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GERANYLPYROPHOSPHATE:*p*-HYDROXYBENZOATE GERANYLTRANSFERASE ACTIVITY IN EXTRACTS OF *LITHOSPERMUM ERYTHRORHIZON* CELL CULTURES

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Key word index—*Lithospermum erythrorhizon*; Boraginaceae; cell culture; biosynthesis; naphthoquinones; shikonin; geranyltransferase.

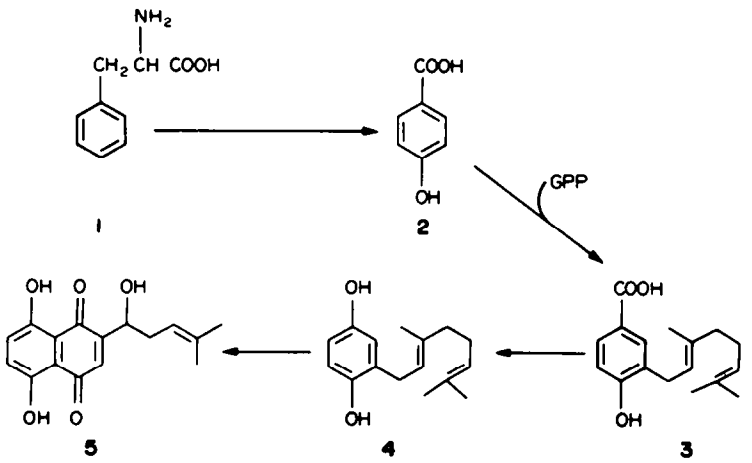
Abstract—The enzymatic formation of *m*-geranyl-*p*-hydroxybenzoic acid from geranylpyrophosphate and *p*-hydroxybenzoic acid was investigated in cell-free extracts of *Lithospermum erythrorhizon* cell cultures. The reaction required the presence of a divalent cation, magnesium being the most effective activator. The enzyme showed a very broad pH optimum between pH 7.1 and 9.3. It was highly specific for both *p*-hydroxybenzoic acid and geranylpyrophosphate, and the apparent K_m values for these two substrates were 0.014 and 0.56 mM, respectively. The activity was located in the pellet of a 100 000 *g* centrifugation, showing that the enzyme is bound to membranes or microsomes. Shikonin-producing cultures contained an activity of this enzyme 35 times higher than non-producing cultures, suggesting that this enzyme is of regulatory importance in shikonin biosynthesis.

INTRODUCTION

The biosynthetic pathway leading to shikonin (Scheme 1) has been elucidated by Inouye *et al.* by tracer experiments using callus cultures of *Lithospermum erythrorhizon* [1]. A key step in this pathway is the formation of *m*-geranyl-*p*-hydroxybenzoic acid (GBA; 3) from *p*-hydroxybenzoic acid (PHB; 2) and geranylpyrophosphate (GPP), linking precursors from the shikimate–phenylalanine pathway and from the isoprenoid pathway which together provide the complete carbon skeleton of shikonin (5).

A similar reaction occurs in ubiquinone biosynthesis, where *m*-polyprenyl-*p*-hydroxybenzoic acid is formed from polyprenylpyrophosphates and *p*-hydroxybenzoic acid [2]. This reaction has been demonstrated in cell-free extracts of bacteria [3–6] and animal tissues [7], as well as in mitochondria isolated from broad beans and yeast [8]. Recently methods have been developed in order to obtain active enzyme preparations from the cultured cells of *Lithospermum* [9]. Using these methods, we could detect the formation of [^{14}C]*m*-geranyl-*p*-hydroxybenzoic acid (3) from [^{14}C]*p*-hydroxybenzoic acid (2) and geranylpyrophosphate in cell-free extracts of shikonin-producing cultures.

This paper describes the further characterization of the geranylpyrophosphate:*p*-hydroxybenzoate geranyltransferase (abbreviation: PHB geranyltransferase) in enzyme extracts of *L. erythrorhizon* cell cultures. The activities of this enzyme in shikonin-producing and shikonin-free cell cultures are compared.



Scheme 1. Biosynthetic route to shikonin proposed by Inouye *et al.* [1].

RESULTS

Radioisotope assay for PHB geranyltransferase activity

As described previously [9], rapid formation of [^{14}C]*m*-geranyl-*p*-hydroxybenzoic acid was observed when enzyme extracts from shikonin-producing cells were incubated with [^{14}C]*p*-hydroxybenzoic acid, geranylpyrophosphate and magnesium chloride. Within 60 min, over 95% conversion of *p*-hydroxybenzoic acid to its geranylated derivative was observed (Fig. 1), and the reaction was linear with the amount of enzyme between 7.5 and 22.5 μg protein per 50 μl incubation volume.

HPLC assay for PHB geranyltransferase activity

The reaction product was partly decomposed on contact with strong acids such as TCA. However, a simple assay procedure involving the termination of the reaction with formic acid and the extraction with a small volume of ethyl acetate finally yielded satisfactory results. Anthracene was used as internal standard. Under the conditions described in the Experimental, retention times were 5.6 min for *m*-geranyl-*p*-hydroxybenzoic acid and 10.6 min for anthracene.

At 37°, the reaction was not only linearly dependent on time for 90 min, but also on the protein amount between 15 and 45 μg per 100 μl incubation volume. No product formation was observed in the absence of GPP or *p*-hydroxybenzoic acid, or with heat-denaturated enzyme. Incubation temperature between 37° and 40° yielded a maximum amount of product.

Stability

The enzyme extract lost more than 90% of its activity upon storage at 4° for 72 hr. If stored frozen at −20°, however, it retained 68% of its activity for 3 days, and 7% for 2 months. Frozen extracts often showed some turbidity after thawing; in such cases, the activity could be increased by homogenization with a Potter homogenizer.

Optimal pH

As Fig. 2 shows, the enzyme exhibits an unusually broad pH optimum between pH 7.1 and 9.3; pH 7.5 was used in the following experiments.

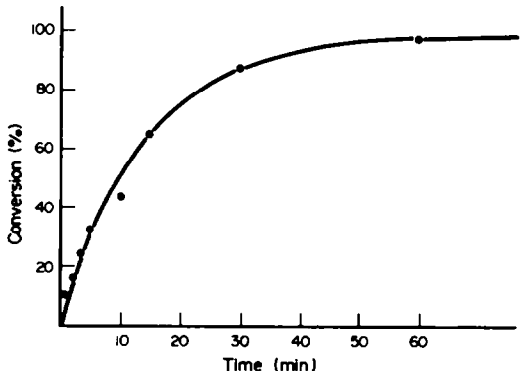


Fig. 1. Time course of the conversion of [^{14}C]*p*-hydroxybenzoic acid into [^{14}C]*m*-geranyl-*p*-hydroxybenzoic acid by cell-free extracts of *L. erythrorhizon* cell cultures. See Experimental for assay conditions.

Optimal concentrations of substrates and magnesium

The optimal concentration of *p*-hydroxybenzoic acid was 1 mM, with some inhibition occurring at higher concentrations (Fig. 3). As for the substrate GPP, the optimal reaction velocity was achieved at concentrations between 2 and 10 mM, whereas the rate of product formation was reduced at 20 mM. From the data shown in Fig. 3, the apparent K_m values for the substrates were calculated as 0.014 mM for PHB and 0.56 mM for GPP. The optimal concentration of magnesium was 10 mM.

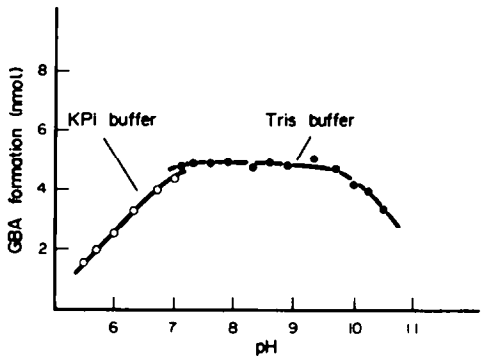


Fig. 2. pH dependency of the PHB geranyltransferase reaction. See Experimental for assay conditions. GBA = *m*-geranyl-*p*-hydroxybenzoic acid.

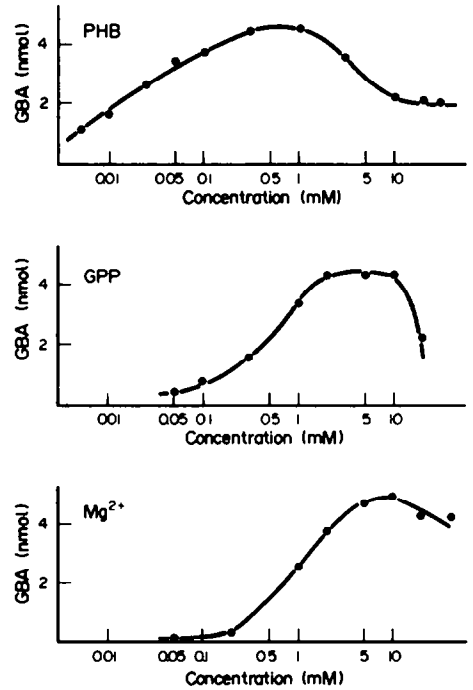


Fig. 3. Dependency of the PHB geranyltransferase reaction on the concentrations of *p*-hydroxybenzoic acid, geranylpyrophosphate and magnesium. Reaction velocities for PHB concentrations below 0.1 mM were determined by radioisotope assay (incubation time 5 min), all others by HPLC assay (incubation time 30 min). See Experimental for assay conditions. Product formation is expressed as nmol product/30 min/100 μl standard incubation mixture. GBA = *m*-geranyl-*p*-hydroxybenzoic acid.

Metal ion requirements

Table 1 shows that the presence of a divalent metal cation was essential for the reaction. Magnesium led to the highest product formation. Cobalt and, to a lesser extent, nickel also activated the PHB geranyltransferase, whereas manganese had only a very slight effect, and no product formation was observed with zinc, copper, iron or calcium. All cations except magnesium caused some precipitation in the incubation mixture (pH 7.5), which may be responsible for the fact that some ions reduced product formation below the level found in the complete absence of any metal cation.

Sedimentation properties

Supernatants obtained from the homogenized cells by centrifugation at 300 *g* and 10 000 *g*, respectively, showed similar activities of PHB geranyltransferase (data not shown). Centrifugation at 100 000 *g*, however, resulted in a nearly complete lack of the activity in the supernatant (Table 2), suggesting that the enzyme is bound to membrane fragments or microsomes.

Substrate specificity for p-*hydroxybenzoic acid*

Several aromatic acids and phenolics were tested as substrates for the enzyme, using each compound at a concentration of 1 mM. The formation of a GPP-dependent product was observed by HPLC. Except for PHB, only salicylic acid was to some degree converted into an unidentified product with GPP (retention time 10.3 min under the conditions described in the Experimental), but the reaction velocity, calculated from the molar extinction of salicylic acid, appeared to be only 3.3% of the rate obtained with PHB. No product formation was observed with *m*-hydroxybenzoic acid, benzoic acid, *trans*-cinnamic acid or *p*-coumaric acid. Also homogentisic acid, the substrate of prenylation in the biosynthesis of plastoquinone [10] and tocopherol [11, 12], was not accepted as substrate for the PHB geranyltransferase, nor was hydroquinone.

Substrate specificity for geranylpyrophosphate

When crude enzyme extracts were incubated with PHB, $MgCl_2$, dimethylallylpyrophosphate (DMAPP) and isopentenylpyrophosphate (IPP), *m*-geranyl-*p*-hydroxybenzoic acid was formed at the same rate as in the presence of GPP (Table 3), suggesting the presence of a very active prenyltransferase that synthesizes GPP from DMAPP and IPP. When either DMAPP or IPP was used singly, *m*-geranyl-*p*-hydroxybenzoic acid was still formed (identified by TLC in solvent A and by HPLC), but the reaction velocity was reduced to 35–40% of the value obtained with GPP. This indicates that the extract also contained an IPP isomerase, converting IPP into DMAPP and vice versa. These enzymes were soluble, in contrast to PHB geranyltransferase, and the enzyme fraction obtained from the pellet of 100 000 *g* centrifugation formed only traces of *m*-geranyl-*p*-hydroxybenzoic acid when incubated with DMAPP, IPP or both (Table 3).

On the other hand, the radioactive assay revealed that an enzyme sedimented at 100 000 *g* utilized DMAPP to form, besides traces of *m*-geranyl-*p*-hydroxybenzoic acid, a minute amount of an unidentified product slightly more polar than the former acid, likely to be *m*-dimethylallyl-*p*-hydroxybenzoic acid. No corresponding product could be detected when using IPP, and also farnesylpyrophosphate (FPP) was not accepted as a substrate (data not shown).

PHB geranyltransferase activity in shikonin-producing and shikonin-free cell cultures

Extracts of the shikonin-producing strain M18 on M9 medium and the shikonin-free strain LY on LS medium were prepared and assayed as described in the Experimental. The extract of the shikonin-producing culture showed a much higher activity (68.4 pKat/mg protein) of the PHB geranyltransferase than the one of the non-producing culture (1.94 pKat/mg protein). Some activity, however, could still be detected in the completely shikonin-free LY strain.

Table 1. Influence of different metal ions on the PHB geranyltransferase reaction

Metal added (10 mM)	GBA* formation (nmol)	Relative activity (%)
None	0.09	1.5
$MgCl_2$	6.05	100
$CoCl_2$	2.86	47.2
Ni $(OAc)_2$	1.40	23.2
$MnCl_2$	0.18	2.9
$ZnCl_2$	< 0.03	< 0.5
$CuCl_2$	< 0.03	<0.5
$CaCl_2$	< 0.03	< 0.5
$FeSO_4$	< 0.03	< 0.5

See Experimental for assay conditions.
*GBA = *m*-geranyl-*p*-hydroxybenzoic acid.

Table 2. Sedimentation properties of the PHB geranyltransferase

Enzyme solution	GBA* formation (nmol)	Relative activity (%)
Crude enzyme extract	6.71	100
Supernatant 100 000 *g*	0.27	4
Pellet 100 000 *g*	6.06	90
Pellet + supernatant	6.44	96

Five ml of crude enzyme extract (0.54 mg protein/ml) were centrifuged at 100 000*g* for 60 min. The pellet was resuspended in 5 ml Tris–HCl buffer (0.1 M, pH 7.5) containing 10 mM DTT, and centrifuged again under the same conditions. The resulting pellet was resuspended in 1.25 ml of the same buffer (final protein concentration 0.42 mg/ml). The incubation mixtures (100 µl) contained 64 µl of the crude enzyme extract, or 64 µl of the supernatant, or 16 µl of the suspended pellet, or 64 µl supernatant plus 16 µl suspended pellet, and substrates as described in the Experimental.
*GBA = *m*-geranyl-*p*-hydroxybenzoic acid.

Table 3. Formation of *m*-geranyl-*p*-hydroxybenzoic acid (GBA) from PHB and different prenylpyrophosphates in cell-free extracts of *L. erythrorhizon* cell cultures

Prenyl pyrophosphate (2 mM)	Crude enzyme extract GBA* formation (nmol)	Crude enzyme extract Relative activity (%)	Pellet 100000 *g*† GBA formation (nmol)	Pellet 100000 *g*† Relative activity (%)
Geranylpyrophosphate	6.91	100	6.06	100
Dimethylallylpyrophosphate	2.47	36	0.09	1.5
Isopentenylpyrophosphate	2.75	40	< 0.03	< 0.5
Dimethylallylpyrophosphate + isopentenylpyrophospate	6.84	99	0.11	1.8

*GBA = *m*-geranyl-*p*-hydroxybenzoic acid.
†See Table 2 for conditions of 100000 *g* centrifugation.

DISCUSSION

The geranylpyrophosphate: *p*-hydroxybenzoate geranyltransferase (PHB geranyltransferase) reported in this paper is the first enzyme of shikonin biosynthesis to be detected *in vitro*. Its demonstration has become possible by the development of methods which avoid the irreversible enzyme inhibition usually encountered during the preparation of cell-free extracts from this culture, and to obtain active enzyme solutions [9]. The detection of PHB geranyltransferase in *L. erythrorhizon* cultures confirms the current concept of shikonin biosynthesis [1].

The PHB geranyltransferase showed high substrate specificity for both GPP and PHB. This proves that the activity detected is in fact due to a specific enzyme involved in shikonin biosynthesis, and not to enzymes catalysing other prenylation reactions, such as *p*-hydroxybenzoate polyprenyltransferase involved in ubiquinone biosynthesis [8], homogentisate polyprenyltransferase participating in the biosynthesis of plastoquinone and tocopherol [10–12] and 1,4-dihydroxynaphthoate polyprenyltransferase of phylloquinone biosynthesis [13].

The PHB geranyltransferase did not sediment at 10000 *g*, but nearly completely in 100000 *g* centrifugation (Table 2), suggesting that it is bound to membrane fragments or microsomes. Earlier electron microscopic studies of cultured cells of *L. erythrorhizon* have shown that shikonin production is associated with the development of a highly elongated endoplasmic reticulum, and it has been suggested that the ER is actually the site of shikonin biosynthesis [14]. The sedimentation properties of the PHB geranyltransferase are in accordance with this hypothesis. Since the earlier intermediates in shikonin biosynthesis are fairly hydrophilic, and some evidence was obtained in the present study that the enzymes providing geranylpyrophosphate for this pathway are soluble, it is possible that the prenylation of *p*-hydroxybenzoic acid is the first step in shikonin biosynthesis to be carried out by a membrane-bound enzyme. In the following reaction steps leading to shikonin (Scheme 1), intermediates become more lipophilic, and the reported localized swellings of the ER [14] might be the place where these reactions are carried out.

Extensive studies have been made on various factors promoting or inhibiting the production of shikonin in *L. erythrorhizon* cell cultures [15–22]. However, since no enzyme activities involved in shikonin biosynthesis could be measured, evidence as to which steps in this pathway are of regulatory importance has been sparse. The PHB geranyltransferase reported now in this paper catalyses a key step in shikonin biosynthesis, linking precursors of the shikimate pathway and the isoprenoid pathway, which together provide all carbon atoms of the shikonin molecule. Interestingly, the activity of this enzyme in shikonin-producing cultures was found to be approximately 35 times higher than in non-producing cultures. This strongly suggests that the enzyme is of regulatory importance for the formation of shikonin.

The presence of a low level of PHB geranyltransferase even in a completely shikonin-free culture corresponds to the results of earlier studies [1, 23] which revealed that *m*-geranyl-*p*-hydroxybenzoic acid is still formed in shikonin-free cultures, though in minute quantities. The non-producing cultures, therefore, must lack one or several of the enzymes converting *m*-geranyl-*p*-hydroxybenzoic acid into shikonin. Some evidence has been presented [1] that the blocked step may be the conversion of *m*-geranyl-*p*-hydroxybenzoic acid into geranylhydroquinone.

Further studies on PHB geranyltransferase will be necessary in order to clarify its role in the regulation of shikonin biosynthesis, as well as its exact intracellular localization. Attempts to purify the enzyme and to characterize it in detail will first require its solubilization from the membranes to which it is bound.

EXPERIMENTAL

Radiochemicals. [Carboxyl-^{14}C]*p*-hydroxybenzoic acid (50 Ci/ mol) was obtained from CEA, France.

TLC solvent systems. A = $CHCl_3$–MeOH–HOAc (100:2:1); B = hexane–MeCOEt (8:2).

HPLC. Column TSK-Gel ODS 120 A 10 μm (Toyo Soda, Japan), 150 × 4.6 mm; solvent system $CHCl_3$–MeOH–HOAc (75:25:0.3); flow rate 1.5 ml/min; detection: absorption at 257 nm.

Cell cultures. The callus cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. were derived from germinating seeds [15]. By selection from the heterogeneous callus culture, strain M18 capable of producing shikonin and strain LY incapable of producing this pigment were obtained [24]. Cell suspension cultures were initiated and maintained as described elsewhere [20]. To obtain shikonin-producing cultures, cells of the strain M18 (inoculum size: 1.1 g) were transferred from LS medium into 100 ml Erlenmeyer flasks containing 30 ml of M9 medium

[17, 18] supplemented with 10^{-6} M IAA, 10^{-5} M kinetin, and 5 ml liquid paraffin (ampoule quality, Merck) [9]. To obtain shikonin-free cultures, cells of strain LY were cultured in LS medium supplemented with the same hormones.

Preparation of cell-free extracts. Cells (5 g) were suspended in KPi buffer (10 ml, 0.1 M, pH 6.5), containing 10 mM DTT and 0.5 g PVPP. They were ruptured in a Potter homogenizer and centrifuged at 10000 *g* for 10 min. The supernatant was gel-filtered through Sephadex G 25 (PD 10 column) equilibrated with Tris-HCl buffer (0.1 M, pH 7.5) containing 10 mM DTT.

Protein content. Determined according to the method of Bradford [25].

HPLC assay for PHB geranyltransferase activity. Unless stated otherwise, the HPLC assay was used. The incubation mixture contained in a total vol. of 100 μl: *p*-hydroxybenzoic acid, 0.1 μmol; GPP, 0.2 μmol; $MgCl_2$, 1 μmol; and enzyme extract, 80 μl (= 40 μg protein). After incubation for 30 min at 37°, the reaction was stopped by cooling the reaction mixture to 0° and addition of 5 μl formic acid. 2 nmol anthracene were added as internal standard, and the reaction mixture was extracted with 150 μl EtOAc. After centrifugation, 20 μl of the EtOAc layer were examined by HPLC.

Radioisotope assay for PHB geranyltransferase activity. The incubation mixture contained in a total vol. of 50 μl: [carboxyl-^{14}C]*p*-hydroxybenzoic acid, 0.5 nmol (25 nCi); geranylpyrophosphate, 100 nmol; $MgCl_2$, 500 nmol; enzyme extract, 40 μl (= 20 μg protein). After incubation for 30 min at 37°, the reaction was terminated by cooling to 0° and addition of 5 μl formic acid. Unlabelled *p*-hydroxybenzoic acid and *m*-geranyl-*p*-hydroxybenzoic acid (0.1 mg each) were added as carriers, and the soln was directly applied to TLC on silica gel in solvent system A. Chromatograms were examined with a radio scanner.

Chemical synthesis of m-*geranyl*-p-*hydroxybenzoic acid.* Performed according to the method of Inouye *et al.* [1].

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