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Characterisation of anthocyanins derived from carrot (*Daucus carota*) cell culture

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Abstract

Two anthocyanin pigments were isolated from cell cultures of Carrot, Nentes scarlet-104 local variety. Chemical hydrolysis, column and paper chromatography, HPLC, proton and ^{13}C NMR and mass spectroscopic studies indicated the presence of cyanidin-3-lathyroside [cyanidin-3-0{β-D-xylopyranosyl (1→2) β-D-galacto pyranoside}] (90%) and cyanidin-3-β-D-glucopyranoside (10%) in the callus cultures, whereas only cyanidin-3-lathyroside (0.05%) was found in the explant carrot. The significant difference was that there was no acylated anthocyanin present as reported in other varieties of carrot.

1. Introduction

Anthocyanins are widely distributed in nature in various plant species. They are mainly found in flowers and fruits. They have high potential as food colorants because of their low toxicity (Markakis, 1982). Anthocyanins have been investigated from a ''black carrot'' variety. It was reported to have six anthocyanins with only cyanidin as an aglycone (Glabgen, Wray, Strack, Metzger & Seitz, 1992). In another study, malvidin and/or peonidin glucosides have also been reported (Harborne, 1967). We report here an anthocyanin from the local variety carrot, Nentes scarlet-104, which is pale orange in colour. The characterization of the stucture of anthocyanin from callus cultures and explant was undertaken by chemical analysis. Significantly, the production of anthocyanin by cell culture could be increased by almost 85–90% as compared to the explant by hormonal influence.

2. Materials and methods

Carrot (*Daucus carota*) seeds, Nentes scarlet-104 variety, were purchased from the Indo-American Seed Corporation. The carrot of the same variety, which is pale orange in colour, was purchased from the local market and used for extraction and quantification of anthocyanin pigment.

Green callus from the seedlings of carrot was obtained in MS media (Murashige & Skoog, 1962) containing 2.0 mg l^{-1} 2,4-dichloro phenoxyacetic acid and 0.2 mg l^{-1} kinetin and 3% sucrose. The callus did not produce any pigmentation. When green the callus was transfered to MS media containing indole-3-acetic acid and kinetin pigmentation was observed. By selecting the pigmented portion of the callus and subculturing it, by the 8th passage, maximum anthocyanin production was achieved with 2.15 mg l^{-1} indole-3-acetic acid and 0.15 mg l^{-1} kinetin in the MS media.

The anthocyanin pigment was extracted from the callus, and also explant, using acidified methanol (1% HCl), overnight at 4°C. The extracts were concentrated at reduced pressure. The concentrate was washed with chloroform and diethylether in order to remove chlorophyll and lipid materials. The concentrate was loaded on a Dowex 50 W-4X (H^+ type) resin column. The column was washed with water and methanol and then the anthocyanin was eluted from the column using acidified methanol. The eluate was concentrated and then separated by paper chromatography and column chromatography using Sephadex LH-20. The column was eluted using methanol:acetic acid:water (10:1:9 v/v) solvent when two fractions were obtained in the case of callus extract and one fraction in the case of explant.

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The anthocyanin fractions **I** and **II** of callus extract and from the explant fraction were hydrolysed using 2 N HCl for 1 h (Francis, 1982). The acyl groups were extracted with diethylether. The aglycones were extracted with amyl alcohol and the aqueous solution was used for the carbohydrate analysis.

The anthocyanidins (aglycones) were analysed using a bondapack C_{18} column (250×4.6 mm, 10 μm) with methanol:acetic acid:water (7:1:2 v/v) as solvent at a flow rate of 1 ml/min at 530 nm (Wilkinson, Sweeny & Lacebucci, 1977). The carbohydrates were identified by paper chromatography on a Whatman No 3 paper using *n*-butanol:benzene:pyridine:water (5:1:3:3 v/v), *n*-butanol:ethanol:water (4:1:2 v/v) and isopropanal: pyridine:water (12:4:4 v/v) as solvents.

The mass spectra was recorded for all the three anthocyanins on a Jeol SX 102/ DA- 6000 mass spectrophotometer with Xenon as FAB gas (6 kv 10 mA). Thioglycerol was used as the matrix.

NMR (400 MHz) spectra were obtained on a Bruker WM-400 spectrometer at 20°C using $CD_3OD + 10\%$ TFA-d as solvent. Chemical shifts were recorded for both proton and ^{13}C NMR as parts per million (ppm) down field from CD_3OD as reference.

3. Results and discussion

Paper chromatography with BAW (*n*-butanol:acetic acid:water (4:1:5 v/v upper phase), HCl:water (3:97 v/v), *n*-butanol:2N HCl (1:1 v/v) and acetic acid:HCl:water (15:3:82 v/v) solvents (Table 1) and column chromatographic separation showed one major spot (90%) and one minor spot (10%) corresponding to two different anthocyanin pigments, **I** and **II** in the case of callus cultures and one spot in the case of explant, which corresponded to the anthocyanin **I** of the callus extract. The crude pigments were further purified by column chromatography using Dowex 50W-4X (H^+ type) and separated by Sephadex LH- 20 gel filtration and HPLC.

The UV–visible spectra showed maximal absorption at 535 and 281 nm. The extinction coefficient $E_{440\ nm}/E_{VIS\ Max}$ 0.26, was similar for all the anthocyanins, indicating it to be a cyanidin-3-glycoside in both callus extract and explant (Harborne, 1967). Alkaline hydrolysis and TLC analysis indicated the absence of any acyl groups in the anthocyanins. This was the major difference found in the explant and also callus extract, when compared to the black carrot pigment. The NMR and MS study also did not indicate the presence of any acyl group in the explant or in callus extract anthocyanin. The acid hydrolysis and TLC analysis indicated cyanidin as the aglycone, which was further authenticated by HPLC, using methanol:acetic acid:water (7:1:2 v/v) as solvent. The paper chromatographic analysis of hydrolysate indicated galactose and xylose as sugars in anthocyanin **I** of callus extract and explant and glucose in anthocyanin **II** of callus extract.

The structures of anthocyanins **I** and **II** and the explant anthocyanin were determined by proton and ^{13}C NMR and Mass spectra.

Table 2
Proton NMR spectral data of cyanidin-3-lathyroside (**I**) and cyanidin-3-glucoside (**II**)

Anthocyanins	**I** δ (ppm)	**II** δ (ppm)
Cyanidin		
4	8.96	8.97
6	6.65	6.82
8	6.89	6.86
2′	8.06	8.15
5′	7.00	6.98
6′	8.28	8.33
3-O-β Galactopyranoside		
1″	5.40	
2″	4.22	
3″	3.89	
4″	3.96	
5″	3.74	
6″	3.84	
β-xylopyranoside		
1‴	4.69	
2‴	3.15	
3‴	3.28	
4‴	3.36	
5a‴	3.01	
5b‴	3.61	
3-O-β-glucopyranoside		
1″		5.37
2″		3.97
3″		3.61
4″		3.56
5″		3.67
6a″		4.02
6b″		3.82

Table 1
R_f values of anthocyanin of callus extract and explant in different solvents

Anthocyanin	Butanol:acetic acid:water	HCL:water	Butanol:2 N HCl	Acetic acid:HCl:water
Anthocyanin **I** from callus extract	31	29	20	35
Anthocyanin **II** from callus extract	38	–	25	26
Anthocyanin from explant	31	28	21	36

Table 3
^{13}C NMR spectral data of cyanidin-3-lathyroside (**I**) and cyanidin-3-glucoside (**II**)

Anthocyanins	**I** δ (ppm)	**II** δ (ppm)
Cyanidin		
2	164.7	164.3
3	145.7	145.6
4	136.5	137.0
5	159.5	159.5
6	103.6	103.5
7	170.6	170.5
8	95.3	95.1
9	157.9	157.7
10	113.5	113.4
1′	121.6	121.3
2′	119.0	118.5
3′	147.7	147.4
4′	156.1	155.7
5′	117.7	117.4
6′	128.9	128.2
3-O-β Galactopyranoside		
1″	102.4	
2″	80.3	
3″	75.4	
4″	70.3	
5″	78.0	
6″	62.6	
3-O-β-xylopyranoside		
1‴	106.3	
2‴	76.1	
3‴	78.1	
4‴	71.3	
5‴	67.4	
3-O-β-glucopyranoside		
1″		103.7
2″’		74.8
3″		78.1
4″		71.1
5″		78.2
6″		62.3

The FAB Mass spectra of the anthocyanin **I** of the callus extract and the explant gave a molecular mass *m*/*z* 581, corresponding to cyanidin-3-lathyroside {cyanidin-3-0-[β-D-xylopyranosyl (1→2)-β-D-galactopyranoside} in addition to *m*/*z* 281 to cyanidin. The anthocyanin **II** of callus extract gave *m*/*z* 449, corresponding to cyanidin-3-glucoside and *m*/*z* 281, corresponding to cyanidin.

The detailed structure of these two anthocyanins **I** and **II** and the explant were elucidated by proton and ^{13}C NMR, as shown in Tables 2 and 3. In the aromatic region, the characteristic signals were easily correlated to cyanidin nuclear protons. The signals of the sugar moieties were observed in the region of δ 5.67–2.70. The two anomeric proton signals were observed at δ 5.40 (J = 8.0 Hz) and δ 4.70 (J = 7.5 Hz), suggesting these sugar units to be β-forms, and vicinal couplings of their ring protons were J = 4.7–11 Hz. Thus, these units must be β-D-pyranose forms (Johansen, Andersen, Nerdal & Aksnes, 1991; Sakamoto et al., 1993). The proton and ^{13}C NMR suggested that the anthocyanin **I** of the callus extract and the anthocyanin derived from explant were identical in structure, whereas anthocyanin **II** in callus extract was not found in the explant. This indicates that there is a hormonal effect on callus cultures in the production of anthocyanins. It was also observed that the pigmentation of the callus was achieved only in IAA media and not in 2,4-D media, as reported earlier by Glabgen et al. (1992). The quantitative analysis of total anthocyanin production indicated only about 0.05% in the explant, whereas it was increased by 85–90% in callus cultures. This is significant for commercial production as a food colorant.

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