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Bio-catalytic Synthesis of Isoprenoids by Cultured Cells of *Cucurbita maxima*

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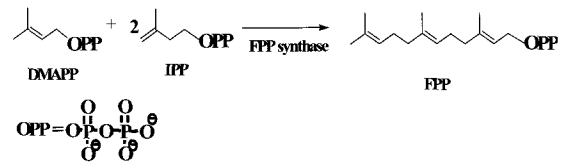
We previously demonstrated the biotransformation of prenyl alcohols into the corresponding carboxylic acids by cultured cells of *Cucurbita maxima*. Here, we further investigated two kinds of biotransformation by these cultured cells using combinations of prenyl alcohols and isopentenol and of prenyl diphosphate and isopentenyl diphosphate. The major material formed in the biocatalytic reaction of geranyl diphosphate with isopentenyl diphosphate as substrates was geranylgeraniol, with not only farnesol but also geranylfarnesol also formed, although as minor materials. With the combination of geraniol and isopentenol, in contrast, geranylgeraniol was formed, but neither farnesol nor geranylfarnesol was detected.
Key words: *Cucurbita maxima*, cultured plant cells, biotransformation, prenyltransferases, geranylgeraniol

1. INTRODUCTION

Many researchers have used cultured plant cells and bacteria to investigate bioorganic synthesis and biotransformation, including oxidation, reduction, hydroxylation, isomerization, alkylation, and glycosylation [1-4]. In particular, biotransformation with tissue culture is considered a useful and important method for one-pot synthesis of many useful materials [5-7]. So far, the isoprenoid biosynthetic pathway in which isoprenyl allylic and homoallylic compounds are used has been studied in details. However, little research has appeared on the biotransformation of allylic and homoallylic substrates using cultured cells [8-10].

Naturally occurring isoprenoids such as steroids, ubiquinones, prenyl proteins, carotenoids, dolichols, and natural rubber play an important role in daily life. These isoprenoids are all synthesized from the fundamental linear prenyl diphosphates, which are synthesized by the action of prenyltransferase from two simple precursors, namely isopentenyl diphosphate (IPP) as a homoallylic substrate and dimethylallyl diphosphate (DMAPP) as an allylic primer (Scheme 1.) [11-16].

We previously reported the biotransformation of prenyl alcohols into the corresponding carboxylic acids using cultured pumpkin cells[17].



Scheme 1.

Expecting that prenyltransferases such as farneysl diphosphate (FPP) synthase and geranylgeranyl diphosphate (GGPP) synthase would occur in these cells, we were interested to determine their usefulness in the biotransformation of shorter-chain prenyl compounds into longer-chain prenyl compounds via the action of endogenously occurring prenyltransferases.

2. EXPERIMENTAL

2.1 General

Prenyl alcohols obtained by alkaline phosphatase treatment of reaction products and prenyl carboxylic acids from biocatalytic reactions were analyzed by HPLC using a Hitachi type L-6000 equipped with a Hitachi L-7420 (LaChrom) type UV-vis detector, with a ChromatoDAQ II (ULVAC) and a LichroCART (Merck) column. The eluent used was a mixture of hexane and 2-propanol at 40:1 (A) or 80:1

(v/v) (B) [17-18]. Identification of the reaction products was performed by GC-MS using a JMS-AM II 50 type GCG mass spectrometer connected to a HP 5890 series II gas chromatograph equipped with ultra-alloy-1 (S). The column temperature was set to increase from 90 to 280 °C with a linear temperature gradient at a rate of 15 °C/min and then to remain at 280 °C for 3 min.

2.2 Substrates

Syntheses of isopentenyl diphosphate (IPP) and geranyl diphosphate (GPP)

Homoallylic tosylate and allylic chloride, derived from 3-methyl-3-buten-1-ol (IOH) and geraniol (GOH), respectively, were each converted to the corresponding diphosphates by Davisson's method [18].

Farnesol (FOH) as an authentic sample was purchased from Aldrich, and GGOH was supplied by TAMA Biochemical Co., Ltd.

2.3 Cell line and culture conditions

Callus tissues of pumpkin were induced as described previously [17]. Cell suspension cultures were cultivated with 100 ml of liquid MS medium on an orbital shaker at 70 (or 100) rpm at 24 °C in the dark. And pH was adjusted to 5.7 – 5.8 before autoclaving for 10 min at 120 °C. In addition, magnesium ion required for enzymatic reaction was contained in the MS medium.

2.4 Biocatalytic reactions of isoprenoids

Biocatalytic reactions of GPP with IPP

With a total volume of 20 ml of cultured cells in the medium, the incubation mixture for the biocatalytic reaction contained 5.0 μmol of IPP and 2.5 μmol of GPP as subject substrates. The cultures were incubated on an orbital shaker at 70 (or 100) rpm and 24 °C in the dark. After 15 days' incubation, the reaction mixture was treated with alkaline phosphatase for 12 h, and the cells and medium were separated by filtration with a nylon mesh. The medium and homogenized parts of the cells were extracted with pentane and analyzed by HPLC and GC-MS. To compare the relative yields, the reaction of GPP with IPP as a blank test was performed in the medium under the same conditions as described for incubation but without cultured cells. A second blank test was conducted by incubating cultured cell liquid without the substrates.

Biocatalytic reactions of GOH with IOH

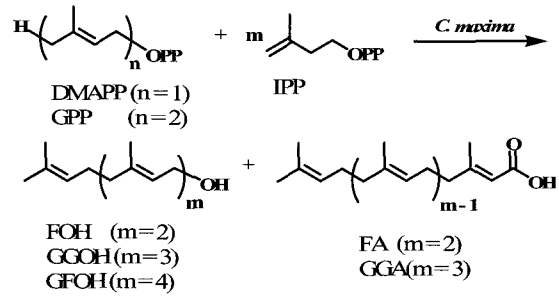
With a total volume of 20 ml of cultured cells in the medium, the incubation mixture for the biocatalytic reaction contained 5.0 μmol of IOH and 2.5 μmol of GOH as the subject substrates. The reaction and work-up were performed as above.

2.5 Time course Experiments

The two substrates, 2.5 μmol of GPP and 5.0 μmol of IPP, were added to a suspension culture of pumpkin cells containing 2, 4-dichlorophenoxyacetic acid (2, 4-D) ($5x10^{-3}$ μM) and kinetin ($5x10^{-5}$ μM) as a hormone. At a total volume of 20 ml, incubation was performed on a rotary shaker (100 rpm) for 15 days at 24 °C in darkness. Samples (1 ml) of the cultured mixture were removed under sterile conditions at two-day intervals and extracted with hexane, and the extracts were subjected to HPLC followed by GC–MS. Investigation of the time course of biocatalytic synthesis of isoprenoids was performed to analyze reaction products which were treated and untreated with alkaline phosphatase.

3. RESULT AND DISCUSSIONS

To study the biocatalytic synthesis of isoprenoids, we analyzed the reaction products from GPP and IPP using cultured pumpkin cells as shown in Scheme 2.



Scheme 2.

3.1 Biocatalytic reaction of GPP with IPP using cultured cells of *C. maxima*

The biocatalytic reactions of GPP and IPP were carried out in tissue culture, and the reaction products released from the cells were hydrolyzed with alkaline phosphatase to the corresponding three kinds of alcohols. The major alcohol (yield: 25%) was eluted on the HPLC at 27.0 min using eluent B. The GC-Mass spectrum showed a molecular ion peak at *m/z* 290 (rel. int. 2.9%), corresponding to $C_{20}H_{34}O$; other fragment ions were observed at *m/z* 272 ($M^+ - 18$) (2.2), 203 ($M^+ - 18 - 69$) (8.4), 135 ($M^+ - 18 - 69 - 68$) (12.3), 69 (68.5), 67 ($M^+ - 18 - 69 - 68 - 68$) (42.2), and 55 (base peak), indicating that the alcohol is geranylgeraniol (GGOH). Finally, we identified the alcohol as GGOH by HPLC comparison with an authentic sample.

GC-MS was used to detect other minor products derived from the biocatalytic products. Two minor alcohols were detected with GC retention times at 8.4 min (4% yield) and 13.5 min (1% yield). The mass spectrum of first minor alcohol showed similar to that of authentic (all-*E*)-farnesol (FOH). Then, the product together with authentic FOH showed one peak at 29.1 min by HPLC. Based on this, the former was identified as FOH.

The spectrum of the second minor alcohol showed a molecular ion peak at *m/z* 358 (rel. int. 8.4%), corresponding to $C_{25}H_{42}O$; other fragment ions were observed at *m/z* 340 ($M^+ - 18$) (3.2), 271 ($M^+ - 18 - 69$) (8.2), 203 ($M^+ - 18 - 69 - 68$) (14.1), 135 ($M^+ - 18 - 69 - 68 - 68$) (27.9), 69

(16.3), and 57 (base peak). This alcohol was identified as geranylfarnesol (GFOH) by comparison with authentic sample.

We also analyzed the reaction products untreated with alkaline phosphatase. GGOH was obtained in 20% yield, while FOH (4%), GFOH (1%), farnesoic acid (FA, 1%), and geranylgeranoic acid (GGA, 1%) were present as minor products.

In a previous study [17], we showed that GGA is formed by biotransformation of GGOH. In addition, we noted that FA is formed from FOH and geranoic acid from geraniol (GOH). The peaks of GGOH and FOH in the extract of the culture solution were confirmed by HPLC and GC-MS, and the presence of GFOH, FA, and GGA was confirmed by GC. We expect that endogenously occurring oxidation enzymes such as alcohol dehydrogenase and aldehyde dehydrogenase are also likely operative in this biotransformation.

3.2 Biocatalytic reaction of GOH with IOH using cultured cells of *C. maxima*

We examined the biocatalytic reactions of GOH and IOH with the tissue culture. Results showed that GGOH was formed in a 7% yield. A longer-chain prenyl alcohol (C_{20}) was synthesized from the shorter-chain prenyl alcohol (C_{10}). We expect that GOH incorporated into the intracellular matrix was phosphorylated to GPP, which was then converted to FPP or GGPP in the culture cells. The present experiment showed that prenyl alcohols are permeated into cultured cells containing prenyltransferases such as GPP-, FPP-, and GGPP-synthases. We expect that oxidative enzymes occurring in pumpkin likely act in the biotransformation, because the prenyl alcohols were oxidized to prenyl carboxylic acids.

3.3 Time course of the biocatalytic reaction of GPP with IPP by tissue culture cells of *C. maxima*

The time course of the biocatalytic reaction of GPP with IPP by tissue culture was examined using two kinds of experiment to allow the determination of concentrations. The first experiment measured products obtained by treatment of the reaction mixture with alkaline phosphatase (Figure 1.), while the second measured products untreated with alkaline phosphatase, on the basis of the notion that an endogenous phosphatase occurs in pumpkin tissue culture (Figure 2.).

The first experiment using treatment with alkaline phosphatase showed that GGOH is slowly afforded after three days, and reached a 25% yield at 20 days (Figure 1). With regard to FOH, formation was observed in markedly small quantities at three days, and could not be detected thereafter. This finding can be explained as follows: FPP is afforded by single-condensation of GPP and IPP. A second IPP molecule is then consumed in the formation of GGPP.

For the time course experiment conducted without alkaline phosphatase, the formation of GGOH started slowly after three days, and then increased from the 9th day, to finally reach a 20% yield.

Interestingly, affording FOH slightly was checked from the time course experiments as shown Figure 1. and 2. However, formation of GGOH had an upward tendency gradually.

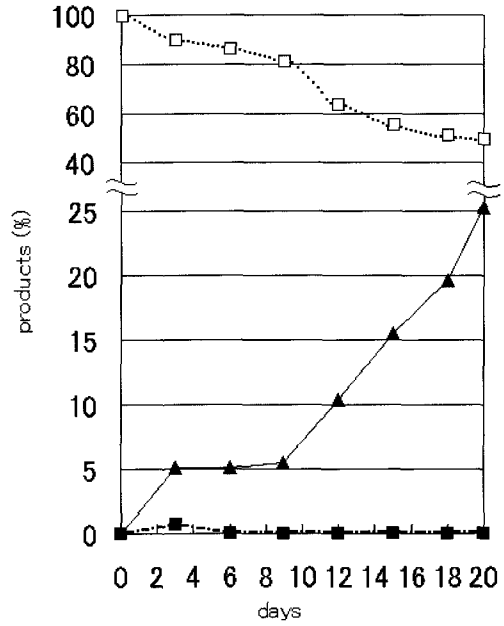


Figure 1. Time-course of the biotransformation of GPP with IPP by cultured cells of *C. maxima*. Yields of GOH (□), FOH (■), and GGOH (▲) are plotted after treatment with alkaline phosphatase.

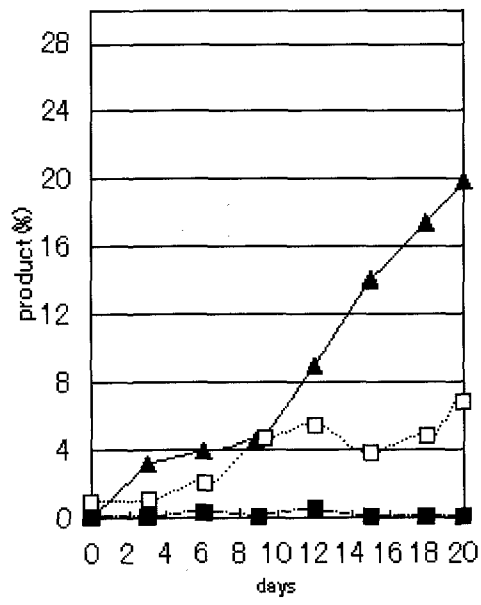


Figure 2. Time-course of the biotransformation of GPP with IPP by cultured cells of *C. maxima*. Prenylalcohols obtained without alkaline phosphatase treatment were analyzed. Yields of GOH (□), FOH (■), and GGOH (▲) are plotted.

4. CONCLUSIONS

Catalytic reaction of geranyl diphosphate/geraniol with isopentenyl diphosphate/isopentenol using cultured pumpkin cells gave geranylgeraniol (yield: 25%), together with small amounts of geranylfarnesol and farnesol and two kinds of carboxylic acid, farnesoic acid and geranylgeranoic acid.

5. ACKNOWLEDGMENTS

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