Measurement of Phenolic Compounds and Their Effect on Shikonin Production in Lithospermum Cultured Cells

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Shikonin production by Lithospermum cell cultures is induced by transferring the cells into production medium. Six phenolic compounds, p-hydroxybenzoic acid, caffeic acid, sinapic acid, ferulic acid, syringaldehyde, and salicylic acid, were detected in both shikonin-producing and non-producing cells. Their contents in the former were much lower than those in the latter except for salicylic acid, the content of which strongly increased when cells were producing shikonin. The cell wall fraction, after alkaline hydrolysis, gave two phenolic compounds, p-hydroxybenzoic acid and caffeic acid. Their contents were much higher in shikonin-producing cells than in shikonin-free cells. Of these compounds, exogenous addition of p-hydroxybenzoic acid increased shikonin production in the production medium. Although it is a precursor of shikonin, the increment of shikonin produced was much larger than the administered p-hydroxybenzoic acid, suggesting this compound has a stimulatory effect on shikonin biosynthesis at a low concentration.

Key words: Lithospermum erythrorhizon; p-hydroxybenzoic acid; salicylic acid; shikonin; biosynthesis

Cell suspension cultures of Lithospermum erythrorhizon, which produce large amounts of red napthoquinone pigments (a mixture of shikonin derivatives) in “production medium” (M9).1-3 are unable to synthesize the pigments in Linsmaier–Skoog (LS) liquid medium4 mainly owing to the presence of ammonium ion supplied as a nitrogen source in the medium. We have demonstrated that the shikonin-free cells accumulated p-hydroxybenzoic acid, a biosynthetic precursor of shikonin,5 mainly in the form of the O-glucoside, which rapidly decreases in quantity when the cells are transferred into M9 medium to produce shikonin derivatives.5

Tani et al. reported that the shikonin biosynthesis in Lithospermum cell cultures is regulated by internal modulators such as acidic oligogalacturonides,6,7 which are derived from the cell wall, when the cells were inoculated in shikonin production medium. We have also attempted to identify low molecular weight substrates acting as such internal modulators on shikonin biosynthesis in Lithospermum cell cultures. In this study, we report the differences in phenolic components between the shikonin-free and the shikonin-producing cells. The effects of these phenolic compounds on shikonin production are also described in this paper.

Materials and Methods

Culture methods. Cell line M18 of Lithospermum erythrorhizon Sen. et Zucc.8 was used for these experiments. Cell suspension cultures were grown in LS medium9 containing 1 μM indole-3-acetic acid (IAA) and 10 μM kinetin, in 100-ml conical flasks (30 ml of medium) on a reciprocal shaker (100 stroke/min) at 25°C in the dark. These shikonin-free cells cultured in the LS medium were harvested 3 weeks after inoculation, and used for chemical analysis. Shikonin production was induced by transferring the shikonin-free cells (inoculum size: 1.2 g; 30 ml medium) that had been cultured in LS medium for 3 weeks into production medium (M9) containing 1 μM IAA and 10 μM kinetin.1,2 After 2 weeks of culture in the M9 medium under the same conditions as mentioned above, the pigmented cells of the early stationary growth stage (2 weeks after inoculation) were harvested to analyze the phenolic components.

Isolation and identification of phenolic compounds. Fresh non-pigmented cells (150 g) cultured in LS medium for 3 weeks were extracted with methanol (300 ml) thrice. The extract concentrated in vacuo was extracted with ether (50 ml × 5) under acidic condition (pH 2.5). The ether extract was separated by prep. TLC (silica gel, CHCl3; methanol=9:1) to yield p-hydroxybenzoic acid (4.6 mg), caffeic acid (2.1 mg), sinapic acid (0.5 mg), syringaldehyde (0.3 mg), and ferulic acid (0.5 mg). The shikonin-producing cells (150 g fresh wt) cultured in M9 medium for 2 weeks were treated in a similar way to give salicylic acid (4.0 mg) in addition to the same phenolic compounds as those isolated from the non-pigmented cells. These isolated compounds were identified by direct comparison (1H-NMR, TLC, and HPLC) with the authentic compounds.

Isolation of phenolic compounds from cell walls. Cell wall fractions were prepared from both cultures by a modification of the method10 of the neutral detergent procedure.10 The dried cells (8.45 g dry wt, each) were extracted with methanol to remove the soluble compounds, then ground in a mortar with an aqueous solution of the neutral detergent consisting mainly of 3% sodium dodecyl sulfate. The mixture was shaken for 24 h at 25°C, and filtered through a sintered glass filter (pore size No. 1), and then the residue containing cell walls was washed successively with H2O (1 liter), hot H2O (1 liter) acetone (300 ml), and ether (300 ml). The cell walls were dried on P2O5 (yield: 25% of dried cells), and a sample (50 mg) was treated with 1 M NaOH (5 ml) under N2 at 20°C for 20 h. The filtrate and washings (5 ml) of the hydrolysate were combined, acidified (pH 2.5) with 4 M HCl, then extracted with ether (3 ml × 10). The etheral extract was dried over anhydrous Na2SO4 and concentrated under a N2 stream. A sample (10 μl) of the methanol solution (1 ml) was analyzed by HPLC.

Measurement of phenolic compounds. Fresh shikonin-producing and non-producing cells (14 g each), which were harvested 2 and 3 weeks after inoculation, respectively, were treated by the method mentioned above to give etheral extracts containing phenolic compounds. The extracts were dissolved in methanol (1 ml) and analyzed by HPLC under the following conditions; column: TSK-gel ODS 120A, 10 μm (Toso LH, Japan, 4.6 mm i.d. × 150 mm), solvent systems: (I) n-butanol-H2O-acetic acid (10:400:1), (II) methanol-H2O-acetic acid (50:200:0.5), (III) methanol-H2O-acetic acid (45:55:0.5); flow rate. 1.5 ml/min; pressure, 100 kg/cm2; detection, absorption at 280 nm. The quantities of phenolic compounds were calculated by comparing their peak areas with those of the standard samples.

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Administration of phenolic compounds. Each phenolic compound (p-hydroxybenzoic acid, caffeic acid, sinapic acid, ferulic acid, syringaldehyde, and salicylic acid) detected in Lithospermum cells was dissolved in DMSO (0.1 ml), and added aseptically through a membrane filter (pore size: 0.2 μm) to test tubes containing M9 medium (7 ml). For comparison, the effects of several phenolic compounds (cinamic acid, p-coumaric acid, protocatechuic acid, vanillic acid, syringic acid, vanillin, coniferaldehyde, and sinapaldehyde) were also tested. Fresh cells (0.3 g) were inoculated into the test tubes and cultured on a shaker for 2 weeks before harvest. The cell suspension was filtered through Miracloth (Calbiochem) and the shikonin in the cells collected was measured by the method described elsewhere. To prevent the change in pH of the medium owing to the addition of phenolic acids, 10 mM of 2-(N-morpholino)ethanesulfonic acid (MES) was added to the M9 medium. It was confirmed that both DMSO and MES have no effect on cell growth and shikonin synthesis in Lithospermum cell cultures at the concentration used in the experiment.

Preparation of sinapaldehyde and coniferaldehyde. Sinapaldehyde was chemically synthesized from methyl sinapate by the method of Kutsuki et al. The other chemicals used were purchased from Nakalai Tesque and Wako Pure Chemicals.

Results

Shikonin is biosynthesized from phenylalanine via p-hydroxybenzoic acid, which is formed through the general phenylpropanoid pathway (Fig. 1). In the cell cultures, several low molecular weight phenolic compounds were detected in soluble fractions of the harvested cells. Chemical investigation indicated that those phenolic compounds were p-hydroxybenzoic acid, caffeic acid, sinapic acid, ferulic acid, syringaldehyde, and salicylic acid. Their contents in both pigmented (M9 medium) and non-pigmented cells (LS medium) at early stationary growth stage were quantitatively analyzed by HPLC as shown in Table I. The contents of these compounds were higher in the non-pigmented cells than in the pigmented cells, except for salicylic acid. The formation of salicylic acid was markedly increased when shikonin production was initiated in the cells cultured in production medium.

Phenolic acids are known to exist in plant cell walls in an insoluble form. They are mainly bound through ester linkage to hydroxyl groups of polysaccharide of the cell wall. Because some of the phenolic compounds detected in these experiments were expected to be present also in the cell wall of those cultured cells as bound forms, their contents in the cell walls of both kinds of Lithospermum cell cultures were also measured by HPLC. As seen in Table I.

Table I. Contents of Phenolic Compounds in Cells (Soluble Form) and Cell Walls (Bound Form) of Lithospermum Cells Cultured in LS (Non-pigmented Cells) and M9 (Pigmented Cells) Medium

<table>
<thead>
<tr>
<th></th>
<th>Soluble fraction of cell</th>
<th>Cell wall fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LS medium</td>
<td>M9 medium</td>
</tr>
<tr>
<td></td>
<td>(μg/g dry wt)</td>
<td>(μg/g dry wt)</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>361</td>
<td>26</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>56</td>
<td>9</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>880</td>
<td>42</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>trace</td>
<td>113</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>70</td>
<td>52</td>
</tr>
<tr>
<td>Syringaldehyde</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>Shikonin</td>
<td>n.d.</td>
<td>31,700</td>
</tr>
</tbody>
</table>

* Not detectable.

Fig. 1. Biosynthetic Pathway of Shikonin in Lithospermum Cells Cultured in LS and M9 Media.
Solid line shows the metabolic pathway in cells cultured in M9 medium in which a large amount of shikonin is produced. Broken line depicts the metabolism in the cells cultured in LS medium, in which shikonin production fails.
I, only two phenolic acids, \( p \)-hydroxybenzoic acid and caffeic acid, were detected in the alkaline hydrolysates of the cell walls of both pigmented and non-pigmented cell cultures.

This experimental result indicated that there was a marked difference in the contents of phenolic compounds between the non-pigmented and the pigmented cells. In order to clarify the involvement of them in the regulation of shikonin production, those phenolic compounds were administered to the shikonin-producing cell cultures.

Figure 2 shows the effect of each phenolic compound on shikonin production of \textit{Lithospermum} cell cultures. The administration of \( p \)-hydroxybenzoic acid at a concentration of \( 10^{-5} \text{ M} \) increased the shikonin production by about 50% in the cells cultured in M9 medium, without suppressing cell growth as seen in the Fig. 2C. Although \( p \)-hydroxybenzoic acid is a precursor of shikonin biosynthesis, the molar increment of shikonin yield was far greater than the amount of \( p \)-hydroxybenzoic acid administered in the medium (Table II). This observation indicates that exogenous \( p \)-hydroxybenzoic acid at a low concentration (\( 10^{-6} \text{ to } 10^{-3} \text{ M} \)) increased shikonin production in M9 medium. Since \( p \)-hydroxybenzoic acid is a precursor of

![Fig. 2. Effects of Phenolic Compounds on Cell Growth and Production of Shikonin Derivatives in \textit{Lithospermum} Cells in M9 Medium.](image)

Cells were harvested 14 days after inoculation. Each phenolic compound was administered at day 0 as a DMSO solution after sterilization by filtration. A: caffeic acid; B: ferulic acid; C: \( p \)-hydroxybenzoic acid; D: salicylic acid; E: sinapic acid; F: syringaldehyde. Values are means of three replicates.
shikonin, the administration of this compound could stimulate the entire activity of the biosynthetic pathway. Thus, two other aromatic precursors of shikonin, cinnamic acid and p-coumaric acid, were also administered to examine their effects on shikonin biosynthesis. However, they caused no increase in shikonin productivity (data not shown).

Salicylic acid, which was detected in an appreciable amount in pigmented cells, was expected to have a stimulatory effect on shikonin biosynthesis, but it was found to have no significant effect on shikonin production in the range of $10^{-6}$–$10^{-4}$ M (Fig. 2D). Similarly, none of the other related phenolic compounds tested (protocatechuic acid, syringic acid, vanillic acid, vanillin, coniferaldehyde, and sinapaldehyde) had notable effects on shikonin production. These results suggest that $p$-hydroxybenzoic acid is not only a precursor of shikonin but also acts as a modulator of shikonin biosynthesis.

Discussion

This study has demonstrated that phenolic components in *Lithospermum* cells changed in responding to induction of shikonin production. After induction of shikonin biosynthesis, the contents of $p$-hydroxybenzoic acid and caffeic acid, which were high in non-pigmented cells, decreased, while their contents in the cell wall increased. It seems probable that these phenolic acids were combined with polysaccharides of the cell walls through ester linkages. It should be noted that the content of bound $p$-hydroxybenzoic acid in the cell wall was very high in the pigmented cells, indicative that a part of $p$-hydroxybenzoic acid accumulated in a soluble form in the cells of LS medium were incorporated into the cell walls of shikonin-producing cells in M9 medium, although a large part of the $p$-hydroxybenzoic acid was used for the biosynthesis of shikonin as demonstrated previously.\(^ {4,5}\)

The accumulations of phenolic compounds in cell cultures of other plant species were reported in *Rosa* sp. (50–100 \(\mu \text{mol/g fresh wt.}\),\(^ {15}\) *Pinus elliottii* (0.7–3.3 mg/g dry wt.),\(^ {16}\) and *Acer pseudoplatanus* (1–28 mg/g fresh wt.),\(^ {17}\) though the structures of those compounds were not identified. However, Strack and Bokern demonstrated that ferulic acid (2.1–5.7 mg/g dry wt) and $p$-coumaric acid (0.3–3.3 mg/g dry wt) were accumulated in cell suspension cultures of *Chenopodium rubrum* as the glucoside forms.\(^ {18}\) It would be characteristic of *Lithospermum* cells that the content of $p$-hydroxybenzoic acid is higher than the other phenolic compounds in the cells.

Phenolic compounds were also detected as structural components of plant cell walls. Smith and Hartley reported that cell walls of intact plants of *Lolium multiflorum* and *Triticum aestivum* contained ferulic acid (4.89 and 6.60 mg/g cell wall, respectively), $p$-coumaric acid (0.86 and 0.034 mg/g cell wall, respectively), PHB (0.034 mg/g dry wt, latter only), and vanillic acid (trace, latter only).\(^ {13}\) Ferulic acid and $p$-coumaric acid were also found in cell walls of cell suspension cultures of *Spinacia oleracea* by Fry.\(^ {14}\) The cell walls of *Lithospermum* cell cultures contained $p$-hydroxybenzoic acid as the main bound phenolic compounds.

In this paper, we also studied whether or not the internal phenolic compounds in *Lithospermum* cells affected shikonin biosynthesis. In *Phaseolus vulgaris*, for instance, cinnamic acid was reported to act as an internal modulator of chalcone isomerase in vivo.\(^ {19}\) When administered to shikonin-producing cells, only $p$-hydroxybenzoic acid out of six phenolic component isolated from *Lithospermum* cells, stimulated shikonin production by an unknown mechanism. The effective dose of this phenolic acid was as low as that of phytohormones, suggesting the possibility that this compound acts as an internal modulator of shikonin biosynthesis in *Lithospermum* cell cultures. The concentration of $p$-hydroxybenzoic acid in shikonin-free cells in LS medium is high (about 450 \(\mu \text{mol}\)), but no shikonin production is observed in this medium. However, added $p$-hydroxybenzoic acid ($10^{-6}$–$10^{-5}$ M) could increase shikonin production in M9 medium, although ca. 20 \(\mu \text{mol}\) of internal $p$-hydroxybenzoic acid was still detectable in the shikonin-producing cells. One possible explanation for this is that the internal $p$-hydroxybenzoic acid is localized in vacuoles of the cultured cells,\(^ {20}\) while external $p$-hydroxybenzoic acid can come directly into contact with the cell walls and may also be absorbed by the cells to affect enzymes in cytosol or on endoplasmic reticulum where shikonin production occurs.\(^ {21,22}\) The other possibility is that $p$-hydroxybenzoic acid cannot induce the entire shikonin biosynthetic pathway in LS medium, because this medium contains a high concentration of NH\(_4\)\(^ +\), a strong inhibitor of shikonin biosynthesis.\(^ {1,2}\)

Salicylic acid is known to have various physiological activities in plants. For example, salicylic acid was reported to be involved in the hypersensitive response in tobacco,\(^ {23}\) and in *Saurouatum guttatum*, salicylic acid was identified as the natural trigger of heat production.\(^ {24}\) Although salicylic acid was also expected to have a regulatory effect on shikonin biosynthesis in *Lithospermum*, we could not observe a clear influence on shikonin production by this phenolic acid. The salicylic acid would be important in *Lithospermum* cell cultures, but it seems to be independent from shikonin biosynthesis.

References