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Changes of anthocyanin composition by conditioned medium and cell inoculum size using strawberry suspension culture

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SUMMARY

Production of anthocyanin was greater in conditioned medium than in LS medium. The highest production was obtained after 2 days culturing, and the value reached 1100 μg/g cell, which was about twice that of LS medium cultured in each cell inoculum. The highest anthocyanin content was obtained with a 1g cell (fresh weight) inoculation using conditioned medium. The percentage of the main anthocyanin, peonidin-3-glucoside, increased in accordance with the decrease in cell inoculum size, contrary to the results obtained for cyanidin-3-glucoside. This tendency was more clearly observed in LS medium than in conditioned medium.

INTRODUCTION

Anthocyanin is well known as the pigment of flowers and fruits and has been used as a dye and food additive from ancient times. Recently, Igarashi et al. (1990) showed that cyanidin-3-diglucoside-5-monoglucoside, found in the red turnip, *Brassica campestris* L., can decreased the atherogenic index in rats. Also malvidin-3,5-

diglucoside, found in wild grapes, significantly lowers the trioxy glycerols and free fatty acid concentrations, and that the total cholesterol in rat serum was markedly less than that measured in the control (Igarashi and Inagaki, 1991). Moreover, Kamei et al. (1993) reported that anthocyanins inhibit the growth of cancer cells. Studies concerning anthocyanins have therefore become very important not only for their use as food additives but also in the field of pharmacology.

Anthocyanin has also been produced using tissue culture techniques from various plants. However, there are only a few reports that show changes in anthocyanin composition and production using different cell inoculation sizes and conditioned media.

We have identified the structure of anthocyanins produced from suspensions of strawberry, *Fragaria ananassaa* cv Shikinari, cells (Mori et al., 1993), and reported on anthocyanin production under various culture conditions (Mori and Sakurai, 1994; Mori et al.,1994a). While Stuart and Street (1969) used filtered cultured medium of *Acer pseudoplatanus* as the conditioned medium to stimulate cell growth, we have also reported increased anthocyanin production using conditioned medium (Mori et al., 1994b). In the report, we revealed that anthocyanin synthesis is increased according to the concentration of the added conditioned medium which was subcultured 1 week under 800 lux. An increase was also obtained by adding dried conditioned medium. However, the effects of conditioned medium on anthocyanin production are presumed to be connected to cell growth. Therefore, we examined the various effects of cell inoculation size on anthocyanin production using LS medium (Linsmier and Skoog, 1965) and conditioned medium. Further, we studied changes in anthocyanin composition according to different cell inoculum sizes using strawberry suspension cells.

MATERIALS AND METHODS

Plant materials and callus formation

Callus tissue was induced from leaf of *Fragaria ananassa* cv Shikinari (Mori et al, 1993), and the callus was placed on an LS medium containing 3% (W/V) sucrose 0.2% (W/V) Gellangum (Wako Chemical), 0.1mg/L of benzyladenine (BA) and 1mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D). These were incubated at 25°C under 16 hr light, 8 hr dark cycle with fluorescent light of 800 lux.

Cell suspension cultures

Cell suspension cultures were initiated by transferring about 2g (fresh weight) of friable callus tissue to 100 mL of liquid LS medium supplemented with 3% (W/V) sucrose, 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 mL flasks. They were incubated on

a rotary shaker (80 rpm) under continuous fluorescent light of 800 lux at 25°C for 3 weeks, during which the medium was changed every week (Mori et al., 1994). The resulting cell suspension (0.25-2 g fresh) was transferred to freshly prepared LS medium and Conditioned medium (100 mL medium/500 mL flask) and then incubated under continuos fluorescent light of 8000 lux at 25 °C on a rotary shaker to produce anthocyanin.

The conditioned medium was prepared as follows. The third sub-cultured medium under 800 lux was filtered using nylon mesh (30 μm) and used as the conditioned medium (Mori et al., 1994).

HPLC analysis of major anthocyanins

Solution A (acetic acid: acetonitril: water; 20 : 25: 55), diluted to 35 % (V/V) with water and containing 0.1% (V/V) trifluoroacetic acid (TFA) at 4°C was used to extract anthocyanins from fresh callus tissue for HPLC analysis. The extract obtained was then diluted with 35% V/V solution A to a absorbance of 1 at 528 nm. After filteration, 2 μL of the sample was injected and analyzed with HPLC (Waters 600E) using octadecyl silica (ODS) column (Develosil ODS-5, 4.6mm ϕ × 250, Nomura Chemical), which was eluted with 35% (V/V) solution A diluted with water containing 0.1 % (V/V) TFA at 40 °C (Mori et al., 1994).

Determination of anthocyanin content and cell growth

Anthocyanin was extracted overnight from cell using a solution containing 0.1 % HCl-MeOH at 4 °C. After centrifugation at 1000 x g for 5 min, anthocyanin content was calculated with the extinction coefficient ($E^{1\%}_{1cm}$=680 at 528 nm) which was obtained by using purified peonidin-3-glucoside from cultured strawberry cells as a standard (Mori et al., 1993). Total anthocyanin yield was expressed as μg/100 mL, and cells were weighed after filtration of a nylon filter (30 μm).

Statistical analysis.

Data was presented as means ± SE. The statistical significance was determined by one-way analysis of variance followed by a posteriori comparisons on the significant ANOVA results using Fisher PLSD. Statistical significance was set at $p< 0.05$.

RESULTS AND DISCUSSION

Anthocyanin production by different cell inoculum size

Generally, anthocyanin production is affected by cell inoculum size, due to the fact that total anthocyanin production is highly dependent on cell volume. However, cells also produce a conditioning factor into the culture medium according to cell division (Muir et

al.,1954; Stuart and Street,1969; Yamakawa et al., 1985). This may be the cause of the difference in cell growth by various cell inoculum sizes.

Small size of cell inoculum (0.5 and 1.0g cells) showed a drastic increase in cell growth after 5 days (Fig. 1a). While cell growth in the conditioned medium showed a different pattern to that observed in LS medium (Fig. 2a), cell growth rates dropped after 5 days. This may be due to the depletion of nutrients in the culture medium because medium conditioned for 1 week medium was used in this experiment. This is obvious from the resulting difference in cell weight (Figs.1a and 2a).

Anthocyanin synthesis also showed the same tendency regarding cell inoculum size in LS medium (Fig. 1b), however, it differed in the conditioned medium. About 1100 μg/g cell of anthocyanin was obtained by 1g cell inoculum in conditioned medium, and a greater content of anthocyanin was produced by 1g > 0.5g > 2.0g cell inoculum in descending order. The content produced by a 1g-cell was significantly ($p< 0.05$) greater than that of 2g and 0.5g cells, and further it was approximately twice that of LS medium after 2 days of culturing (Fig.1b and 2b). This suggests that there is a maximum cell inoculum size for high stimulation of anthocyanin synthesis.

We have already reported on anthocyanin production using conditioned media (Mori et al., 1994). In the report, we demonstrated anthocyanin synthesis was enhanced by the addition of conditioned medium or dried conditioned medium into freshly prepared LS medium. However, the results of Fig. 2b show that there is threshold to conditioned medium for stimulating anthocyanin. Even with regard to total anthocyanin production, a greater amount of the anthocyanin was obtained in conditioned medium than LS medium after 2 days cluttering (Figs.1c and 2c).

Changes in anthocyanin composition

We have detected about eight pigments, and identified the two major pigments as peonidin-3-glucoside and cyanidin-3-glucoside (Mori et al., 1993). We have already monitored anthocyanin compositions cultured by 0.5g cell inoculation size according to culture time. In the culture condition, the ratio of only the two main pigments changed in the cell. The content of peonidin-3-glucoside increased, while that of cyanidin-3-glucoside decreased as the number of days of culture increased (Mori et al., 1994b).

The ratios of both pigments (Fig. 3a) differed slightly from the results obtained in the former experiment (Mori et al.,1994b), however, the same tendency was observed. Further, the ratios of the pigments changed according to inoculation size when the cells were cultured in LS medium. A decrease in peonidin and increase in cyanidin were evident with increased cell inoculum size. These were significantly ($p<0.05$) changed by the inoculum (Fig. 3a). The tendency seen in Fig. 3a was also observed in conditioned medium (Fig. 3b). However, significant differences were not seen in inoculum greater than 0.5 g. We cannot explain these phenomena. However, the

substance released from the cell must influence not only anthocyanin production but also the composition. There is a possibility of developing production of secondary metabolites using conditioned medium.

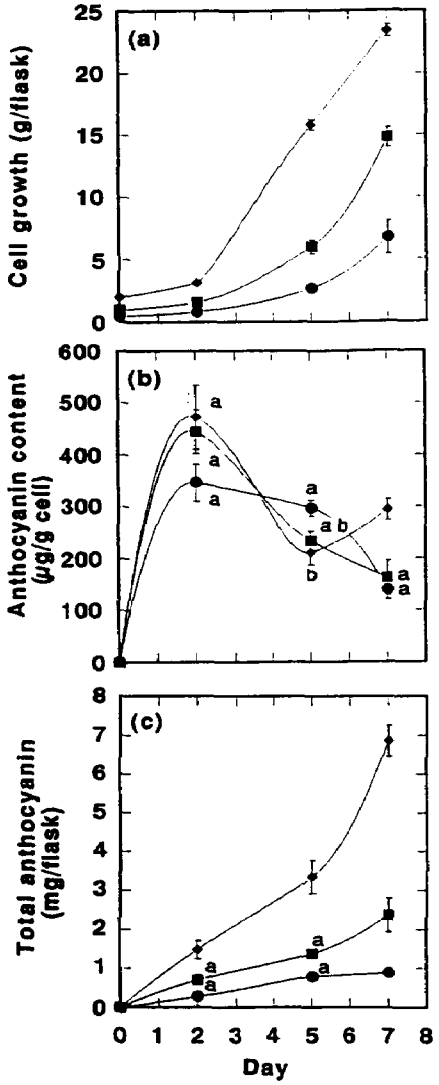

Fig. 1 The effects cell inoculum size on anthocyanin production in LS medium under continuous fluorecent of 8,000 lux using suspension cultures of strawberry cells. Cell inoculum sizes:(●0.5, ■1.0,◆2g fresh weight). Each value represents the averages of three replicates, vertical lines represent standard error of replicates. a: Means with same letter do not show significant difference at $p<0.05$.

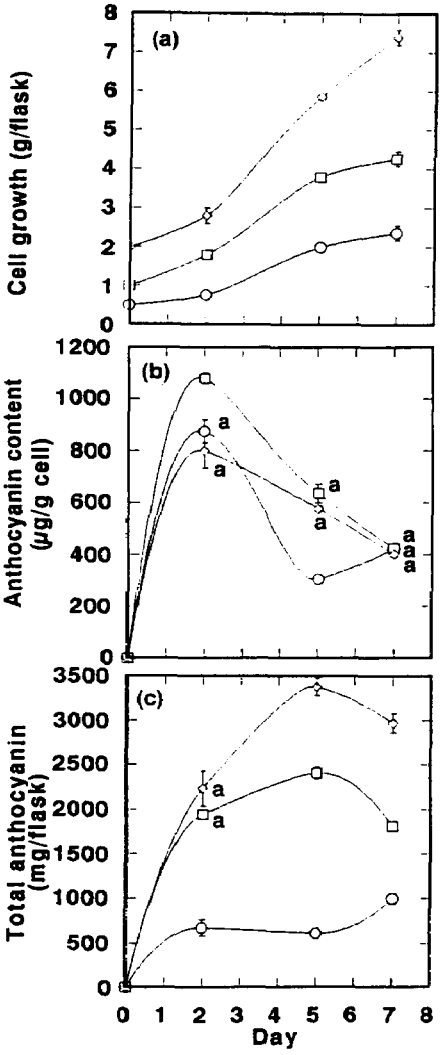

Fig. 2 The effects cell inoculum size on anthocyanin production in Conditioned medium under continuous fluorecent of 8,000 lux using suspension cultures of strawberry cells. Cell inoculum sizes: (○0.5, □1.0, ◇2g fresh weight). Each value represents the averages of three replicates, vertical lines represent standard error of replicates. a-b: Means with same letter do not show significant difference at $p<0.05$.

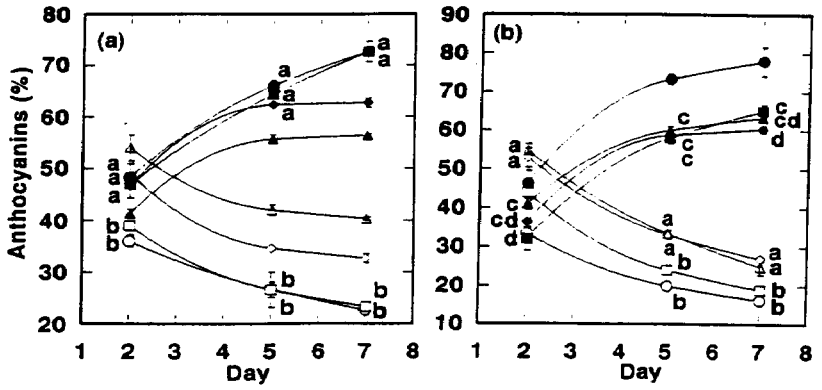

Fig. 3 The changes in the percentage of peonidin-3-glucoside (●—■) and cyanidin-3-glucoside (○—□) in LS (a) and Conditioned medium (b). Cell inoculum sizes (●0.25g; ■ 0.5g; ◆1.0g; ▲2.0g fresh cells) a-d: Means with same letter do not show significant difference at $p < 0.05$.

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