Phytochemistry, Vol. 29, No. 5, pp. 1555–1558, 1990.
Printed in Great Britain.

0031–9422/90 $3.00 + 0.00

GLUCOSYLATION OF ISOMERIC HYDROXYBENZOIC ACIDS BY CELL SUSPENSION CULTURES OF *MALLOTUS JAPONICUS*

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(*Received* 19 *October* 1989)

Key Word Index—*Mallotus japonicus*; Euphorbiaceae; glucosylation; biotransformation; plant cell culture; hydroxybenzoic acid; salicylic acid-β-D-glucoside.

Abstract—Cultured cells of *Mallotus japonicus* converted exogenous *o*-hydroxybenzoic acid into its *O*-glucoside after a lag period of 8 hr during which time the aglycone was taken up rapidly by the cells, partly excreted and then re-absorbed. The glucosylation of the aglycone into *o*-*O*-β-D-glucosylbenzoic acid began almost simultaneously with the induction of glucosyltransferase activity, and *ca* 78% of the aglycone administered was transformed into the glucoside in 12 hr. On the other hand, *m*- and *p*-hydroxybenzoic acids were glucosylated immediately after administration, the latter yielding both its *O*-glucoside and glucose ester. Inhibitor experiments suggested the possible participation of either 70S or 80S ribosomes in the glucosylation of isomeric hydroxybenzoic acids.

INTRODUCTION

It has recently been demonstrated that various plant cell cultures are capable of glucosylating exogenously administered aromatic compounds, such as simple phenols, coumarins, and flavonoids [1–4]. Interestingly, we have found that a culture strain of *Mallotus japonicus* has the ability to glucosylate hydroxybenzoic acids including *o*-hydroxybenzoic acid, an acid which is poorly transformed by the other cultures tested [4]. The present study was undertaken to investigate the kinetics of glucosylation of three isomers of hydroxybenzoic acid in cultured *Mallotus* cells.

RESULTS

The time course of glucosylation for each isomer of hydroxybenzoic acid administered to *Mallotus* cell suspension cultures at the early stationary growth stage (14 days after inoculation) is shown in Fig. 1. About 57% of the *o*-hydroxybenzoic acid (OHB) added to the medium was absorbed by the cells in an hour, after which time its content in the cells gradually decreased (Fig. 1A). This decrease was accompanied by a corresponding increase in the amount of OHB in the medium from 42% at 1 hr to 65% at 4 hr after administration of the substrate, indicating that the cells had released a large part of OHB into the medium after excess absorption. Eight hours after administration of OHB, the cells began to form *o*-*O*-β-D-glucosylbenzoic acid (OHB-OG) exclusively, reabsorbing OHB from the medium slowly over a period of 8 hr. After 20 hr, 78% of the OHB supplied had been converted into OHB-OG and the glucoside was stored unchanged in the cells for at least 72 hr after administration of the substrate. The formation of the glucose ester of OHB, however, was not detected during the culture period.

In the case of *m*-hydroxybenzoic acid (MHB), the cells took up 70% of the MHB administered to the medium in 30 min, and the formation of *m*-*O*-β-D-glucosylbenzoic acid began immediately after administration. Approximately 40% of the MHB administered was converted into its glucoside in 8 hr (Fig. 1B). Unlike OHB, MHB

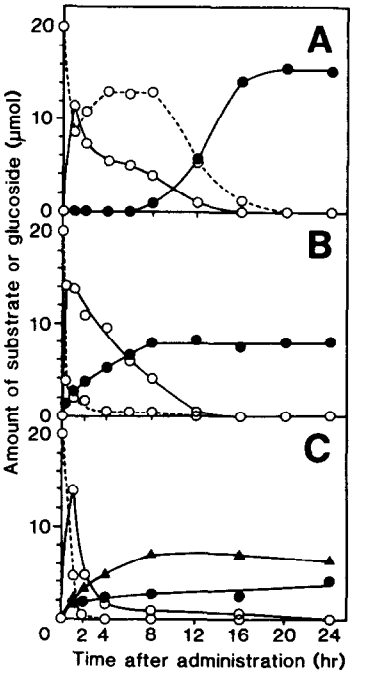

Fig. 1. Changes in the amounts of substrate and glucoside in the cells (—) and the medium (---) of *Mallotus* suspension cultures after the administration of 20 μmol OHB (A), 20 μmol MHB (B) or 20 μmol PHB (C) to 30 ml of medium. Points represent the mean values of 4 replicates. Graph A: ○, OHB; ●, OHB-OG; Graph B: ○, MHB; ●, MHB-OG; Graph C: ○, PHB; ●, PHB-OG; ▲, PHB-COOG.

absorbed by the cells was not released into the medium.

p-Hydroxybenzoic acid (PHB) was completely absorbed by the cells within 4 hr and rapidly transformed not only to its *O*-glucoside (PHB-OG) but simultaneously to its glucose ester (PHB-COOG) (Fig. 1C). About 50% of the total PHB was recovered in the form of the two glucosides 8 hr after administration, the rest apparently having been metabolized into unidentified compounds.

Changes in the glucosyltransferase activity of the cells after the administration of OHB or PHB were studied in relation to the formation of glucosylated products. These glucosyltransferase activities were detected only in the presence of UDP-glucose as a glucose donor. As shown in Fig. 2A, the OHB glucosyltransferase activity induced 8 hr after OHB administration continued to increase until it reached a peak at 16 hr after which time it declined sharply. The time-course of this enzyme activity is in accordance with that of OHB-OG formation.

Glucosyltransferase glucosylating PHB into PHB-OG was found to be constitutive. Its activity was remarkably stimulated 3 hr after PHB administration, even though it was somewhat influenced by the addition of 70% EtOH (0.2 ml/30 ml of medium) used as a vehicle for PHB (Fig. 2B). On the other hand, the activity of another glucosyltransferase yielding PHB-COOG exhibited two peaks at 2 and 10 hr after substrate administration (Fig. 2C). In this case, 70% EtOH had no effect on enzyme activity. These results suggest that the isomeric glucosides of PHB are synthesized by two different position-specific glucosyltransferases.

To investigate whether the glucosylation of hydroxybenzoic acids is regulated at a level of gene expression, transcriptional or translational inhibitors were added to the cell cultures 24 hr prior to the administration of a substrate. The results given in Table 1 show that the glucosylation of OHB was inhibited either by acridine orange, an intercalating agent [5], or by cycloheximide, an inhibitor of protein synthesis on 80S eukaryotic cytoplasmic ribosomes [6]. However, chloramphenicol, an inhibitor of protein synthesis on 70S ribosomes [7], was stimulatory rather than inhibitory to the formation of OHB-OG. These results suggest that a nuclear-encoded cytoplasmic protein-synthesizing system is involved in the synthesis of OHB glucosyltransferase which

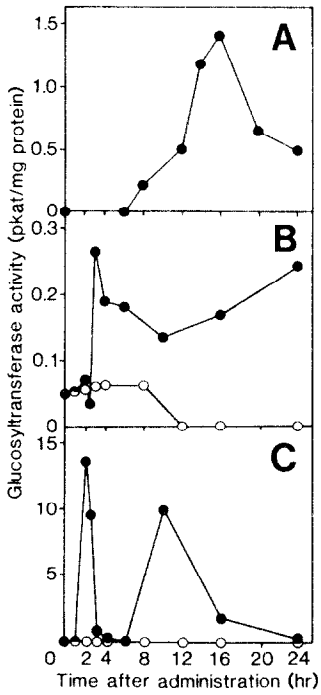

Fig. 2. Changes in glucosyltransferase activities after the administration of 20 μmol substrate (●) or 0.2 ml of 70% EtOH (○) to 30 ml of medium. Points represent the mean values of 2 to 3 replicates. Graph A: OHB (OH) glucosyltransferase; Graph B: PHB(OH) glucosyltransferase; Graph C: PHB(COOH) glucosyltransferase.

Table 1. Effects of transcriptional and translational inhibitors on glucoside formation in cultured *Mallotus* cells

Inhibitor	Concentration (μg/ml)	Relative amount of glucoside formation (%)*			
		OHB-OG	MHB-OG	PHB-OG	PHB-COOG
Acridine orange	250	79	102	117	92
	500	10	94	138	88
	1000	4	87	130	86
	2000	0	82	116	83
Cycloheximide	1	103	97	125	103
	4	69	93	125	100
	16	4	100	117	100
Chloramphenicol	40	102	101	98	104
	80	108	102	97	107
	160	107	84	108	105
	640	117	78	103	104

Each inhibitor was added to the medium 24 hr prior to the administration of substrate (OHB, MHB or PHB).

*Percentage of the control (= 100) without addition of any inhibitor. Values given in the Table represent the means of data obtained from three independent experiments.

is considered to be activated by the administration of OHB to the cells.

Glucosylation of MHB was little affected by cycloheximide but inhibited dose-dependently by either acridine orange or chloramphenicol, suggesting that plastids or mitochondria might participate in the synthesis of MHB glucosyltransferase. In contrast to the conversion of PHB to PHB-OG which unexpectedly was stimulated by either acridine orange or cycloheximide, the formation of PHB-COOG was reduced by the former agent but hardly affected by cycloheximide or by chloramphenicol.

DISCUSSION

The present experiments have shown that a high percentage (57, 81, and 87%) of three isomeric hydroxybenzoic acids (OHB, MHB, and PHB) added to the medium were taken up by *Mallotus* cells within 1 hr after administration. On the assumption that the water content of these cells gives a rough estimate for the volume of cell sap, the intracellular concentrations of OHB, MHB and PHB were calculated to be 2.6, 3.2 and 3.2 mM, respectively, which are at least 10 times higher than the concentrations in the medium. It seems therefore that the cells had actively taken up the hydroxybenzoic acids against a concentration gradient.

The possibility that the peculiar transient release of the absorbed OHB into the medium is due to death or injury of the cells by cytotoxic OHB is unlikely according to the result of an experiment on cell viability estimated by the exclusion of Evan's blue [8]. Furthermore, no decrease in the fresh weight of cells was observed after the administration of OHB at the dosages used in this study, although cell growth was markedly inhibited by the application of a higher dose (100 μmol). Accordingly, the release of OHB appears to be due to an excretory activity of the cells, as reported for other secondary products such as shikonin and berberine [9, 10], although unlike OHB the latter compounds are not re-absorbed by cultured cells of *Lithospermum* and *Thalictrum* respectively. *Mallotus* cells, as shown by the release of OHB but not MHB and PHB, seem to regulate the inflow and outflow of an exogenous substance through an intracellular recognition mechanism of the chemical structure.

The re-absorption of OHB took place concomitantly with the appearance of the glucosyltransferase activity as if the cells had adaptatively prepared for the glucosylation of extrinsic OHB, in order to detoxify or compartmentalize this cytotoxic compound. A preliminary study showed that the glucoside formed was accumulated in the vacuole, as reported for cyanogenic glucosides [11] in sorghum and glucosinolate [12] in horseradish.

The results of inhibitor experiments have suggested that a nuclear-encoded protein-synthesizing system may be involved in the synthesis of OHB-glucosyltransferase, whereas MHB-glucosyltransferase may be synthesized in plastids or mitochondria. On the other hand, PHB, whose transformation to PHB-OG was promoted by cycloheximide, is known to be an intermediate in the biosynthesis of ubiquinone by plants [13], and its glucoside has also been isolated from cultured cells of *Lithospermum erythrorhizon* [14]. Since half of the PHB administered to *Mallotus* cells was metabolized to compounds other than the two glucosides, cycloheximide might have partly inhibited the synthesis of enzymes responsible for ubiquinone formation, directing more PHB to the synthesis of PHB-OG. In this respect, it is of interest that the levels of ubiquinone-6 and of 4-hydroxybenzoate-hexaprenyl transferase activity in derepressed cells of *Saccharomyces* are unaffected by chloramphenicol but inhibited by cycloheximide [15, 16]. Whether such control works also in *Mallotus* cells remains to be verified by further experiments.

EXPERIMENTAL

Plant material and culture. The expts employed a culture strain (MJ-W) established in 1970 from the seedling of *Mallotus japonicus* Muell. Arg., as described previously [4]. Cell suspensions were grown at 25° in the dark in 100 ml Erlenmeyer flasks with 30 ml Linsmaier-Skoog medium [17] containing 10^{-6} M 2,4-D on a reciprocal shaker at 100 strokes/min, and subcultured at an interval of 2 weeks.

Administration of substrate. All the glucosylation expts were carried out with cell suspension cultures cultivated for 14 days after inoculation. OHB, MHB, or PHB (20 μmol) was dissolved in 70% EtOH (0.2 ml) and added to the medium (30 ml). The cultured cells (*ca* 5 g) were harvested by filtration at various intervals after administration and extracted with MeOH (30 ml). The spent medium was immediately frozen and stored in a deep freezer at −20 °. The amounts of aglycone and glucoside in the cells or medium were determined by HPLC.

Preparation of crude enzyme extracts. Cells (60 g) were harvested at intervals (as indicated in Fig. 2) after substrate administration, suspended in 0.2 M Tris–HCl buffer (300 ml, pH 7.0) containing polyvinylpolypyrrolidone (6 g), Amberlite XAD-4 (6 g), dithiothreitol (DTT, 463 mg) and protamine sulphate (300 mg), and homogenized for 3 min (× 3 at intervals of 2 min). The suspension was filtered through Miracloth (Calbiochem), centrifuged at 10000 *g* for 30 min, and the supernatant pptd with 30–70% $(NH_4)_2SO_4$. The ppt. was suspended in 0.2 M Tris–HCl buffer (0.5 ml, pH 7.0) containing DTT (0.77 mg) and dialysed overnight with Spectrapor 6 (Spectrum Medical Industries, Inc.) in the same buffer to remove substances of $M_r < 10000$. Protein content was estimated by the method of ref. [18], after pptn with TCA and dissolution in dilute base. BSA was used as a standard. All operations were performed at 4°.

Glucosyltransferase assay. Glucosyltransferase activity was assayed using a modified method of [19]. A mixture containing 6 μmol Tris–HCl (pH 8.0), 0.3 μmol DTT, 3 μmol UDP-glucose, 3 μmol substrate (OHB or PHB), and enzyme soln in a total vol. of 0.1 ml was incubated at 30° for 30 min. The reaction was terminated by addition of 10 μl 25% TCA, and the pptd material was removed by centrifugation at 1000 *g* for 10 min prior to HPLC.

Measurements of glucosyltransferase activities were carried out under weak alkaline conditions (pH 7.56) to maximize the enzymatic formation of OHB-OG and PHB-OG at the sacrifice of a partial hydrolysis of PHB-COOG formed.

Inhibitor experiments. The inhibitor (acridine orange, cycloheximide, or chloramphenicol) in 25 μl H_2O or 2% DMSO was added to cell suspension cultures (45 mg cells, 450 μl medium) 24 hr prior to the administration of substrate (0.33 μmol/25 μl). The cells were harvested 8, 12 and 10 hr after the administration of MHB, OHB, and PHB, respectively, i.e. at the time of active glucoside formation. The harvested cells were extracted with 0.5 ml MeOH containing umbelliferone as int. standard.

Quantitative analysis. The amounts of hydroxybenzoic acids and their glucosylated products were determined by HPLC

(Shimadzu LC-3A). Conditions for reversed-phase HPLC were as follows; column: TSK GEL ODS LS-410, 10 μm (Toyo Soda Co.), 150 × 4.6 mm, flow rate: 1.0 ml/min, detection: 260 nm for PHB, PHB-O-glucoside, and PHB glucose ester, and 300 nm for OHB, MHB, and their *O*-glucosides, solvent system: MeOH–H_2O–HOAc (10:40:1) for OHB, MeOH–H_2O–HOAc (28:200:5) for MHB, and MeOH–H_2O–HOAc (2:77:1) for PHB derivatives. Each glucosylated product from cultured cells was identified by direct comparison with a synthetic specimen, as described elsewhere [4].

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