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Secondary Metabolite Note

Differential regulation of soyasaponin and betulinic acid production by yeast extract in cultured licorice cells

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Abstract Mode of soyasaponins and betulinic acid accumulation was examined in cultured cells of *Glycyrrhiza glabra* L. (licorice). The time course of their accumulation was different in the cultured cells. Yeast extract promoted betulinic acid accumulation, whereas soyasaponin accumulation was suppressed. These results indicate that soyasaponin and betulinic acid production are differently regulated in cultured cells of *G. glabra*.

Key words: Betulinic acid; *Glycyrrhiza glabra*; licorice; soyasaponin; triterpenoid.

Licorice, root and stolon of *Glycyrrhiza glabra* L., contains a large amount of glycyrrhizin, a sweet oleanane-type triterpenoid saponin which is worldwide used in large quantities as a sweetener and pharmaceutical (Shibata 2000). Cell suspension cultures of *G. glabra* produce no detectable amount of glycyrrhizin (Hayashi et al. 1988), but the cultured cells produce two triterpenoids, soyasaponins (Hayashi et al. 1990) and betulinic acid (Hayashi et al. 1988). Soyasaponins are oleanane-type triterpenoid saponins isolated from various leguminous plants, and reported to possess anti-virus (Nakashima et al. 1989; Hayashi et al. 1997), hepatoprotective (Ohminami et al. 1984) and anti-tumor-promoting (Konoshima et al. 1992) activities. Betulinic acid is a lupane-type triterpene, isolated from a wide range of higher plants, whose derivatives have been reported to be potent anti-HIV agents (Mayaux et al. 1994; Kashiwada et al. 1996). These triterpenoids share the biosynthetic pathway (Figure 1) with phytosterols, which are distributed ubiquitously in the intact plant. Although both soyasaponins and betulinic acid were produced in cultured licorice cells, the mode of their accumulation in cultured licorice cells has not been addressed. In the present study, we examined the time cource of soyasaponin and betulinic acid accumulation and the effects of yeast extract on their accumulation in cultured cells of *G. glabra*.

A culture strain (RNS-1B) was derived from seedlings of *G. glabra*, and maintained in 60 ml of LS medium (Linsmaier and Skoog 1965) containing 100 μM NAA and 1 μM BA in 300 ml Erlenmeyer flask as reported previously (Hayashi et al. 1988; Hayashi et al. 1990). Cells (1 g fresh weight) were inoculated in 30 ml of LS medium containing 100 μM NAA and 1 μM BA (standard medium) or 1 μM NAA and 10 μM BA (production medium) in 100 ml Erlenmeyer flask, and incubated on a reciprocal shaker (100 strokes min^{-1}) at 25°C in the dark. Cultured cells were harvested on Miracloth by filtration, and freeze-dried. Quantitative analysis was conducted by GC according to the method of Hayashi et al. (1990) with a slight modification. Powdered sample (100 mg) of freeze-dried cultured cells was extracted with ethyl acetate (2 ml twice, reflux for 1 hr). Cholesterol (1 mg) was added to the ethyl acetate extract as an internal standard, and the solvent was evaporated *in vacuo*. This dried sample was used for GC analysis of betulinic acid and phytosterols. The residue of the ethyl acetate extraction was re-extracted with methanol (2 ml twice, reflux for 1 hr), and the methanol extract was dried *in vacuo*. The dried residue was next treated with 1 ml of 9% HCl-methanol (reflux for 2 hr). After addition of ether (4 ml) and cholesterol (1 mg), as an internal standard, the solution was neutralized with Ag_2CO_3. The supernatant was dried, and used for GC analysis of soyasaponins. The dried sample was dissolved in a mixture of pyridine (10 μl) and *N*,*O*-bis(trimethylsilyl)acetamide (10 μl), and incubated at 40°C for 1 hr. An aliquot (2 μl) of the solution was analyzed by GC as reported previously (Hayashi et al. 1990). Quantities of triterpenoids and phytosterols were determined by the ratio of the peak area of the respective compound to that of internal standard. The total soyasaponin content was estimated as the content of soyasaponin II which was calculated from the quantity of soyasapogenol B. The sum of stigmasterol, β-sitosterol and campesterol was defined as phytosterol content.

Abbreviations: BA, 6-benzyladenine; GC, gas chromatography; LS medium, Linsmaier and Skoog (1965) medium; NAA, 1-naphthaleneacetic acid.

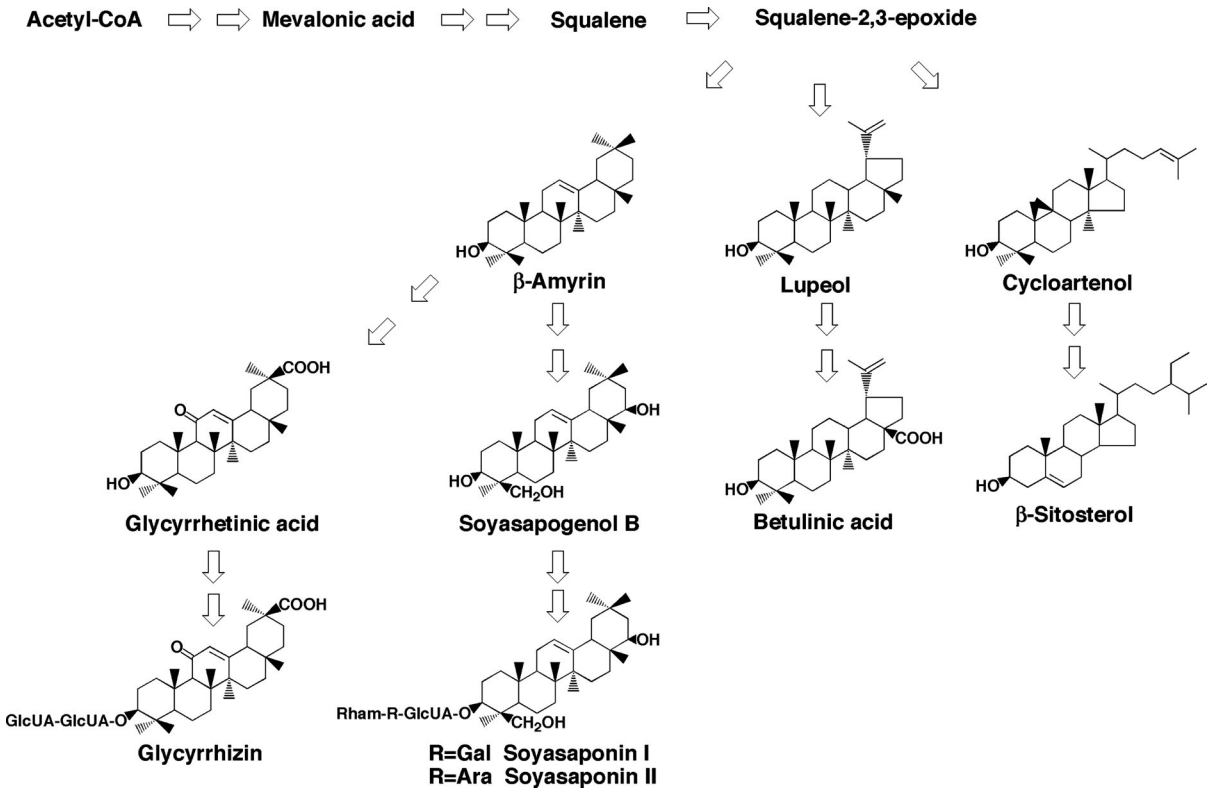

Figure 1. Biosynthetic pathways of triterpenoids and phytosterols in *Glycyrrhiza glabra* L.

In a previous paper (Hayashi et al. 1990), soyasaponin accumulation was reported to be promoted by transfer of cells into the production medium. Figure 2 shows time course of cell growth and the production of soyasaponin, betulinic acid and total phytosterol in cultured licorice cells in the standard medium or the production medium. Although both soyasaponin and betulinic acid accumulation was stimulated in the production medium, the time course of betulinic acid accumulation was different from that of soyasaponins. The amount of betulinic acid began to increase on day 8 in the production medium, whereas soyasaponin and phytosterol accumulation was nearly parallel to cell growth.

Since flavonoid production in the cultured cells of *G. echinata* L., a species of the *Glycyrrhiza* plants, was reported to be promoted by addition of yeast extract (Ayabe et al. 1986), we examined the effect of yeast extract on triterpenoid accumulation in cultured licorice cells in the production medium (Figure 3). Although the cell growth and triterpenoid accumulation were inhibited by the addition of 1% (w/v) of yeast extract, cell growth and total phytosterol levels were not changed by the addition of 0.01–0.1% (w/v) of yeast extract. It is noteworthy that soyasaponin levels were strongly reduced by 0.01–0.1% (w/v) of yeast extract, and this is consistent with the result that mRNA level of β-amyrin synthase, a potential regulatory enzyme controlling the biosynthesis of soyasaponin, was down regulated by

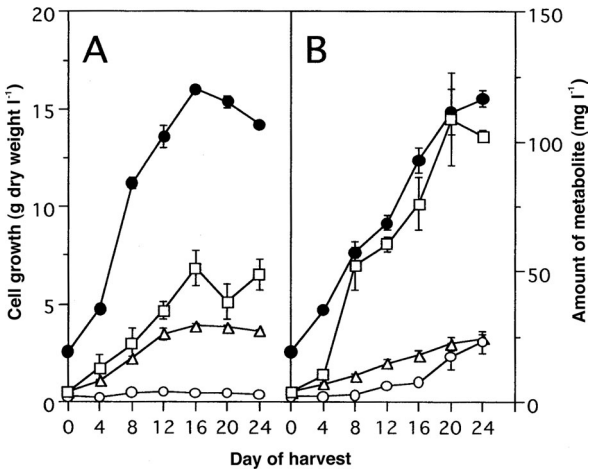

Figure 2. Time course analysis of *G. glabra* cell suspension culture. Cell growth (●) and productions of soyasaponins (□), betulinic acid (○), and phytosterols (△) were monitored in cell suspension culture (strain RNS-1B) grown in standard medium (A) or production medium (B). Data are mean of three replicates, and bars indicate standard errors.

yeast extract in the cutured licorice cells (Hayashi et al. 2003). In contrast, the betulinic acid level was slightly increased by the addition of 0.01–0.1% (w/v) of yeast extract, although the increase of betulinic acid level was much smaller than the decrease of soyasaponin level by yeast extract. Figure 4 shows the time course of cell growth and triterpenoid accumulation in the production medium containing 0.1% (w/v) of yeast extract. The

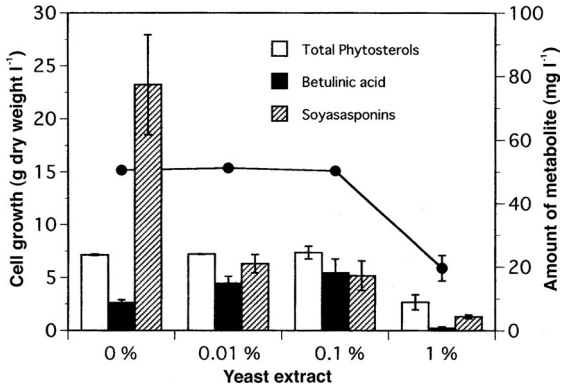

Figure 3. Effect of yeast extract on *G. glabra* cell suspension culture. Cell growth (●) and production of soyasaponins, betulinic acid, and phytosterols were monitored in *G. glabra* cell suspension culture. Yeast extract was added to production medium on day 0. Cells were harvested on day 24. Data are mean of three replicates, and bars indicate standard errors.

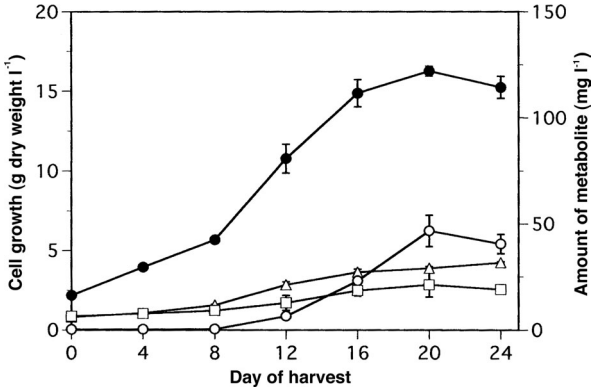

Figure 4. Time course analysis of *G. glabra* cell suspension culture treated with yeast extract. Cell growth (●) and production of soyasaponins (□), betulinic acid (○), and phytosterols (△) were monitored in cell suspension culture (strain RNS-1B) grown in production medium containing 0.1% (w/v) yeast extract. Data are mean of three replicates, and bars indicate standard errors.

amount of betulinic acid began to increase on day 8 in the cultured cells treated with yeast extract, as observed in the cells without yeast extract (Figure 2). The betulinic acid level (42 mg l^{-1}) on day 24 in the time course experiment (Figure 4) was higher than that (19 mg l^{-1}) in the production medium containing 0.1% yeast extract in Figure 3. The betulinic acid level varied in other experiments (data not shown), indicating that the betulinic acid production is not stable in this cultured strain.

The present study indicates that soyasaponin and betulinic acid accumulation is differently regulated in cultured cells of *G. glabra*. These differences suggest different roles of these triterpenoids in the intact plant of *G. glabra*. Distribution of these triterpenoids was reported to be different in the intact plant of *G. glabra* (Hayashi et al. 1988; Hayashi et al. 1993). Soyasaponins are distributed in the seed, rootlet, hypocotyl, and young stolon, and betulinic acid is distributed in the rootlet and cork layer of the underground parts, whereas glycyrrhizin, the sweet constituent in licorice, is located exclusively in the woody portion of the underground parts.

It is of interest that betulinic acid production was slightly promoted by yeast extract, whereas soyasaponin production was inhibited. Many secondary products, which participate in the chemical defense of higher plants, are induced by elicitors such as yeast extract and fungal polysaccharide. In *Tabernaemontana divaricata*, production of pentacyclic triterpenoid phytoalexins, such as ursolic acid and its hydroxylated products, was induced by the *Candida albicans* elicitor (van der Heijden et al. 1988; van der Heijden et al. 1989). Betulinic acid accumulates in the cork layer of the thickening root of *G. glabra* (Hayashi et al. 1988). Since the cork layers play an important role in protecting the plant organ against the environment, betulinic acid might participate in protection of licorice root as a chemical defense substance.

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