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Examination of triterpenoids produced by callus and cell suspension cultures of *Glycyrrhiza glabra*

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ABSTRACT

Callus and cell suspension cultures of Glycyrrhiza glabra failed to produce detectable amounts of glycyrrhizin, the major oleanane-type triterpene glycoside of the thickening root, or of its 11-deoxoderivative. However, betulinic acid, a lupane-type triterpene, which was found in the root bark, and a small amount of β-amyrin, a possible precursor of oleanane-type triterpenes, were detected in cell suspension cultures in addition to lupeol, a fundamental form of lupane-type triterpenes. These findings suggest that the absence of glycyrrhizin in undifferentiated cultured cells may be partly due to interruption of the later reactions leading to the synthesis of glycyrrhizin from a triterpenoid intermediate.

INTRODUCTION

Glycyrrhizin, the main triterpene saponin contained in the root of G. glabra, is used in large quantities as a natural sweetener and also for medical treatments of chronic hepatitis in Japan. It was reported that glycyrrhizin was produced in substantial amounts by callus and cell suspension cultures of G. glabra (Tamaki et al. 1972; Yoo and Kim 1976), but its chemical identification remained ambiguous.

The present study has been undertaken in an attempt to clarify whether glycyrrhizin is accumulated by callus or cell suspension cultures derived from various organs of G. glabra seedlings, and to find out what kinds of triterpenoids are produced by the cultured cells.

MATERIALS AND METHODS

Chemicals

Glycyrrhizin and 11-deoxoglycyrrhizin, synthetically derived from glycyrrhizin, were supplied by Maruzen Kasei Co. Ltd., Japan. The latter compound was identified by ^{1}H- and ^{13}C-NMR in comparisons with an authentic sample of 11-deoxoglycyrrhizin isolated from licorice root by Kitagawa et al.(1987). Authentic samples of betulinic acid, β-amyrin and lupeol were obtained from Dr. W. Kamisako of Mukogawa Women's University, Japan.

Plant material and culture method

The seeds, leaves, stems, and roots of G. glabra L. were collected from fruit-bearing plants (plant height: ca 90 cm) growing wild by the roadside in the suburb of Muş, Turkey in August, 1986.

Callus cultures were derived from various parts (root, hypocotyl, stem, and leaf) of 4-week-old seedlings on nutrient agar medium (Linsmaier and Skoog 1965) containing 100 μM 1-naphthaleneacetic acid (NAA) and 1 μM 6-benzyladenine (BA), and subcultured on the same medium at intervals of 5 weeks for 18 months in the dark at 25°C. Suspension cultures (RNS-strain) initiated from the root callus were subcultured in the above medium (100 ml) without agar in 300ml Erlenmeyer flasks agitated on a reciprocal shaker (100 strokes/min) at intervals of 3 to 4 weeks for a period of 15 months. Suspension cultures (RDS-strain) were also established in LS medium containing 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 μM kinetin.

For studying the time course of growth and triterpene production, cells (1 g) were inoculated in each 100 ml flask (30 ml medium) and incubated under the same conditions as indicated above. Cells were harvested from three flasks every three days during a culture period of 24 days to measure the cell growth and the contents of betulinic acid and phytosterols.

To provide a large sample of cells for chemical analysis, ca. 120 g of cells in 1 l of spent medium were used to inoculate a 4 l-jar fermentor containing 3 l of fresh medium, and cultured at 25°C in the dark. The medium was agitated by a screw-type stirrer (60 rpm) and aerated by bubbling (200 ml air/min). Cells were harvested four weeks after inoculation and air-dried at 65°C for one week.

Isolation and identification of triterpenes and phytosterols

Dried cells (73 g) mentioned above were extracted with MeOH (1 l x 3) by reflux, then the MeOH was evaporated in vacuo at 40 °C.

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The residue (ca. 10g) was suspended in 300 ml of H_2O, and the pH was adjusted to 4 with 0.1N HCl. This solution was extracted with EtOAc (300 ml x3); the EtOAc layer was evaporated in vacuo to give an extract (2.4 g), which was subjected to silica gel column chromatography (80 g Wakogel C-100 in 3 cm x 30 cm column) using admixtures of $CHCl_3$-EtOAc (20:0, 19:1, 18:2, 16:4, 10:10, 500 ml each) and of EtOAc-MeOH (20:0, 19:1, 18:1, 16:4, 10:10, 0:20, 500 ml each) in that order as solvents. A fraction (0.38 g) eluted with $CHCl_3$-EtOAc (18:2) was recrystallized from MeOH to yield colorless needles (92 mg), m.p. 283-286°C. This compound was identified as betulinic acid, 3-hydroxy-lup-20(29)en-28-oic acid, by TLC, MS, GC-MS (as methyl ester), IR, ^{1}H-NMR, and high resolution MS in comparison with an authentic sample.

From a fraction (0.45 g) eluted with $CHCl_3$-EtOAc (19:1), a mixture of triterpenes were obtained by preparative TLC. This sample was analyzed by capillary GC-MS: capillary column (Shimadzu CBP5-M25-025), gas flow: He (0.5 kg/cm^2, split 40 ml/min, purge 2 ml/min), column temp.: 200-300°C (10°C/min). Lupeol and β-amyrin were identified by comparison with authentic samples. Phytosterols (sitosterol and stigmasterol) contained in the above fraction were identified by GC-MS.

Quantitative analysis of betulinic acid and phytosterols

A powdered sample (100 mg) of cultured cells was extracted with $CHCl_3$ (4 ml x2) by reflux. After evaporation of $CHCl_3$, the residue was dissolved in Et_2O and treated with diazomethane to methylate the carboxyl group of betulinic acid. Cholesterol (0.5mg) was added to the solution as an internal standard. An aliquot (20 µl) of this sample (1 ml) was analyzed by GC: glass column (3 mm x 1 m) packed with Silicone OV-17 (1%) on Chromosorb W (AW-DMCS) 80-100 mesh, carrier gas: N_2 (30 ml/min), column temperature: 100-280°C (5°C/min), detector: FID. The contents of betulinic acid and phytosterols (sitosterol, stigmasterol) were calculated from the ratio of the peak area of each compound to that of the internal standard. The minimum quantity detectable by this method was 0.001% w/w of the sample.

Detection of betulinic acid in intact plants

A powdered sample (100 mg) of licorice was extracted with $CHCl_3$ and methylated as mentioned above. The sample was analyzed by GC-MS. Betulinic acid was detected by the fragment ion peak of m/z 189, which is the base peak of betulinic acid methyl ester, as well as by other typical ions.

Quantitative analysis of saponins in intact plants and cultured cells

A powdered sample (100 mg) was extracted with hot 70% MeOH (5 ml x2). The extract was adjusted to 10 ml by MeOH, and subjected to reverse phase ion pair HPLC according to a modified method of Sagara et al.(1985), which is suitable for separating glycyrrhizin from minute amounts of co-existing phenolic substances. The conditions of HPLC were stainless column (4.6 mm x 150 mm) packed with TSK gel ODS 120A (Toyo Soda), solvent system: MeOH - H_2O (60 : 40) containing 20 mM tetra-n-butylammonium bromide adjusted to pH 6.0 with 0.1 N NaOH, flow rate: 1.5 ml/min, column temperature: 50°C, detection: UV detector (254 nm). The minimum quantity of glycyrrhizin detectable was 0.01% w/w of the sample.

Analysis of 11-deoxoglycyrrhizin was made by HPLC under the same conditions as used for glycyrrhizin except for: solvent system: MeCN - H_2O containing 0.1% v/v H_3PO_4 (45 : 55), flow rate: 1 ml/min, column temperature: 40°C, detector: UV detector (205 nm). The detection limit for this compound was 0.05% w/w of the sample.

RESULTS

Formation of betulinic acid

Betulinic acid was isolated from the cells cultured in a jar fermentor and identified by various analytical methods. GC and GC-MS analyses revealed the presence of stigmasterol (0.04-0.07% of cell dry wt), sitosterol (0.06-0.12%) and small amounts of β-amyrin and lupeol. Neither glycyrrhizin nor 11-deoxoglycyrrhizin could be detected by HPLC in the 70% MeOH extracts of any callus and cell suspension cultures examined. Similarly, no glycyrrhetinic acid was detected in both cultures by GC and GC-MS. Furthermore, glycyrrhizin was undetectable by HPLC in the aqueous layer and all the fractions of the EtOAc layer obtained from the MeOH extract of cell suspension cultures (see experimental). By contrast, betulinic acid was detected by GC in all the callus cultures derived from various organs (hypocotyl, root, stem, and leaf) of one-month-old G. glabra seedlings, although its content varied from 0.01% to 0.17% among culture strains (Table 1).

Table 1. Content of betulinic acid in callus and suspension cultures of Glycyrrhiza glabra

Culture strain	Origin of cultures	Content (% of dry wt) of betulinic acid
Callus cultures[1]		
LC-1	Leaf	0.05
LC-2	Leaf	0.14
LC-3	Leaf	0.05
SC-1	Stem	0.01
SC-2	Stem	0.13
HC-1	Hypocotyl	0.01
HC-2	Hypocotyl	0.06
RC-1	Root	0.12
RC-2	Root	0.17
RC-3	Root	0.01
Suspension cultures		
RNS-1[2]	Root	0.15
RNS-2[2]	Root	0.05
RDS-1[3]	Root	0.04

1) callus cultures on LS agar medium containing 100 µM NAA and 1 µM BA for 5 weeks, 2) suspension cultures in LS medium containing 100 µM NAA and 1 µM BA for 3 weeks, 3) suspension cultures in LS medium containing 1 µM 2,4-D and 1 µM kinetin for 4 weeks, glycyrrhizin was not detected in all cultures above (<0.01% of dry wt).

Fig. 1 shows the time course of production of betulinic acid and phytosterols in cell suspension cultures of root origin. The amount of betulinic acid began to increase at the late linear growth stage in contrast to phytosterols, which increased almost in parallel with cell growth. The maximum yield of betulinic acid (28.4 mg/l) was observed on day 21, after the cessation of cell growth.

Distribution of betulinic acid in plant organs

Both GC and GC-MS analyses of the extracts from different organs of G. glabra plants showed that betulinic acid was detectable neither in the aerial parts (leaf and stem) nor in the xylem of the thickening root, but was found only in the root bark and in the radicle (Table 2). This suggests that accumulation of betulinic acid is localized in a particular tissue of the intact plant, although this compound is produced by callus cultures derived from any parts of the seedling. By contrast, glycyrrhizin which was found in the xylem of the thickening root in a large amount (3.4% of dry wt) was barely detected in the root bark and the radicle.

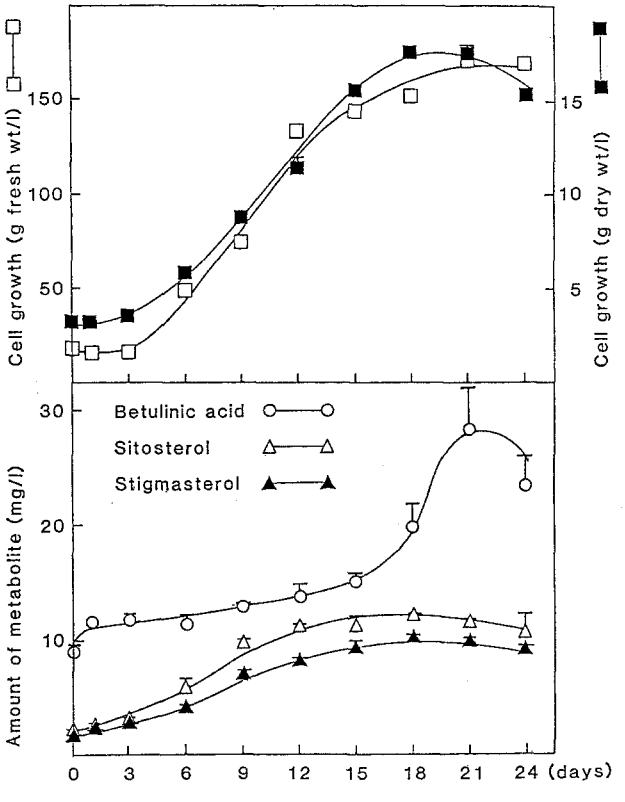

Fig 1. Time course of cell growth and production of betulinic acid and phytosterols in G. glabra cell suspension cultures. Bars represent s.e. (n=3).

Table 2. Distribution of glycyrrhizin and betulinic acid in G. glabra plants

Organ	Content (% of dry wt) of glycyrrhizin	betulinic acid
Leaf[1)]	n.d.[3)]	n.d.[4)]
Stem[1)]	n.d.[3)]	n.d.[4)]
Root[1)] (φ 32 mm)		
Xylem	3.78	n.d.[4)]
Bark	0.06	>0.1
Root[2)]		
Thickening Root (φ 6 mm)	0.28	0.01-0.1
Radicle (φ < 1 mm)	n.d.[3)]	0.01-0.1

1) collected in Turkey, 2) cultivated for 6 months in Kyoto, Japan, 3) not detected (<0.01%), 4) not detected (<0.001%).

DISCUSSION

There have been controversies as to whether glycyrrhizin is produced by licorice cell cultures. According to Tamaki et al. (1972), cell suspension cultures of G. glabra produced high amounts (3-4% of dry wt) of glycyrrhizin. Yoo and Kim (1976) also reported the formation of glycyrrhizin (detected by TLC analysis) by G. glabra callus cultures. On the other hand, Henry et al. (1984) could not detect the accumulation of glycyrrhetinic acid, the aglycone of glycyrrhizin, in callus and suspension cultures of G. glabra. Kobayashi et al. (1985) isolated chalcones and isoflavones from callus cultures of G. uralensis, which contains glycyrrhizin in the root, however, no mention was made of glycyrrhizin.

In the present study, we could detect neither glycyrrhizin nor glycyrrhetinic acid in callus and suspension cultures of G. glabra. The reason for the discrepancies between our observation and the other workers' findings concerning glycyrrhizin production is not clear, but it could be due to differences in either analytical methods or cell lines. In none of the earlier studies was glycyrrhizin isolated from cultured cells for unequivocal identification.

In the present study, glycyrrhizin was detectable only in the thickening roots of G. glabra plants cultivated in the field for 6 months after germination. Therefore, the accumulation of glycyrrhizin appears to be associated with the secondary thickening growth of roots.

Both callus and suspension cultures of G. glabra were shown to accumulate betulinic acid instead of glycyrrhizin. Betulinic acid was isolated from the roots of Glycyrrhiza sp. (Saitoh et al. 1969) and G. uralensis (Hattori et al. 1986), and its occurence has been reported in callus cultures of Solanum sp. (Indrayanto et al. 1983), Datura innoxia (Kamisako et al. 1984), and Solanum aviculare (Vaněk et al. 1985). As evidenced by the accumulation of betulinic acid, the absence of glycyrrhizin in G. glabra cultures may not be caused by lack of precursors, but could be due to inhibition of certain reactions in the

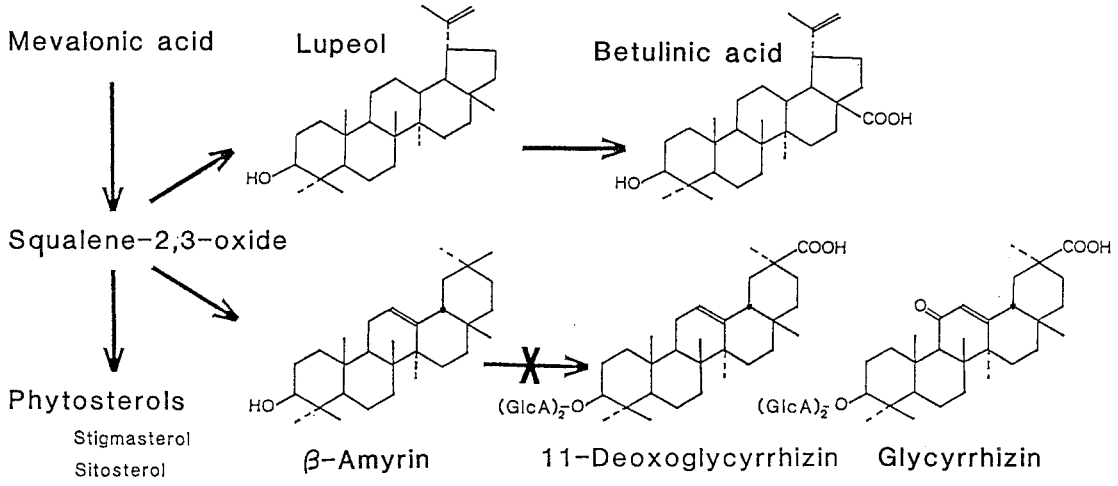

Chart 1. Hypothetical biosynthetic pathways leading to triterpenoids in Glycyrrhiza glabra cell cultures.

biosynthetic steps leading from squalene-2,3-oxide to specific oleanane-type triterpene saponins such as glycyrrhizin and 11-deoxoglycyrrhizin (Chart 1). The latter compound, which might be an intermediate in the synthesis of glycyrrhizin, was isolated as aglycone (Canonica et al. 1966) and glycoside (Kitagawa et al. 1987) from licorice roots. The presence of β-amyrin, a fundamental skeleton of oleanane-type pentacyclic triterpenes, in G. glabra cultures suggests that an enzyme system involved in the oxidation and glycosylation processes of an oleanane-type intermediate might be inhibited in undifferentiated cultured cells. Experiments are under way to substantiate this possibility.

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