Production of Anthocyanins by Habituated Cultured Cells of Nyoho Strawberry (Fragaria ananassa Duch.)

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Received August 22, 2001; Accepted November 22, 2001

Cultured leaf cells of Nyoho strawberry (Fragaria ananassa Duch.) produced 4 times more anthocyanins than fruit, with additions of 2.0 mg/l of 1-naphthaleneacetic acid (NAA) and 0.2 mg/l of benzyladenine (BA) for 2 weeks. They were habituated after 6 passages at NAA 1.25 and BA 0.125 μg/l in 107 cultures with stepwise decrease of the additions at 2-week intervals, and supervision of a gradual decrease in anthocyanin contents of 80%. They fully recovered through the following 43 passages without the additions. Finally, 3 types of cell line rich in cyanidin 3-glucoside (62%), peonidin 3-glucoside (55%) or both (49 and 36%, respectively) were obtained. They also produced pelargonidin 3-glucoside (0.6 to 1.8%), which was the main component in the fruit. The fruit produced peonidin 3-glucoside (0.4%), as well as pelargonidin 3-O-(6-O-malonyl-β-D-glucoside) and cyanidin 3-glucoside. The habituation seems to have caused some activation or inhibition of the cell’s anthocyanin formation, which originated from the mother plant.

Keywords: habituation, strawberry, cell culture, anthocyanins

Anthocyanins are widely used as natural food colorants, and have been reported to have functional effects, such as antioxidant (Tsuda et al., 1998; Noda et al., 1998) antitumor (Koide et al., 1996; Koide et al., 1997), antimutagenicity (Yoshimoto et al., 1999), and others (Tsuda et al., 1999); they are also expected to be functional food ingredients. Research on production of secondary plant metabolites by plant cell and tissue cultures is increasing to find an effective system, or as one aspect of food biotechnology (Koda et al., 1992; Koda et al., 1993; Nawa & Ohtani, 1992).

There have been many other studies on the production of anthocyanin by cultured cells, such as grape (Yamakawa et al., 1983), sweet potato (Nozue et al., 1987), Euphorbia millii (Yamamoto et al., 1989), and Rabbiteye blueberry (Nawa et al., 1993) as well as strawberry (Hong et al., 1989; Kurata et al., 2000). Cyanidin 3-glucoside, pelargonidin 3-glucoside, pelargonidin 3-O-(6-O-malonyl-β-D-glucoside), and other cyanidin and pelargonidin glycosides, except peonidin glycosides, were identified in strawberry fruits (Fuleki, 1969; Wrolstad et al., 1979; Christou, 1988), sugarbeet (Kevers et al., 1986; Bakker et al., 1994; Tamura et al., 1995). These studies indicated that the cultured cells required one or more kinds of added plant growth regulators for the production of anthocyanins. Although habituation of cultured cells to the regulators for growth and metabolite production has been reported in soybean (Ikeda et al., 1979; Christou, 1988), sugarbeet (Kevers et al., 1981), tobacco (Syono & Furuya, 1974), ginseng (Nishio et al., 1976), lily (Sheridan, 1968), maize (Hawes et al., 1985), and others (Kevers et al., 1996; Park et al., 1989), no report is available on their anthocyanin production, as far as we know. The habituation must be efficient for simplification of the cell culture, and of the anthocyanin extraction and purification.

Recently, strawberry fruit was reported to be an effective antioxidant-rich food for retardation of aging of rats (Joseph et al., 1998), and the production of anthocyanins by cultured cells of strawberry is very interesting. In this paper, we report habituation of cultured red cells of Nyoho strawberry (F. ananassa Duch.), and the production of anthocyanins.

Materials and Methods

Materials Authentic peonidin 3-glucoside, delphynidin, cyanidin, pelargonidin and peonidin were obtained commercially from Extrasynthese S. A. (Impasse Jacquard, Genay, France). Petunidin and malvidin were prepared from the skin of Kyoho grapes (Vitis vinifera L. x V. labrusca L.) by the method of Shiraiishi et al. (1986).

Callus induction and selection of the red cell line Calluses were induced from sterilized young leaf sections (5x5 mm) of Nyoho strawberry, and cultured at 25°C in the dark on Murashige-Skoog medium (Murashige & Skoog, 1962), (pH5.7) supplemented with 1.0 mg/l of NAA and 1.0 mg/l of kinetin, 3% (w/v) sucrose, and 0.25% (w/v) Gellan gum (Wako Pure Chemical Industries, Ltd., Osaka). The induced calluses (5–6 g fresh weight), slightly yellowish white and soft, were subcultured on Murashige-Skoog medium and then on a modified Gamborg’s B5 (MB5) medium which was composed of the basal salts of Gamborg’s B5 medium (Gamborg et al., 1968) and the organic elements of Murashige-Skoog medium, with 2.0 mg/l of NAA and...
0.2 mg/l of BA under white fluorescent lighting (5000 lux-24 h) at 3- or 4-week intervals.

The first subculture on the MB5 medium was tentatively designated as the 1st generation in this study. After 31 subculturings and selections of red cells with the naked eye, cell lines, which always showed a red exterior, were selected out, and then were subjected to habituation to NAA and BA.

**Habituation of red cells** Selected red cell lines were subcultured at 2-week intervals on the MB5 solid medium including half concentrations of NAA and BA in the preceding subculture mediums (see Table 1 on the stepwise dilution process from 1/2 to 1/1600). Subculturing at each concentration was repeated until the red cells achieved a stable growth and anthocyanin formation. Before proceeding to the next dilution stage, a trial subculture to 1/1600. Subculturing at each concentration was repeated until the red cells grew stably and formed anthocyanins without NAA or BA.

After habituation, with HPLC analysis, several red cell lines were selected. The three best were selected and used for the analyses.

**Estimation of total anthocyanins** The cultured cells and fruit were freeze-dried and stored until used. The stored cells and fruit were homogenized with 0.1% (v/v) HCl-90% (v/v) methanol, and with 0.5% (v/v) trifluoroacetic acid (TFA)-90% (v/v) acetonitrile (CH₃CN), respectively, at 4˚C overnight in the dark. The homogenates were centrifuged, and absorbance at 530 nm of the clear supernatants was measured for anthocyanins. The contents were expressed as A₅₃₀ per g dry weight.

**Extraction and purification of anthocyanins** The cultured fresh cells and the mashed fruit were treated with 0.5% (v/v) TFA-90% (v/v) CH₃CN at 4˚C overnight in the dark, and the homogenates were filtered. The organic solvents were removed from each filtrate by evaporation at 40˚C, and the whole was centrifuged. The supernatants were applied onto Sep-Pak C18 cartridges (Waters Corporation, Milford, Massachusetts). After washing with water, the pigments were eluted with a small volume of 0.1% HCl-methanol.

The obtained pigments were purified by HPLC with linked preparative ODS columns [20x(80+250) mm, Wakosil-II 5C18 AR, particle size 5 μm, Wako Pure Chemical Industries, Ltd.] by eluting with a linear gradient from 7.5 to 27.5% of a solvent B [TFA-acetic acid-CH₃CN-water (0.5:40:50:9.5, v/v)] in a solvent A [TFA-water (0.5:99.5, v/v)] for 80 min at a flow rate of 6.0 ml/min at 40˚C. The elution pattern was monitored at 520 nm. Each peak fraction was concentrated under reduced pressure, and then rechromatographed with the same columns and conditions.

**Analytical HPLC of anthocyanins** Purified anthocyanins or hydrolysates described hereafter were analyzed by HPLC with linked analytical ODS columns [4.6x(50+250) mm, Wakosil-II 5C18 AR, particle size 5 μm] by eluting with 7.5% of solvent B for the first 10 min, followed by elution with a linear gradient from 7.5 to 30% of solvent B in solvent A for 45 min, at a flow rate of 1.0 ml/min at 40˚C. The elution pattern was monitored at 520 nm.

**Hydrolysis of anthocyanins** Anthocyanins were fully or partially hydrolyzed with 2 N HCl for 30 min or 3 min, respectively, at 100˚C. They were hydrolyzed also with hydrogen peroxide and NH₄OH for analysis of the sugar moiety at the C-3 position of anthocyanins (Strack & Wray, 1989).

**TLC of sugar moiety** Sugar moiety was analyzed with a cellulose thin-layer (0.1 mm thick, 10 cm, Merck KGaA, Darmstadt, Germany) with a solvent mixture of ethyl acetate-pyridine-water (40:11.6, v/v), and compared with the authentic rhamnose (Rₐ 1.00), xylose (Rₐ 0.67), arabinose (Rₐ 0.53), glucose (Rₐ 0.32) and galactose (Rₐ 0.21).

**Instrumental analysis of anthocyanins** Anthocyanins and anthocyanidins were identified by analysis with LC-MS (LCMS-QP8000, Shimadzu Corporation, Kyoto) with the aforementioned analytical ODS coupled columns. The analytical conditions for MS were as follows: atmospheric pressure chemical ionization probe, 4500 V, 400˚C; nebulizing gas flow, 2.5 l/min; curved desolvation line, -50 V, 250˚C. The pigments were eluted with a linear gradient of CH₃CN from 5 to 40% (v/v) in 0.5% TFA for 80 min at a flow rate of 0.6 ml/min at 40˚C. The elution pattern was monitored by measuring absorption spectra from 240 to 600 nm with a photodiode array detector (SPD-M10AVP, Shimadzu Corporation). Delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin were eluted in that order with the LC-MS system.

| Table 1. Processes of NAA and BA dilutions, and of subculturing for habituation of Nyoho strawberry red cells |
|-----------------|-----------------|-----------------|-----------------|
| Dilation level  | Concentration (mg/l) | Times of subculturing | Generation number |
| 1               | 2.000           | 0.2000          | 31              | 1–31             |
| 1/2             | 1.000           | 0.1000          | 6               | 32–37            |
| 1/4             | 0.500           | 0.0500          | 6               | 38–43            |
| 1/8             | 0.250           | 0.0250          | 12              | 44–55            |
| 1/16            | 0.125           | 0.0125          | 15              | 56–70            |
| 1/32            | 0.0625          | 0.00625         | 12              | 71–82            |
| 1/64            | 0.03125         | 0.003125        | 22              | 83–104           |
| 1/128           | 0.015625        | 0.0015625       | 4               | 105–108          |
| 1/256           | 0.0078125       | 0.00078125      | 7               | 109–115          |
| 1/512           | 0.00390625      | 0.000390625     | 11              | 116–126          |
| 1/1024          | 0.001953125     | 0.0001953125    | 6               | 127–132          |
| 1/2048         | 0.0009765625    | 0.00009765625   | 6               | 133–138           |

*Culture medium is modified Gamborg’s B5 medium (refer to Materials and Methods).*

0.2 mg/l of BA under white fluorescent lighting (5000 lux-24 h) at 3- or 4-week intervals.
3.43 (1H, t, J=9.2, H-4’); peak 3, dH 9.04 (1H, s, H-4), 8.25 (1H, dd, J=2.2, 8.7, H-6’), 8.21 (1H, d, J=2.2, H-2’), 7.05 (1H, d, J=8.7, H-5’), 6.93 (1H, d’, J=0.9, 2.0, H-8), 6.68 (1H, d, J=2.0, H-6), 5.33 (1H, d, J=7.7, H-1’), 4.02 (3H, s, OCH3), 3.93 (1H, dd, J=2.1, 12.1, H-6’a), 3.72 (1H, dd, J=6.0, 12.0, H-6”a), 3.66 (1H, dd, J=7.7, 9.2, H-2”), 3.61–3.55 (1H, m), 3.56 (1H, t, J=8.9, H-3”), 3.44 (1H, t, J=8.9, H-4”). Data of peak 3’ were very similar to those of peak 3.

Results and Discussion

Habituation of red cells  Nyoho strawberry red cells proliferated and produced anthocyanins 4 to 5 times by culturing for 2 weeks when cultured on the MB5 solid medium supplemented with 2.0 mg/l NAA and 0.2 mg/l BA. The anthocyanin content (420 A530/g dry wt.) was 4 times more than Nyoho fruit (104 A530/g dry wt.). When the red cells were transferred and subcultured on the medium with no addition of NAA and BA, they browned seriously and died (refer to Figs. 2A and 2B). Therefore, the red cells were subjected to gentle habituation to NAA and BA.

Table 1 shows the outline of the dilution process of NAA and BA concentrations for the habituation, and Fig. 1 shows change in anthocyanin formation in the red cells during the habituation process. With progression of the dilution of NAA and BA concentrations, the red cells gradually lost the ability for anthocyanin formation and growth (Fig. 1). When the red cells were transferred and subcultured on the new medium of the next dilution stage, they were seriously damaged. Therefore, several or more subculturings at the same concentration were needed for their certain recovery. Six passages were needed at the weak-dilution stages (1/2- and 1/4-concentrations), and more (12 to 22) passages were needed at the further dilution stages (1/8- to 1/64-concentration), followed by a decrease in passages at the following stages (Table 1).

The declining tendency of anthocyanin formation continued until the dilution stage of 1/400 concentration (5 μg/l NAA and 0.5 μg/l BA). Anthocyanin content at the trough was near 20%...
(91 A_{530}/g dry wt.) of the initial level at the stage of no dilution. However, the ability for anthocyanin formation was somewhat recovered at the stage of 1/800 concentration (2.5 µg/l NAA and 0.25 µg/l BA). After 6 subcultures at the stage of 1/1600 concentration (1.25 µg/l NAA and 0.125 µg/l BA), the red cells acquired the ability for anthocyanin formation and growth without additions of NAA and BA, namely the red cells were habituated to NAA and BA (Figs. 1 and 2C). The anthocyanin productivity gradually increased through the following 43 subcultures to NAA and BA (Figs. 1 and 2C). The anthocyanin productivity gradually increased through the following 43 subculturings, and returned to near the initial level (477 A_{530}/g dry wt.) at 181st generation (Figs. 1 and 2D). Finally, the 3 best red cell lines were obtained (refer to Table 3). A decline in the productivity of compounds caused by the habituation has also been reported for saponin in Panax ginseng (Nishio et al., 1976), and brassinosteroids in Catharanthus roseus (Park et al., 1989); however, such a remarkable recovery of the productivity as in the present red cells seems rare.

There have been many reports on habituation to plant growth regulators, for example, to indole-3-acetic acid, 2,4-dichlorophenoxyacetic (2,4-D) acid and kinetin in soybean calluses by treatments of 2,4-dinitrophenol and phenoxyisobutyric acid (Christou, 1988); to indole-3-acetic acid and kinetin in sugarbeet callus by Gibberellin A3, and cold treatment (Kevers et al., 1981); to 2,4-D in Nicotiana tabacum callus by treatment of a high concentration of synthetic auxin (Syono & Furuya, 1974); to 2,4-D in Panax ginseng callus by dilution of 2,4-D (Nishio et al., 1976); and to indole-3-acetic acid in Lilium callus by no addition (Sheridan, 1968). But these habituations were not for anthocyanin production.

An enhancement of endogenous indole-3-acetic acid (IAA) level was reported for the auxin habituated soybean cultured cell line (Wyndaele et al., 1988). The results suggest a possibility of similar enhancement of IAA also in the habituated Nyoho red cells. The present results of the very long-term process for the habituation in Nyoho red cells suggest that acquisition of the ability to synthesize the endogenous hormones at levels adequate for the anthocyanin formation was very difficult for the red cells.

Properties of anthocyanins of in habituated red cells Figure 3 shows a reversed phase HPLC analysis of anthocyanins from one of the 3 habituated red cell lines (A) and from fruit (B) of Nyoho strawberry, and Table 2 shows properties of the pigments. The HPLC analysis of the red cell’s anthocyanins gave about 12 peaks including two main peaks 1 (t_R 24.31) and 3 (t_R 31.58), and a small peak 2 (t_R 28.35). Two other lines showed the same elution pattern except for the composition percentages (refer to Table 3). Mori et al. (1993) reported peonidin 3-glucoside and cyanidin 3-glucoside in cultured leaf cells of Shikinari strawberry (F. ananassa Duch.), while HPLC analysis of the Nyoho fruit showed 12 or more peaks including two main peaks 2’ (t_R 28.76) and 5’ (t_R 37.50), small peaks 1’ (t_R 24.26) and 4’ (t_R 33.25), and a very small peak 3’ (t_R 31.62). Tamura et al. (1995) identified pelargonidin 3-glucoside, pelargonidin 3-O-(6-O-malonyl-β-D-glucoside), and cyanidin 3-glucoside in Nyoho fruit. HCl hydrolysis of peaks 1 and 1’, peaks 2 and 2’, or peaks 3 and 3’ followed by HPLC analysis gave cyanidin, pelargonidin or peonidin, respectively. Partial hydrolysis of the peaks with HCl revealed the intermediate products to be only corresponding aglycons (Table 2). Furthermore, complete hydrolysis with HCl and partial hydrolysis of those peaks with hydrogen peroxide and NH_4OH followed by TLC analysis revealed the sugar moiety to be only glucose (Table 2). From the above results and each peak’s 1H-NMR spectrum data, peaks 1 and 1’, peaks 2 and 2’,

**Fig. 3.** HPLC analysis of anthocyanins from red cells (A) and fruit (B) of nyoho strawberry.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Peak No</th>
<th>Anthocyanin</th>
<th>Aglycon</th>
<th>m/z</th>
<th>Number of HPLC peaks after partial HCl hydrolysis of anthocyanin</th>
<th>Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Cells</td>
<td>1</td>
<td>24.31</td>
<td>449</td>
<td>Cyanidin</td>
<td>287</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>28.35</td>
<td>433</td>
<td>Pelargonidin</td>
<td>271</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>31.58</td>
<td>463</td>
<td>Peonidin</td>
<td>301</td>
<td>2</td>
</tr>
<tr>
<td>Fruit</td>
<td>1’</td>
<td>24.26</td>
<td>449</td>
<td>Cyanidin</td>
<td>287</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2’</td>
<td>28.76</td>
<td>433</td>
<td>Pelargonidin</td>
<td>271</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td>31.62</td>
<td>463</td>
<td>Peonidin</td>
<td>301</td>
<td>2</td>
</tr>
<tr>
<td>Peonidin 3-glucoside</td>
<td>31.56</td>
<td>463</td>
<td>Peonidin</td>
<td>301</td>
<td>2</td>
<td>glucose</td>
</tr>
</tbody>
</table>

*See Figs. 3 (A) and (B).
 Obtained by hydrolysis of each peak with H_2O_2 and NH_4OH.
Peak corresponding to 3 or 3’ is not shown.
and peaks 3 and 3’ were identified as cyanidin 3-glucoside, pelargonidin 3-glucoside, and peonidin 3-glucoside, respectively. Peak 5’ of fruit was also identified as pelargonidin 3-O-(6-O-malonyl-β-D-glucoside). Coincidence in absorption spectra between peaks 1 and 1’, peaks 2 and 2’ or peaks 3 and 3’ (or peonidin 3-glucoside), supports the above confirmations (Fig. 4). Peak 4’ of the fruit remains to be elucidated.

The present HPLC analysis of the red cells showed some peaks preceding cyanidin 3-glucoside. Delphynidin glycosides were reported to be eluted earlier than some types of cyanidin glycoside in the fruit of *Vaccinium myrtillus* by HPLC with ODS column (Baj et al., 1983), but delphynidin was not detected in the present study. It seems that some of the preceding peaks may be anthocyanidin di- or tri-glycosides or polymerized proanthocyanidins with anthocyanins (Edwin & Terence, 1988).

**Anthocyanin compositions of habituated red cell lines**

Anthocyanin compositions of the best 3 habituated red cell lines are summarized in Table 3 with that of the fruit. There was a remarkable difference in the compositions between the cultured cells and the fruit as reported by Mori et al. (1993). The red cell’s anthocyanin, cyanidin 3-glucoside and peonidin 3-glucoside amounted to 78 to 85% of the total pigment. On the other hand, the fruit’s anthocyanins, pelargonidin 3-glucoside and pelargonidin 3-O-(6-O-malonyl-β-D-glucoside) amounted to about 96% of the total, as reported by Tamura et al. (1995), who had shown that pelargonidin 3-glucoside and pelargonidin 3-O-(6-O-malonyl-β-D-glucoside) accounted for about 64 and 31%, respectively, of the total. A remarkable difference in the anthocyanin composition was also noticed among the 3 cell lines, cell lines A and B were rich in cyanidin 3-glucoside, peonidin 3-glucoside, and cell line C was comparatively rich in pelargonidin 3-glucoside. It seems that cell line C may be a ramification of cell lines A and B.

Cell line B contained about 55 and 23% of peonidin 3-glucoside and cyanidin 3-glucoside, respectively, and was somewhat close to the Shikinari cultured cells (Mori et al., 1993) in peonidin 3-glucoside domination, which showed relative percentages of peonidin 3-glucoside (70%) and cyanidin 3-glucoside (15%) of the total anthocyanin.

The present results on the habituated red cell lines showed the presence of pelargonidin 3-glucoside, which is the main anthocyanin in Nyoho fruit (Tamura et al., 1995). The synthesis of pelargonidin glycosides seemed to be seriously inhibited or suppressed in the habituated cells by some unknown factor. In contrast, it appeared that the synthesis of peonidin 3-glucoside was highly activated especially in cell line B, because the present results on the fruit showed the occurrence of a trace of peonidin 3-glucoside. Similarly, cyanidin 3-glucoside also seems to have been remarkably activated especially in cell line A. These results suggest that variation of the anthocyanin compositions in the cell

**Table 3. Anthocyanin compositions of habituated red cell lines of Nyoho strawberry.**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cyanidin 3-glucoside</th>
<th>Pelargonidin 3-glucoside</th>
<th>Peonidin 3-glucoside</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>line A 61.5 0.6 18.6 19.3</td>
<td>B 23.2 0.7 55.0 21.0</td>
<td>C 48.9 1.8 35.6 13.6</td>
<td></td>
</tr>
<tr>
<td>Fruit</td>
<td>3.5 57.5 0.4 38.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Calculated from corresponding peak area of HPLC monitored at 520 nm (refer to Fig. 3).
- Correspond to peaks 1 and 1’, peaks 2 and 2’, and peaks 3 and 3’, respectively, in Fig. 3.
- Sum of corresponding peak 5’ (pelargonidin 3-O-(6-O-malonyl-β-D-glucoside), 37.1%), and peak 4’ (unknown, 1.5%) in Fig. 3 (B).

**Fig. 4.** Absorption spectra of anthocyanins from habituated red cells and fruit of nyoho strawberry. (A): peaks 1 and 1’ of Fig. 3, (B): peaks 2 and 2’ of Fig. 3, (C): peaks 3 and 3’ of Fig. 3 and peonidin 3-glucoside (-----), ----- red cells, ----- fruit.
lines may be due to some difference in activation or inhibition of anthocyanin synthetic ability originated from the mother tissue. The difference in anthocyanin compositions between the cultured cells and the intact organs suggested some important changes in the anthocyanin synthetic pathway. Mori et al. (1993) suggested that the shikimic acid channel for producing anthocyanin was inhibited by an unknown factor during callus formation.

Three types of habituated red cell lines from the Nyoho strawberry are almost established, but possibilities of further variation in them cannot be denied. The habituated red cell lines easily multiply in suspension culture, and further investigations on the production of anthocyanins and on direct utilization of these cells as a foodstuff, are in progress.

References


