Synthesis of 3,4-Dimethoxyphenyl β-D-Glucopyranoside and Its Related Glycosides by Cultured Plant Cells

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3,4-Dimethoxyphenol (3,4-DMP) was converted into the corresponding glucoside in suspension-cultured cells of Coffea arabica. The maximum efficiency of glucosylation was attained, more than 40%, within 96 h after the addition of 1 mM 3,4-DMP when cultured in a modified Murashige and Skoog’s medium with 5 μM 2,4-dichlorophenoxyacetic acid and 0.5 μM kinetin. The glucoside was identified as 3,4-dimethoxyphenyl β-D-glucopyranoside (3,4-DMP glucoside) by 1H-NMR and hydrolysis with α- and β-glucosidases. As the application of the glycosylation by C. arabica cells, eleven glycosides were obtained from seven methoxyphenols including 3,4-DMP.

3,4-Dimethoxyphenol β-D-glucopyranoside (3,4-DMP glucoside) was purified from the non-sugar fraction of crude black sugar prepared from sugar cane, and has an activity that reduced the level of plasma insulin without elevating plasma glucose in the glucose tolerance test, suggesting that it might inhibit the absorption of glucose from the small intestines. It is expected that the glucoside may protect pathological changes induced by taking a large amount of refined sugar, such as hyperlipemia, obesity, diabetes, and arteriosclerosis. However, as there is only a little quantity of 3,4-DMP glucoside in sugar cane, it has not been used in medicines or food materials.

Glycosylation is considered to be an important method for the synthesis and the structural modification of compounds with useful biological activities in the case of salicylic acid. And recently, it has been demonstrated that the cultures of various plant cells have the ability to glycosylate many kinds of exogenously administered compounds, such as phenolics, steroids, flavonoids, and steviol.

In this report, we described the synthesis of a large amount of 3,4-DMP glucoside by glycosylation of 3,4-DMP, and application of this method to synthesis of other related methoxyphenol glycosides.

Materials and Methods

Materials. α-Methoxyphenol (α-MMP), m-methoxyphenol (m-MMP), p-methoxyphenol (p-MMP), 2,3-dimethoxyphenol (2,3-DMP), 2,6-di-methoxyphenol (2,6-DMP), 3,4-dimethoxyphenol (3,4-DMP), 3,5-di-methoxyphenol (3,5-DMP), and 3,4,5-trimethoxyphenol (3,4,5-TMP) were purchased from Sigma Chemical Co.

Cell culture. Five culture strains used in this study were derived from the following plant materials in the years indicated, and were maintained in a modified Murashige and Skoog’s (MS) medium with 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 μM kinetin (Kin) (adjusted to pH 5.7 before autoclaving) for Coffea arabica (leaf, 1988), Diospyros kaki (hypocotyl, 1991), Gardenia jasminoides (leaf, 1988), and Lupinus polyphyllus (hypocotyl, 1991) and with 5 μM isopentyladenine (2iP) and 0.5 μM indole-3-butyric acid (IBA) for Theobroma cacao (leaf, 1987). Cultures were agitated in 300-ml conical flasks containing 100 ml of the medium on a rotary shaker at 100 strokes/min at 25°C in the dark, and the cells were subcultured at two-week intervals.

Glycosylation experiments. In glycosylation experiments, cultured cells (1 ml packed cell volume) were inoculated into 50-ml conical flasks containing 10 ml of the medium described above and incubated under the same conditions. After two weeks of culture (stationary phase), the cultured cells (1 ml packed cell volume) were transferred to fresh medium with each methoxyphenol derivative (1 mM as a final concentration) aseptically added, and their cultures were continued.

To examine the effects of phytohormones in the medium on glycosylation, C. arabica cells (1 ml packed cell volume) were transferred to fresh media containing various concentrations of auxins and/or cytokinins. The sorts and concentrations of phytohormones used are described in Table I. One mM 3,4-DMP was added aseptically to the cells and cultured for 96 h.

Quantitative assay. After culturing with each methoxyphenol derivative for 48 or 96 h, the harvested cells (1 ml packed cell volume) were homogenized. The formed glycoside in the supernatant (500 μl) was hydrolyzed with 10 units/ml of almond β-glucosidase (Sigma Chemical Co.) at 40°C and pH 5.5 for 16 h. Each released methoxyphenol was measured by HPLC on an ODS column (E. Merck) that was eluted with water-H2O (25:75, v/v) at a flow rate of 0.5 ml/min at 40°C and monitored by measuring absorbance at 270 nm. The amount of free methoxyphenol before β-glucosidase treatment was also determined by HPLC on an ODS as a blank. The amount of glycoside formed was calculated by subtraction of the amount of free methoxyphenol from that of methoxyphenol in the supernatant after β-glucosidase treatment. The efficiency of glycosylation was expressed as the percent of each methoxyphenol that had been glycosylated by cultured cells. All values represented the means of results from three independent experiments.

Figure 1 shows typical HPLC chromatograms. After culturing for 48 h without the addition of 3,4-DMP to C. arabica cells, no methoxyphenol was detected in the culture (Fig. 1-a). Culturing with 3,4-DMP, a new peak (indicated by a letter G) and that of free 3,4-DMP (indicated by a letter A) were detected (Fig. 1-b). After β-glucosidase treatment, the new peak disappeared and the peak corresponding to 3,4-DMP was increased (Fig. 1-c). As the 3,4-DMP glucoside was reported to be a bifunctional protein capable of hydrolyzing β-D-glucopyranosides as well as β-D-galactopyranosides, the newly appeared peak, therefore, was recognized as a glycoside, and the amount of 3,4-DMP increased after β-glucosidase treatment was originated from the glycosylated 3,4-DMP.

Extraction and purification of the glycoside. 3,4-DMP (154 mg) was added to a culture of C. arabica cells (200 ml packed cell volume) in a modified MS medium with 5 μM 2,4-D and 0.5 μM Kin. After 96 h, harvested cells were washed with H2O, then homogenized in the culture medium. The homogenate was boiled at 100°C for 15 min. The supernatant was lyophilized and the glycoside was extracted with hot methanol. The extract was put on a preparative HPLC on an ODS column that was eluted with methanol-H2O (35:65, v/v) at a flow rate of 7.0 ml/min at room temp.
temperature.

Glucosidase treatment. To identify the structure of the glycoside, the puried glycoside was hydrolyzed by 10 units/ml of \( \alpha \)- or \( \beta \)-glucosidase (Sigma Chemical Co.) at 40°C for 16 h. After hydrolysis, the released glucose was measured by the glucose oxidase method,\(^{13}\) and the released 3,4-DMP was measured by HPLC, as described above.

\(^{1}H\)-NMR NMR spectra were recorded with a JEOL JNM-GX270 (270 MHz) spectrometer (JEOL) in CD\(_{3}\)OD with TMS (tetramethylsilane) as the internal reference.

Results

Glycosylation of 3,4-dimethoxyphenol in cultures of various plant cells

Five different lines of cultured cells, from C. arabica, D. caryophyllus, L. polyphyllus, T. cacao, and G. jasminoides, were tested for the ability to glycosylate 3,4-DMP. As shown in Fig. 2, the efficiency of glycosylation of C. arabica cells was the highest, more than 40% after culturing for 96 h. The values for D. caryophyllus, L. polyphyllus, T. cacao, and G. jasminoides cells were less than 15% for 48-h culture, and they could not accumulate the glycosides any more during 2 weeks of culture.

This experiment showed that various cultured cells can glycosylate foreign compounds, such as 3,4-DMP, which are not originally present in the cultured cells. However, the amount of glycosylated 3,4-DMP varied with the cultured plant cells.

In this study, because of their high glycosylation ability, we used suspension cultures of C. arabica for glycosylation of 3,4-DMP.

Concentration of 3,4-dimethoxyphenol and the efficiency of glycosylation

The effects of the concentration of 3,4-DMP on its glycosylation by C. arabica cells after culturing for 96 h are shown in Fig. 3. The maximum efficiency of glycosylation reached more than 40% at 1–2 mM 3,4-DMP and decreased at higher concentrations. Since excessive 3,4-DMP is toxic to the cells, 2 mM 3,4-DMP seems to be the critical concentration at which the cellular capacity for glycosylation and the toxicity of 3,4-DMP are balanced.

Fig. 1. HPLC Chromatograms for Glycosylation of 3,4-Dimethoxyphenol by C. arabica Cells.

C. arabica cells were cultured for 48 h, without (a) and with (b) administration of 3,4-DMP, and assayed with HPLC (ODS) described in Materials and Methods. (c) After C. arabica cells were cultured for 48 h with administration of 3,4-DMP, glycoside formed was treated with \( \beta \)-glucosidase and assayed with HPLC (ODS). (A), 3,4-dimethoxyphenol; (G), glycoside formed; (△), starting point of the chromatogram.

Fig. 2. Glycosylation of 3,4-Dimethoxyphenol by Cultures of Various Plant Cells.

Each plant cells was cultured on the medium described in Materials and Methods. The efficiencies of glycosylation were measured at 96-h culture for C. arabica cells and at 48-h culture for the other cells.

Fig. 3. Effects of Concentration of 3,4-Dimethoxyphenol on Glycosylation by C. arabica Cells.

Cells were harvested at 96 h after administration of 3,4-DMP, and 3,4-DMP glycoside was measured by HPLC.

Fig. 4. Course of Glycosylation by C. arabica Cells.

(O), cell growth (ml packed cell volume); (•), the amount of glycoside in the cells (mmol); (△), the amount of glycoside in the medium (mmol). Cells were harvested at various intervals after administration of 1 mM 3,4-DMP, and 3,4-DMP glycoside was measured by HPLC.
Course of glycoside formation
The course of glycosylation of 3,4-DMP was investigated. One mM 3,4-DMP was added to C. arabica cells at the beginning of the culture. As shown in Fig. 4, the suspension cultures of C. arabica cells converted 3,4-DMP into its glycoside, which accumulated in the cells. One small quantities were released into the medium. The conversion into the glycoside was observed from an early time (at 10 h) after administration. The extent of conversion reached more than 40% within 96 h and then decreased gradually.

Optimum conditions for glycosylation
As shown in Table I, among auxins, 2,4-D was the most effective on both cell growth and glycoside formation. The amount of glycoside formed per ml packed cell volume was higher in a medium with 5 μM 2,4-D and 0.5 μM Kin than in a medium with 5 μM 2,4-D only for 96-h culture, and cell growth during 2 weeks of culture was also higher in the former medium.

Purification and structure of the glycoside
The pure glycoside was obtained as a white powder in a yield of about 25% (79 mg) from C. arabica cells when 154 mg of 3,4-DMP was administered in the culture medium.

After hydrolysis by β-glucosidase, the glycoside (as a concentration of 11 mg/ml) yielded 3,4-DMP (35.0 mm) and glucose (33.4 mm). The molar ratio of 3,4-DMP to glucose was 1.05. Alpha-glucosidase did not hydrolyze the glycoside. The results suggested that the glycoside was 3,4-DMP β-monoglycoside.

In its 1H-NMR spectrum (Table II), the aromatic proton signals were observed at δ 6.95–6.76, and the signals assignable to two methoxyl groups were also observed at δ 3.90 and 3.87. The signal at δ 4.88 assignable to anomic proton of glucose were also observed. The large coupling constant (J = 7.3 Hz) of the anomic proton in the glucoside suggests the β configuration for the anomeric center.

Consequently, the glucoside can be identified as 3,4-dimethoxyphenyl β-D-glucopyranoside (Fig. 5).

<table>
<thead>
<tr>
<th>Phytohormones</th>
<th>Cell growth (ml PCV/Flask)</th>
<th>Glycoside formed (μM/ml PCV)</th>
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<tr>
<td>5 μM IAA</td>
<td>1.40 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.18 ± 0.28</td>
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<tr>
<td>5 μM 1BA</td>
<td>1.60 ± 1.83</td>
<td>2.24 ± 0.25</td>
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<tr>
<td>5 μM NAA</td>
<td>1.83 ± 0.12</td>
<td>9.96 ± 3.03</td>
</tr>
<tr>
<td>0.5 μM 2,4-D</td>
<td>2.00 ± 0.08</td>
<td>6.62 ± 1.93</td>
</tr>
<tr>
<td>5 μM 2,4-D</td>
<td>4.03 ± 0.12</td>
<td>17.30 ± 0.52</td>
</tr>
<tr>
<td>50 μM 2,4-D</td>
<td>2.97 ± 0.05</td>
<td>19.19 ± 1.14</td>
</tr>
<tr>
<td>5 μM 2,4-D + 0.5 μM Kin</td>
<td>4.27 ± 0.05</td>
<td>51.53 ± 2.51</td>
</tr>
<tr>
<td>5 μM 2,4-D + 5 μM Kin</td>
<td>3.33 ± 0.05</td>
<td>17.05 ± 0.76</td>
</tr>
</tbody>
</table>

<sup>a</sup> PCV: packed cell volume.
<sup>b</sup> Standard error.

Table II. 1H-NMR Spectral Data for 3,4-DMP Glucoside

δ

<table>
<thead>
<tr>
<th>δ</th>
<th>2</th>
<th>6.92</th>
<th>(IH, d., J = 2.8 Hz)</th>
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<tr>
<td></td>
<td>5</td>
<td>6.95</td>
<td>(IH, d., J = 8.9 Hz)</td>
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<tr>
<td></td>
<td>6</td>
<td>6.76</td>
<td>(IH, q., J = 8.9, 2.8 Hz)</td>
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<tr>
<td>1&lt;sup&gt;α&lt;/sup&gt;</td>
<td>4.88</td>
<td>(IH, d., J = 7.3 Hz)</td>
<td></td>
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<tr>
<td>2–5&lt;sup&gt;α&lt;/sup&gt;</td>
<td>3.38–3.70</td>
<td>(4H, m.)</td>
<td></td>
</tr>
<tr>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.02</td>
<td>(IH, dd., J = 12.0, 2.0 Hz)</td>
<td></td>
</tr>
<tr>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.98</td>
<td>(IH, dd., J = 12.0, 5.5 Hz)</td>
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<tr>
<td>3-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>3.90</td>
<td>(3H, s.)</td>
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<tr>
<td>4-OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>3.87</td>
<td>(3H, s.)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. Structure of 3,4-Dimethoxyphenyl β-D-glucopyranoside.

Fig. 6. HPLC Chromatograms in Glycosylation of Methoxyphenol Derivatives by C. arabica Cells.
C. arabica cells were cultured for 48 h, with administration of (a), 3,5-DMP; (b), 2,3-DMP; (c), 2,6-DMP; (d), 3,4,5-TMP. (A), methoxyphenol; (G), glycoside formed; ( ), starting point of the chromatogram.
Glycosylation of methoxyphenol derivatives

To evaluate the substrate specificity of glycosylation of *C. arabica* cells, eight kinds of methoxyphenols including 3,4-DMP were tested as substrates.

Figures 1-b and 6 show typical HPLC chromatograms of glycosylation of various methoxyphenols. As for di and trimethoxyphenols, the efficiency of glycosylation at 48 h of culture was greatest for 3,4-DMP, and was 36% for 3,4-DMP, 35% for 3,5-DMP, 23% for 2,3-DMP, 13% for 2,6-DMP, and 26% for 3,4,5-TMP. In the case of 3,4-DMP and 3,5-DMP, one glycoside was detected; for 2,3-DMP and 2,6-DMP, two glycosides were detected; for 3,4,5-TMP, three glycosides were detected. In addition, the values for monomethoxyphenols (o-MMP: 9%, m-MMP: 12%, p-MMP: 0%) were smaller than those for dimethoxyphenols. The cells could not glycosylate p-MMP during 2 weeks of culture.

Discussion

Many plants contain useful glycosides, for example, pharmacologically active components of Chinese medicines. Many of them exist in very small quantities in the plants and very difficult to purify on an industrial scale. To produce such useful glycosides, these are thought to be three methods. The first is chemical synthesis, but it needs a multiple-step reactions consisting acetylation, glycosylation, and deacetylation, and it always accompanied with some by-products. The second is the enzymatic synthesis by using microbial enzymes or plant UDP-sugar glycosyltransferases. There are some reports on glucosylation by a microbial enzyme, but the resultant glucosides had α-linkages. The latter enzymes can not be available for industry, because UDP-sugar is very expensive. The third is glycosylation by cultured plant cells, which is a one-step and position-specific reaction. They made the glucosides with β-linkages, which was the same structure as those in nature. Therefore, glycosylation by cultured plant cells seems to be suitable for our purpose. We tried to produce 3,4-DMP glucoside, which inhibited glucose absorption from the small intestines, by plant cell cultures from 3,4-DMP, which are easily and cheaply obtained.

In this report, five different plant cell cultures were tested for their abilities to glycosylate 3,4-DMP. Remarkable differences in glycosylation ability were found among these cultured cells. This result suggested the necessity of selecting cells that are able to glycosylate an administered compound at a high rate. *C. arabica* cells were found to be suitable for our purpose, because they had a superior glycosylating ability and normally lack phenolic glycosides, such as 3,4-DMP glucoside. These experiments showed that more than 40% of 3,4-DMP was converted into the corresponding glucoside, when it was applied to *C. arabica* cells cultured on a modified MS medium with 5 μM 2,4-D and 0.5 μM Kin. The maximum accumulation of the glucoside in the cells was at 96 h after the administration, and then gradually decreased until it was almost 20% at the end of the growth cycle (2 weeks of culture). The glucoside seemed to be metabolized to other unknown compounds. This view was supported by the evidence that neither release of the glucoside from the cells into the medium nor reappearance of 3,4-DMP in the cultures was observed during culturing.

We got 79 mg of purified 3,4-DMP glucoside from 2000 ml of culture of *C. arabica* cells (200 ml packed cell volume), in a yield of about 25%, and purification of the glucoside from *C. arabica* cells was very simple. It consisted of only two steps: extraction with methanol and HPLC on an ODS column. Furthermore, the substrate, 3,4-DMP, is commercially available in a large quantity and this preparation method for its β-glucoside can be scaled up. Therefore, 3,4-DMP glucoside might be used as an ingredient in a diet.

Eight methoxyphenol derivatives were used as substrates for glycosylation by *C. arabica* cells. 3,4-DMP and 3,5-DMP were effectively converted into their glycosides. The efficiency of glycosylation for dimethoxyphenols tended to be greater than that for monomethoxyphenols. After the administration of monomethoxyphenols, cells developed necrosis within 24 h. On the other hand, cells administered dimethoxyphenols or trimethoxyphenols were not changed even in 96 h of culture. Monomethoxyphenols are suggestively to be more toxic for cells than dimethoxyphenols or trimethoxyphenol. This phenomenon seems to refer to the efficiency of glycosylation. As regards dimethoxyphenols, the efficiency of glycosylation tended to decrease in the order of 3,4-DMP > 3,5-DMP > 2,3-DMP > 2,6-DMP. The result suggests that the position of methoxy groups in the substrate molecules has influence on glycosylation. The efficiency of glycosylation seems to be decreased by steric hindrance of the methoxy groups in an ortho-configuration. On the other hand, the efficiency of glycosylation into o-glucoside in suspension cultures of *Mallotus japonicus* tended to decrease in order of o-hydroxybenzoic acids (HBA), m-HBA, and p-HBA. One of the reasons for such differences seems to depend on the configuration of groups close to the hydroxyl group to be glycosylated between methoxyphenols and hydroxybenzoic acids, which is attributed to the substrate specificity of each glycosyltransferase.

In this study, we obtained at least eleven glycosides including 3,4-DMP glucoside, which reduced the level of plasma insulin, from eight kinds of methoxyphenols. Further study of this biological activity of these glycosides is needed.

References