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ENZYMATIC REGULATION OF SHIKONIN BIOSYNTHESIS IN *LITHOSPERMUM ERYTHRORHIZON* CELL CULTURES

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; cell culture; biosynthesis; naphthoquinones; shikonin; geranyltransferase; glucosyltransferase; phenylalanine ammonia lyase; photoregulation.

Abstract—The activities of *p*-hydroxybenzoic acid geranyltransferase, *p*-hydroxybenzoic acid-*O*-glucosyltransferase, *p*-hydroxybenzoic acid-*O*-glucosidase and phenylalanine ammonia lyase (PAL) were investigated in relation to the accumulation of shikonin and *p*-hydroxybenzoic acid-*O*-glucoside in *Lithospermum erythrorhizon* cell suspension cultures under different conditions of irradiation with white light.

Light strongly inhibited the activity of *p*-hydroxybenzoic acid geranyltransferase, whereas it stimulated that of *p*-hydroxybenzoic acid-*O*-glucosyltransferase. The activity of PAL as well as of *p*-hydroxybenzoic acid-*O*-glucosidase, however, was little influenced by irradiation of the cultures with light. These results suggest that one of the regulatory principles of shikonin biosynthesis in these cultures is the ratio of the activities of *p*-hydroxybenzoic acid geranyltransferase and *p*-hydroxybenzoic acid glucosyltransferase.

INTRODUCTION

Cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. are used in Japan for the commercial production of the naphthoquinone pigment shikonin [1]. The biosynthesis of shikonin in these cultures can be influenced by various chemical and physical parameters; e.g. NH_4^+ [2, 3], glutamine [4] and blue light [5] strongly inhibit shikonin formation, whereas Cu^{2+} [6], activated charcoal [7] and certain polysaccharides [8] induce pigment formation. Cell lines capable of producing high amounts of shikonin have been selected, as well as cell lines completely lacking this biosynthetic capability [9]. The biosynthetic pathway leading to shikonin [10, 11] is shown in Scheme 1. It was hitherto unknown at which steps of this pathway regulation may take place, since none of the secondary metabolic enzymes was known. Recently, methods have been developed for enzymatic studies in these cultures [12], and a key enzyme of shikonin biosynthesis, i.e. *p*-hydroxybenzoic acid geranyltransferase (PHB geranyltransferase), has been detected *in vitro* [13].

The present study was undertaken in order to investigate the role of PHB geranyltransferase and of other secondary metabolic enzymes in the regulation of shikonin biosynthesis.

RESULTS

PHB geranyltransferase

The activity of PHB geranyltransferase as well as the accumulation of shikonin and PHB-*O*-glucoside [14] were measured during a culture period of 20 days in LS medium [15], which inhibits shikonin production, and in M9 medium, which strongly stimulates the formation of the pigment [6]. The cultures in the latter medium were exposed to four different conditions of irradiation with white light (6000 lux): (i) 20 days in the dark; (ii) 20 days under irradiation; (iii) 8 days in the dark, followed by 12 days of irradiation; (iv) 8 days under irradiation, followed by 12 days in the dark.

The results are shown in Fig. 1. In accordance with previous findings [2], cells cultured in LS medium (Fig. 1A) did not produce shikonin, but accumulated PHB-*O*-glucoside. In M9 medium in the dark, however, the PHB-*O*-glucoside present in the inoculum was catabolized, and shikonin was produced (Fig. 1B). PHB geranyltransferase activity, which is essential for shikonin production, was high in M9 medium throughout the culture period. By contrast, it was very low in LS medium; the small increase of activity in the first days after inoculation may be interpreted as a stress reaction owing to the transfer of the precultured cells to unconditioned medium.

The dramatic influence of light on secondary metabolism in these cultures is shown in Fig. 1C–E. If cells in M9 medium were irradiated throughout the culture period (Fig. 1C), a pattern similar to that observed in LS medium appeared; no shikonin but PHB-*O*-glucoside was formed, and PHB geranyltransferase activity was iow, except for a temporary increase in the earliest days. Changes of the light condition during the culture period caused an immediate change in the pattern of secondary metabolism. When cells were transferred from the dark to the light (Fig. 1D), shikonin accumulation was reduced while PHB-*O*-glucoside was accumulated instead; at the same time, the PHB geranyltransferase activity fell sharply. By contrast, when cultures were transferred from the light to the dark (Fig. 2E), the content of PHB-*O*-glucoside decreased while PHB geranyltransferase activ-

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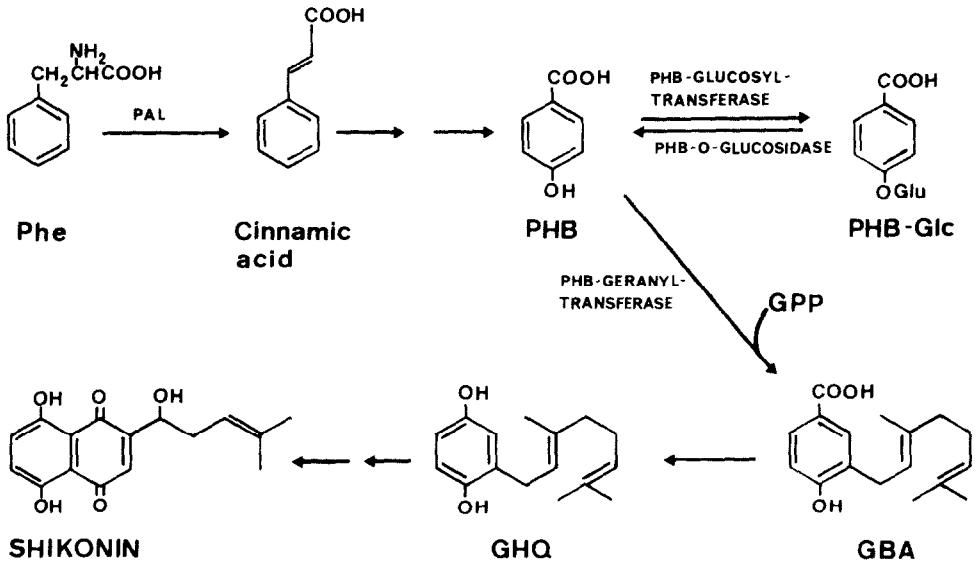
Abbreviations: PHB, *p*-hydroxybenzoic acid; PVPP, polyvinylpolypyrrolidone; DTT, dithiothreitol; PAL, L-phenylalanine ammonia lyase; LS medium, Linsmaier–Skoog medium.



Scheme 1. Biosynthetic pathway of shikonin [10, 11].

ity increased sharply, although the rise in the enzymatic activity resulted only in a relatively low production of shikonin. PHB geranyltransferase activity began to rise as early as two hours after the transfer of cultures from the light to the dark (data not shown), showing a 13-fold increase within 48 hr. To determine whether this increase is due to a *de novo* synthesis of the enzyme, inhibitors of protein synthesis (cycloheximide and puromycin) and of nucleic acid synthesis (5-fluorouracil) were added to the cultures immediately before transfer to the dark, and enzyme activity was measured after 16 hr (Table 1). All three inhibitors inhibited the increase of PHB geranyltransferase activity, although the cells were still alive, as shown by staining tests with Evans blue and neutral red. This suggests that *de novo* synthesis of the enzyme takes place as soon as irradiation is stopped.

Earlier studies [16, 17] have suggested that the inhibition of shikonin biosynthesis by white or blue light may be due to photodegradation of flavin mononucleotide (FMN), since the degradation product lumiflavin proved to be a strong inhibitor of shikonin production. However, we have observed no inhibitory effect of lumiflavin (10^{-6} and 10^{-5} M) on PHB geranyltransferase activity *in vitro*, suggesting that the *in vivo* effect of lumiflavin is not due to its direct action on this enzyme.

Table 1. Influence of inhibitors of protein biosynthesis and nucleic acid biosynthesis on the induction of PHB geranyltransferase activity

Inhibitor	PHB geranyltransferase activity (pKat/mg protein)
None	6.75
Puromycin, 2.5×10^{-4} M	3.80
Cycloheximide, 10^{-5} M	2.25
5-Fluorouracil, 10^{-5} M	1.20

Cultures were grown in M9 medium under white light (6000 lux) for eight days. After aseptic addition of the inhibitor, they were transferred to the dark. After 16 hr, enzyme activity was measured as described in the Experimental.

Effect of light on phenylalanine ammonia lyase, PHB-O-glucosidase and PHB-O-glucosyltransferase

It is considered that, in addition to PHB geranyltransferase, such enzymes as PAL, PHB-*O*-glucosyltransferase, and PHB-*O*-glucosidase might play important roles in the regulation of shikonin synthesis through the supply and storage of precursors (Scheme 1). We have examined the effect of white light (6000 lux) on their activities in cell suspension cultures.

The results are shown in Fig. 2. As expected, no shikonin but PHB-*O*-glucoside was accumulated in cell cultures grown either in LS medium in the dark or in M9 medium under irradiation, where the activity of PHB geranyltransferase was extremely low, in contrast to a high activity of PHB-*O*-glucosyltransferase. On the other hand, light hardly affected the activity of PHB-*O*-glucosidase; however, the glucosidase activity of the enzyme extract used in the present experiment has been found to be non-specific to PHB-*O*-glucoside (data not shown). Unexpectedly, PAL, which is a typical example of the light-inducible enzyme [18], was not influenced by light in *Lithospermum* cultures. However, this finding is compatible with the secondary metabolism observed in *Lithospermum* cultures, since shikonin derived from *t*-cinnamic acid is accumulated in the dark.

DISCUSSION

PHB geranyltransferase is a key enzyme in shikonin biosynthesis, linking a precursor derived from the shikimate pathway, i.e. PHB, to a precursor from the isoprenoid pathway, i.e. geranylpyrophosphate, thus forming the complete carbon skeleton of shikonin. The present study has shown that the activity of this enzyme is

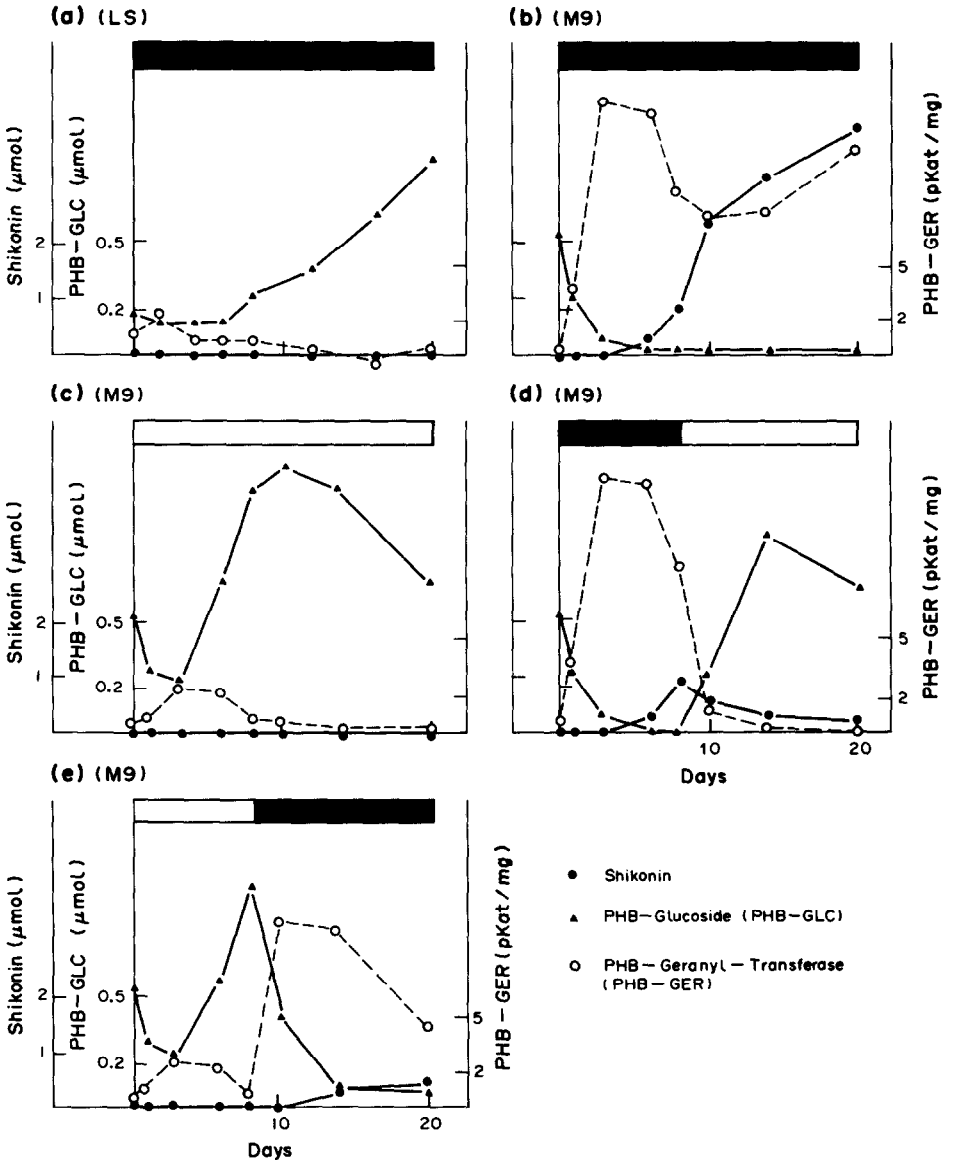


Fig. 1. PHB geranyltransferase activity and accumulation of shikonin and PHB-*O*-glucoside in cell suspension cultures of *L. erythrorhizon*. A: LS medium, 20 days in the dark; B: M9 medium, 20 days in the dark; C: M9 medium, 20 days under irradiation; D: M9 medium, 8 days in the dark, followed by 12 days of irradiation; E: M9 medium, 8 days under irradiation, followed by 12 days in the dark. □: Irradiation with white light (6000 lux). ■: Dark period. Contents of secondary metabolites are given per 30 ml medium, and enzyme activity is expressed in pKat/mg protein.

strongly reduced under the influence of light as well as by LS medium. The induction of shikonin biosynthesis in M9 medium is completely inhibited by light. Previous examinations [5] have shown that blue light is responsible for this inhibition, red light being ineffective. However, the receptor of the light stimulus still remains unknown.

Although high shikonin production is closely associated with high PHB geranyltransferase activity, the reverse is not necessarily true. For instance, when cultures in M9 medium were transferred from the dark to the light (Fig. 1E), PHB geranyltransferase activity rose rapidly to a high level, but shikonin formation remained at a low level. This finding seems to be in accordance with earlier experimental results [5] that callus cultures pretreated with light were able to produce only a small quantity of shikonin even when returned to the dark. Apparently, there must be another photocontrolled step that is possibly related to the photodegradation of FMN, which appears to play an important role in shikonin biosynthesis [16, 17].

As shown by Yazaki *et al.* [14], *L. erythrorhizon* cultures that are not producing shikonin, either in M9 medium under irradiation or in LS medium, accumulate

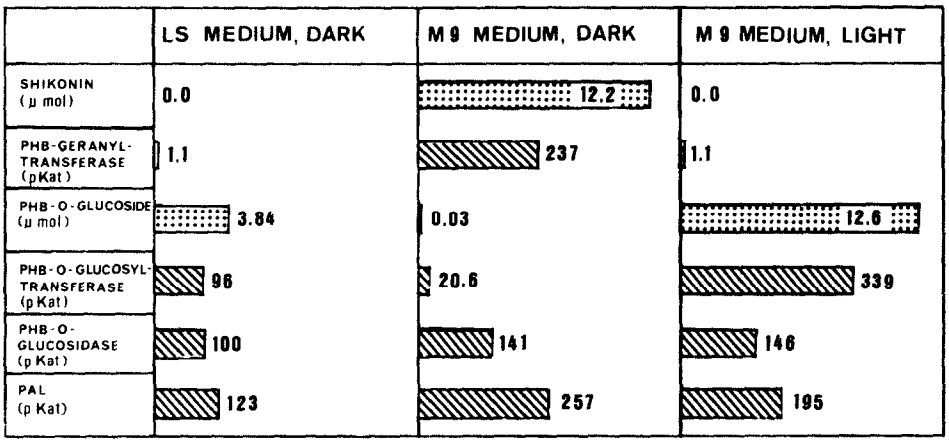


Fig. 2. Activities of enzymes related to shikonin biosynthesis and the amounts of shikonin and PHB-*O*-glucoside in cell suspension cultures of *L. erythrorhizon*. Cells in LS medium and M9 medium were harvested 16 and 14 days after inoculation, respectively. White light, 6000 lux. Enzyme activities and contents of secondary metabolites were determined as described in the Experimental. All data are given per 30 ml medium, and represent averages of four replicates.

PHB-*O*-glucoside in an amount (in moles) corresponding to 25–105% of the amount of shikonin produced in the M9 medium in the dark. Furthermore, the present finding with regard to the correlation between PHB-*O*-glucosyltransferase activity and PHB-*O*-glucoside accumulation, as well as between PHB geranyltransferase activity and shikonin accumulation, suggests that the biosynthesis of shikonin is alternatively regulated by the two enzymes involved in the metabolism of the key intermediate PHB. The ratio of the activities of PHB geranyltransferase and PHB-*O*-glucosyltransferase may determine whether PHB is converted into shikonin, which is excreted out of the cell [19], or into its glucoside, which is likely to be stored in the vacuole.

Definite proof for a regulatory role of PHB-*O*-glucosyltransferase would require purification of this enzyme and the demonstration of its specifity for PHB. This is now under investigation.

EXPERIMENTAL

Radiochemicals. *p*-Hydroxy[*carboxyl*-^{14}C]benzoic acid (1.83 MBq/μmol) was obtained from CEA, France.

HPLC. TSK-Gel ODS 120 A 10 μm (Toyo Soda, Japan), 150 × 4.6 mm.

Cell cultures. The callus cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. were derived from germinating seeds [5]. By selection from the heterogeneous callus culture, strain M18 capable of producing a large amount of shikonin was obtained [9]. Cell suspension cultures were initiated and maintained as described elsewhere [7]. To obtain shikonin-producing cultures, cells of the strain M18 (inoculum size 1.1 g) were transferred from LS medium [15] into 100 ml Erlenmeyer flasks containing 30 ml of M9 medium [2, 6] supplemented with 10^{-6} M IAA, 10^{-5} M kinetin, and 5 ml liquid paraffin (ampoule quality, Merck) [12]. To obtain shikonin-free cultures, cells were cultured in LS medium supplemented with the same hormones.

Preparation of cell-free extracts. Cells (2 g) were suspended in K-Pi buffer (4 ml, 0.1 M, pH 6.5) containing 10 mM DTT and 0.2 g PVPP. The suspension was homogenized (Potter homogenizer) and then centrifuged at 10000 *g* for 10 min. The supernatant was passed through Sephadex G 25 (PD 10 column) equilibrated with a 0.05 M NaCl containing 10 mM DTT.

Protein content. Determined according to the method of ref. [20].

Determination of shikonin content. The amounts of shikonin in the paraffin layer and in the cells were determined as described elsewhere [7, 12].

Determination of PHB-O-glucoside content. To 100 μl of the cell-free extract obtained before gel filtration, 100 μl of MeOH and 100 nmol of *m*-hydroxybenzoic acid (as internal standard) were added. After centrifugation, a sample (20 μl) of the soln was examined by HPLC. Solvent: H_2O–MeOH–HOAc (77:2:1); detection: 254 nm; R_t: PHB-*O*-glucoside 4.5 min, *m*-hydroxybenzoic acid 12.9 min.

Assay for PHB geranyltransferase activity. The incubation mixture contained in a total vol. of 100 μl: Tris–HCl, pH 7.5, 10 μmol; *p*-hydroxybenzoic acid, 0.1 μmol; GPP, 0.2 μmol; $MgCl_2$, 1 μmol; and enzyme extract, 80 μl. After incubation for 60 min at 30°, the reaction was stopped by addition of 5 μl HCO_2H. Diethylstilbestrol (20 nmol) was added as int. standard, and the reaction mixture was extracted with 150 μl EtOAc. After centrifugation, a sample (20 μl) of the EtOAc layer was subjected to HPLC analysis. Solvent: H_2O–MeOH–HOAc (750:250:3); detection: 254 nm; R_t: *m*-geranyl-*p*-hydroxybenzoic acid 7.3 min, diethylstilbestrol 3.8 min.

HPLC assay for PHB-O-glucosyltransferase activity. The incubation mixture contained in a total vol. of 300 μl: Tris–HCl, pH 7.5, 30 μmol; *p*-hydroxybenzoic acid, 1.5 μmol; UDPG, 3 μmol; and enzyme extract, 240 μl. Before and after incubation for 90 min at 30°, samples of 100 μl were taken and 100 μl of MeOH and 100 nmol of *m*-hydroxybenzoic acid (as int. standard) were added. After centrifugation, the soln (20 μl) was examined by HPLC. Solvent: H_2O–MeOH–HOAc (77:2:1); detection: 254 nm. A small amount of PHB-*O*-glucoside present in the enzyme extract before incubation was subtracted from the final amount.

Assay for PHB-O-glucosidase activity. The incubation mixture contained in a total vol. of 100 μl: potassium citrate, 5 μmol, and K-Pi, 5 μmol, pH 5.0; *p*-hydroxybenzoic acid-*O*-glucoside, 0.5 μmol; and enzyme extract, 80 μl. After incubation for 120 min at 30°, 100 μl of MeOH and 100 nmol *m*-hydroxybenzoic acid (as

int. standard) were added. After centrifugation, the soln (20 μl) was examined by HPLC. Solvent: H_2O–MeOH–HOAc (77:2:1); detection: 254 nm; R_f: *p*-hydroxybenzoic acid 9.5 min, *m*-hydroxybenzoic acid 12.8 min.

Assay for PAL activity. The incubation mixture contained in a total vol. of 100 μl: sodium borate, pH 8.7, 3.3 μmol; phenylalanine, 0.667 μmol; and enzyme extract, 80 μl. After incubation for 75 min at 30°, the reaction was stopped by addition of 10 μl 5N TCA. 4-Methylumbelliferone (50 nmol) was added as int. standard. After centrifugation, a sample (20 μl) of the soln was examined by HPLC. Solvent: H_2O–MeOH–HOAc (40:60:1); detection: 272 nm; R_f: *t*-cinnamic acid 9.8 min, 4-methylumbelliferone 5.1 min.

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