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Menthol biosynthesis pathway in *Mentha piperita* suspension cells

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Abstract : The metabolic capability of the cultured cells of peppermint was tested with whole intact cells by feeding appropriate exogenous substrates to the suspension cultures. Conversion of (−)-limonene into any other monoterpenes was not observed with the suspension cultures. (+)-Pulegone was converted into (+)-isomenthone and (−)-menthone, and (−)-menthone into (−)-menthol. The experiments confirmed that the suspension retained most of the menthol biosynthesis pathway in the cell except for a few loci. (−)-Isopiperitenone was transformed into (+)-pulegone, piperitenone, (−)-7-hydroxyisopiperitenone and two unidentified products (Received August 17, 1993; accepted September 21, 1993).

Introduction

Mentha piperita, a member of the genus *Mentha* (Lamiaceae), commonly referred to as peppermint, is among the most well-known essential oil-producing plants. The fragrant oil of peppermint, major components being (−)-menthone and (−)-menthol, contains complex mixtures of monoterpenes produced and accumulated in highly specialized secretory structures, the glandular trichomes. Investigation of Croteau's group demonstrated that (+)-isopulegone is the key intermediate in the conversion of (−)-isopiperitenone to (+)-pulegone and finally established the sequence of menthol biosynthetic sequence[1,2].

Many studies have been performed to produce monoterpenoids by cell cultures[3,4]. However, secondary metabolites tend to be synthesized in specialized cell types, and the cell cultures were found not to produce monoterpenoid because cultured cells could not differentiate[5]. In recent years, Lee reported that suspension cells of peppermint produced essential oil upto 528 mg/l[6] and elicitor treatment increased (−)-menthol content, but the composition of the oil was different from that of the natural peppermint oil. Suspension cells of peppermint produced more (+)-pulegone and (+)-piperitone, precursors of (−)-menthol and (−)-menthone, than (−)-menthol and (−)-menthone, the major components of the natural peppermint oil. Peppermint oil from the field-grown peppermint contains 49.6% (−)-menthol, 21.8% (−)-menthone, 5.0% (+)-pulgone and 0.2% (+)-piperitone, while oil from the suspension cultured cells contains 19.6%, 0.9%, 26.1 % and 12.7%, respectively[6]. In the biosynthetic pathway of the menthol, the reduction of $\Delta^{4,8}$-double bond of (+)-pulegone produces (−)-menthone. Therefore, it is possible that the reduction step from (+)-pulegone to (−)-menthone was inhibited in the suspension cells which resulted in the accumulation of (+)-pulegone in the suspension cultured cells. Piperitone is produced through the iso-

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merization-reducion of (−)-isopiperitenone instead of the reduction-isomerization producing (+)-pulegone. Biochemical understanding for menthol producing enzyme is thus needed to produce more menthol and menthone in the peppermint oil by manipulating the cell culture system.

This work was performed to understand the metabolism of terpenoids in cultured peppermint cells. To achieve the goal, biotransformation technique was employed to study the metabolism of the terpenoids in peppermint cells. The study would eventually contribute to the production of the peppermint oil similar to the natural peppermint oil in its composition by peppermint suspension cultures.

Materials and Methods

Plant materials

The suspension cells of *Mentha piperita* were maintained in the laboratory of HJL. The suspension cells were subcultured every month and maintained at 27℃ with 16~8 hrs light-dark cycle. The shaking speed of the suspension cells was 120 rpm on a rotary shaker. The culture media were Lin-Staba (LS) medium without agar for the suspension cells.

Solvents and reagents

For cell and tissue cultures, the "plant cell culture tested grade" reagents were purchased from Sigma (St. Louis, MO). (−)-Limonene (96%, $[\alpha]_D = -94°$), (+)-limonene (97%, $[\alpha]_D = +123°$), (+)-camphor (99%, $[\alpha]_D = +44°$), (−)-menthol (99%, $[\alpha]_D = -50°$), and (+)-isomenthol (99%, $[\alpha]_D = +24°$) were obtained from Aldrich (Milwaukee, WIS). (+)-Pulegone (95%, $[\alpha]_D = +22°$) was obtained from Merck (Darmstadt, Germany). Preparation of (−)-isopiperitenone, piperitenone, (+)-piperitone, (−)-menthone, (+)-isomenthone is reported elsewhere[7]. The solvents were GR grade or redistilled EP grade. Other reagents were GR grade.

General methods

Gas chromatograms were obtained on a Hewlett-Packard 5980 GC using HP-FFAP capillary column (50 m×0.33 μm×0.2 mm), a Carlo Erba series-II GC using SE-30 capillary column (15 m×0.33 μm×0.2 mm) with flame ionization detector. TLC was performed on the pre-coated Silica gel 60 F254 plates (0.25 mm, Merck) using hexane-diethyl ether (2 : 1) as the solvent. The plate was visualized under short UV light, by iodine vapor or by spraying with anisaldehyde spray reagent (*p*-anisaldehhyde : glacial acetic acid : methanol : sulfuric acid = 0.5 : 10 : 85 : 5). The adsorbent used for VLC was a Merck Kiesegel 60G (Art. 7731).

Biotransformation

For biotransformation with whole cell system, following procedure was employed. Ten milligrams of the monoterpene in 20 μl of ethanol was added to a 250 ml baffled Erlenmeyer flask containing 100 ml of 3 weeks old cell suspension and incubated under the previously described condition for 24 hrs. Each suspension was extracted with 100 ml of methylene chloride. The extract was dried with anhydrous $MgSO_4$ and evaporated to a small volume to be analyzed for monoterpene with GLC.

Isolation of biotransformation product

The suspension cells (800 ml) were incubated with 80 mg (−)-isopiperitenone for 24 hrs and were extracted with methylene chloride. The extract was dried with anhydrous $MgSO_4$ and evaporated to a small volume. The mixture was fractionated with preparative TLC (solvent; acetone : hexane = 3 : 1).

Results and Discussion

Metabolism study with whole cell

(−)-Limonene, (−)-isopiperitenone, (+)-pulegone or (−)-menthone was administered to the 3 weeks old peppermint suspension cell cultures. A time-course study was performed to determine the adequate incubation time. A twenty-four hour incubation was found to be satisfactory in effecting enough biotransformation of the fed terpene as exemplified with (−)-isopiperitenone (Fig. 1). After incubation, the methylene chloride extracts of the suspensions were analyzed by GLC. The results

are summarized in Table 1.

Conversion of (−)-limonene into other monoterpenes was not observed with the suspension cultures. In peppermint leaves, (−)-limonene is converted to (−)-isopiperitenol and subsequently to (−)-isopiperitenone by a cytochrome P-450 and a dehydrogenase. However, (−)-limonene was not converted into other monoterpenes by suspension cells in this experiment. The inertness of (−)-limonene in suspensions may have been caused by a low solubility of the compound in the suspension. The other monoterpenes used as exogenous substrates have a somewhat hydrophilic character due to their carbonyl or hydroxyl functional groups. When (−)-limonene (10 mg) was administered to the suspension as DMSO solution to increase the solubility of the terpene, it was converted to several unknown compounds. However, the known terpenoids in the menthol biosynthetic pathway were not detected (detection limit; ~1 nmol). This finding suggested that the suspension cells lacked or were low in cytochrome P-450 activity to convert limonene to isopiperitenol. Low limonene permeability of the cell wall could be an alternative explanation.

The suspension cultures converted (−)-isopiperitenone to not only (+)-pulegone(~235 nmol) but also piperitenone (~45 nmol) and (+)-piperitone (~3 nmol). In leaves, (−)-isopiperitenone is converted to (+)-pulegone, (−)-menthone and finally (−)-menthol, the major constituents in essential oil. In suspension, however, (−)-isopiperitenone was converted to (+)-pulegone as well as piperitenone and (+)-piperitone. In addition, suspension cells converted (−)-isopiperitenone to several unknown

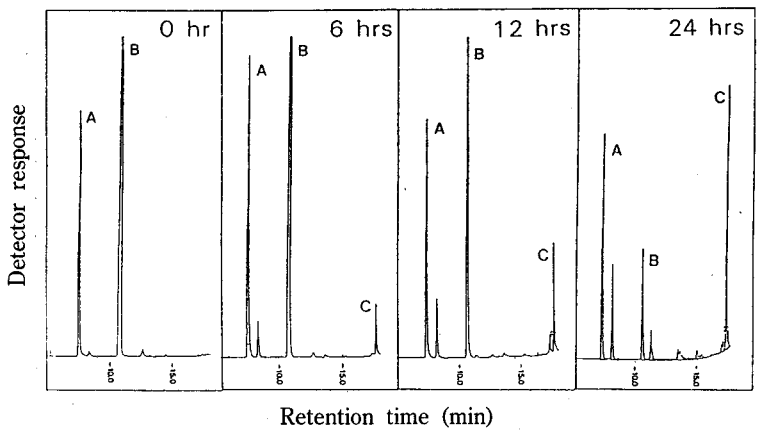

Fig. 1. Time-course incubation of (−)-isopiperitenone with suspension cells. A, camphor; B, (−)-isopiperitenone; C, (−)-7-hydroxyisopiperitenone.

Table 1. Conversion of monoterpenes by biotransformation of the peppermint suspension cultures. (Detection limit was 1 nmol)

Products Formed (nmol)	Administered Substrates			
	(−)-Limone	(−)-Isopiperitenone	(+)-Pulegione	(−)-Menthone
(−)-Limonene	–	<1	<1	<1
(−)-Isopiperitenone	<1	–	<1	<1
Piperitenone	<1	45	<1	<1
(+)-Piperitone	<1	3	<1	<1
(+)-Pulegone	<1	235	–	<1
(−)-Menthone	<1	21	21	–
(+)-Isomenthone	<1	<1	44	<1
(−)-Menthol	<1	<1	<1	3

compounds, which were identified as (−)-7-hydroxyisopiperitenone, (*R*)- and (*S*)-6-hydroxyisopiperitenone (unpublished result in this lab). (+)-Pulegone, formed from (−)-isopiperitenone by the $\Delta^{1,2}$-reduction and the subsequent $\Delta^{4,8}:\Delta^{8,9}$-isomerization, was accumulated in suspension but did not further metabolize to (−)-menthone or (+)-isomenthone. These observations are illustrated in Fig. 2.

Piperitenone is a product of the $\Delta^{4,8}:\Delta^{8,9}$-isomerization of (−)-isopiperitenone, and piperitenone is reduced to yield (+)-piperitone. The results from the leaf enzyme preparation and the report by Croteau's group[1] suggest that the $\Delta^{1,2}$-reduction of (−)-isopiperitenone is dominant and the $\Delta^{4,8}:\Delta^{8,9}$-isomerization of (−)-isopiperitenone is weak in the peppermint epidermal glands. But, in suspensions, the $\Delta^{4,8}:\Delta^{8,9}$-isomerization does occur competitively to $\Delta^{1,2}$-reduction. The $\Delta^{4,8}:\Delta^{8,9}$-isomerization activity of (−)-isopiperitenone in suspensions explains the accumulation of piperitenone and (+)-piperitone in the suspension cell cultures because (+)-piperitone is the end product and is not metabolized further. As the activity of (+)-pulegone reductase in suspension cells was lower than the leaves, (+)-pulegone accumulated in the suspension cell cultures while (+)-pulegone is mainly converted to (−)-menthone in leaves. Two stereospecific reductases are responsible for $\Delta^{4,8}$-reduction of (+)-pulegone. (−)-Menthone-specific reductase is predominant in leaves whereas the activity of (+)-isomenthone specific reductase was found to be higher than (−)-menthone specific reductase in suspension. Nevertheless, as the activity of $\Delta^{4,8}$-reductases producing (−)-menthone or (+)-isomenthone in suspension are low compare to that of the leaves, these emzyme activities were not detected at low concentration of (+)-pulegone.

Incubation of the suspension cells with (+)-pulegone afforded more (+)-isomenthone (~44 nmol), a minor component in the naturally occurring peppermint oils, than (−)-menthone (~21 nmol), the normal product of (+)-pulegone in peppermint leaves. Incubation of the suspension cells with (−)-menthone gave rise to a small amount of (−)-men-

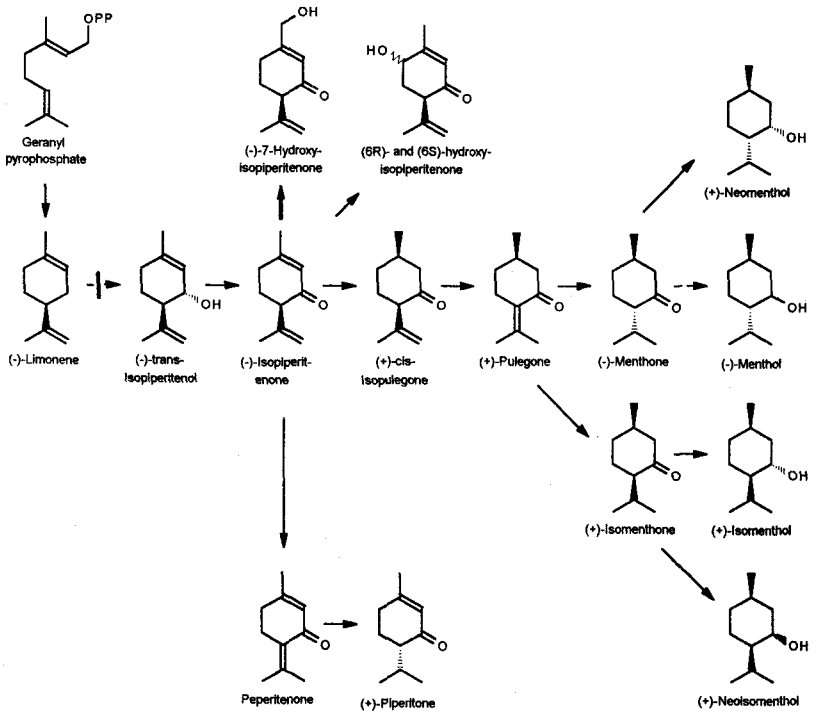

Fig. 2. Bioconversion of the intermediates in the menthol pathway by suspension culture of *Mentha piperita*.

thol (~3 nmol). These results imply that suspension cultured cells have the metabolic pattern of both mesophyll and glandular trichome cells. It is possible that the undifferentiated one cell type may have both metabolic patterns and thus functions in menthol producing pathway and in piperitenone pathway. Another possibility is that the suspension cultured cells are actually consisted of two cell types, mesophyll cell and epidermal cell.

Discussion

The experiment with the suspension culture confirmed that the suspension cells retained most of the menthol biosynthetic machinery in the suspension cells. But the enzyme activities in the suspension cells are rather low compared to that of the epidermal glands, and the preference for the the regioselectivity and the stereoselectivity are different from that of the peppermint leaves at the biosynthetic branching points. The low activities of the menthol biosynthetic enzymes may be caused by the low translation and transcription of the genes encoding these enzymes. These low activities again may be caused by other factors. As the physical and biochemical status of the suspension cells are different from that of the leaves, so are the operating activities different. The cytochrome P-450 activity responsible for oxidation of (−)-limonene to (−)-*trans*-isopiperitenol was not observed in this experiment, while oxidation of C-6 and C-7 on (−)-isopiperitenone was found. The present findings implies that the oxidation is the rate limiting step in menthol pathway for the suspension cell.

Several hundreds of biotransformations of exogenous substrates by plant cell cultures have been reported[13]. However, biotransformation using peppermint cells are scarce. Aviv's group reported the biotransformation of monoterpenes by the peppermint suspension cell culture[13]. Their reports indicated that biotransformation furnishes an effective tool to investigate the conversion of the secondary metabolites *in vitro* by the cultured plant cells. They tested (+)-pulegone and (−)-menthone and found that administered (+)-pulegone is biotransformed to (+)-isomenthone[14], and (−)-menthone is biotransformed to (+)-neomenthol[15] by the peppermint suspension cell culture.

The present report, however, deals with a complete set of intermediary terpenes in the menthol biosynthetic pathway. We have tested the biotransformation capabilities of the peppermint suspension culures with the established cell-line maintained at one of the authors (HJL) laboratory by successive subculture because the biotransformation capabilities differ according to the cultured cell lines and culture conditions. The peppermint cell lines converted (−)-isopiperitenone to 6- and 7-hydroxyisopiperitenone, (+)-pulegone, piperitenone, (−)-menthone. This step could be a branching point leading to the waste products, 6- and 7-hydroxyisoperitenone, which are not normal constituents in the plant.

(+)-Pulegone was converted to (−)-menthone as well as (+)-isomenthone as reported by Aviv[14]. These results suggested that the suspension cultured peppermint cells have most of the menthol biosynthetic enzyme systems but different activities from that of the epidermal glands. Therefore, in order to use the peppermint suspension cell cultures as the means of producing the peppermint oil, it is necessary to enhance the activity of the enzymes, not operating as high as in the leaves, and to prevent the waste pathway by manipulating the physiology or genetics of the cells.

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박하(*Mentha piperita*) 세포 현탁배양에서 멘톨생합성 경로

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초록 : 박하(*Mentha piperita*) 세포현탁배양에(−)-menthol 생합성 중간체를 투여하여 배양된 세포의 대사경로를 연구하였다. (−)-Limonene을 투여 하였을 때 이는 다른 대사물로 변환되지 않는 것으로 관찰 되었다. (+)-Pulegone은 (+)-isomenthone 및 (−)-menthone으로 변환되었으며, (−)-menthone은 (−)-menthol로 변환되었다. 이 실험은 현탁배양세포가 대부분의 생합성 활성을 유지하고 있으며 (−)-limonene hydroxylase의 활성이 제한적임을 보여 주었다. (−)-Isopiperitenone을 투여하였을 때는 (+)-pulegone, piperitenone, (−)-7-hydroxyisopiperitenone, (*R*)- 및 (*S*)-6-hydroxy isopiperitenone이 생성되었다.