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Flavanone 3-Hydroxylase Activity in Cultured Cells of Roselle (*Hibiscus sabdariffa L.*)

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Roselle (*Hibiscus sabdariffa* L.) is an annual shrub distributing in tropical and subtropical countries which accumulates anthocyanin pigments in calyces. These pigments have been used for making various foods and as food colorants. *In vitro* cultured cells of roselle are capable of producing anthocyanins tentatively identified as cyanidin 3-monoglucoside and cyanidin 3-xylosylglucoside[1]. Important factors influencing the anthocyanin accumulation in roselle cell cultures are 2, 4-D and light irradiation both of which remarkably enhanced the anthocyanin production. In contrast, the anthocyanin accumulation was almost completely inhibited when the cells were cultured in the dark or in a medium containing IAA instead of 2, 4-D[1]. The activity of phenylalanine ammonia-lyase (PAL) catalyzing the initial step of phenylpropanoid pathway, was not affected by either the kind of auxin added to the medium or by the light regime[2]. Although chalcone synthase (CHS), which catalyzes the initial reaction of flavonoid pathway, was shown to play an important role in anthocyanin biosynthesis regulated by light irradiation, the site of regulation by auxin remains to be found[3].

Naringenin, formed from malonyl CoA and *p*-coumaroyl CoA by the action of CHS and chalcone isomerase, is then converted to dihydroflavonols by flavanone 3-hydroxylase. This reaction has been shown to be a key step in the biosynthesis of flavonols and anthocyanidins[4]. In the present communication, we describe detection of flavanone 3-hydroxylase activity in cultured roselle cells and comparison of its activity in the cells cultured in 2, 4-D medium and those cultured in IAA medium.

Suspension cultures of roselle were maintained in Linsmaier and Skoog's medium[5] supplemented with 1 μM 2, 4-D and 1 μM kinetin (2, 4-D medium). Usually the cells (*ca.* 0.5 g) were inoculated in 10 m*l* of medium in a test tube (27×130 mm) and cultured on a reciprocal shaker at 25°C under continuous light irradiation (about 40 μmol • m^{-2} • s^{-1}) with a transfer interval of 14 days. The cells collected by vacuum filtration were immediately frozen in liquid nitrogen and stored at −70°C. All the following operations were carried out at 4°C. The frozen cells (*ca.* 3 g) were mixed with polyvinylpolypyrrolidone and Dowex 1-X2 in 15 m*l* extraction buffer (100 mM tris-HCl, pH 7.5, containing 14 mM 2-mercaptoethanol and 1 mM phenylmethylsulfony fluoride) and stirred for 15 min. The slurry was centrifuged at 17,000 xg for 20 min. The ammonium sulfate fraction (0-75% saturation) was obtained from the supernatant and used as an enzyme preparation after being desalted according to the method of Penefsky[6].

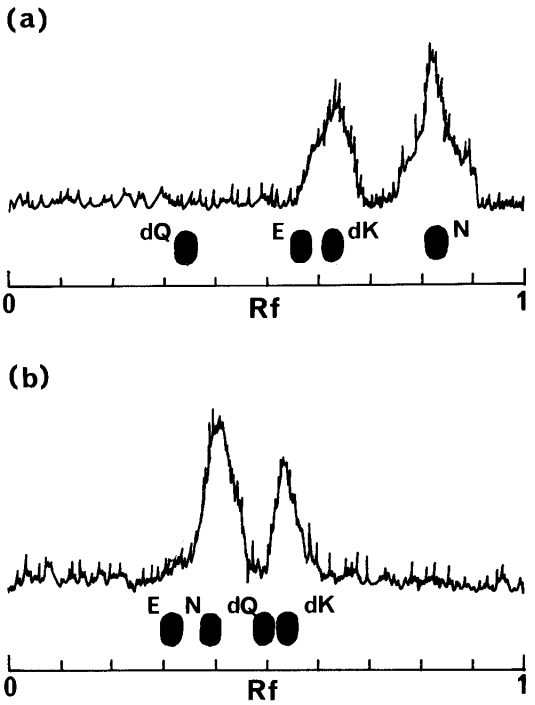

Fig. 1 Radio-chromatograms of ethyl acetate extract from reaction mixture. Development with solvent systems; (a) chloroform-acetic acid-water (9 : 2 : 1), (b) 15% acetic acid. Radioactivity was recorded with Aloka TLC chromato-scanner. N, naringenin; E, eriodictyol; dK, dihydrokaempferol; dQ, dihydroquercetin.

An enzyme preparation (100 μg protein) thus obtained from the cells grown in 2, 4-D medium and actively accumulating anthocyanins (6 days after cell inoculation) were incubated with 42 μmol 2-mercaptoethanol, 25 nmol 2-oxoglutaric acid, 5 nmol ferrous sulfate, 1 μmol sodium ascorbate and 5. 5 nmol [4a, 6, 8$-^{14}$C] naringenin (20, 000 dpm) in total 0. 3 ml of 100 mM tris-HCI, pH 7. 5, at 30° C for 30 min. [4a, 6, 8$-^{14}$C] Naringenin was synthesized from [2$-^{14}$C] malonyl CoA (Amersham) and *p*-coumaroyl CoA (synthesized by the method of Stöckigt and Zenk[7]) by using partially purified CHS preparation obtained from cultured roselle cells according to the method of Britsch and Grisebach[8]. The reaction mixture was then extracted with 250 μl ethyl acetate containing narigenin, dihydrokaempferol, eriodictyol and dihydroquercetin (20 μg each) as cold carriers to locate possible flavonoid products on a TLC plate.

Fig. 1 shows radio-chromatograms of the ethyl acetate extract on cellulose F plates (Merck) developed with (a) chloroform-acetic acid-water (10 : 9 : 1) and (b) 15% acetic acid. The only radioactive product formed from naringenin corresponded to dihydrokaempferol in the two different solvent systems. The identity of the radioactive product with dihydrokaempferol was also confirmed by both autoradiography and radioactivity measurement of the band corresponding to dihydrokaempferol after scraping off the TLC plates (data not shown). The reaction was found to be linear with protein concentration up to 150 μg protein and with time up to 30 min. Incubations without either 2-oxoglutaric acid or ferrous sulfate showed a considerably lower 3-hydroxylation activity than incubation in the presence of both cofactors. These results indicate the occurrence of flavanone 3-hydroxylase in the anthocyanin producing roselle cells cultured in 2, 4-D medium.

Then we compared flavanone 3-hydroxylase activity in the cells cultured in 2, 4-D medium and

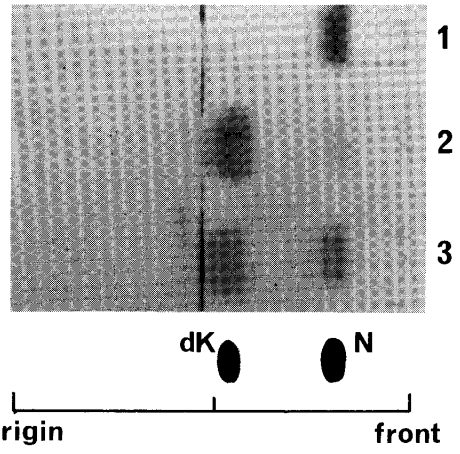

Fig. 2 Autoradiogram of ethyl acetate extract from reaction mixtures incubated with enzyme preparations obtained from the cells grown in 2, 4-D medium (Lane 2) and from the cells cultured in IAA medium (Lane 3). Lane 1 indicates result of control incubation with boiled enzyme.Development with the solvent sytem chloroform-acetic acid-water (9 : 2 : 1). N, naringenin; dK, dihydrokaempferol.

those in IAA medium where 1 μM IAA was added to the medium instead of 2, 4-D. In order to eliminate any carry-over effect of 2, 4-D used in the subculture medium, suspension cells (6 days after cell inoculation) which had been maintained in IAA medium for two successive subcultures and showed no anthocyanin accumulation were used for enzyme preparation. As shown in **Fig. 2**, enzymatic formation of dihydrokaempferol from naringenin could also be detected in the enzyme extract obtained from the cells cultured in IAA medium. The activity of flavanone 3-hydroxylase in the cells in IAA medium was comparable to that of the cells in 2, 4-D medium (**Table 1**).

In conclusion, flavanone 3-hydroxylase activity was expressed in the anthocyanin lacking cells cultured in IAA medium to the same extent as in the anthocyanin producing cells cultured in 2, 4 -D medium. Thus, this enzyme is unlikely to by involved in the regulation of anthocyanin biosynthesis by auxin.

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Table 1. Flavanone 3-hydroxylase activity in suspension cultures of roselle

Enzyme source	Protein (μg/assay)	3-Hydroxylation[4] (dpm in dihydrokeaempferol)
2, 4-D[1]	139	4562
IAA[2]	128	4011
Boiled enzyme[3]	—	228[5]

[1]Enzyme was obtained from the cells grown in 2, 4-D medium.
[2]Enzyme was obtained from the cells grown in IAA medium.
[3]Enzyme obtained from the 2, 4-D medium-grown cells was heated in boiling water for 5 min before used for assay.
[4]Incubated mixture was extracted with ethyl acetate. The extract was applied to a cellulose F plate which was then developed with chloroform-acetic acid-water (9 : 2 : 1). The band corresponding to dihydrokaempferol was detected under UV (254 nm), scraped off, and the radioactivity was determined.
[5]The activity is probably due to tailing of the radioactive naringenin spot on a TLC plate.

generous gift of dihydrokaempferol.

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《和文要約》

ローゼル（*Hibiscus sabdariffa* L.）培養細胞のフラバノン 3-ヒドロキシラーゼ活性

水上　元・荒金真理子・大橋　裕

長崎大学薬学部

ローゼル培養細胞のフラノバノン 3-ヒドロキシラーゼ活性を検討した．IAA を含む培地で培養しアントシアニン生合成を抑制した細胞においても，2, 4-D 培地中で活発にアントシアニンを生産している細胞と同程度の活性を発現しており，本酵素がオーキシンによるアントシアニン生合成の調節に関与しているとは考えられない．