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Characterization of γ-terpinene synthase from *Citrus unshiu* (Satsuma mandarin)

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Abstract. γ-Terpinene is a monoterpene and a major component of essential oils made from citrus fruits and shows strong antioxidant activity in various assay systems. Plant γ-terpinene synthase is a member of the monoterpene cyclase family, which produces a specific monoterpene through cyclization of geranyl diphosphate (GPP), but the monoterpene cyclases have not been fully characterized. It is necessary to prepare large amounts of γ-terpinene synthase from *Citrus unshiu* (Satsuma mandarin) for the characterization, on this purpose we expressed the protein in *Escherichia coli* (*E. coli*) cells. As most monoterpene synthases have plastid-targeting signals, a gene lacking these signals was prepared and functionally expressed in *E. coli* cells harboring extra copies of the argU gene. The purified enzyme was incubated with GPP and the main product was confirmed to be γ-terpinene by GC/MS.

Keywords: γ-Terpinene, monoterpene synthase, *Citrus unshiu*, functional expression

1. Introduction

Some essential oils prepared from peel or flavedo of fruits are known to have antibacterial, antifungal, sedative, and anxiolytic activities [1,2]. Recently, their antioxidant activity has also been observed; as a result, proposals have been made to use the substances as natural antioxidants for food and cosmetics. Essential oils consist of many compounds, such as monoterpene, sesquiterpene, benzene, alochols, and aldehydes. Among them, phenols and some kinds of monoterpenes, especially γ-terpinene, show high antioxidant activity [3,4].

Monoterpene is synthesized from GPP via several reactions including isomerization and cyclization (Fig. 1). Those complex reactions are catalyzed by one specific monoterpene synthase corresponding to each monoterpene. However, the enzymatic mechanisms of these synthases have not been fully elucidated.

Citrus unshiu (Satsuma mandarin), one of the most popular citrus fruits in Japan, is a major product of Shizuoka, Japan. The main monoterpenes of essential oils from *Citrus unshiu* are limonene, γ-terpinene and β-pinene [5].

We tried to functionally express a significant amount of γ-terpinene synthase in *E. coli* to elucidate the mechanism of monoterpene synthase.

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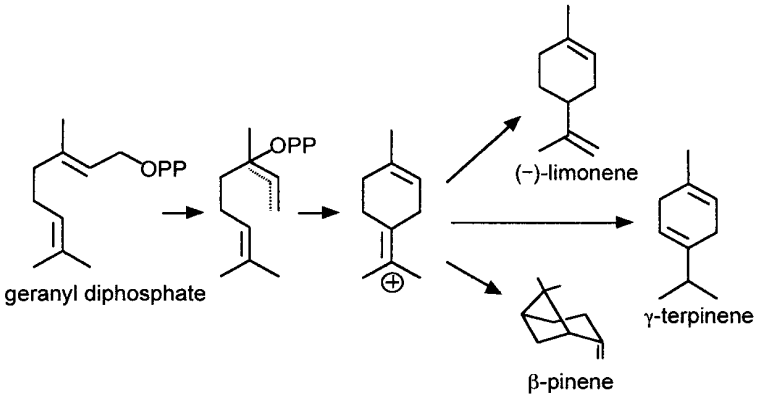

Fig. 1. Biosynthesis of three monoterpenes.

2. Materials and methods

2.1. Materials

γ-Terpinene synthase cDNA, CitMTSL3 (DDBJ accession number: AB110639), was isolated from *Citrus unshiu* (Satsuma mandarin). Geranyl pyrophosphate ammonium salt was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). γ-Terpinene, β-pinene, and $(-)$-limonene were purchased from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan).

2.2. DNA manipulation, expression in E. coli cells, and protein purification

The full-length CitMTSL3 was subcloned into an expression vector pET-9a (Novagen, Damstadt, Germany) by introducing the *Nde* I site overlapping the initiation codon of the gene. This construct was designated as N1. As monoterpene synthases bear a plastid-targeting signal [6], the signal of CitMTSL3 was removed. The gene was subcloned into pET-9a and the construct was designated as N2. *E. coli* BL21 (DE3) -CodonPlus-RIL strain (Stratagene, La Jolla, CA, USA) cells were transformed by N1 or N2. This strain carries extra copies of the argU (AGA, AGG) tRNA genes, that recognize the arginine codons AGA and AGG, which are rare in *E. coli* [7]. A single colony was picked and transferred to 3 mL of an LB broth with 100 mg/L ampicillin and grown overnight at 37°C. Aliquots of 1 mL were inoculated into 100 mL of an LB broth containing 100 mg/L ampicillin and kept for 2 h at 37°C. For the induction of expression, isopropyl thio-β-galactoside (IPTG) was added to a final concentration of 0.5 mM. The growth was continued for 2 h at 37°C, 4 h at 30°C, or 4 h at 20°C. Cells were collected by centrifugation, resuspended in 50 mM Tris-HCl (pH 7.5) buffer containing 0.1 M NaCl, 0.1 mM EDTA, and 1 mM DTT, and disrupted by sonication. The debris was removed by centrifugation. The supernatant was used for further analysis as a crude enzyme preparation. MALDI-TOF MS analysis of the expressed protein was performed using an ultraflex mass spectrometer (Bruker Daltonics, Bremen, Germany) in a linear and positive ion mode. Sinapinic acid was used as a matrix for MALDI-TOF MS analysis.

2.3. Enzyme assay and GC/MS analysis

The enzyme was assayed in a total volume of 100 mL of a reaction mixture containing 20 mM Tris-HCl (pH 7.8), 2 mM $MgCl_2$, 2 mM $MnSO_4$, 1 mM DTT, and 767 μM GPP. After the addition of 100 mL

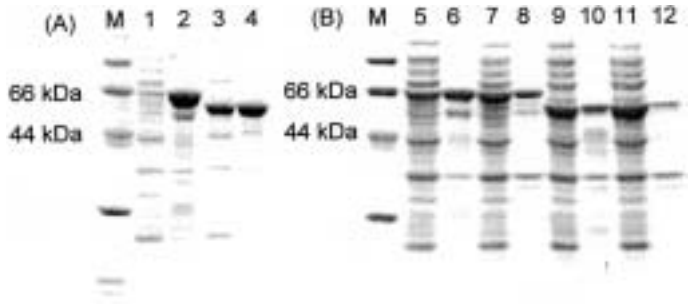

Fig. 2. SDS-PAGE analysis of expressed protein. The proteins formed in *E. coli* cells harboring N1 were fractionated into soluble (lanes 1, 5, and 7) and insoluble (lanes 2, 6, and 8) fractions. The proteins formed in *E. coli* cells harboring N2 were fractionated into soluble (lanes 3, 9, and 11) and insoluble (lanes 4, 10, and 12) fractions. After induction, the growth was continued for 2 h at 37°C (lanes 1, 2, 3, and 4), 4 h at 30°C (lanes 5, 6, 7, and 8), or 4 h at 20°C (lanes 9, 10, 11, and 12).

hexane, the solution was mixed and incubated for 2–4 h at 30 °C. The solution was mixed well again and an aliquot of the hexane layer was removed. The hexane fraction was injected into an HP-6890 series gas chromatograph system equipped with an HP-6890 series mass selective detector (Hewlett Packard, Palo Alto, CA, USA) and an OV-1 column (25 m × 0.25 mm, Hewlett Packard). The elution program was as follows; the initial temperature was 50°C for 5 min, with a ramp of 10°C/min to 200°C, and it was held for 5 min. The products were identified according to the retention times, and the mass spectra compared with those of authentic reference compounds.

3. Results and discussion

3.1. Functional expression in E. coli

The expressed proteins in *E. coli* were fractionated into insoluble and soluble fractions. When the cells were cultivated at 37°C, a major part of the protein expressed in *E. coli* cells harboring N1 was in the insoluble fraction and the protein expressed in *E. coli* harboring N2 was found almost equally in the two fractions (Fig. 2(A)). This suggests that the formation of the inclusion body is reduced by removing a plastid-targeting signal. Moreover the amount of soluble-form enzyme was increased by lowering the temperature to 30°C or 20°C after the IPTG induction (Fig. 2(B)). The optimal cultivation conditions tentatively used were 2 h at 37°C before IPTG induction and 16 h at 20°C after the induction. MALDI-TOF MS analysis of the protein expressed in *E. coli* harboring N2 showed that the molecular weight of the product of N2 was 63894.8, which agreed well with that calculated from the amino acid sequence (data not shown).

3.2. Enzyme assay and GC/MS analysis

The products of N2 were analyzed by GC/MS (Fig. 3). The main product was identified as γ-terpinene ($t_R = 10.13$ min). The formation of α-pinene ($t_R = 7.81$ min), α-pinene ($t_R = 8.58$ min), and limonene ($t_R = 9.64$ min) were also observed, but their amounts were very small. Because the selectivities for the main products are not so strict, such side products are widely observed in monoterpene synthases. As an explanation, it is suggested that the *E. coli* host could proteolytically process the enzyme to a form that could compromise substrate and intermediate binding conformations [8].

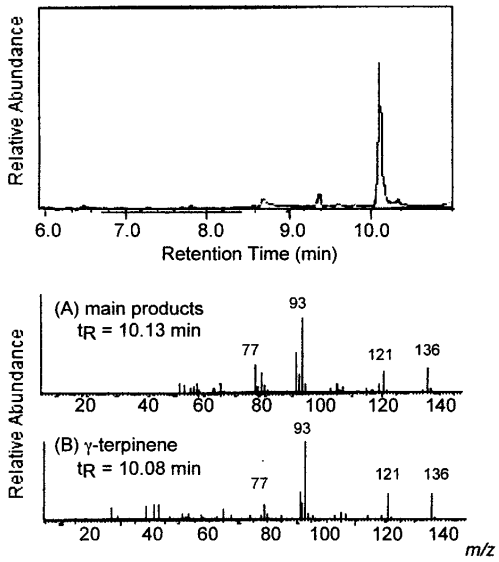

Fig. 3. GC-MS chromatogram of the compounds produced by the expressed protein by N2, and MS spectra of the main product (A) and that of γ-terpinene (B).

In this study, a large amount of γ-terpinene synthase was functionally expressed in *E. coli*, and the main product was identified as γ-terpinene. The site-directed mutagenesis and crystal structure of the enzyme is now under investigation to elucidate the specificity of the products of enzyme.

Acknowledgement

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