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Glucosylation of Salicyl Alcohol by Cell Suspension Cultures of *Solanum mammosum*

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Abstract – Cell suspension cultures of *Solanum mammosum* transformed inoculated salicyl alcohol into salicin (salicyl alcohol 2-O-β-D-glucopyranoside). The highest level of salicin (59.3 mg/flask) in the cells was formed within 3 days after inoculating with salicyl alcohol (50 mg /flask containing 50 ml medium). The glucosylation capability of salicyl alcohol by cell suspension cultures of *S. mammosum* was relatively higher than that reported previously.

Keywords – *Solanum mammosum*, Solanaceae, cell suspension cultures; glucosylation, biotransformation, salicyl alcohol, salicin, salicyl alcohol 2-O-β-D-glucopyranoside.

Introduction

Plant cells cultured *in vitro* can be used not only for production of secondary metabolites but also for biotransformation of various compounds; both natural and synthetic substances can be used as substrates. Various plant cell cultures were capable of glucosylating a variety of exogenously supplied compounds (Suga and Hirata, 1990).

The glucosylation of simple phenols (e.g. salicylic acid, salicyl alcohol) by various cell suspension cultures have been published by Umetami *et al.* (1982). Mizukami *et al.* (1983) reported that the formation of salicin from salicyl alcohol in cell suspension cultures of *Lithospermum erythrorhizon* and *Gardenia jasminoides*. Dombrowksi (1993) reported the transformation of salicyl alcohol into salicin and isosalicin in the cell suspension cultures of *Salix matsudana*. Audette *et al.* (1966) cited that the phenolic glycoside salicin is of wide spread occurrence in the genus *Salix* and has been used in medicine for the treatment of acute rheumatism and influenza.

Although some cell suspensions of *Solanum* species (eg. *S. aviculare, S. mammosum*) could not produce solasodine (Galenes *et al.*, 1984, Indrayanto *et al.*, 1986), the cultures could transform some exogenously inoculated substrates. Cell suspension cultures of *S. aviculare* could convert some exogenous substrates, such as (+)-3-carene, citronella, thujol, thujone, cis and trans verbenol, verbenone, and (-)-limonene (Vanek *et al.*, 1990; 1992). Callus cultures of *S. mammosum* could transform progesterone into 5-α-pregnane-3,20-dione (Sondakh, 1989)

In this report, we describe the formation of salicin (1) from the exogenous supply of salicyl alcohol (2) in the cell suspension cultures of *S. mammosum*.

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Experimental

General procedure – One and two dimensional ^1H (300.13 MHz) and ^{13}C NMR (75.47 MHz) spectra were determined at 300° K in DMSO-d_6 using a Bruker AC 300 spectrometer fitted with a 5 mm probe head. Chemical shifts are reported relative to the solvent peaks DMSO-d_6 (δ ^1H=2.60; ^{13}C=39.5 ppm). ^{13}NMR signal multiplicities (s, d, t or q) were determined using the distortionless enhancement by polarization transfer (DEPT) 135° sequence. Full assignment of **1** were substantiated by nuclear overhauser enhancement (NOE) difference and two dimensional correlated spectrocopy (H-H COSY Spectra). The negative ion electronspray mass spectrum (ESMS) of **1** was determined using a VG Platform instrument. The concentration of glucose in the medium was determined spectrometrically (Hewlett Packard Spectrophotometer HP 8452) using enzymatic colorimeter test Peridochrom® reagent (Boehringer Mannheim). The differential scanning colorimeter (DSC) thermogram of glucoside 1 was determined using a Shimadzu DT 30 Differential Thermal Analyzer.

Cell suspension cultures – Clone sm. of the cell suspension cultures used in these studies were initiated from previously established callus cultures of *S. mammosum* (Indrayanto *et al.*, 1986). The calli were cultivated in 300 ml Erlenmeyer flask containing 50 ml of modified Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with sucrose (30 g/l), kinetin (2 mg/l), NAA (1 mg/l) and caseinhydrolisate (1 g/l) on a gyrotary shaker (120 rounds per minute) at 25±1℃ under continuos light (ca. 2000 lux).

Toxicity and biotransformation experiments – Cells (10 g fresh weight) were inoculated into liquid medium (50 ml) without **2** (control culture) and into liquid medium containing various concentration of **2** (500, 750, 1000, 1250 and 1500 mg/l). After 7 day's incubation, the cultured cells were harvested, collected, filtered, weighed, dried and powdered. The standard protocol for the biotransformation experiment was as follows: cells (10 g fresh weight) were inoculated into a 300 ml Erlenmeyer flask containing 50 ml of medium, **2** (1000 mg/l) was added and the mixture cultured for 1 to 7 days.

Extraction, isolation and purification of 1 – The oven dried (40℃ powdered biomass (6.25 g) was refluxed (2 hr) in MeOH. The MeOH extract concentrated under reduction pressure was submitted to silicagel 40 (70-230 Mesh ASTM) (E. Merck) column chromatography and preparative TLC silicagel 60 GF 254 (E. Merck) (Solvent system; EtOAc:MeOH:H_2O=77:13:10) to give **1** (304 mg). A sharp endothermic peak of DSC thermogram of **1** was observed at 185.5℃

Compound 1 – The negative ion.(-20 volts) ESMS (NH_4COOH assisted matrix) of **1** showed peaks at m/z (% rel.int.): 331.0 [M+COOH]$^-$ (100.0) and 285.2 [M-H]$^-$ (3.6). ^1H-NMR (300.13 MHz, DMSO-d_6) δ ppm: 7.44 (1H, dd, J=7.3, 1.8 Hz, H-6), 7.27 (1H, ddd, J=8.3, 7.3, 1.8 Hz, H-4), 7.17 (1H, dd, J=8.3, 1.2 Hz, H-3), 7.08 (1H, td, J=7.3, 1.2 Hz, H-5), 5.40 (1H, br s, OH at C-2'), 5.40 (1H, br s, OH at C-3'), 5.13 (1H, br s, OH at C-4'), 5.05 (1H, br t, J=5.4 Hz, OH at C-7), 4.84 (1H, d, J=7.6 Hz, H-1'), 4.72 (1H, dd, J=14.1, 5.0 Hz, H-7^B), 4.65 (1H, br t, OH at C-6'). 4.54 (1H, dd, J=14.1, 5.7 Hz, H-7^A), 3.79 (1H, m, H-6'^{B}), 3.55 (1H, m, H-6'^{A}), 3.39 (1H, m, H-5'), 3.34 (1H, m, H-2' and H-3'), 3.25 (1H, m, H-4'). ^{13}C-NMR (75.47 MHz, DMSO-d_6) δ ppm: 154.6 (C-2), 131.4 (C-1), 127.6 (C-4), 127.1 (C-6), 121.7 (C-5), 114.7 (C-3), 101.4 (C-1'), 77.0 (C-5'), 76.4 (C-3'), 73.4 (C-2'), 58.2 (C-7), 69.7 (C-4'), and 60.7 (C-6').

Quantitative analysis of 1 and 2 – The concentration of **1** and **2** in the medium and the biomass were determined densitometrically using a Shimadzu CS 930 TLC Scanner on silicagel 60 GF 254 precoated plates (E. Merck) following elution with the EtOAc/

MeOH/H_2O (77/13/10). Quantitation was performed by measuring the absorbance reflectance (at λ_{max}) of the analyt spots (270 nm for **1**; 275 nm for **2**). The concentration of **1** and **2** were determined from calibration graphs obtained using salicyl alcohol and salicin (Sigma) as external standards on the same plate. The method of validation was according to Funk *et al.* (1992). The linearity was achieved from 0.8 to 20 µg/spot (for **1** and **2**), with relative standard deviations of process (n=6) (V_{xO})=3.0% (for **1**) and 3.8% (for **2**). The accuracy by standard addition was 91.51±2.66% (for **1**) and 104.16±2.15% (for **2**), confidence range of the intercept of the recovery curve (V_{Baf})=0.132±0.458 (for **1**) and 0.238±0.305 (for **2**); slope (V_{Bbf})=1.105± 0.176 (for **1**) and 1.001±0.109 (for **2**), at $p<0.05$.

Results and Discussion

Toxicity of 2 toward *S. mammosum* cell suspension cultures was investigated for concentrations in the range 500-1500 mg/l. Cells death was observed at higher concentration (1500 and 1250 mg/l). At concentration lower than 1000 mg/l, cells survived but did not grow. In all of the experiments, 1000 mg/l of **2** was administered. The experiments were indicated that the cell suspension cultures of *S. mammosum* were able to tolerate a higher concentration of the substrat **2** than the others cell cultures reported by previous authors i.e.; 310 mg/l (Mizukami *et al.* 1983) and 620 mg/l (Dombrowski,1993).

TLC analysis showed the presence of metabolite **1** (Rf 0.32) in the cells. Two control experiments showed that in the absence of cells, **2** (Rf 0.70) remained unchanged in the control medium, and 1 was only produced when 2 was added to the suspension cultures.

The DSC thermogram of **1** indicated that the products was reasonably pure. The NH $_4$COOH matrix assisted negative ion ESMS of **1** showed peaks at m/z [negative ions]: 331 [M+COOH]$^-$ and 285 [M-H]$^-$, suggesting **1** to have a molecule weight of 286 Daltons, and to be a glucosyl analogue of salicyl alcohol. One and two-dimensional NMR spectral data were consistent with the identification of **1** as a salicyl alcohol 2-O-β-D-glucopyra-noside. In particular, the coupling constant of the H-1' glucosyl proton (J=7.6 Hz) established the configuration at this center to be equatorial (β) (Agrawal, 1992), while irradiation of the glucosyl H-1' resonance (5.02 ppm) in an NOE-difference experiment enhanced H-3' (3.34 ppm, signal also coincident with H-2'), H-5' (3.39 ppm) and H-3 (7.17 ppm). The identification of **1** as 2-O-β-glucoside (salicin) was confirmed by a comparison of the ^{13}C NMR spectrum of **1** with that of an authentic specimen of salicin (Sigma).

The time course of formation of **1** from **2** in cell suspension cultures of *S. mammosum* was studied (Fig. 1). Quantitative analysis showed that the greatest level of **1** (59.3 mg/flask equivalent to 118.6 mg/g dry weight) was formed in the cells in 3 days after the inoculation of 50 mg of **2** in a flask contained 50 ml of medium each (i.e. an inoculation level 1000 mg/l). Glucoside **1** was not detected in the medium during the 7 day inoculation period. On the other hand, salicyl alcohol was detected in both the cells and the medium during the inoculation period.

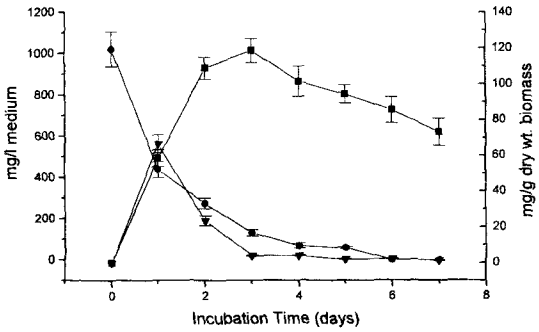

Fig. 1. The time course of the biotransformation of salicyl alcohol (SA) to salicin by cell suspension cultures of *Solanum mammosum*. Value represent mean±SD (n=4).
—●— SA in medium, —■— Salicin in biomass, —▼— SA in biomass.

The glucosylation capabilty of cell suspension cultures of *S. mammosum* reported here, namely a 51.8% conversion **2** to **1**, is higher than that reported previously for *Gardenia jasminoides* (30%; Mizukami *et al.*, 1983) and *Salix matsudana* (48%; Dombrowksi, 1993). To our knowledge this is the first report of the biotransformation of salicyl alcohol into its glucoside **1** (salicin) by cell suspension cultures of *S. mammosum*. The result showed that the cell suspension cultures of *S. mammosum* could glucosylate the exogenous substrates. Some biotransformation studies using other exogenous substrates are in progress in our laboratory.

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