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Change of Maximal Cellular Productivity of Arbutin by Biotransformation Depending on the Culture Stage of *Catharanthus roseus* Cells

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Maximal productivity of arbutin per cell at various culture stages was examined. *Catharanthus roseus* cells from early log phase to stationary phase were collected on nylon mesh, then aliquots of cells were suspended in fresh medium or the original medium from which the cells had been removed, for comparison, and hydroquinone was added at 2 mM every day until the cells died, in order to obtain the maximal yield. Biotransforming ability based on dry weight of cells in fresh medium dramatically increased with advancing cell stage. In the original medium, however, the biotransforming ability decreased in cells older than day 5. When sucrose (1%) was added to 6-day cells in the original medium, arbutin yield drastically increased. These results suggest that older cells possess higher ability to produce arbutin, but can not manifest this in batch culture because of depletion of essential component(s) in the medium; one of these components was sucrose.

UDPG (uridine 5′-diphosphate glucose) was strongly induced in 6-day cells in fresh medium after the addition of hydroquinone even though it was barely detected before. UDPG had originally been supplied on the 3rd day. In those young cells, the hydroquinone supplied UDPG molecules were presumed to be used competitively both for the glucosylation of hydroquinone and for synthesizing cell wall polysaccharide. The enzyme activity to glucosylate hydroquinone was sufficient in cells on the 3rd, 6th and 8th day. Cell volume of 8-day cells was 1. 4-fold as much as that of 3-day cells with the increament of vacuole volume; this might be related to arbutin accumulation.

In conclusion, older cells have a greater ability to produce arbutin by biotransformation. The larger amount of UDPG which is available for the glucosylation of hydroquinone, as well as the larger vacuole, contribute to its ability at least partly.

Introduction

Cultured plant cells biotransform many kinds of substrates supplied exogenously via a wide range of reactions, such as glucosylation, glucosyl esterification, hydroxylation, oxido-reduction between alcohol and ketone, reduction of carbon double bonds, hydrolysis, isomerization, epoxidation, dehydrogenation, methylation, and others[1-3]. Among such biotransformation reactions, glucose conjugations (glucosylation and glucosyl esterification) are major reactions; they are the subject of

45% of published reports on biotransformation[1].

The glucosylated derivative of hydroquinone, arbutin, is a potent suppressor of the synthesis of melanin in human skin[4], without any apparent side effects[5]. We have studied the industrial application of glucosylation of exogenous hydroquinone using *Catharanthus roseus* cell culture, establishing the effects of sugars on the glucosylation of exogenous hydroquinone[6], the importance of the concentration of hydroquinone in the medium[7], and the difference of glucosylating ability between strains of *Catharanthus roseus*[8]. In this study, we deal with differences in the ability of cells to glucosylate exogenous hydroquinone depending on culture stage in batch culture.

Several reports have been published on differences in biotransformation ability of the cells at different culture stages; the inductional and exponential phases were better in soybean cell culture[9], *Gardenia jasminoides* cultured cells have higher glucosylation ability in the exponential phase[10], and *Datura innoxia* cell culture gives good results in the late exponential stage[11]. In those experiments, however, the cultured cell suspensions were used as such; changes in the glycosylating ability of the cell itself could not be established because the cell density and the components of the medium changed with the culture time. We tried to clarify the cellular ability to glucosylate exogenous hydroquinone by collecting the cells at various stages of culture and transferring them to fresh medium.

Materials and Methods

1. *Cell strain and culture conditions*

Strain A of *Catharanthus roseus*, established at Tohoku University, was used as a cell suspension in Linsmaier and Skoog's (LS) medium[12] containing 2.2×10^{-6} M 2, 4-dichlorophenoxyacetic acid and 30 g/*l* sucrose. Cells were subcultured every week in the dark at 26°C on a gyratory shaker (110 rpm) by the transfer of 20 m*l* of the suspension to 180 m*l* of fresh medium in a 500-m*l* flask.

In experiments, cells at various culture stages were collected by filtration on a nylon mesh (pore size 148 μm). A 2.5 g (fresh weight) aliquot was transferred to 20 m*l* (in a 100 m*l* flask) of the fresh LS medium or the original medium from which the cells had been removed. That density of fresh weight was approximately equal to the density at 6 day culture. HQ (0.2 m*l* at 200 mM), 40 μg/flask, was added to the cell suspension every day untill the cells died completely; hydroquinone was added 3 times to 3-, 4-, 5- and 6-day cells while 4 times to 7-, 8- and 9-day cells.

2. *Analysis of arbutin*

Next day after the last addition of hydroquinone, the cell suspension was homogenized in a Polytron (Kinematica) and centrifuged. Arbutin in the resultant supernatant was analyzed by HPLC (LC 100 system, Yokogawa Electrics Inc., Tokyo, Japan) on a Capcell Pak C_{18} column (Shiseido, Tokyo, Japan) in 5% methyl alcohol (adjusted to pH 2.5 with phosphoric acid) with monitoring at 230 nm. Synthesized arbutin was stable in cells at least for several days.

3. *Analysis of UDPG*

UDPG (uridine 5′-diphosphate glucose) was analyzed by HPLC (LC 100 system) according to the method of Ashihara *et al.*[13]. About 1 g fresh weight of cells was collected by filtration on a paper filter (No. 2, Advantech, Tokyo, Japan) and transferred to four volumes of cold perchloric acid solution (6% w/v), followed by disruption with a sonicator (Bioruptor, Cosmo Bio Inc., Tokyo, Japan). After centrifugation at 18,000 rpm for 15 min., the supernatant was neutralized using 20% and 5% KOH solution. The neutralized solution was evaporated *in vacuo* and the residue was dissolved in 2 m*l* of phosphate buffer (20 mM, pH 7). After standing at 20°C for 20 min., the solution was filtered through a paper filter and a guard column (Millipore Inc., USA), then analyzed

by HPLC (LC 100 system) on a Shimpack Wax-1 column (4 mmϕ×50 mm, 100 Å, Shimadzu Inc., Kyoto, Japan) in 20 mM phosphate buffer (pH 7.0) at 1 m*l*/min. with monitoring at 260 nm.

4. *Assay of the enzyme activity to glucosylate hydroquinone*

Enzyme assay was performed according to the method of Yokoyama *et al.*[6]. Crude enzyme solution was prepared after homogenization of cells in 50 mM phosphate buffer (pH 5.0) containing 10 mM mercaptoethanol and centrifugation of the homogenate. An aliquot of the preparation of crude enzyme (100 μl) was added to 80 μl of a reaction mixture that contained 10 mM UDP[U-^{14}C] glucose (0.05 μCi), 10 mM hydroquinone, 10 mM mercaptoethanol and 50 mM phosphate buffer (pH 5.0), and the mixture was incubated at 37°C for 60 min. The reaction was stopped by addition of 0.2 m*l* of ethanol containing 0.01 mM cold arbutin and a 20 μl aliquot of the mixture was developed by TLC. The arbutin fraction detected under UV light (254 nm) was scraped off the plate, and the radioactivity was measured in a scintillation counter.

Results and Discussion

1. *Change of the ability to produce arbutin*

In order to examine the ability to produce arbutin of cells at each culture stage, aliquots of cells were transferred to fresh medium, or, as comparison, to the original medium from which cells had been removed at each culture stage. Added sucrose (3%) was consumed via conversion to glucose and fructose, and disappeared in the medium at 8 th day of the culture. We examined the change in the ability to produce arbutin during that period, 8 days. Hydroquinone was added as described in **Materials and Methods** in order to measure the maximal production of arbutin. As is clear from **Fig. 1**, the arbutin-producing ability of cells in fresh medium began to increase sharply in cells from cultures older than 3 days (when cell growth changed from the induction to the exponential

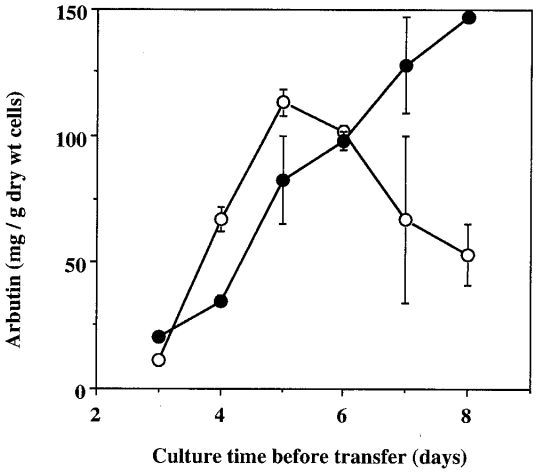

Fig. 1 Change of the ability to produce arbutin during culture.
Catharanthus roseus cells from early log phase to stationary phase were collected on a 148 μm nylon mesh. Aliquots of 2.5 g of cells (fresh weight) were suspended in 20 m*l* of fresh medium (●) or the original medium (○) from which the cells had been removed. Every day, 0.2 m*l* of 200 mM hydroquinone was added until the cells died. After incubation for the biotransformation, the cell suspensions were homogenized and arbutin in the resultant supernatant after centrifugation was analyzed by HPLC. Arbutin content was calculated per dry weight cells. Each value represents an average of 3 determinations, with SD. The experiments were repeated at least 4 times with similar results.

phase) and kept increasing, while that of the cells transferred to the original medium was maximal with 5-day cells. These results suggest that cells increase in ability to produce arbutin in later stages, but in the original medium they can not manifest their full ability owing to an insufficient amount of component(s) in the medium in the later stages. When 1% sucrose was added to 6-day cells in the original medium which still had a sugar concentration of more than 1%, the productivity of arbutin drastically increassed from 103±11 to 184±1 (mg/g dry cells) (the average of 3 determinations, with SD). Therefore, sugar is at least one of the essetial components of the biotransformation. We have already reported in more detail the importance of the sugar on the glucosylation of hydroquinone[6].

2. *Changes of UDPG in the cells, the enzyme activity to glucosylate hydroquinone and cell volume*

The amounts of UDPG were assayed in cells from 3-day and 6-day cultures. The control level, before hydroquinone addition, was very high in 3-day cells while in 6-day cells UDPG was hardly detected (**Fig. 2**), in accordance with previously reported data[14]. This suggests that younger cells require more UDPG, presumably for synthesizing the cell wall.

The control level of UDPG does not necessarily reflect the amount which is available for

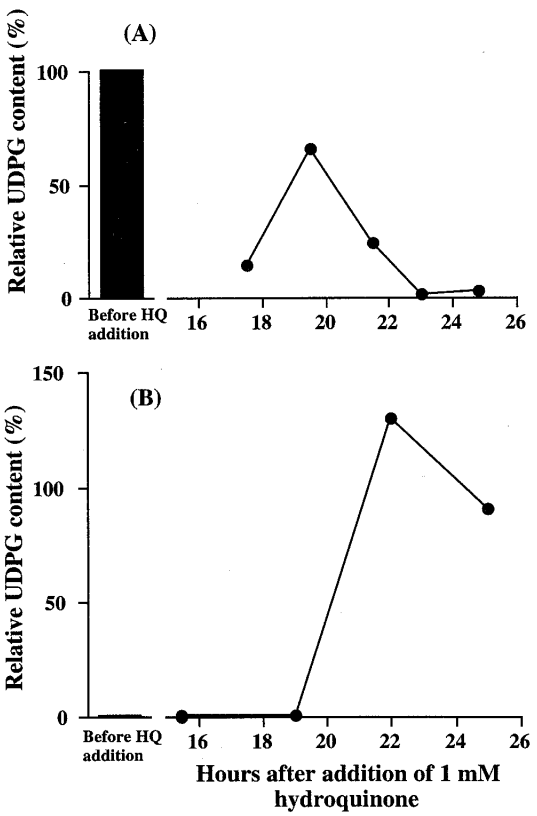

Fig. 2 Change of the UDPG content in cells which had been cultured for 3(A) or 6(B) days. *Catharanthus roseus* cells were collected after culture for 3 or 6 days and resuspended in fresh medium by the same method as mentioned in the legend to **Fig. 1**. Hydroquinone was added at 1 mM. UDPG content in the cells was measured before and at various times after addition of hydroquinone(●). Closed columns at the left side represent original UDPG content in 3-day(A) or 6-day(B) cells before addition of hydroquinone. UDPG content is represented relative to the original content in 3-day cells, taken as 100%. The experiments were repeated 2 times, with similar results.

Table 1. The enzyme activity to glucosylate hydroquinone.

Culture time (days)	The enzyme activity (dpm $\times 10^{-3}$/g dry wt)
3	71±40
6	124±27
8	78±14

Enzyme activities were assayed 24 hr after addition of 2 mM HQ.
Each value represents the average of 3 determinations, with SD.
The experiments were repeated 3 times, with similar results.

bioconversion to arbutin. Added hydroquinone may induce new UDPG formation. Therefore, we assayed the amount of UDPG just after the added hydroquinone (1 mM) was completely consumed. That amount was expected to reflect not only the control level, but also the level induced by adding hydroquinone. **Fig. 2** shows that UDPG was clearly induced in the 6-day cells, which originally hardly contained UDPG when hydroquinone was added; the total supplied amount was larger in 6-day cells than 3-day cells. In 3-day cells, the supplied UDPG molecules may be used competitively both for the glucosylation of hydroquinone and for synthesizing cell wall polysaccharide.

The activity of the enzyme to glucosylate hydroquinone was assayed in cells from 3-day, 6-day and 8-day cultures. The activity tended to be highest on the 6th day (**Table 1**). The lag time for the synthesis of this enzyme, which is fully inducible, was unchanged at different culture stages, being about 3 hr (data not shown).

Generally, older cells are larger in volume with larger vacuole. We measured a cell diameter of strain A of *Catharanthus roseus*. Average ones were 27.9 (n=1019) and 31.3 μm (n=815) on 3-day and 8-day cells respectively; they were estimated to be 11.3 and 16.1 $mm^3 \times 10^{-6}$ in cell volume. The increment in cell volume depended on one in vacuole volume (neutral red dye test). Those data coincided with the previous report that strain A of *Catharanthus roseus* increased in fresh weight after 2 days of incubation in batch culture[15]. Vacuole-development has been reported to relate with a increment of accumulation of a second metabolite[16]. We also compared two morphologically different lines (A and B) with each other for their abilitities to biotransform hydroquinone to arbutin[8]. In vacuole-larger strain B, arbutin which had been synthesized from hydroquinone was accumulated much more largely; arbutin yield was much higher than in strain A. Older cells of strain A are also understood to accumulate arbutin in a larger amount with larger vacuole.

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《和文要約》

バイオトランスフォーメーションによるアルブチン生産において培養エイジの違いによる細胞自身の生産能の変化

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ハイドロキノン(HQ)を *Catharanthus roseus* 培養細胞に与え，バイオトランスフォーメーションによりアルブチンを生産する系において，培養エイジの違いによるアルブチン生産能の変化を調べた．同じ培地成分下での細胞自体の生産性の違いをみるために，各エイジの細胞をナイロンメッシュで集め，あらためて新鮮なLS培地に移しアルブチン生産能を調べた．比較として元の培地に移した場合の生産性も調べた．元の培地に移した場合，培養5日目(対数増殖期)で生産性がピークになるのに比べ，新鮮培地に移すとアルブチン生産性はエイジを経るに従い顕著に増加した．元の培地に移した場合，生産性が減り始めた培養6日目の細胞に蔗糖(1%)を補充すると，生産性は大きく回復した．結局，細胞自体は培養を経るほどアルブチンの生産能力が高まることがわかった．細胞内のUDPG供給量，ハイドロキノンの配糖化酵素活性，及び細胞体積を測定したところ，アルブチンの生産に利用できるUDPG量の増大，及び液胞の発達によりアルブチンの蓄積場所が大きくなることがHQの変換効率を上げる一要因になっていることが考えられた．