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Identification and production of flavonoids in a cell suspension culture of *Scutellaria baicalensis* G.

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ABSTRACT

A high yielding cell line of *Scutellaria baicalensis* G. has successfully been developed to produce flavonoids. Major components of the flavonoids were identified as baicalin and wogonin-7-O-glucuronic acid by a series of instrumental analyses using UV, IR, MASS, and NMR. After 12 days in suspension culture, the cell growth reached 14 g^{DW}/l, and baicalin and wogonin-7-O-glucuronic acid were obtained in concentrations of 2.9 g/l and 1.07 g/l, respectively. The culture temperature was found to be an important parameter for improving production yield of the flavonoids. The yield of baicalin was observed to increase to 4.2 g/l by shifting the temperature from 30 °C to 25 °C after 72 h of suspension culture.

Key words: *Scutellaria baicalensis* G., cell suspension culture, flavonoids (baicalin and wogonin-7-O-glucuonic acid) identification and production.

Abbreviations: DW = cell dry weight; FW = cell fresh weight; 2,4-D = 2,4-dichlorophenoxyacetic acid; PSH medium = phytohormone added Schenk and Hildebrandt medium; FPM = a modified Schenk and Hildebrandt medium for flavonoid production.

INTRODUCTION

Roots of *Scutellaria baicalensis* G. have been used for a long time as an active principle of herbal prescriptions for diseases such as inflammation and pyrexia (Koda 1973). It has also been reported that it can be used as a liver detoxicant (Gi and Won 1975) and its ether extract shows a synergistic effect with anticancer drugs (Lee *et al.* 1991). Roots of *S. baicalensis* contain many flavonoid compounds, of which baicalin, baicalein, wogonin-7-O-glucuronic acid, and wogonin (Fig. 1) are known to be the major components with biological activity (Takido 1973).

We have been successful in developing a high yielding cell line for flavonoid production. Baicalin and wogonin-7-O-glucuronic acid were identified as the major products. In this study, structure identification and a feasibilty of mass production of these flavonoid compounds by cell suspension culture were investigated.

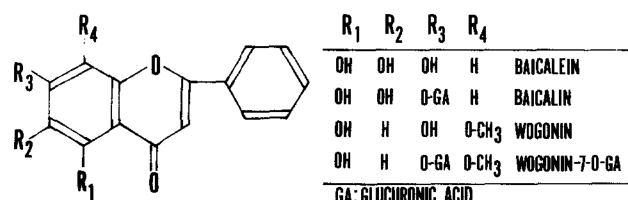

Fig. 1. Flavonoids of *Scutellaria baicalensis*

MATERIALS AND METHODS

Plant Material and Culture Method.

Seeds of *Scutellaria baicalensis* G. (family Labiatae; Kim 1989) were obtained from local herb suppliers (Seoul, Korea). Seeds were surface sterilised by immersing in 70 % ethanol for 60 sec followed by a treatment with 20 % commercial sodium hypochlorite solution for 20 min. They were aseptically germinated on Schenk and Hildebrandt basal salt agar medium (Schenk and Hildebrandt 1972) and then grown in a growth chamber for 4 weeks at 25 °C, with photoperiods of 12 h and a light intensity of 2500 lux. Calli were induced with aseptic seedlings on phytohormone added (2 mg/l 2,4-D and 1 mg/l kinetin) Schenk and Hildebrandt (PSH) medium supplemented with 9 g/l agar at 25 °C in the dark. After one month, calli formed were subcultured on the same medium. Greenish yellow colonies were selected repeatedly. Finally, a cell line, H3, which originated from the hypocotyl of a seedling, was selected. It was maintained by subculture every 4 weeks on PSH medium with agar.

Approximately one gram of fresh callus was transferred to 250 ml Erlenmeyer flasks containing 40 ml of the PSH medium and cultivated on a gyratory incubator (100 rpm) at 25 °C in the dark. The cells grown in suspension were transferred to fresh PSH medium. After 8 to 10 days of culture, they were used as seed inoculum (10 $\%^{v/v}$) for flavonoid production. A modified Schenk and Hildebrandt medium (FPM) was used for flavonoid production, which contained 40 g/l glucose, 3 g/l KNO_3, 200 mg/l $MgSO_4 \cdot 7H_2O$, 1 mg/l $MnSO_4 . H_2O$, 1 mg/l $ZnSO_4 \cdot 7H_2O$, 5 mg/l $CuSO_4 \cdot 5H_2O$, 100 mg/l $CaCl_2 \cdot 2H_2O$, 1 mg/l KI, 200 mg/l $NH_4H_2PO_4$, 1 mg/l H_3BO_3, 0.1 mg/l $NaMoO_4 . 2H_2O$, 15 mg/l $FeSO_4 . 7H_2O$, 20 mg/l Na_2-EDTA, 5 mg/l vitamin B_1, 0.5 mg/l vitamin B_6, 5 mg/l nicotinic acid, 1 g/l myo-inositol and 0.1 mg/l kinetin.

Cell aggregates developed in suspension culture were embedded and sectioned (JB-4 Embedding Kit, Polysciences, Inc., Warrington, PA) to visualize the cell morphology. Thin slices (10 μm thick) were observed under a light microscope after they were stained with toluidine blue.

Isolation of Flavonoids.

Freeze-dried cells were extracted with methanol by reflux. The extract was dried on a rotary evaporator and then re-extracted with ether by reflux. Major portions of flavonoids were obtained from the ether insoluble part by applying a reverse phase column chromatography with

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LiChroprep® column (Merk), which was then eluted with eluting solution containing 145 ml tetrahydrofuran, 125 ml dioxan, 50 ml MeOH, 20 ml AcOH, 2 ml 5 % H_3PO_4, and 658 ml deionized water. Two major fractions were obtained from the column chromatography. Compounds 1 and 2 from each fraction were then crystallized in methanol.

Analytical Methods.
After cells were separated on a stainless steel mesh (pore size: 30 μm), cell fresh weight (FW) was measured and cell dry weight(DW) was then determined by drying the wet cell cake in an oven at 95 °C to a constant weight. The glucose concentration was determined by the dinitrosalicylic acid assay method (Chaplin and Kennedy 1986) and the conductivity of the culture broth was measured with a conductance meter. Concentrations of flavonoids were determined by using HPLC (Waters with an UV detector, 270 nm) equipped with a stainless steel column (μBondapak™ C18, 3.9 x 300 mm). The mobile phase was the same as the eluting solution of the reverse phase column chromatography. Calibration curves for determination of baicalin and wogonin-7-O-glucuronic acid were prepared from the peak areas of HPLC data. Melting point of crystalline flavonoids was determined by using an electrothermal digital melting point apparatus and their structures were confirmed through a series of instrumental analyses including UV (Uvikon-930), MASS (Kratos,Concept 1s,FAB), IR (Analtech RFX-65), and NMR (Bruker AMX FT 500 MHz).

RESULTS AND DISCUSSION

The suspension culture of *Scutellaria baicalensis* was established in 250 ml Erlenmayer flasks containing 40 ml PSH medium. Cell growth and flavonoid production are shown in Fig. 2. Both reached their maximum 14 days after inoculation. Maximum cell mass and flavonoid yield were 9.5 g^{DW}/l and 2.5 g/l, respectively. The product level was two times higher than that of scutellaria radix (dried root of *S. baicalensis*) since the root contains only about 13 % of flavonoids on a dry basis (Tadato *et al.* 1985).
Flavonoids were separated by reverse phase HPLC with μBondapak™ C18 column. The HPLC patterns of the cultured cell extract were similar to those of the scutellaria radix extract (Fig. 3). This may indicate that the flavonoid mixture produced by cell line, H3, used in this study, had almost the same composition as a scutellaria radix.
Major portions of the flavonoids were obtained from

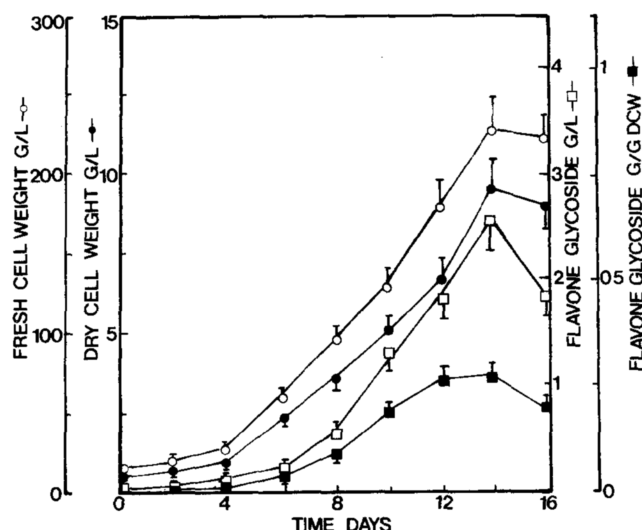

Fig. 2. Time course of cell growth and flavonoid production in the phytohormone added Schenk and Hildebrandt medium (PSH medium)

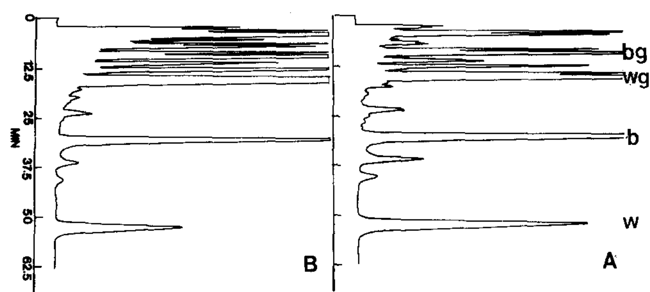

Fig. 3. HPLC patterns of scutellaria root and suspension cultured cell extracts (A: root extract, B: extract of cells from 14 day old suspension culture, bg: baicalin, wg: wogonin-7-O-glucuronic acid, b: baicalein, w: wogonin)

the ether-insoluble part of the methanol extract of the cultured cells. Two flavonoid components, compound 1 and 2, were obtained in crystallized form from two major fractions in reverse phase column chromatography of the ether-insoluble part. From the ether-soluble part, flavonoids such as baicalein, wogonin and oroxylin A were also detectable, but their production titres were negligibly small.
Instrumental analyses were conducted to determine the structures of these crystalline compounds.
Compound 1 (mp: 222-224 decomp.), which was crystalized in the form of yellow prisms in methanol, was obtained from the first fraction. From the acid hydrolysate of compound 1, glucuronic acid lactone and baicalein were detected by TLC analysis. From the UV spectrum two major absorbance bands were observed at 317 nm and 276 nm in methanol. Bathochromic shifts of methanol spectrum in band I by addition of $AlCl_3$ and flavone from its hydrolysate by addition of $NaOAc/H_3BO_3$ indicated that 5,6-dihydroxy-7-substituted structure was present (Mabry *et al.* 1970). The mass spectrum, which showed the molecular ion peak at m/e=446, was consistent with a molecular formula of $C_{21}H_{18}O_{11}$. Fragment ion peaks at m/e=270, 168, 105 and 78 were attributable to the A-ring and B-ring fragmentation, respectively. The ^1H-NMR and ^{13}C-NMR data of this substance showed typical patterns of flavonoid (Table 1) and it was thereby identified as 5,6-dihydroxyflavone-7-O-glucuronic acid, that is, baicalin.
Compound 2, which was crystallized in the form of yellow needles (mp: 212-214, decomp.) in methanol, was obtained from the second fraction. Glucuronic acid lactone and wogonin were detected by TLC analysis of the acid hydrolysate. From its UV spectrum two major absorbance bands were observed at 338 nm and 272 nm in methanol. Bathochromic shift, by addition of $AlCl_3$, of the methanol spectrum in band I indicate that 5-hydroxly group was present. No shift, by addition of $NaOAc/H_3BO_3$, in band I of flavone from its hydrolysate indicated that 6-hydroxyl group was absent (Mabry *et al.* 1970). The mass spectrum, which showed the molecular ion peak at m/e=460, was consistent with a molecular formula of $C_{22}H_{20}O_{11}$. Fragment ions at m/e=284, 182, 132 and 78 were attributable to the A-ring and B-ring fragmentation, respectively. The ^1H-NMR and ^{13}C-NMR data of this substance showed typical patterns of flavonoid and it was thereby identified as 5-hydroxy-8-methoxyflavone -7-O-glucuronic acid lactone, that is wogonin-7 -O-glucuronic acid. The analytical data for compound 1 and 2 are summarized in Table 1.
Typical time courses of cell growth and flavonoid

Table 1. UV, IR, MASS, ^{1}H-NMR and ^{13}C-NMR data of compound 1 and compound 2

Compound 1 (baicalin):

UV λmax, MeOH, nm: 276, 316; λmax, MeOH+NaOMe, nm: 286, 357; λmax, MeOH+$AlCl_3$, nm: 288, 343; λmax, MeOH+$AlCl_3$/HCl, nm: 289, 338; λmax, MeOH+NaOAc, nm: 277, 308; λmax, MeOH+NaOAc/H_3BO_3, nm: 283, 318.

MASS m/e(%): 446(M^+, 18), 270(100), 102(8), 78(10).

IR υmax, KBr, cm^{-1}: 3370(OH), 1743(COOH), 1666(C=O), 1612(C=C).

^{1}H-NMR(in DMSO-d_6), ppm: 12.58(1H, s, C_5-OH), 8.64(1H, s, C_6-OH), 8.08(2H, dd, J=1.5, 8.4Hz, $C_{2',6'}$-H), 7.6(3H, m, $C_{3',4',5'}$-H), 7.04(1H, s, C_8-H), 7.0(1H, s, C_3-H), 5.48(1H, d, J=4.7Hz, $C_{2''}$-OH), 5.26(1H, d, = 5.2Hz, $C_{3''}$-OH), 5.24(1H, d, J=7.7Hz, anomeric-H), 3.5-3.3(3H, m, $C_{2'',3'',4''}$-OH), 3.17(1H, d, J= 4.7Hz, $C_{4''}$ -OH).

^{13}C-NMR(in DMSO-d_6) ppm: 182.4(C_4), 169.8($C_{6''}$), 163.5(C_2), 151.1(C_9), 149.2(C_7), 146.4(C_5), 132(C_6 or $C_{4'}$), 130.7($C_{4'}$ or C_6), 130.3($C_{1'}$), 129.1($C_{3',5'}$), 126.3($C_{2',6'}$), 106(C_{10}), 104.7(C_3), 99.9 ($C_{1''}$), 93.7(C_8), 75.3($C_{5''}$ or $C_{3''}$), 74.9($C_{3''}$ or $C_{5''}$), 72.5($C_{2''}$), 71.1($C_{4''}$).

Compound 2 (wogonin-7-O-glucuronic acid):

UV λmax, MeOH, nm: 272, 338; λmax, MeOH+NaOMe, nm: 281, 387; λmax, MeOH+$AlCl_3$, nm: 292, 398; λmax, MeOH+$AlCl_3$/HCl, nm: 292, 409; λmax, MeOH+NaOAc, nm: 272, 338; λmax, MeOH+NaOAc/H_3BO_3, nm: 272, 338.

MASS m/e(%): 460(M^+, 20), 284(100), 182(4), 132(12), 78(10).

IR υmax, KBr, cm^{-1}: 3417(OH), 1743(COOH), 1654(C=O), 1612(C=C).

^{1}H-NMR(in DMSO-d_6), ppm: 12.58(1H, s, C_5-OH), 8.14(2H, dd, J=1.5, 8.4Hz, $C_{2',6'}$-H), 7.67(3H, m, $C_{3',4',5'}$-H), 7.13(1H, s, C_3-H), 6.78(1H, s, C_6-H), 5.66(1H, s, anomeric-H), 5.33(1H, d, J=4.7Hz, $C_{2''}$-OH), 4.08(1H, d, J=5.2Hz, $C_{3''}$-OH), 3.95(3H, s, Ar-OCHA3), 3.4-3.7(3H, m, $C_{2'',4'',5''}$-H), 3.23(1H, d, J=4.7Hz, $C_{4''}$-OH).

^{13}C-NMR(in DMSO-d_6), ppm: 182.29(C_4), 169.96($C_{6''}$), 163.53(C_2), 155.91(C_9 or C_7), 155.88(C_7 or C_9), 149.14(C_5), 132.2($C_{1'}$), 130.61($C_{4'}$), 129.21($C_{3',5'}$), 126.32($C_{2',6'}$), 105.3(C_{10}), 105.15(C_3), 98.55(C_6), 75.71($C_{5''}$), 75.24($C_{3''}$), 72.8($C_{2''}$), 71.14($C_{4''}$), 61.33(C_8-OCH_3).

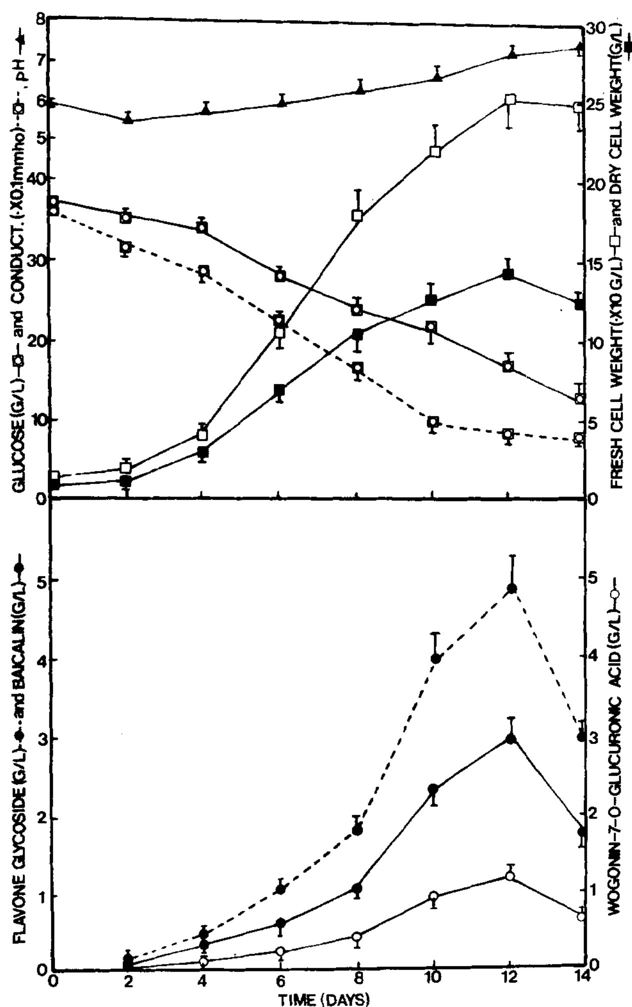

Fig. 4. Time course of cell growth and flavone glycoside production in flavonoid production medium (FPM)

production in FPM are shown in Fig. 4. Flavonoid production was positively correlated to cell growth; as cell growth increased flavonoid production also increased in a similar pattern. After 12 days of cultivation, the cell mass of 14 g^{DW}/l, and titres of 2.9 *g/l* and 1.07 *g/l* of baicalin and wogonin-7-O-glucuronic acid were obtained, respectively. It was found that the content of the flavone glycosides of these cultured cell was about two times higher than that of four-year old scutellaria radix.

The flavonoid yield reported here for *S. baicalensis* is rather high compared to other cases of plant cell suspension culture in which flavonoid titers of only a few milligram per liter of culture volume were reported (Yamakawa *et al.* 1983, Yamamoto *et al.* 1992).

The cells of *S. baicalensis* formed aggregates in FPM yielding yellow and compact cell clumps. Microscopic analysis of the cell aggregates showed that they contained many porous channels inside the cell clumps. The cell aggregates may have been structurally organized to facilitate nutrient transport and also cell to cell contact.

Temperature played an important role in production of the flavonoids. The effect of temperature on flavoid production is summarized in Fig. 5. While cell growth was most favored at 30 ℃, higher flavonoid production was attained at 25 ℃ (Fig. 5A). Thus, a two-stage temperature regime was employed; 30 ℃ and 25 ℃ for growth and production stages, respectively. The temperature was shifted from 30 ℃ to 25 ℃ after 72 h of suspension culture, and it was observed that baicalin production increased to 4.2 *g/l* (Fig. 5B). It is not clear at present how the temperature affects the cellular metabolism. One may assume that the activities of enzymes involved in the flavonoid biosynthesis, e.g. phenylalanine ammonia-lyase (Hahlbrock *et al.* 1976) are strongly affected by culture temperature. A more detailed study of the temperature effect is currently under investigation as a part of the process optimization.

In conclusion, the major flavonoids produced by *S. baicalensis* cell line, H3, were identified as baicalin and wogonin-7-O-glucuronic acid. This cell line produced in much higher yields the same flavonoids as those of scutellaria radix. It is believed that further

improvement may be possible by optimizing culture conditions, including the temperature effect.

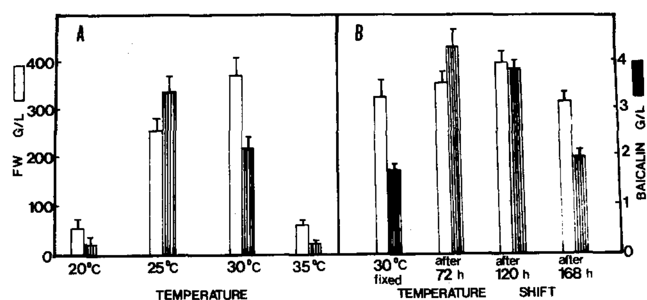

Fig. 5. Effect of temperature on cell growth and baicalin production (A: Effect of culture temperature, B: Effect of temperature shift. Temperature was shifted from 30 °C to 25 °C at the specified time. Cells were harvested at 288 h)

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