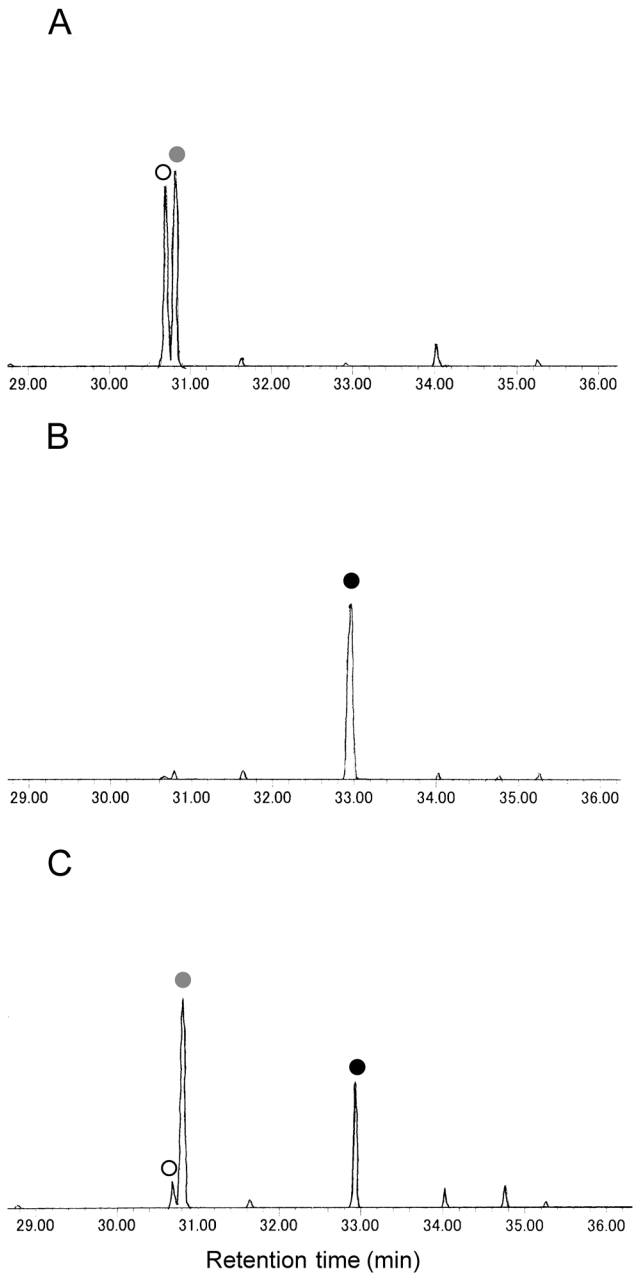

Fig. 3. GC Total Ion Chromatograms of the Reaction Products of PcPK-ISPD1 and PcPT-ISPD2 When (−)-Isopiperitenol Was Used as Substrate

(A) Control assay of PcPT-ISPD2 without cofactor; (B) PcPK-ISPD1; and (C) PcPT-ISPD2. Open circles are (−)-*cis*-isopiperitenol, gray circles are (−)-*trans*-isopiperitenol, and closed circles are (−)-isopiperitenone.

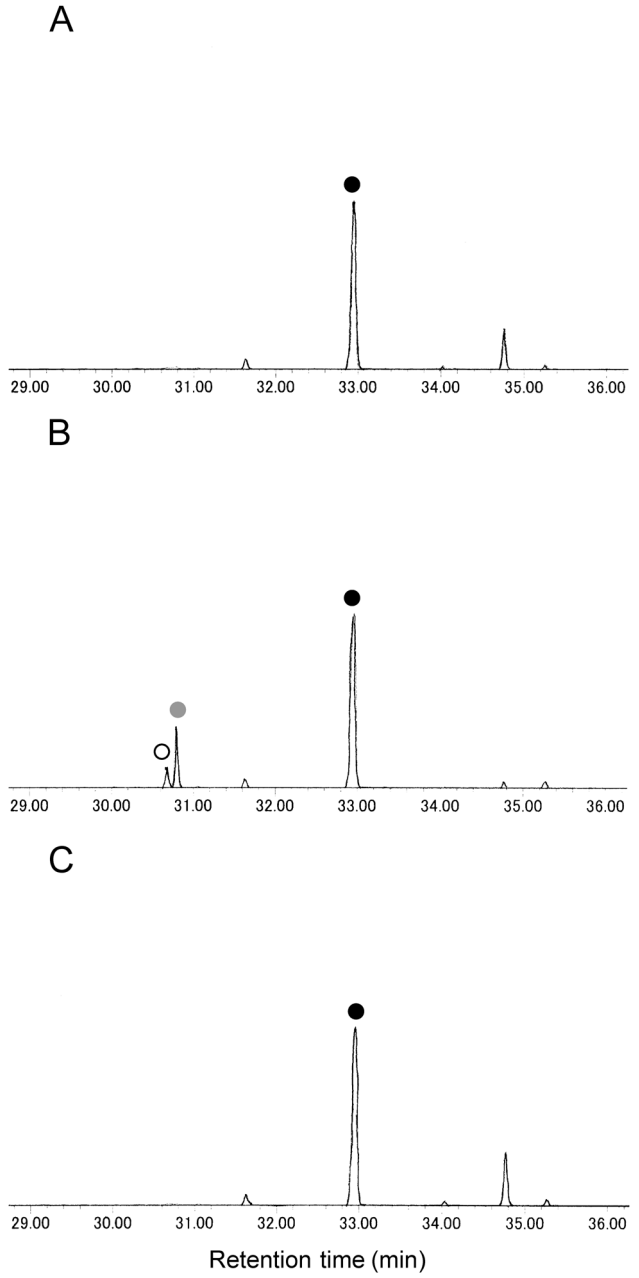

Fig. 4. GC Total Ion Chromatograms of the Reaction Products of PcPK-ISPD1 and PcPT-ISPD2 When (−)-Isopiperitenone Was Used as Substrate

(A) Control assay of PcPT-ISPD2 without cofactor; (B) PcPK-ISPD1; and (C) PcPT-ISPD2. Open circles are (−)-*cis*-isopiperitenol, gray circles are (−)-*trans*-isopiperitenol, and closed circles are (−)-isopiperitenone.

the reverse of alcohol oxidation, such as the reduction of ketones to alcohols.[25] PcPK-ISPD1 was incubated with (−)-isopiperitenone and NADH as cofactor, which produced (−)-*cis*-isopiperitenol and (−)-*trans*-isopiperitenol (Fig. 4B).

Functional Analysis of PcPT-ISPD2 Three ISPD2s isolated in this study shared greater than 99.6% amino acid sequence identity. It was reported in the previous study that enzymes relevant to biosynthesis of essential oil in perilla showed the same enzymatic function regardless of their oil types and strains when they share more than 96.8% amino acid identity.[11–14] Therefor, PcPT-ISPD2 was characterized further in this study.

PcPT-ISPD2 oxidized one of the isomers of (−)-isopiperitenol (a mixture of diastereoisomers) into (−)-isopiperitenone (Fig. 3C). (−)-*cis*-Isopiperitenol is eluted prior to (−)-*trans*-isopiperitenol in GC analysis using a capillary column with liquid phase of polyethyleneglycol.[26,27] The compound that PcPT-ISPD2 consumed was the isomer that eluted earlier, showing that PcPT-ISPD2 oxidizes (−)-*cis*-isopiperitenol. The control solutions without cofactors produced no reaction products (Fig. 3A).

The reverse-enzymatic reaction of PcPT-ISPD2 was also carried out by incubating the enzyme with (−)-isopiperitenone and NADH. However, no reaction product was detected (Fig. 4C). The control solutions without cofactors also produced no reaction products (Fig. 4A).

Comparison of ISPD1 and ISPD2 ISPD2 from perilla selectively converted (−)-*cis*-isopiperitenol into (−)-isopiperitenone and did not catalyze the reverse reaction. ISPD from peppermint, which is involved in the biosynthesis of (−)-menthol, also oxidized one of the (−)-isopiperitenol diastereoisomers and did not catalyze ketone reduction.[17] Although these two enzymes appear similar, ISPD from peppermint consumed the *trans*-isomer,[17] whereas ISPD2 from perilla transformed the *cis*-isomer. These results imply that (−)-*cis*-isopiperitenol is an intermediate for the PT biosynthesis of perilla. On the other hand, ISPD1 oxidized both diastereoisomers of (−)-isopiperitenol, and also catalyzed the reduction of (−)-isopiperitenone. The substrate preferences of ISPD1 and ISPD2 were different from that of ISPD from peppermint.

ISPD2 of greater substrate specificity was also cloned from PK-type perilla (PcPK-ISPD2) and was expected to use isopiperitenol as a substrate. However, none of the oil constituents found in PK-type perilla could be rationalized to be synthesized *via* isopiperitenol. These may indicate that PcPK-IPSD2 was not likely to be associated with the biosynthesis of essential oil. Constituents of perilla plant other than oil components could be another possibility for substrate/product of PcPK-IPSD2; however narrow substrate specificity of PcPK-ISPD2 makes it less likely.

ISPD1 has broader substrate specificity than ISPD2. ISPD1 (analogous sequence to contig no. 3976) was only isolated from PK-type strain no. 5712, and was not found in PT-type strains nos. 5598 and 5601. The reads per kilobase per million reads (RPKM) value corresponds to the gene expression level. The RPKM value of contig no. 3976 (corresponding to ISPD1) was large in strain no. 5712, whereas it was almost zero in strain no. 5601. These results are consistent with the absence of ISPD1 from strain 5601 and with the expression of ISPD1 in the PK-type but not PT-type strains of perilla. However, the PK biosynthetic pathways do not include the oxidation of isopiperitenol (Fig. 1). These results suggest that ISPD1 is less likely to be involved in oxidation of isopiperitenol. The RPKM value of contig no. 3976 was high in elsholtziaketone-type and PK-type perilla strains, and their putative biosynthetic pathways involve the oxidation of secondary monoterpene alcohols (Fig. 1). There remains an interesting possibility that ISPD1 catalyzes these oxidation steps.

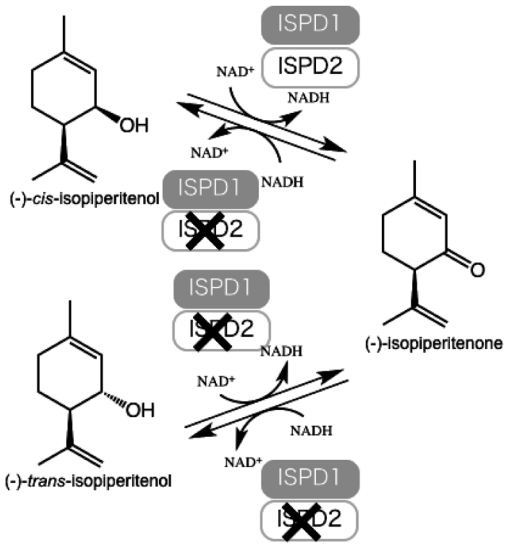

Fig. 5. Reactions of the ISPD1 and ISPD2 Enzymes Characterized in This Study

CONCLUSION

In this study, two types of isopiperitenol dehydrogenases, ISPD1 and ISPD2, were isolated from perilla. ISPD1 oxidized both *cis*- and *trans*-isopiperitenol to (−)-isopiperitenone and also consumed (−)-isopiperitenone as a substrate for reduction (Fig. 5). In contrast to ISPD1, ISPD2 converted (−)-*cis*-isopiperitenol into (−)-isopiperitenone, and did not catalyze the reduction of (−)-isopiperitenone (Fig. 5). The low RPKM of the corresponding contig for *ISPD1* (contig no. 3976) in PT-type perilla, and the absence of *ISPD1* in PT-type perilla, suggests that ISPD2 rather than ISPD1 is involved in the biosynthetic pathway of PT.

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