Pergamon 0031-9422(94)00821-3 Phytochemistry, Vol. 38, No. 5, pp. 1127-1130, 1995

0031-9422/95 $9.50 + 0.00

INTRACELLULAR LOCALIZATION OF UDPG: *p*-HYDROXYBENZOATE GLUCOSYLTRANSFERASE AND ITS REACTION PRODUCT IN *LITHOSPERMUM* CELL CULTURES

K. YAZAKI,* K. INUSHIMA, M. KATAOKA and M. TABATA

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida, Kyoto 606-01, Japan

(*Received* 15 *August* 1994)

Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; biosynthesis; subcellular localization; *p*-*O*-*β*-D-glucosylbenzoic acid; *p*-hydroxybenzoic acid; glucosyltransferase; *β*-glucosidase; cell culture.

Abstract—Intracellular localization of *p*-*O*-*β*-D-glucosylbenzoic acid (PHBOG) and its aglycone, *p*-hydroxybenzoic acid (PHB), a key intermediate of shikonin biosynthesis, was investigated in *Lithospermum erythrorhizon* cell cultures. The glucoside, which is characteristically accumulated in shikonin-free *Lithospermum* cell cultures, proved to be localized mainly in the vacuoles of cells. *p*-Hydroxybenzoate glucosyltransferase, however, was not detected in the vacuoles but exclusively in the cytosol of cells. Furthermore, *β*-D-glucosidase, which hydrolyses PHBOG to yield PHB, was also located in the cytosol. These results suggest that PHB glucosylated in the cytosol is transported into the vacuole to be stored in a large quantity under protection from *β*-D-glucosidase in shikonin non-producing cells, until utilization as a precursor upon induction of shikonin biosynthesis.

INTRODUCTION

Cell cultures of *Lithospermum erythrorhizon* are capable of producing large amounts of red naphthoquinone shikonin pigments and its derivatives, when cultured in M9 production medium [1–3]. Shikonin production, however, is completely suppressed when the cells are subcultured in Linsmaier–Skoog's (LS) liquid medium [4] as a growth medium containing ammonium in place of nitrate [1, 2]. Under such conditions, shikonin biosynthesis is halted mainly at the prenylation step of *p*-hydroxybenzoic acid (PHB), which results in the accumulation of the *p*-*O*-*β*-D-glucoside of the phenolic acid (PHBOG) [5]. When shikonin production is induced in M9 medium or by addition of certain acidic oligosaccharide [6], the glucoside is increasingly hydrolysed to yield PHB, which is used for the synthesis of shikonin and other quinone derivatives, including dihydroechinofuran [7]. Although the enzymes involved in the early steps of shikonin biosynthesis, e.g. phenylalanine ammonia lyase (PAL) [8], are thought to be localized in the soluble fraction, such as the cytosol, PHB: geranyltransferase has been detected in the microsomal fraction containing specific vesicle membranes derived from the endoplasmic reticulum (ER) [9]. In this connection, it is of interest to know where PHBOG might be synthesized or accumulated and how the glucoside linkage would be cleaved to supply the substrate PHB to geranyltransferase. This paper deals with the subcellular localization of PHBOG and of the two enzymes catalysing synthesis and hydrolysis of this compound.

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Protoplasts were prepared from *Lithospermum* cells, which had been cultured in LS liquid medium supplemented with 10^{-6} M 3-indoleacetic acid and 10^{-5} M kinetin for two weeks in the dark, by incubating them in 0.7 M mannitol solution containing 2% cellulase Onozuka RS and 0.3% Pectolyase Y-23 for 2 hr. In order to obtain a higher yield of protoplasts, basal inorganic elements of the LS medium were added to the enzyme solution. After filtering the protoplast suspension through nylon mesh, protoplasts were collected by centrifugation, followed by washing with an enzyme-free LS-mannitol solution. Vacuoles were liberated from protoplasts by incubating them in a high concentration of potassium phosphate buffer at pH 8. Under these conditions, intact protoplasts were rarely observed under the microscope. After filtering the vacuole suspension through a nylon mesh, vacuoles were collected by gentle centrifugation.

The contents of PHBOG and PHB in the protoplasts were compared with those in the vacuoles by HPLC analysis. The amounts of PHBOG were 116 nmol 10^{-6} protoplasts and 104 nmol 10^{-6} vacuoles (Table 1), implying that *ca* 90% of PHBOG in the cell was located in the vacuoles. Interestingly, the aglycone, PHB, was also found exclusively in the vacuoles, although its content

Table 1. Intracellular localization of PHBOG and PHB in *Lithospermum* cells cultured in LS liquid medium

	Protoplasts (*A*)	Vacuoles (*B*)	$\frac{B \times 100(\%)}{A + B}$
PHBOG content	116 nmol 10^{-6}	104 nmol 10^{-6}	89.7
Concn of PHBOG*	12.6 mM	11.3 mM	
PHB content	4.15 nmol 10^{-6}	4.40 nmol 10^{-6}	106
Concn of PHB*	451 μM	478 μM	

*Concentrations of compounds in protoplasts or vacuoles calculated on the basis of their volumes determined by microscopic measurements.

Table 2. Intracellular localization of PHB: glucosyltransferase and β-glucosidase in *Lithospermum* cells cultured in LS liquid medium

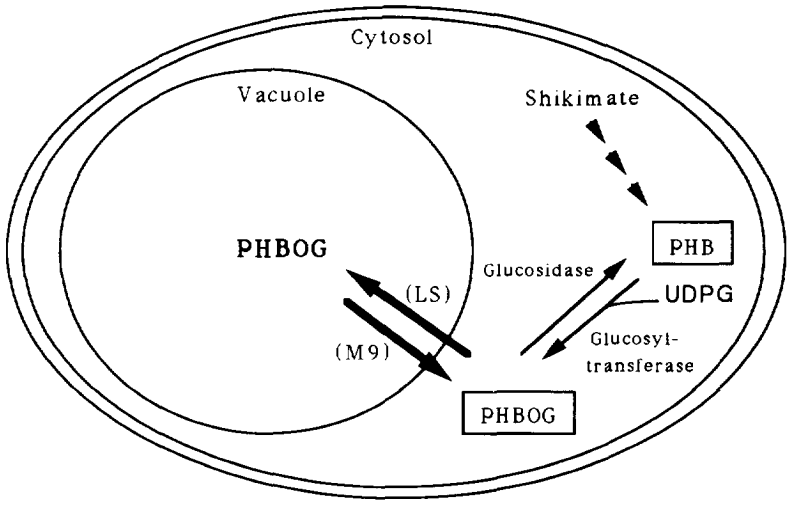
	Protoplasts (*A*)	Vacuoles (*B*)	$\frac{B \times 100(\%)}{A + B}$
PHB: glucosyltransferase (pkat 10^{-6})	1.69	0.163	9.64
β-Glucosidase (pkat 10^{-6})	24.6	2.04	8.29

was as small as 4% of the PHBOG content, as previously observed in freshly cultured cells [5].

To investigate the intracellular localization of PHB: glucosyltransferase and β-glucosidase [10], crude enzyme solutions were prepared from protoplasts and vacuoles of *Lithospermum* cell cultures, respectively. Plasma membranes and tonoplast membranes were burst by sonication after resuspending protoplasts or vacuolar pellets in an extraction buffer, which was followed by centrifugation. To obtain a protein fraction, the supernatant was gel filtered on a column which had been equilibrated with either Tris–HCl buffer (pH 7.5) for glucosyltransferase or with sodium acetate buffer (pH 5) for β-glucosidase. In sharp contrast to the substrate compounds, both enzyme activities were mainly found in protoplasts, whereas vacuoles exhibited only 8–9% of these activities (Table 2). These enzyme activities detected in the vacuoles are considered to be owing to contamination by some protoplasts, which could not be eliminated by centrifugation. Furthermore, the vacuolar pellet could not be washed thoroughly because of the instability of the very delicate vacuoles, so that it must have contained an appreciable contamination of the cytosol showing high activities of both glucosyltransferase and β-glucosidase.

The present study has clearly demonstrated that PHBOG is accumulated chiefly in the vacuoles of shikonin-free *Lithospermum* cells cultured in LS medium, while its aglycone, PHB, was hardly detected in the protoplasts. This observation is in agreement with the finding that PHB: glucosyltransferase activity [10] is primarily located in the cytosol fraction of the cell. It suggests that PHB molecules synthesized in the cytosol would immediately be glucosylated and then rapidly transported into vacuoles when *Lithospermum* cells are not producing shikonin in LS medium (Scheme 1). When the cells are transferred to the production medium M9 [1, 2], however, PHBOG would be quickly hydrolysed to yield PHB, which is further prenylated to form *m*-geranyl-*p*-hydroxybenzoic acid [8, 11], a key intermediate in the biosynthetic pathway leading to shikonin and dihydroechinofuran [6]. The fact that β-glucosidase activity to liberate PHB from PHBOG was largely found in the cytosol seems to suggest that the tonoplast membrane protects PHBOG stored in the vacuole from attack by β-glucosidase present in the cytosol. Upon the induction of shikonin production, on the other hand, the glucoside would probably be transported to the outside of the vacuole, exposing itself to cytosolic β-glucosidase to release PHB used for shikonin synthesis.

The presence of various glucosyltransferases in the cytosol have been reported for *Melilotus alba* [12], *Hordeum vulgare* [13], *Hippeastrum* and *Tulipa* [14], whereas glucosides of phenolic compounds, such as glucosyloxycinnamic acid [12], esculin [13] and anthocyanin [14], have been shown to be localized in vacuolar fractions as 'the end products' of the biosynthetic activities. In the case of phenolic acid esters of malic acid, although the biosynthetic enzyme, L-malate hydroxycinnamoyltransferase, has been reported to be delocalized in the vacuoles of *Raphanus sativus* [15, 16], the products are also accumulated in the vacuoles; they are not further metabolized. In *Lithospermum* cells, however, PHBOG once accumulated in the vacuoles would be reutilized with the induction of shikonin biosynthesis, suggesting that the vacuole is not only an organelle for accumulating waste products, but a flexible compartment for storing a biosynthetic intermediate. Thus, the *Lithospermum* culture system seems to offer an interesting



Scheme 1. Hypothetical model illustrating the compartmentation of *p*-hydroxybenzoic acid (PHB) and its glucoside (PHBOG) in *Lithospermum* cells and the localization of the biosynthetic enzymes. LS: LS medium; M9: M9 medium.

model for investigating the mechanisms of biochemical compartmentation relevant to secondary metabolism in plant cells.

EXPERIMENTAL

Culture method. Culture strain M18 of *L. erythrorhizon* Sieb. et Zucc. [17] was grown in a 500 ml-flask containing 120 ml LS liquid medium [4] supplemented with 10^{-6} M 3-indoleacetic acid and 10^{-5} M kinetin on a reciprocal shaker (100 strokes min^{-1}) at 25° in the dark. They were subcultured to the same medium at intervals of 2 weeks.

Prepn of protoplasts and vacuoles. Fr. cells (30 g) were harvested by filtration through Miracloth (Calbiochem) 14 days after inoculation. These cells were incubated in 300 ml of an enzyme soln composed of 2% Cellulase Onozuka RS (Yakult Co. Ltd), 0.3% Pectolyase Y-23 (Seishin Co.) and 0.7 M mannitol in basal elements of LS medium, adjusted to pH 6.5, at 25° and agitated on a reciprocal shaker (40 strokes min^{-1}) for 2 hr. The suspension was then filtered through a nylon mesh (62 μm) under gravity to remove cell aggregates, followed by centrifugation at 145 *g* for 3 min in 25 Falcon tubes (15 ml), each containing a layer of 22% sucrose soln (2 ml) as a cushion for protoplasts. After collecting protoplasts on the sucrose layer, they were washed (× 3) with 0.7 M mannitol in LS basal soln by centrifugation. An aliquot of the protoplast pellet was used for quantitative analysis of PHBOG and for enzyme assay, while the rest of the pellet was gently resuspended in a vacuole-liberating buffer (0.15 M K-Pi, pH 8) and the number of protoplasts remaining in the suspension was monitored by microscopic observation. During standing for 30 min at 4°, plasma membranes were burst by the shock of a high concn of Pi, as well as a change in pH. The vacuole suspension was gently stirred with a Pasteur pipette and filtered through nylon mesh (62 μm) to remove slimy cell debris. Vacuoles thus isolated were collected by centrifugation at 120 *g* for 3 min. The number of protoplasts or vacuoles were counted microscopically with the aid of a hematocytometer (0.2 mm deep, Erma).

Quantitative analyses of PHBOG and PHB in protoplasts and vacuoles. To the pellet of isolated protoplasts or vacuoles (10 μl), 40 μl of MeOH was added and suspension was mixed by Vortex mixer to destroy both plasma and tonoplast membranes completely. After centrifugation to remove cell debris, the supernatant was subjected to reversed phase HPLC, which was conducted under the following conditions: column: TSK-gel ODS 120A (φ 10 μm, 4.6 mm i.d. × 150 mm); flow rate 1.5 ml min^{-1}; detection: *A* at 254 nm; solvent system: H_2O–HOAc (79:1).

Enzyme assay. To the pellet of protoplasts or vacuoles, 500 μl of 0.1 M K-Pi buffer (pH 6.5) containing 10 mM dithiothreitol (DTT) and 30 mg PVPP was added, and the suspension homogenized using a sonicator with a microtip at intervals of 1 sec pause per 4 sec impose for a total period of 1 min. The homogenate was centrifuged at 12 000 *g* for 15 min at 4° to obtain a supernatant (500 μl), which was subsequently loaded on to a NAP-5 column (Pharmacia) equilibrated with 0.2 M Tris–HCl buffer (pH 7.5) containing 2 mM DTT for glucosyltransferase activity, or with 0.1 M Na-acetate buffer (pH 5) containing the same concn of DTT as above for β-glucosidase [10]. These gel-filtered protein frs were used as cell-free extracts for assaying the activities of the two enzymes as follows. (1) PHB glucosyltransferase activity was measured in an incubation mixt. of 100 μl containing 2.5 μmol PHB, 20 μmol UDP-glucose and 80 μl cell-free extract. (2) β-Glucosidase activity was measured in an incubation mixt. (100 μl) of 2.5 μmol PHBOG and cell-free extract 90 μl. Both mixts were incubated at 30° for 90 min and the enzyme reactions terminated by adding 20% TCA. After centrifugation to remove protein ppt., the supernatant

was subjected to HPLC in order to quantitate the enzyme reaction product, PHBOG or PHB.

REFERENCES

1. Fujita, Y., Hara, Y., Ogino, T. and Suga, C. (1981) *Plant Cell Rep.* **1**, 59.
2. Fujita, Y., Hara, Y., Suga, C. and Morimoto, T. (1981) *Plant Cell Rep.* **1**, 61.
3. Tabata, M. and Fujita, Y. (1985) in *Biotechnology in Plant Science* (Day, P., Zaitlin, M. and Hollaender, A., eds), p. 207. Academic Press, Orland.
4. Linsmaier, E. F. and Skoog, F. (1965) *Physiol. Plant.* **18**, 100.
5. Yazaki, K., Fukui, H. and Tabata, M. (1986) *Phytochemistry* **25**, 1629.
6. Tani, M., Takeda, K., Yazaki, K. and Tabata, M. (1993) *Phytochemistry* **34**, 1285.
7. Fukui, H., Tani, M. and Tabata, M. (1992) *Phytochemistry* **31**, 519.
8. Heide, L., Nishioka, N., Fukui, H. and Tabata, M. (1989) *Phytochemistry* **28**, 1873.
9. Yamaga, Y., Nakanishi, K., Fukui, H. and Tabata, M. (1993) *Phytochemistry* **32**, 633.
10. Bechthold, A., Berger, U. and Heide, L. (1991) *Arch. Biochem. Biophys.* **288**, 39.
11. Yazaki, K., Fukui, H. and Tabata, M. (1986) *Chem. Pharm. Bull.* **34**, 2290.
12. Oba, K., Conn, E. E., Canut, H. and Boudet, A. M. (1981) *Plant Physiol.* **68**, 1359.
13. Werner, C. and Matile, P. (1985) *J. Plant. Physiol.* **118**, 237.
14. Hrazdina, G., Wagner, G. J. and Siegelman, H. W. (1978) *Phytochemistry* **17**, 53.
15. Sharma, V. and Strack, D. (1985) *Planta* **163**, 563.
16. Strack, D. and Sharma, V. (1985) *Physiol. Plant.* **65**, 45.
17. Mizukami, H., Konoshima, M. and Tabata, M. (1978) *Phytochemistry* **17**, 95.