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GLUCOSYLATION OF PHENOLIC COMPOUNDS BY *DATURA INNOXIA* SUSPENSION CULTURES

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Key Word Index—*Datura innoxia*; Solanaceae; plant cell culture; biotransformation; phenolic glucosides.

Abstract—*Datura innoxia* grown in suspension cultures can glucosylate simple phenols. Three isomers of dihydroxybenzene (hydroquinone, resorcinol and catechol) were readily converted into their corresponding mono-β-glucosides. Both salicyl alcohol and salicylaldehyde fed to the cells were transformed specifically to isosalicin instead of salicin. Furthermore, the analysis of the cells treated with salicylic acid suggested the formation of its glucose ester in addition to the corresponding monoglucoside. Feeding experiments showed that the cultured cells possess a remarkably high capacity for glucosylation of hydroquinone, which was totally converted into arbutin within 10 hr after administration. The *in vitro* glucosylation of hydroquinone carried out by the cell-free extract demonstrated that this enzymic reaction requires the presence of UDPG as a high energy donor of glucose.

INTRODUCTION

Since Yamaha and Cardini [1] found an enzyme from wheat germ that catalyzes the formation of glucosides from phenols and uridine diphosphate glucose (UDPG), it has been demonstrated that a wide variety of higher plants [2] and ferns [3] possess the capacity for glucosylation of administered phenols. Recently, Pilgrim [4] has suggested that the callus cultures of 3 plant species are able to convert some simple phenols including hydroquinone into the corresponding monoglucosides. A basic study of glucosylation in cultured cells is of interest in order to gain a better understanding of the biochemical processes. Furthermore, a quantitative investigation on phenolic glucosylation is of great importance in view of the possible application of plant cell culture to an efficient production of useful glucosides from natural or synthetic compounds. In this connection, the glucosylation of certain steroids and cardenolides has been reported in several plant cell suspension cultures [5].

This paper deals with identification of monoglucosides produced from simple phenols administered to *Datura innoxia* suspension cultures, the efficiency of arbutin formation from hydroquinone fed to the cultured cells and the demonstration of the enzyme system catalyzing the glucosylation of hydroquinone in the cell-free extracts. Undifferentiated cultured cells of *D. innoxia* were suitable for these experiments, because they show a superior growth in a synthetic liquid medium and normally lack phenolic glucosides such as dihydroxybenzene glucosides, salicin and isosalicin, unless their aglycones are supplied to the culture medium externally.

RESULTS

Formation of phenolic glucosides

The suspension cultures of *D. innoxia* at the late exponential growth phase (14 days after inoculation) were fed aseptically with a phenolic compound and subsequently incubated for 3 days before harvest. The glucosylated compounds isolated from the EtOH extracts of the harvested cells were characterized by TLC analyses, UV absorption measurements and analyses of the acid and β-glucosidase hydrolysis products. In all cases, the mono-β-glucosides produced were found mostly in the cells and negligible quantities were released into the culture medium.

Administration of *p*-dihydroxybenzene (hydroquinone, 10^{-3} M) to cell suspension cultures yielded its mono-β-D-glucoside arbutin, which was obtained in a crystalline form in high yield. Similarly, cells fed with *m*-dihydroxybenzene (resorcinol, 10^{-3} M) or *o*-dihydroxybenzene (catechol, 10^{-3} M) gave *m*- or *o*-hydroxyphenyl β-glucoside, respectively.

The monoglucosides formed by feeding salicyl alcohol (saligenin, 5×10^{-3} M) to the cultures were identified by cellulose acetate membrane electrophoresis in addition to the analytical methods mentioned above. The results indicated that salicyl alcohol was predominantly converted to isosalicin (*o*-hydroxybenzyl β-D-glucoside), whereas only a trace of salicin (salicyl alcohol β-D-glucoside) was formed in the *Datura* cells. Salicylaldehyde (5×10^{-3} M), when administered also gave rise to isosalicin, but helicin (salicylaldehyde β-D-glucoside) was not detected.

Analyses of the products obtained from the cells treated with *p*- or *o*-hydroxybenzoic acid (salicylic acid) revealed the formation of 2 different derivatives in each case. It has been suggested that these are the corresponding glucose esters and mono-β-glucosides on the basis of the color reaction with diazotized *p*-nitroaniline and the analyses of the hydrolysates, but the structures remain to be determined unequivocally.

Efficiency and time course of arbutin formation

For considering the quantitative biotransformation of phenols the glucosylation of hydroquinone was chosen. A preliminary culture experiment indicated that the

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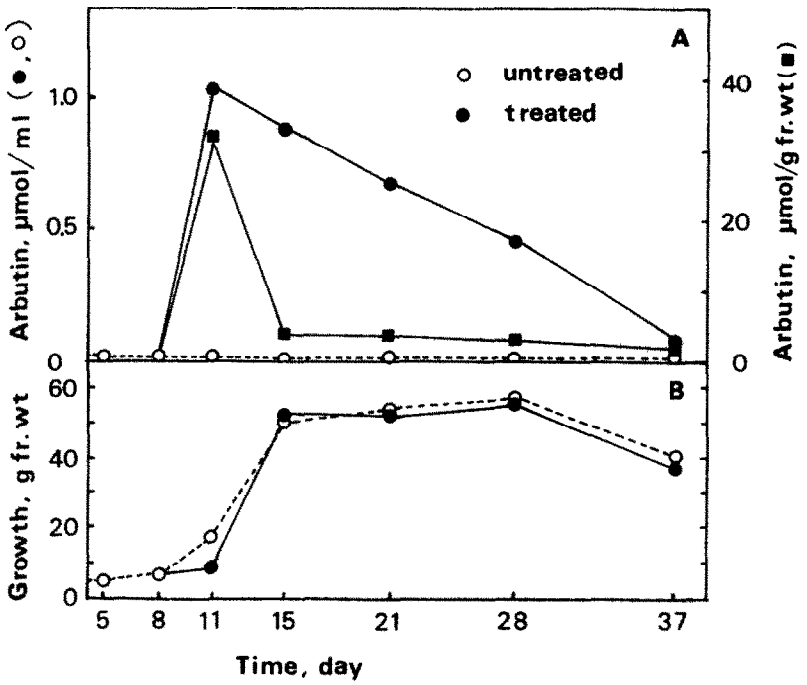

Fig. 1. Time course of arbutin production (A) and growth curve (B) in the cultured cells administered with hydroquinone (250 μmol) 8 days after the initiation of culture. The amounts of arbutrin found in the treated cells are expressed in terms of μmol per ml (●—●) and the content on the basis of fr. wt of cells (■—■).

growth of *Datura* cells was strongly inhibited if hydroquinone (10^{-3} M) was added to the culture medium at the beginning of the culture period. However, when hydroquinone of the same concentration was administered to 8-day-old culture at the exponential growth phase, the cells resumed normal growth after a few days while the glucosylation of hydroquinone occurred rapidly as shown in Fig. 1. The accumulation of arbutin in the treated cells was at its maximum after 3 days, and then gradually decreased until it was almost undetectable at the end of the growth cycle. A sharp fall in the arbutin content on the basis of cell fr. wt is observed at the linear phase of growth, probably because the cells continued to grow when free hydroquinone was no longer available in the environment. On the other hand, the subsequent disappearance of arbutin in the stationary phase strongly suggests that arbutin was gradually metabolized to other compounds. This view is supported by the evidence that neither release of arbutin from the cells into the medium nor reappearance of hydroquinone in the cultures was observed throughout the stationary phase. In control cultures there was essentially no accumulation of arbutin at any stage during the culture period of 37 days.

The relationship between the time of hydroquinone supply and the efficiency of arbutin formation, was examined by administering hydroquinone to separate cultures at the 1st, 2nd, and 3rd week of the growth cycle corresponding to the exponential, progressive deceleration, and stationary phases, respectively. The rate of conversion into arbutin was *ca* 100% in 24 hr, whenever hydroquinone was added to the culture medium (Fig. 2). However, the turnover of arbutin took place at a lower rate when hydroquinone was supplied at later stages of the growth cycle instead of the exponential phase.

The time sequence of arbutin formation was examined by sampling cells at short intervals after feeding with hydroquinone at the exponential growth phase (1 week

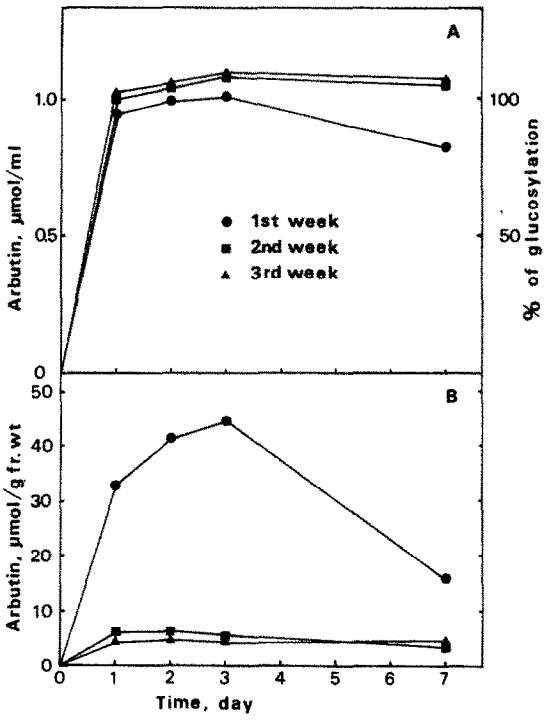

Fig. 2. Time course of arbutin production in the cultured cells administered with hydroquinone (250 μmol) at 3 different stages of growth: (A) amount of arbutin in μmol/ml; (B) arbutin content on the basis of fr. wt of cells.

after the initiation of culture). Glucosylation proceeded linearly between the 2nd and the 6th hr, reaching a 100% level in 10 hr after the introduction of hydroquinone. Furthermore, the simultaneous addition of glucono-δ-lactone, which is known as a competitive inhibitor of β-glucosidase in almonds [6], did not inhibit glucosylation significantly even at a growth-limiting concentration of 2×10^{-3} M.

In an attempt to investigate the cellular capacity of glucosylation in the continuous presence of substrate in the cell environment, each culture that had been pretreated with 250 μmol of hydroquinone was supplied repeatedly with 125 μmol of the same compound every 24 hr from the 8th to the 12th day of culture. In this feeding system, the cell growth was considerably reduced because of the toxicity of excess hydroqinone, increasing only 40% in fr. wt during the 6-day period of incubation. Although the daily rate of glucosylation was very high (100%) on the 1st supply, it was eventually decelerated by the successive feeding of the substrate until there was no more arbutin formation after the 6th supply (Fig. 3). As a consequence of this feeding, 70% of the total hydroquinone was converted to arbutin within 6 days.

Enzymic glucosylation of hydroquinone in cell-free extracts

In order to obtain direct evidence for the existence of an enzymic system for the glucosylation of hydroquinone *in vitro*, cell-free extracts prepared from 8-day-old suspension cultures were assayed for enzymic activity. No satisfactory enzyme solution could be obtained by conventional procedures such as the preparation of cell homogenates or of acetone powders and the ultrasonic treatment of cells in the absence or presence of polyclar AT, polyethylene glycol 4000, or Triton X-100.

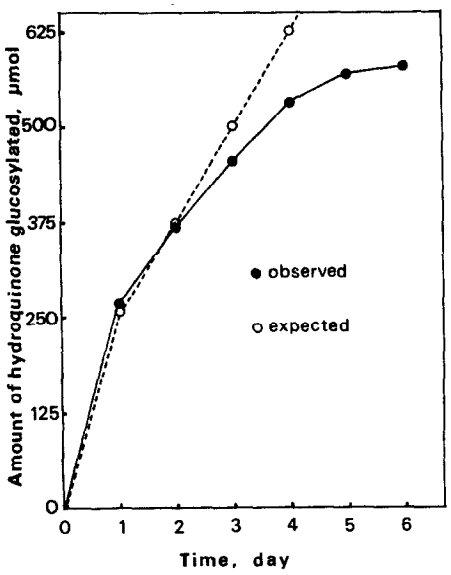

Fig. 3. Accumulation of arbutin in the cultured cells supplied with hydroquinone at successive intervals of 24 hr. Hydroquinone at a concentration of 10^{-3} M was first administered to 7-day-old suspension cultures (at time 0 in the figure), which was followed by a daily administration of 5×10^{-4} M hydroquinone for 5 days. (●—●): observed amounts of arbutin produced by the cells; ○—○: amounts of arbutin expected from a 100% conversion of hydroquinone added to the medium at 24 hr-intervals.

However, active enzyme solutions could be obtained by rapid treatment of cell homogenates with the ion exchange resin Dowex 1×2 (Cl^- form) [7], followed by purification by the Sephadex G-25 column centrifugation method described by Kohl [8].

The enzymic glucosylation of hydroquinone was successfully carried out using the cell-free preparations under the conditions specified in the Experimental and the experiments proved that this reaction requires UDPG as a high energy donor of glucose. A quantitative assay on 3 samples of cultures treated with hydroquinone for 24 hr at the exponential growth phase gave an average enzymic activity of 2.25 unit/g fr. wt of cells. In contrast, the enzymic activity was as low as 0–0.2 unit in the untreated cells of the control cultures which were assayed by the same procedures as used for the treated cells.

DISCUSSION

The present experiments have shown that foreign dihydroxybenzenes when introduced into the cultured cells of *D. innoxia* are readily converted to the corresponding mono-β-glucosides. It follows as a consequence of glucosylation that the cells could survive, even if treated with such a toxic phenol as hydroquinone. It has often been suggested that glucosylation serves as a method for the detoxification of harmful phenolic compounds which could either arise from normal plant metabolism or from the environment [9]. Although there has been little work on the quantitative estimation of glucoside formation in plants, the present experiments have demonstrated that the efficiency of hydroquinone glucosylation in the *Datura* cells is remarkably high when compared with that reported for the glucosylation of steroids and cardenolides in other plant suspension cultures [5].

The feeding experiments have shown that salicyl alcohol forms little salicin in *Datura* cells, but is predominantly metabolized to the isomeric glucoside isosalicin, which was first isolated from *Filipendula ulmaria* [10]. This result is in agreement with those obtained by Pridham and Saltmarsh [11] and also by Zenk [12] who showed that salicyl alcohol cannot be the direct precursor of salicin in various plants, but is instead converted to isosalicin. In contrast to the conversion of salicylaldehyde into salicin via helicin in *Salix* leaf tissues [12], salicylaldehyde fed to the *Datura* suspension culture is transformed into isosalicin probably via salicyl alcohol. Thus the metabolism of salicylaldehyde appears to be different, depending upon the plant species.

As regards the biotransformation of *o*- and *p*-hydroxybenzoic acids, the structures of their glucosylated products isolated from the cultured cells have not been fully established. However, it is of interest that the cells produce not only a glucoside but also a glucose ester from these phenolic acids. The formation of glucose esters has been reported by Harborne and Corner [13] in leaves of various plants fed with cinnamic acid and its derivatives. On the other hand, Klämbt [14] has reported that benzoic acid can be converted into salicylic acid and salicylic acid β-glucoside by several species of plants.

In considering the possible application of biotransformation of phenolic compounds by plant cell cultures, the high efficiency of glucosylation such as that observed

for hydroquinone might be promising in producing pharmaceutically useful glucosides from a variety of phenols. However, such practical problems as the substrate specificity of glucosyltransferase, the rate of biotransformation and an adequate method of substrate feeding must be elucidated for a wide variety of phenolic compounds by further studies.

EXPERIMENTAL

Callus tissues were originally derived from a stem segment of *Datura innoxia* Mill. and maintained for 6 yr in suspension culture of ref. [18] supplemented with 10^{-6} M 2,4-D. The detailed procedure of cell culture is described in ref [19].

Qualitative analysis of glucosides. Each phenolic substrate (10^{-3} M or 5×10^{-3} M) was supplied aseptically to suspension cultures in 500 ml flasks containing 200 ml of medium through a millipore membrane filter 2 weeks after cell inoculation, and the cells were cultured for 3 additional days. The cultured cells were collected by filtration, mixed with $CaCO_3$ (1 wt % of cells), homogenized with hot EtOH, and extracted for 2 hr at 100°. The extract was adsorbed on a Si gel (5 g) column and eluted with EtOAc–MeOH (9:1). The eluate was submitted to Si gel GF_{254} preparative-TLC using the following solvent systems: (A) xylene–MeOH–HOAc–H_2O (1:4:1:4, upper layer), (B) EtOAc–MeOH–H_2O (95:22:13) or (C) EtOAc–MeOH–H_2O (100:17:13). A band corresponding to each glucoside was removed from the plate and the glucoside eluted with MeOH. Each glucoside was hydrolyzed with 1% almond β-glucosidase in acetate buffer pH 5.5 for 3 hr at 37° or with N HCl for 1 hr at 100°. Glucose in the hydrolysate was identified by PC (*n*-BuOH–Py–H_2O, 6:4:3) and its content was determined by the *p*-anisidine–HCl method [20]. The aglycones and the standard samples were chromatographed on Si gel GF_{254} plates with 3 solvent systems B, C, and D (C_6H_6–dioxane–HOAc, 90:25:4) and detected by means of UV light, I_2 vapor, Millon's reagent, phloroglucinol–HCl, diazotized *p*-nitroaniline and Gibb's reagent. Each aglycone was eluted from Si gel with MeOH and the solvent was evaporated. The residue was taken up in EtOH and the amount of the aglycone was determined by UV spectrophotometry. For further verification of the aglycones, UV absorption spectra of the 0.02 N NaOH and H_2SO_4-acidic MeOH solns were measured, and cellulose acetate membrane electrophoresis in 0.02 N NaOH at 0.4 mA/cm was performed. For isolation of arbutin, cells fed with 10^{-3} M hydroquinone were collected by filtration, extracted with hot MeOH, and the solvent evaporated. The Et_2O-insoluble portion of the residue was subjected to polyamide (H_2O) and Si gel (EtOAc–MeOH, 9:1) column chromatography and Si gel GF_{254} preparative TLC (system C) successively in order to isolate arbutin, mp 199.5° (uncorr), identical (mmp, UV, IR, NMR) with an authentic sample. The EtOH extract of the cells fed with 5×10^{-3} M salicylaldehyde or salicyl alcohol was chromatographed on a Si gel column (EtOAc–MeOH, 9:1). In each case chromatography of the eluate on a Si gel column with a stepwise gradient of $CHCl_3$–MeOH gave isosalicin, mp 68° (uncorr), λ_{max}^{EtOH} nm:274, $\lambda_{max}^{0.02N\ NaOH}$ nm:294, identical (mmp, UV and TLC) with an authentic specimen. The two cellular products (compounds I and II) obtained by feeding *o*-hydroxybenzoic acid were separated by a Si gel column (EtOAc–MeOH, 9:1) and preparative TLC (system C). Compound I, R_f 0.5 (system A), R_f 0.43 (C), diazotized *p*-nitroaniline (DPNA) positive; compound II, R_f 0.21 (A), R_f 0.28 (C), DPNA negative. On hydrolysis with acid or β-glucosidase, both compounds I and II yielded equimolar amounts of salicylic acid and glucose. It is known that reactions of various phenolic acids with DPNA are always positive when the hydroxy group is free, whereas reactions are negative when the hydroxy group is glucosylated [12, 21]. Thus, compounds I and II are estimated as β-D-glucosyl 1-*o*-hydroxybenzoate and *o*-hydroxybenzoyl β-D-glucoside, respectively. Compounds III and IV produced from *p*-hydroxybenzoic acid administered to cultured cells were separated by the chromatographic methods mentioned above. Compounds III, R_f 0.46 (A), R_f 0.48 (C), DPNA positive; compound IV, R_f 0.38 (A) R_f 0.31 (C), DPNA negative. Both compounds afforded equimolar amounts of *p*-hydroxybenzoic acid and glucose on acid or β-glucosidase hydrolysis. Thus, compounds III and IV were estimated as β-D-glucosyl 1-*p*-hydroxybenzoate and *p*-hydroxybenzoyl β-D-glucoside, respectively.

Quantitative analysis of hydroquinone and arbutin. The condensed culture medium and the hot EtOH extract of harvested cells (2–70 g fr. wt) were combined, and the mixture was adsorbed on Si gel (5 g), dried, and eluted with EtOAc–MeOH (9:1, 300 ml). The eluate was concentrated to 5 ml and an aliquot chromatographed on Si gel GF_{254} (system D). Hydroquinone and arbutin were eluted separately with MeOH from the Si gel and each soln was made up to 5 ml with MeOH. A known vol of the hydroquinone soln was concentrated to dryness. To the residue dissolved in H_2O (2 ml) was added 0.5% phloroglucinol (0.2 ml) and 0.56% KOH (2 ml), and the soln made up to 4 ml with H_2O. After 1 hr, the A was measured at 420 nm [22]. An aliquot of the arbutin soln was concentrated to dryness and to the residue dissolved in H_2O (0.5 ml) was added 3.2% 4-aminopyrine (0.25 ml), 0.42% NH_4OH (0.25 ml), 0.2% $K_3Fe(CN)_6$ (0.5 ml), and $CHCl_3$ (3.5 ml). The aq layer was discarded after centrifugation and the A of the $CHCl_3$ soln was measured at 455 nm to estimate the amount of arbutin [23].

Assay of glucosyltransferase activity. All the procedures were carried out at 0–4°, unless otherwise stated. Fresh cultured cells (8-day-old) harvested after 24 hr-incubation with hydroquinone were homogenized in a blender for 90 sec with a mixture of acid-washed sea sand (1:1 by wt) immersed in 0.05 M KPi buffer pH 7 containing 10^{-3} M glutathione (reduced form) and 10^{-2} M EDTA, and passed through a glass filter. The filtrate mixed with Dowex 1×2 (Cl^-) (5 w/v %) was stirred for 3 min and then filtered. This filtrate was placed on top of a Sephadex G-25 column (5×1.4 cm) which had been equilibrated with 0.05 M KPi buffer. The whole column was centrifuged at 86 *g* for 3 min in order to remove low MW substances [7, 8]. The eluate thus obtained by centrifugation is referred to as the enzyme prepn. A reaction mixture consisting of UDPG (0.8 μmol), hydroquinone (0.8 μmol), EDTA (0.2 μmol), cysteine (0.2 μmol), Tris–malate pH 6.5 (40 μmol), and the enzyme prepn (90 μl) in a total vol of 0.2 ml was incubated under N_2 at 37° for 1.5 hr. The reaction was stopped by the consecutive addition of 5% $ZnSO_4$ (0.2 ml) and 3 N $Ba(OH)_2$ (0.2 ml). After centrifugation, the clear supernatant was deionized with a mixed resin of IR-120 (H^+) and IRA-410 (AcO^-). The reaction product was separated by TLC (Si gel GF_{254}, system A). For quantitative estimation of UDP produced by the enzyme reaction, the reaction mixture incubated under the above conditions was heated at 100° for 3 min and cooled to room temp. This soln (0.1 ml) was mixed with 0.5 μmol phosphoenolpyruvate (25 μl), 2.5 μmol $MgSO_4 \cdot 7H_2O$ (25 μl) and rabbit muscle pyruvate kinase soln (50 μl), and incubated for 15 min at 37°. After treatment of the reaction mixture with 10% TCA (1 ml), the amount of pyruvate in the supernatant was assayed spectrophotometrically at 420 nm by the 2,4-DNPH method [24, 25]. A unit of enzyme activity is defined as μmol of UDP produced per 15 min at 37°.

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