http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 9 (15): 2905-2908, 2006
ISSN 1028-8880

Isolation and Structure Charactrisation of Anthocyanin Pigments in Black Carrot (*Daucus carota* L.)

[1]Ghasemifar Elham, [1]Heydari Reza, [2]Khalafi Jabbar, [1]Setareh Parisa and [1]Jameii Rashid
[1]Department of Biology, [2]Department of Chemistry, Faculty of Science,
Uromia University, Uromia, Iran

Abstract: Three anthocyanin pigments were isolated from black Carrot root *(Daucus carota* L.*)*. Cationexchange resin column, TLC analysis, UV-visible spectrophotometry and proton NMR spectroscopy studies indicated the presence of nonacylated compound 1 is β-D-glucopyranosyl-(1>6)-[β-D-xylopyranosyl-(1>2)]-β-D-galactopyranosyl-(1>O^3)-cyanidin. Acylated compounds 2 and 3 are 6-O-acyl-β-D-glucopyranosyl-(1>6)-[β-D-xylopyranosyl-(1>2)]-β-D-galactopyranosyl-(1>O^3)-cyanidins. Anthocyanins 2 and 3, respectively, were acylated with 2-methoxycinnamic acid and 4-hydroxy-3, 5-dimethoxycinnamic acid (sinapic acid). Monomeric anthocyanin content was determined by pH-differential. There was 434.85±1.3 mg kg^{-1} dry matter (cyanidine-3-glucoside base).

Key words: Black carrot, anthocyanin, acylated, TLC, UV-visible, ^{1}H-NMR

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). The so-called black or purple carrot (*Daucus carota* L.) originated from Central Asia, where it has been known for ~3000 years. The identification of the anthocyanins that are responsible for the color of black carrots has been the subject of several studies (Baker *et al*., 1994; Dougall *et al*., 1998; Glässgen *et al*., 1992a, b; Harborne *et al*., 1983). Major anthocyanins were found in black carrot plant tissues and cell cultures possess cyanidin as an aglycon. Five major pigments in black carrot root were reported, three of anthocyanins were acylated with sinapic, ferulic, or p-coumaric acid (Dougall *et al*., 1998; Glässgen *et al*., 1992b; Kammerer *et al*., 2004). Only recently, anthocyanins processing a Peonidin or pelargonidoin type aglycon were also identified in black carrots, but only in trace amounts (Kammerer *et al*., 2003a, b). In another study, up to seven cyanidin glycosides, which, five of them were acylated with hydroxycinnamic and hydroxybenzoic acids, were identified and quantified in 15 black carrot roots. Total anthocyanin amounts were ranged from 45.5 mg kg^{-1} dry matter to 17.4 g kg^{-1} dry matter (Kammerer *et al*., 2004). We report here three anthocyanins from black or purple carrot extract (*Daucus carota* L.). Structure of pigments were characterized by ^{1}H-NMR spectroscopy and UV-visible spectrophotometry. Significantly, two compounds of anthocyanins were acylated with cinnamic acid. Monomeric anthocyanin content was determined by pH-differential at pH 1 and 4.5.

MATERIALS AND METHODS

Black carrot samples: Black carrots has been grown wildly and cultured in Sadaghian village of Salmas at West Azarbayejan (WA) province in the west of Iran. Black carrot roots were harvested from Sadaghian village. The samples were washed in cold tap water and sealed in polyten bags, frozen and stored at -18°C.

Extraction procedures: Carrot (*Daucus carota* L.) roots (500 g) were thawed at room temperature and marcerated in a Waring blender with methanol (250 mL) containing 0.1% HCl (37.7% v v^{-1}) under a nitrogen atmosphere for 5 min. The macerate was filtered through wathman No. 1 filter paper under vacuum and the residue was extracted with the same solvent until most of the pigments were extracted. The combined extract were mixed with Dowex 50 W-X8 cation-exchange resin (H^+ type) column. After a short time, the resin was thoroughly washed with distilled water to remove sugars and then with pure methanol to remove other organic compounds from anthocyanins. The pigments were eluted from the resin using methanol

Corresponding Author: Ghasemifar Elham, Department of Biology, Faculty of Science, Uromia University, Uromia, Iran
Tel: 0098-0872-4226652

containing HCl at concentrations varying from 0.1 to 1.0% (v v^{-1}). The eluates were combined and concentrated under vacuum in a flash evaporator at 34°C almost to dryness. The concentrate was redissolved in a small volume of 0.01% HCl in methanol and stored at -2°C in the dark, until further analysis.

Monomeric anthocyanin content: Monomeric anthocyanin content was determined by using a pH-differential method. WAP biowave S2100 Diode Array were used for spectral measurements at 525 and 700 nm. Pigment content which was calculated, based on cyanidine -3-glucoside with a molecular weight (MW) of 445.2 g L^{-1} and molar absorbance (ε) of 29600 cm^{-1} mg^{-1} (Giust and Wrolstad, 2001). The monomeric anthocyanin content of black carrot was 434.85±1.3 mg kg^{-1} dry matter.

One and two dimensional cellulose TLC: For Thin-layer Choromatography (TLC) on cellulose, about 20 μg of the concentrated extract was spotted on each cellulose plate (20×20 cm). For, Two-dimension TLC, the first solvent was BAW(n-buthanol-acetic acide-water, 4: 1: 5 v v^{-1}) and the second solvent was AWH (acetic acid-water-HCl, 15: 82: 3 v v^{-1}). The One-dimension TLC done was carried out in BuH (n-buthanol-HCl,1: 1)solvent system (Narayan and Venkataraman, 2000).

Separation and purification of anthocyanin: The isolated bands of TLC were eluted with 0.01% HCl in methanol and the solvent was concentrated under vacuum condition in a flash evaporator at 34°C almost to dryness and stored at -2°C.

Spectroscopy analysis: ^{1}H-NMR spectral data were recorded on an instrument operating at Bruker Avance-AQS 300 MHZ in CD_3OD/TFA-d_1 (98: 2 v v^{-1}) or CD_3OD When the signals of the anomeric protons of the investigated anthocyanins were partially overlapped by the signal of the dissolved H_2O, an additional shift to lower magnetic field of this signal was produced by adding TFA-d_1. UV-Visible spectral data were recorded on 0.01% HCl in methanol and vis spectra were measured in 0.1 M acetate+0.1 M phosphate adjusted to pH 2.0 and to pH 6.0 (HCl) within an hour of preparing the solution using UV-Vis spectrophotometer.

RESULTS AND DISCUSSION

Isolation, purification and characterization of anthocyanins: Compounds 1-3 of black carrot roots were isolated, purified and characterized (saw Materials and methods section). The nonacylated anthocyanin is β-D-glucopyranosyl-(1>6)-[β-D-xylopyranosyl-(1>2)]-β-D-galactopyranosyl-(1>O^3)-cyanidin (1). Compound 1 have been partially characterized (Baker *et al*., 1994; Giust and Wrolstad, 2001; Glässgen *et al*., 1992a, Kammerer *et al*., 2004). Anthocyanins 2 and 3 are 6-O-acyl-β-D-glucopyranosyl-(1>6)-[β-D-xylopyranosyl-(1>2)]-β-D-galactopyranosyl-(1>O^3)-cyanidins. Compounds 2 and 3, respectively, were acylated with 2-methoxycinnamic acid and 4-hydroxy-3,5-dimethoxycinnamic acid (sinapic acid) (Baker *et al*., 1994; Glässgen *et al*., 1992a; Kammerer *et al*., 2004). The structures of three anthocyanin are shown in Fig. 1.

NMR studies: The ^{1}H-NMR data for anthocyanins 1, 2 and 3 are given in Table 1. By using of the ^{1}H-NMR chemical shifts and coupling constants for oligosaccharides in general, together, with data that have been reported from NMR spectroscopy of anthocyanins

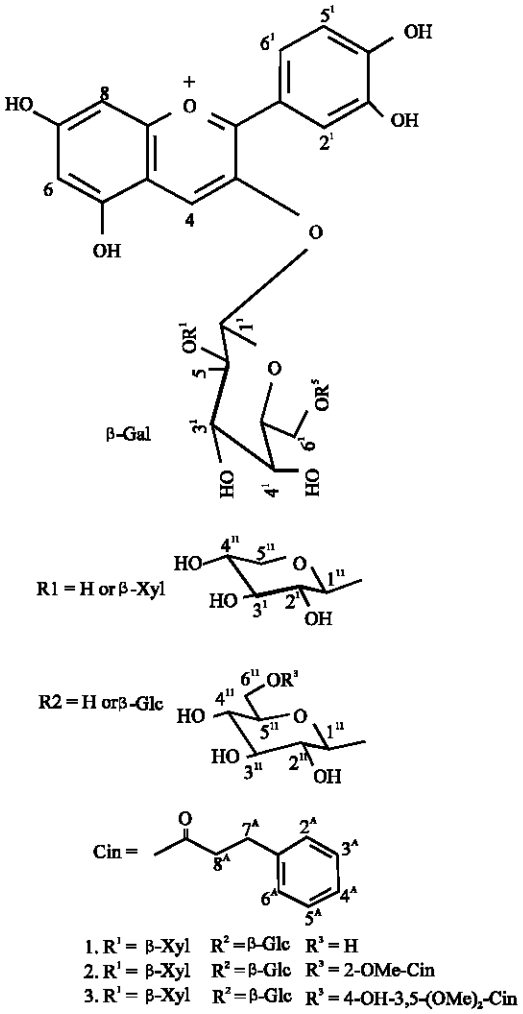

Fig. 1: Structure of anthocyanins in black carrot

Table 1: H NMR data of anthocyanins 1, 2 and 3

	H	1	2	3
Flav	4	8.98s	8.46d	8.48d
	6	6.73d	6.69d	6.67d
	8	6.93d	6.50d	6.56d
	2′	8.09d	7.92d	7.95d
	5′	7.05d	7.01d	7.02d
	6′	8.29	8.15	8.19
Gal	1^{I}	5.40d	5.25d	5.30d
	2^{I}	4.21	4.27	4.27
	3^{I}	3.91	4.18	4.20
	4^{I}	4.01d	3.93d	3.91d
	5^{I}	4.03	4.50	4.46
	6^{IA}	3.99	4.23	4.22
	6^{IB}	3.85	3.76	3.75
Xyl	1^{II}	4.70d	4.83d	4.87d
	2^{II}	3.17	3.19	3.16
	3^{II}	3.31	3.32	3.32
	4^{II}	3.39	3.23	3.27
	5^{IIA}	3.65	3.65	3.64
	5^{IIB}	3.02	3.12	3.16
Glc	1^{III}	4.26d	4.48d	4.47d
	2^{III}	3.17	3.44	3.40
	3^{III}	3.22	3.44	3.40
	4^{III}	3.30	3.76	3.75
	5^{III}	3.31	3.44	3.40
	6^{IIIA}	3.85	5.34	5.33
	6^{IIIB}	3.60	4.11	4.12
Acyl	2^{A}		7.22	6.20s
	3^{A}			
	4^{A}			
	5^{A}			
	6^{A}			
	7^{A}			6.20s
	8^{A}		7.55d	7.28d
	MeO		6.5d	6.20d
			3.58s	3.40s
			2^{A}	$3^{A},5^{A}$

(Glässgen *et al*., 1992a; Strack and Wary, 1989), specific protons in this anthocyanins were assigened. For example, the anomeric proton of each unit of the aglycone was observed in regions typical for that particular sugar moiety. D-galactose subunit for 1, 2 and 3, respectively, were δ 5.40, 5.25 and 5.30; D-xylose subunit, δ 4.70, 4.83 and 4.87; D-glucose subunit, δ 4.26, 4.48 and 4.47. All these protons exhibited H-1–H-2 coupling constants between 7.3 and 7.8 Hz, showing that all the sugar linkages are in the β-D-configuration. When xylose is present, H-2^{e} is found downfield at more than δ 4.169, confirming the attachment of xylose at the 2-position of the galactose residue (Dougall *et al*., 1998). Comparison of the NMR data of a monoacylated compounds with its nonacylated counterpart, e.g., 2, 3 and 1, respectively, indicates that acylation has occurred on C-6^{III} of the glucose moiety as the chemical shift of the H-6^{III} of 2 and 3, they are observed at δ 5.34 and 5.33 that for 1 at δ 3.85.

The presence of a cinnamic acid residue on the glucose moiety has a marked effect on the chemical shift of the cyanidin H-4 proton in the acylated compounds. The chemical shift for H-4 in nonacylated 1 is δ 8.98, while those for the acylated 2 and 3 appear at δ 8.46 and δ 8.48.

Table 2: UV-visible data of anthocyanins 1, 2 and 3

Compounds	λ_{max} (nm) MeOH/HCl	λ_{max} (nm) pH 2.0	λ_{max} (nm) pH 6.0	Ratio of ApH 6:A pH 2
1	529,282	513	537	0.09
2	537,326,281	524	545	0.41
3	538,297,319	527	547	0.49

Also, there is a change in the chemical shift of H-8 from the region of the δ 6.93 in the nonacylated 1 to δ 6.50 and δ 6.56 in the cinnamoylated compounds 2 and 3.

Glässgen *et al*. (1992a) have noted a pronounce upfield shift of H-4 in carrot anthocyanins acylated with sinapic, ferulic, 4-coumaric and 4-hydroxybenzoic acids relative to that for the nonacylated compound. However, the specific nature of the interactions of the acyl groups with the chromophore are not clear at this time.

UV-vis spectral data: In MeOH-HCl the $\lambda_{vis.max}$ of the nonacylated 1 was at 529 nm. The acylated compounds except 2 and 3, respectively, had $\lambda_{vis.max}$ at 537 and 538 nm. Compounds 2 and 3 in MeOH-HCl all had either a shoulder or a peak at 319 and 326 nm. These compound are acylated with cinnamic acid bearing an electron-donating substituent. Anthocyanins undergo a pH-dependent hydration reaction to give colorless hemiacetals, the ratio of absorbance at pH 6: pH 2 is a semiquantitive measure of color retention by an anthocyanin at near-neutral pH.

For the anthocyanins examined here the $\lambda_{vis.max}$ at pH 6 is increased by 20-24 nm compared to those values a pH 2. The ratio of absorbance at the visible peak at pH 6: pH 2 is smallest with 1 and tends to be greatest with acyl groups bearing electron-donating substituents. In general, color retention at pH 6 is greater in the anthocyanins were acylated with cinnamic acid compared to the nonacylated one Table 2.

REFERENCES

Baker, D.C., D.K. Dougall, W.E. Glässgen, S.C. Johanson, J.W. Metzger, A. Rose and H.U. Seitz, 1994. Effects of supplied cinnamic acid and biosynthetic intermediates on the anthocyanins accumulated by wild carrot suspension cultures. Plant Cell. Tiss. Organ. Cult., 39: 79-91.

Dougall, D.K., D.C. Baker, E.G. Gakh, M.A. Redus and N.A. Whittemore, 1998. Studies on the stability and conformation of monoacylated anthocyanins. Part 2-Anthocyanins from wild carrot suspension cultures acylated with supplied carboxylic acids. Carbohydr. Res., 310: 177-189.

Giust, M.M. and R.E. Wrolstad, 2001. Unit F1. 2. Anthocyanins Characterization and Measurement with UV-Visible Spectroscopy. In: Current Protocols in Food Analytical Chemistry. Wrolstad, R.E. and S.J. Schwartz (Eds.), New York: Wiley.

Glässgen, W.E., V. Wary, Strack, J.W. Metzger and H.U. Seitz, 1992a. Anthocyanins from cell suspension cultures of *Daucus carota*. Phytochemistry, 31: 1593-1601.

Glässgen, W.E., H.U. Seitz and J.W. Metzger, 1992a. High-performance liquid chromatography/electrospray mass spectrometry and tandem mass spectrumetry of anthocyanins from plant tissues and cell culture of *Daucus carota* L. Biol. Mass Spectrom, 21: 271-277.

Harborne, J.B., A.M. Mayer and N. Bar-Nun, 1983. Identification of the major anthocyanin of carrot cells in tissue culture as cyanidin3-(sinapoylxylosyglucosylgalactoside). Z. Naturforsch., C: Biosci., 38: 1055-1056.

Kammerer, D., N. Bersrdini, A. Schieber and R. Carle, 2003a. Charakterisierung von Anthocyanen und phenolishen Säuren aus Schwarzen Karotten mittels HPLC-MS. Lebensmittelchemie, 57: 28-29.

Kammerer, D., R. Carl and A. Schieber, 2003b. Detection of peonidin and pelargonidin glycosides in black carrots (*Daucus carota* ssp. sativus var. atrorubens Alef.) by high-performance liquid chromatography/ electrospary ionization mass spectrometry. Rapid Commun. Mass Spectrom, 17: 2407-2412.

Kammerer, D., R. Carl and A. Schieber, 2004. Quantification of anthocyanins un black carrot extracts (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) and evaluation of their color properties. Eur. Food Res. Technol., 219: 479-486.

Markakis, P., 1982. Anthocyanin as food colours. New York: Academic Press.

Narayan, M.S. and L.V. Venkataraman, 2000. Characterisation of anthocyanins derived from carrot (*Daucus carota*) cell culture. Food Chem., 70: 361-363.

Strack, D. and V. Wary, 1989. Anthocyanins. In: Methods in Plant Biochemistry. Dey, P.M. and J.B. Harborne (Eds.), Vol. 1, Academic Press, New York, Chapter, 9: 325-356.