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SHORT COMMUNICATION

Biotransformation of Eugenol to Bis-eugenol by *Kalopanax pictus* Cell Culture

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Abstract So far, the production of bis-eugenol has been conducted by chemical synthesis. However, because chemical synthesis of bis-eugenol has disadvantages such as regioselectivity and low yield, an environmentally friendly production of bis-eugenol by biological synthesis has become attractive. In the present study, bis-eugenol was produced using *Kalopanax pictus* callus culture. High-performance liquid chromatography analysis of reaction product revealed a new peak, whose structure was determined to be bis-eugenol by nuclear magnetic resonance spectroscopy. After 18 h of biotransformation using *K. pictus* cell, 16.3 mg/L of bis-eugenol was produced without any byproducts. This is the first report on production of bis-eugenol by biotransformation using plant cell culture.

Keywords biotransformation · bis-eugenol · eugenol · *Kalopanax pictus* Thunb. Nakai

Phytochemicals are valuable sources for developments of flavors, agrochemicals, and medicinal foods. However, biosynthetic pathway of phytochemicals is complex, and total synthesis of phytochemical is not easily achieved. Instead, plant cell cultures have been used as suitable biocatalysts to perform the complex biosynthesis. Until now, a wide variety of compounds including aromatics, steroids, alkaloids, coumarins, and terpenoids were synthesized through biotransformation using plant cells, and this approach is effective in regiospecific and stereospecific reactions (Suga and Hirata, 1990; Archana et al., 2001). The biotransformation by cultured plant cells includes oxidation, reduction, hydroxylation, esterification, methylation, isomerization, hydrolysis, and glycosylation (Ishihara et al., 2003).

Eugenol (4-allyl-2-methoxyphenol) is a member of the phenylpropanoid class compounds, and commonly used as a flavoring agent in cosmetic and food products and as a dental material such as temporary filling and root canal sealer by zinc oxide eugenol cements. In addition, several studies demonstrated that eugenol show the positive properties of antioxidant and anti-inflammatory activities, as well as antigenotoxic and anticarcinogenic potentials (Fotos et al., 1986; Feng and Lipton, 1987; Hashimoto et al., 1988; Zheng et al., 1992; Rompelberg et al., 1996; Nagababu and Lakshmaiah, 1997). However, eugenol has adverse effects of causing inflammatory and allergic reactions such as allergic contact dermatitis at higher concentrations, due to the formation of phenoxyl radicals, quinone methide intermediates via its pro-oxidant activity (Atsumi et al., 2000). To reduce the adverse effects, some researches proposed that eugenol was synthesized into various dimers of eugenol-related compounds, and the dimers showed lower cytotoxicity and had a significantly higher antioxidant activity than eugenol (Satoh et al., 1998; Atsumi et al., 2000). Up to now, chemical synthesis has been used to produce bis-eugenol; however, it has limiting factors such as regioselectivity of the synthesis products and usage of harmful reagents. If it is possible to produce the rational amount of bis-eugenol by biotransformation with cell derived from plants, this method could be a solution that can replace chemical synthesis. Therefore, we attempted to produce bis-eugenol by biotransformation using *Kalopanax. pictus* callus (KACs) and successfully achieved the production of bis-eugenol from eugenol (Fig. 1).

K. pictus Thunb. Nakai is a perennials woody plant belonging

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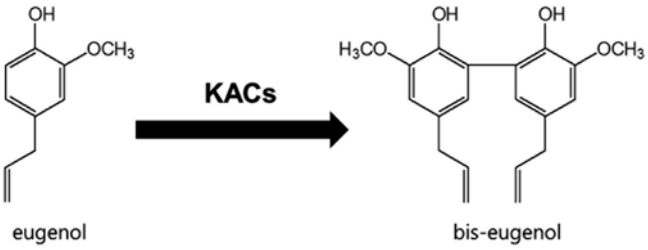

Fig. 1 A schematic representation of eugenol conversion into bis-eugenol by *Kalopanax pictus* cells

to the family *Araliaceae* distributed in northeast Asia, Korea, China, and Japan (Krussmann, 1985). It has been used as a traditionally important medicine, which contains high amounts of chemical constituents such as phenolic glycosides of liridendrin, syringing, and hederagenin glycosides of kalopanaxsaponin as triterpene (Sano et al., 1991; Park, et al., 1998). KACs were used as potential catalyst to produce bis-gugenol by biotransformation. KACs, which were cultured on MS medium supplemented with 4.4 μM 2,4-D, 2% sucrose, and 1.5 % agar, were transferred to liquid MS medium supplemented with 4.4 μM 2,4-D and 2 % sucrose, and cultured on a shaking incubator at 120 rpm for 21 days at 25±1°C in the dark. Some compounds were tested as to whether the KACs could metabolize, as substrates, eugenol. carvacrol, and thymol. When cells reached at double the fresh weight, each substrate was added to the liquid medium, and cultured for 2 days. The cells cultured with eugenol, carvacrol, or thymol were extracted twice with an equal volume of ethyl acetate and dried under speed vacuum dryer. The reaction product was initially analyzed using thin layer chromatograph (TLC; Silica gel $60F_{254}$, Merck) with a solvent of benzene (3): EtOAc (1).

Among substrates used, eugenol gave a new product with different Rf value compared with that of control, with no added eugenol to KACs culture medium. However, carvacrol and thymol did not show any difference with control, indicating that KACs can only use eugenol as a substrate. The reaction product was additionally analyzed by high performance liquid chromatography (HPLC) equipped with a PDA detector. For the analysis of reaction product, separation of the extracts was conducted by a gradient elution of mobile phase A (water containing 0.1% formic acid) and mobile phase b (acetonitrile containing 0.1% formic acid). Gradient condition was: initial 10% B, 0–10 min; 30% B, 10–25 min; 40% B, 25–40 min; 50% B, 40–50 min;. The flow rate was 1.0 mL/min and UV detection at 280 nm. Injection volume was 10 μL. The reaction product showed new peak at different retention time when compared with that of control (Fig 2B). Retention time of eugenol was at 18.2 min, whereas new product eluted at 24.5 min. This result indicated that KACs could specifically produce a single product. The molecular mass of eugenol and the reaction product were determined by liquid chromatograph-mass spectrometer equipped with a photodiode array detector (Varain355), an ion trap spectrometer, at flow rate of 0.2 mL/min. The mobile phase consisted of A (0.1% formic

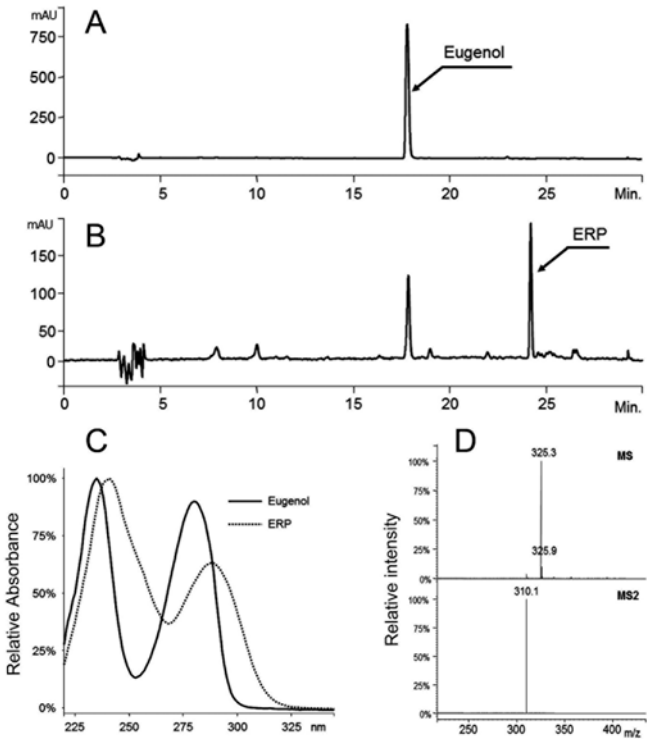

Fig. 2 HPLC and MS analyses of compound produced by *K. pictus* cell culture. A, MS medium supplemented with eugenol without *K. pictus* cell. B, MS medium supplemented with eugenol with *K. pictus* cell. C, UV absorbance of eugenol and reaction product (ERP). D: LC-MS/MS analysis of reaction product (ERP)

acid in water) and B (0.1% formic acid in acetonitrile). The gradient increased linearly from 0% to 10% B in 2 min, to 40% B in 10 min, and 70% in 20 min. Mass spectra were acquired simultaneously using an electrospray ionization (ESI) source in positive and negative ionization modes at 600 V. The spray needle voltage was set at 5 kV, the capillary voltage was 80 V, and the capillary temperature 220°C. The sheath gas pressure was set at 35 psi and the auxiliary gas pressure at 10 psi. Full scan mass spectra were recorded for the range of m/z 50–2000. The molecular mass of the reaction product was increased by 162 Da when compared with that of bis-eugenol. It is likely that two eugenol molecules were dimerized through the carbon bridge (Fig. 2D).

To determine the structure of the reaction product, ^{1}H-NMR analysis was carried out. NMR was performed as described by Kim et al. (2010a). Structure of the reaction product was determined by comparison of the data published by Ogata et al. (2008). NMR data was analyzed as follows: NMR (DMSO-d6, 400 MHz); δ 2.51 (t, 2H, *J*57.9 Hz, 7-H), 1.61 (m, 2H, *J*57.3, 7.9 Hz, 8-H), 0.93 (t, 3H, *J*57.3 Hz, 9-H). These results matched with previously published data (Ogata et al., 2008), an indication that KACs produced bis-eugenol when eugenol is used as a substrate.

Bis-eugenol, known as antioxidant and anti-inflammatory agent, is used as a flavoring agent in food products and cosmetic (Murakamin et al., 2003; Fujisawa et al., 2004). Mainly, bis-

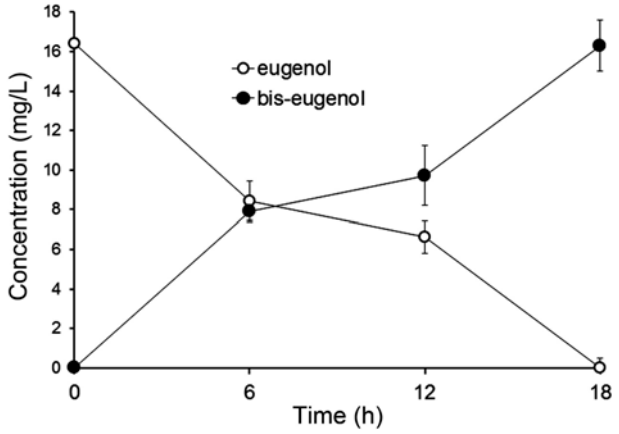

Fig. 3 Production of bis-eugenol by biotransformation using *K. pictus* cell.

eugenol has been chemically synthesized. To circumvent chemical synthesis approach, biotransformain of eugenol using KACs was carried out. After the culturing of KACs for 21 days, a substrate (eugenol) was added at the final concentration of 16.4 mg/L, and the production of bis-eugenol was monitored periodically. While the amount of eugenol continued to decrease throughout the incubation time, the reaction product (bis-eugenol) increased with increasing incubation time. After 18 h of incubation, the production of bis-eugenol reached a maximum of 16.3 mg/L, whereas the remaining amount of eugenol was not detectable, indicating that all of the eugenol added was efficiently metabolized at this time point (Fig. 3).

Biotransformation is a useful tool for regioselective and stereoselective modifications of phytochemicals, which exert pharmaceutical effects to human such as anticancer, antivirus, and anti-inflammatory, and reduces risk of heart disease. This tool has advantages of saving expensive cofactors and of extracting reaction products by simple step such as an ethyl acetate partitioning (Kim et al., 2010b; Lim et al., 2011). In the present study, biotransformation method was employed and conducted successfully to produce bis-eugenol using KACs. Although *Escherichia coli* and baking yeast are very useful hosts of biotransformation, several problems exixt in adopting this tool; 1) genes related to specific compound biosynthesis must be cloned and characterized. 2) genes such as P450s are difficult to express functionally in *E. coli*. On the contrary, the cell culture using plant cells has advantages over *E. coli* and baking yeast; this method does not require cloning and characterizing all genes involved in the biosynthesis of certain compounds, as well as does not require functional expression of gene such as P450s (Kim et al., 2010c; Cui et al., 2011).

Pure isolation of valuable chemical from plant extract involves multifarious refining process, and the amount of compound extracted from plants could be affected by seasonal and spatial limitation. However, cell culture using plants cell could overcome these problems due to the simple process such as ethyl acetate extraction and artificial fermentation system. Using biotransformation with KACs, we successfully produced bis-eugenol from eugenol as a substrate.

To the best of our knowledge, this is the first report of biological synthesis with plant cells derived from young leaf of *K. pictus*. Further studies of the reaction product by incorporating abiotic or biotic stress such as yeast extract, UV irradiation, and *Streptomyces* strains, are now in progress.

In conclusion, our study demonstrated that adventitious shoot-derived callus can be efficiently utilized in the conversion of eugenol into bis-eugenol after 18 h of cell suspension culture. This approach to produce bis-eugenol using KACs is an environmentally friendly method compared with that of chemical synthesis.

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