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Journal of Molecular Catalysis B: Enzymatic 54 (2008) 72–75
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Biotransformation of *p*-, *m*-, and *o*-hydroxybenzoic acids by *Panax ginseng* hairy root cultures

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Received 16 July 2007; received in revised form 18 December 2007; accepted 19 December 2007
Available online 28 December 2007

Abstract

The regioselective glycosylation of three isomers of hydroxybenzoic acids was observed in *Panax ginseng* hairy root cultures. *p*-Hydroxybenzoic acid (**1**) and *m*-hydroxybenzoic acid (**2**) were converted into their corresponding glycosides (**1a** and **2a**) and glycosyl esters (**1b** and **2b**) while no metabolite of *o*-hydroxybenzoic acid (**3**) was detected. A new compound, *m*-hydroxybenzoic acid β-D-xylopyranosyl (1 → 6)-β-D-glycopyranosyl ester (**2c**) was identified as a biotransformation product of **2**. Further time-course studies of the biotransformation reactions showed that the glycosides were major products in the latter stage. The addition of carbohydrates or antioxidants increased glycosyl esters formation.

Keywords: Biotransformation; Glycosylation; Hydroxybenzoic acid; *Panax ginseng*; Hairy root cultures

1. Introduction

Glycosylation reactions are of special interest because they facilitate the conversion of water-insoluble compounds to those that are more water-soluble [1]. A great number of glycosylation studies using plant cell cultures or hairy root cultures have been carried out [2–16] since such reactions are very difficult by microbial transformations or by chemical means [1].

Panax ginseng cell and hairy root cultures are widely used and versatile biotransformation tools. Such cultures enzymatically transform phenolics [6,17–20], coumarin [21], digitoxigenin [22,23] and 18β-glycyrrhetinic acid [12] to their corresponding glycosides. In our previous studies, we reported the glycosylation of phenolic hydroxyl groups on hydroquinone [24,25] and *p*-hydroxybenzyl alcohol [26] by *Panax ginseng* hairy roots induced from ginseng tender stems [27]. *p*-Hydroxybenzyl alcohol was efficiently transformed to gastrodin, the active component of the famous anti-headache Chinese herb, *Gastrodia elata* Bl.

As a continuation of our work on the specificities and selectivities of *Panax ginseng* hairy root culture biotransformations of phenolic hydroxyl and carboxyl groups, *o*-, *m*- and *p*-hydroxybenzoic acids were undertaken. These substrates were chosen because (1) it is possible to assess the regioselectivity and the group priority selection of the glycosylation reaction for phenolic hydroxyl and carboxylic acid groups within a single structure; (2) these substrates are building blocks of some complex active natural products such as rhein, pseudopurpurin, and sennidin; and (3) the glycosylation of such phenolic benzoates may reveal novel characteristics of glycosylation reactions.

2. Material and methods

2.1. Instruments and general methodology

HPLC analyses were performed using an Agilent 1100 instrument equipped with a HP chemstation, model G1312A binary pump, model G1313A micro autosampler, model G1316A thermostated column compartment, and a model G1314A variable wavelength detector. ESI-LC/MS was obtained using an Agilent 1100 LC-MSD series trap. The column was a 4.6 mm × 250 mm, 5 μm, Alltima C_{18} analytical column (Alltech Associates, Inc., USA) maintained at 25 °C equipped with a 3.0 mm × 7.5 mm C_{18} precolumn. ^{1}H and ^{13}C NMR spectra were measured with

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doi:10.1016/j.molcatb.2007.12.015

a Bruker Avance 400 spectrometer in methanol-d_4 solution with TMS as the internal reference, and chemical shifts are expressed in δ (ppm). Thin layer chromatography (TLC) was conducted using silica gel GF_{254} plates purchased from Qingdao Marine Chemical Group, China. All of the substrates were purchased from Shanghai Chemical Co. Ltd., and the purities were above 98% as determined by HPLC analyses.

Compounds **1**, **2** and their metabolites were analyzed by HPLC using 5% acetonitrile and 95% water (containing 1% formic acid) as solvent at a flow rate of 1.0 ml/min with UV detection at 242 nm. Retention times (min) under these conditions were as follows: **1a**, 9.2; **1b**, 15.1; **1**, 30.5; **2a**, 16.3; **2b**, 24.9; **2c**, 37.8; **2**, 45.6.

2.2. Plant materials and culture methods

Panax ginseng hairy root cultures were subcultured as previously described on B_5 medium supplemented with 2% (w/v) sucrose and 0.5 g/l lactalbumin hydrolysate on a rotary shaker (100 rpm) at 25 °C in the dark [24].

2.3. Biotransformation of p-, m- and o-hydroxybenzoic acids by Panax ginseng hairy root cultures

The substrate (20 mg) was dissolved in 0.5 ml ethanol and added to a 75 ml suspension of hairy roots pre-cultured for 4 weeks in a 150 ml Erlenmeyer flask. Controls received only 0.5 ml of the vehicle, ethanol. Following 7 days of incubation, the cultures were filtered, the roots were lyophilized and then extracted by boiling with methanol at 68 °C for 2 h. The methanol extract was concentrated to 10 ml in a rotary evaporator. The culture filtrate was extracted with equal volumes of *n*-butyl alcohol for four times, and the extract concentrated by evaporation in vacuo to a residue that was dissolved with 10 ml methanol. Extracts from roots and filtrate were examined by HPLC.

Structures of products were established by ESI-LC/MS and 2D NMR spectroscopic techniques. ^{13}C NMR spectral data of glycosylation products are shown in Table 1.

2.4. Administered additional carbohydrates and antioxidants

Addition of 1.5 g of various carbohydrates (sucrose, glucose, lactose or mannitol) or 20 mg of antioxidants (gallic acid or ascorbic acid) were made to hairy root cultures together with **2**. Incubations were continued for another 7 days. For this experiment, the control used only **2** without any additional compounds.

3. Results and discussion

3.1. Biotransformation of p-hydroxybenzoic acid

Addition of **1** to *Panax ginseng* hairy root cultures gave two compounds, which were found in both roots and the medium but not in the control. Purification by C_{18} column chromatography and preparative HPLC gave products **1a** and **1b** in 17.7 and 14.9% yields, respectively. Their structures were respectively identified as *p*-carboxyphenyl β-D-glycopyranoside (**1a**) and *p*-hydroxybenzoic acid β-D-glycopyranosyl ester (**1b**) according to the MS, ^1H NMR, ^{13}C NMR, DEPT, HSQC and HMBC spectra analysis (Fig. 1).

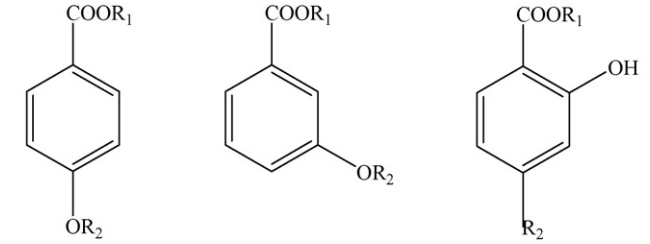
Table 1
^{13}C NMR data of for **1a**, **1b**, **2a**, **2b**, **2c** obtained by biotransformations of hydroxybenzoic acids by hairy root cultures of *Panax ginseng*

C	1a	1b	2a	2b	2c
1	120.1	120.2	123.3	120.6	121.3
2	131.6	132.4	132.1	130.6	130.8
3	116.3	115.8	157.7	157.5	158.0
4	161.3	162.9	120.9	120.3	120.7
5	116.3	115.8	117.5	116.0	116.4
6	131.6	132.4	129.1	129.2	130.3
7	167.4	164.9	168.2	165.4	165.0
1′	100.2	95.0	100.9	94.8	95.3
2′	73.6	73.0	73.4	72.7	72.9
3′	77.6	78.4	76.8	77.5	77.0
4′	70.0	70.0	69.8	69.7	69.9
5′	77.0	76.9	76.5	76.7	76.6
6′	61.0	61.0	61.0	60.9	68.5
1″					104.2
2″					73.7
3″					77.0
4″					69.7
5″					66.1

A time-course study of the bioconversion of **1** to products showed that **1** was rapidly converted into **1a** and **1b** after 6 h (Fig. 2). Substrate **1** was rapidly consumed during the first 12 h, a plateau was reached for the next 12 h and substrate consumption continued for another day. The yield of both products gradually increased over the initial 5-day incubation with higher levels of **1b** initially, and roughly equal yields at about 15% for each compound after 6 days.

3.2. Biotransformation of m-hydroxybenzoic acid

Panax ginseng hairy root cultures converted **2** into three metabolites designated as **2a**, **2b**, and **2c**, which were purified by C_{18} column chromatography and preparative HPLC. Both **2a** and **2c** were found in the roots whereas **2b** was detected in both the medium and roots. None of them was



1: R_1=R_2=H
1a: R_1=H, R_2=Glc
1b: R_1=Glc, R_2=H

2: R_1=R_2=H
2a: R_1=H, R_2=Glc
2b: R_1=Glc, R_2=H
2c: R_1=Glc(6-1)Xyl, R_2=H

3: R_1=R_2=H
4: R_1=Na, R_2=H
5: R_1=H, R_2=SO_3H

Fig. 1. Structures of substrates and their glycosylation products.