PRODUCTION OF AN UNUSUALLY STABLE ANTHOCYANIN
BY DIOSCOREA CIRRHOSA CULTURED CELLS

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A main anthocyanin accumulated in tubers of Dioscorea cirrhosa was identified as alatanin C isolated from purple yam Dioscorea alata. The accumulation of anthocyanins inside the tubers of the former suggests that the cultured cells are capable of producing anthocyanins without illumination. The cultured cells were found to produce the same anthocyanins as the tubers in the dark at a content of ca. 3% of the dried cells. The anthocyanins were unusually stable in a phosphate buffer (0.05M, pH 6) due probably to the cooperation of self-association and intramolecular stacking between anthocyanindins and aromatic nuclei of phenolic acids combined with the anthocyanin glycoside moiety. It was found that the anthocyanins combined with more sinapic acids were more stable in the same buffer.

Key Words: Dioscorea cirrhosa, Dioscoreaceae, anthocyanin, stability, biotechnological production.

Introduction

Anthocyanins are very important natural pigments used for food and drink coloration\(^{(1)}\). However, most of the pigments are known to be unstable in neutral or weakly acidic aqueous conditions. It has been reported that some of acylated anthocyanin pigments are very stable to decoloration\(^{(2\text{--}3)}\). In addition, the biotechnological production of the pigments by cultured cells usually needs the costly illumination to induce and enhance the productivity\(^{(4\text{--}5)}\), because many of the pigments are formed in plant tissues exposed to sun light that is an important inducing factor of the biosynthesis.

In this study, in order to biosynthesize the red pigments in plant cultured cells in the dark, we selected Dioscorea cirrhosa that is known to accumulate the pigments inside the tubers used as stable colorant in the Southeast Asia. This paper reports that the cultured cells have the capability of producing the red pigments in the dark, and that the pigments are very stable even in a weakly acidic condition.

Materials and Methods

Plant Material

The plant used was a perennial, twining plant, Dioscorea cirrhosa (Dioscoreaceae, Japanese name: SOMEMONOIMO). The plant was cultivated in a green house of our Department and harvested in late autumn. The young purple petioles were used for callus induction on Linmaier-Skoog agar medium containing 3% sucrose, 1 μM 2,4-D and 1 μM kinetin in the dark. The dedifferentiated cultured cells were subcultured on various agar media containing a combination of auxin (1 μM 2,4-D, 1 μM and 10 μM NAA, and 10 μM IAA) and cytokinin (1 μM kinetin) in the dark at 25°C.

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The red-colored cell aggregates in the callus on the agar medium containing 10 μM NAA and 1 μM kinetin were selected and transferred onto the agar medium containing 100 μM NAA and 1 μM kinetin for more effective pigment production. The dark-red cells were harvested and kept in a freezer (−20 ℃) for further chemical investigation.

Isolation of anthocyanins

The red cells (120 g fresh wt) were freeze-dried and extracted, 3 times, with MeOH containing 1% HCl (500 ml) for 24 hrs. The combined extracts were concentrated under reduced pressure at ca. 40 ℃. The concentrate was subjected to polyvinylpyrrolidone (PVPP) column (3 × 5 cm) eluted with water containing 1% AcOH. The fractions were collected in the order of elution of 4 red pigments. Each eluate was re-chromatographed on ODS column (10 µm, 8 × 300 mm, elution: 0.5 ml/min of AcOH: CH,CN: H₂O: TFA=14: 17.5: 120: 0.5) detected at 520 nm to purify the red pigment.

The tubers (200 g fresh wt) of the original plant grown in a green house of our Department were sliced and treated with almost the same procedures with the cultured cells to give the red pigments.

Chemical analysis

The isolated pigment was subjected to ¹H-NMR (400MHz, JEOL JNM-A400) and ¹³C-NMR (100 MHz, JEOL JMN-A400) in 10%TFA-MeOH-d₄ containing TMS as an internal standard and to MS (JOEL, JMS-SX102AQQ Hybrid Mass Spectrometer, positive FAB in a mixture of acetic acid and glycerine). UV/visible spectra were measured with Hitachi A2000 Recording Spectrometer.

Results and Discussion

Growth and pigment formation

The cultured cells of *D. cirrhosa* grew very slowly on the Linsmaier-Skoog agar medium tested. The red pigments were produced on the medium containing NAA as an auxin in the dark; a combination of 100 μM NAA and 1 μM kinetin were best for pigment production among the combinations tested.

Chemical analysis of the pigments

The MeOH-1%HCl extract of the cultured cells was compared with that of the tubers of the original plants by HPLC (Fig. 1). The HPLC profile of the former was qualitatively identical with that of the latter, indicating the presence of several red pigments in both tissues. The total contents of the red pigments in the cultured cells were estimated to be ca. 3% of the dried weight as keracyanin. The MS and NMR spectral data of the main pigment (Dc1) isolated was identical with those of alatanin C (Fig. 2) isolated from *Dioscorea alata* by Yoshida et al.²⁰ The other three pigments were estimated to be the derivatives of alatanin C based on the NMR data, although the chemical structures of these pigments were not elucidated. Their NMR spectra in the aromatic region (Fig. 3) indicate that, while Dc1 contains one residue of sinapate per anthocyanidin nucleus, Dc2 and Dc3 are combined with two sinapate residues per anthocyanidin nucleus and Dc4 has no sinapate residue in the molecule; the signals characteristic for a group of 2, 6-2H of sinapate were assigned as a broad signal at δ 6.28 for Dc1, two broad singlets at δ 6.04 and δ 6.12 for Dc2, and two signals at δ 6.05 and δ 6.10 for Dc3, and no signal for Dc4, though their positions in the molecule were not determined. The tubers were treated with almost the same methods with the cultured cells to give a main pigment. The NMR data were identical with that of Dc1. These data suggest that most of the anthocyanins in the tubers of *D. cirrhosa* are esterified with sinapic acid, and that alatanin C could be one of the common anthocyanins among the tubers of *Dioscorea* plants.
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Fig. 1. HPLC profiles of the MeOH - 1%HCl extracts from the cultured cells and the tubers of D. cirrhosa.

Fig. 2. The chemical structure of Dc1 (alatanin C) isolated from the tubers of D. cirrhosa.
Stability of the pigments in the tubers

The MeOH-1%HCl extract of the tubers was concentrated and dissolved in a phosphate buffer (0.05M, pH 6.0) and the absorbancy of the red solution at 520 nm was measured at some intervals (Fig. 4). The crude anthocyanin solution of the tubers was found very stable compared with keracyanin (cyanidin-3-O-rutinoside) as a standard. Although keracyanin decomposed in one hour, the crude anthocyanin solution from the tubers kept the absorbancy at 520 nm very high for over 20 hours, suggesting that the anthocyanins in the tubers are very stable even in a weakly acidic solution.

Each anthocyanin (Dc1~4) isolated from the tubers was also dissolved in the same phosphate buffer (0.05M, pH 6.0) and the absorbancy was compared for over 40 hours (Fig. 5). Dc4 and keracyanin which are not esterified with phenolic acids decomposed quickly. However, the anthocyanins (Dc1, Dc2 and Dc3), the glycoside moiety of which is esterified with sinapic acid, were found to more stable than keracyanin and Dc4; Dc1 containing one molecule of sinapic acid as phenolic acids were medium stable, and Dc2 and Dc3 including two molecules of sinapic acid based on the NMR data were highly stable among the anthocyanins tested here. It is reported, by Yoshida et al. (6) that phenolic residues combined with the glycoside moiety are able to make anthocyanins highly stable through the cooperation of unique self-association and intramolecular stacking between anthocyanidin and phenolic nucleus. It is suggested that the esterification of anthocyanin glycoside moiety with more phenolic acids like sinapic acid makes anthocyanins more stable.
Fig. 3. NMR spectra of Dc1, Dc2, Dc3 and Dc4 purified from the tubers of *D. cirrhosa*.

Fig. 4. The stability of keracyanin and the crude extract from the tubers of *D. cirrhosa* in a phosphate buffer (0.05 M, pH 6).

Fig. 5. The stability of the red pigments (Dc1 ~ Dc4 and keracyanin) in a phosphate buffer (0.05 M, pH 6).
ソメモノイモ培养細胞による安定なアントシアニンの生成

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ソメモノイモ（Dioscorea cirrhosa, ヤマモノイモ科）は塊茎に多量のアントシアニン系色素を蓄積し、沖縄や東南アジアでは染料として使われている。その主色素は、赤色ダイジョ（Dioscorea alata, 英名: purple yam）から単離されたalatinin Cと同定された。ソメモノイモは太陽光の当たらない塊茎の内部にアントシアニンを蓄積することから、その培養細胞は暗黒下で光刺激を必要とせずにアントシアニンを生成できると推測された。暗黒下で培養した細胞は乾燥重当たり約3%のアントシアニンを生成・蓄積し、そのアントシアニンは、リン酸緩衝液（pH6.0, 0.05M）中で40時間以上安定であった。これは、このアントシアニンの糖部分に結合するシナピン酸とアントシアニジン核が自己同士あるいは互いにスタッキングし、安定化するためと考えられ、さらに、誘導体の比較から、結合するシナピン酸の数が増えると安定性が増すことが判明した。

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