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Full Length Research Paper

Anthocyanins from leaf stalks of cassava (*Manihot esculenta* Crantz)

Robert Byamukama*, Jane Namukobe and Bernard Kiremire

Chemistry Department, Makerere University, P.O. Box 7062, Kampala, Uganda.

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The anthocyanins, cyanidin 3-*O*-(6''-*O*-α-rhamnopyranosyl-β-glucopyranoside) (1) and delphinidin 3-*O*-(6''-*O*-α-rhamnopyranosyl-β-glucopyranoside) (2) isolated from the leaf stalks of cassava (*Manihot esculenta* Crantz) by a combination of chromatographic techniques, and their structures were elucidated mainly by the use of homo- and heteronuclear magnetic resonance spectroscopy. The relative amounts of 1 and 2 in the extracts were approximately 92 and 4%, respectively. The absolute amount was 124 mg/100 g.

Key words: Cassava, *Manihot esculenta,* anthocyanins, cyanidin 3-*O*-(6''-*O*-α-rhamnopyranosyl-β-glucopyranoside, delphinidin 3-*O*-(6''-*O*-α-rhamnopyranosyl-β-glucopyranoside).

INTRODUCTION

Cassava, (*Manihot esculenta* Crantz) is a perennial crop native to tropical America with its center of origin in North-eastern and Central Brazil (Lenis et al., 2006; Allen, 2002). In the tropics, the roots of cassava are an important source of carbohydrates for human consumption. Cassava plants also produce green leaves that are consumed as vegetables in some parts of sub-Saharan Africa such as Democratic Republic of Congo (DRC), Uganda, Nigeria and some Asian countries, the Philippines, Indonesia and Malaysia (Almazan et al., 1989). The dry pounded cassava leaf vegetable has reached commercial exploitation in the DRC, being exported to Belgium and France (Ngudi et al., 2003 a). The leaves are known to be rich in vitamin A and proteins (Chandrika et al., 2006; Ngudi et al., 2003 a, b). In addition to use as food, the crop has taken on more importance as a source of starch for industry and food processing and as an animal feed (Lenis et al., 2006; Eruvbetine et al., 2003; Eruvbetine and Afolami, 1992).

Cassava was introduced in Uganda sometime after 1862, and it rapidly spread to most parts of the country (Jameson, 1964) where it provided a basic daily source of dietary energy. Roots are processed into a wide variety of granules, paste, flour, etc., or consumed freshly boiled or raw (Bua et al., 1997). The leaves are also consumed as a green vegetable, which provides proteins and vitamins A and B. Cassava has been recognised as one of the most important food crops in Uganda, second to bananas in terms of area cultivated, total production and per capita consumption ((Bua et al., 1997).

The anthocyanins constitute a major flavonoid group which are responsible for colours ranging from salmon pink through red and violet to dark blue of most flowers, fruits, stems, and leaves of angiosperms (Andersen and Jordheim, 2006). In addition to their potential as food colorants, anthocyanins are nowadays regarded as important nutraceuticals mainly due to their possible antioxidant effects, and they have been given a potential therapeutic role related to some cardiovascular diseases, cancer treatment, inhibition of certain types of virus including Human Immunodeficiency Virus type 1 (HIV-1) and improvement of visual acuity (Stintzing et al., 2002; Talavera et al., 2006; Sandvik et al., 2004; Beattie et al., 2005; Jang et al., 2005; Cooke et al., 2005; Andersen et al., 1997; Nakaishi et al., 2000).

Prawat et al. (1995) identified 3-rutinosides of kaempferol and quercetin; the cyanogenic glycosides, lotaustralin and linamarin, from the fresh leaves of cassava. There has been no report, so far, on the anthocyanin content from the leaf stalks of cassava. The objective of this work was to isolate and determine the structure of anthocyanins responsible for the colour in cassava leaf stalks. The leaf stalks of cassava can be used as a

*Corresponding author. E-mail: rbyamukama@chemistry.mak.ac.ug. Tel: +256 392 548 585. Fax: +256-41 531 061.

Table 1. Calibration equation for determination of anthocyanin concentrations by HPLC.

Cy3gal[a] (mM)	Regression equation[b]	r^c	S.D[d]		S_r^e
			Slope	Intercept	
0.026-4.194	$C_{anthocyanin}=8.7\times10^{-5}$Area$-3.2\times10^{-2}$	1.0	7.3×10^{-7}	1.7×10^{-2}	0.035

[a] = Cyanidin 3-galactoside, isolated from *Aronia melanocarpa*, black chokeberry
[b] = Ratios of peak area of cyanidin 3-galactoside vs. the appropriate concentration in mM; seven standards (0.026-4.194 mM)
[c] = Correlation coefficient
[d] = Standard deviation of slope and intercept
[e] = Standard error of the estimate.

source of anthocyanins since they are thrown away after harvesting the tubers and the leaves.

MATERIALS AND METHODS

Plant material

Leaf stalks of the improved variety of *M. esculenta* Crantz (Cassava) were collected in a home garden in Kisaasi, a Kampala suburb, in Uganda. The identification of the plant was carried out in the Botany Department Herbarium at Makerere University, and voucher specimen has been deposited in the herbarium of the same Department, voucher No. RB34/2007. The leaf stalks were kept in a freezer before extraction.

Isolation of anthocyanins

The leaf stalks of cassava (200 g) were extracted with 1% trifluoroacetic acid (TFA) in methanol. The filtered extract was concentrated under reduced pressure, purified by partition (several times) against ethyl acetate and applied to an Amberlite XAD-7 column. The anthocyanins adsorbed to the column were washed with water, and eluted from the column with methanol containing 1% TFA. The concentrated anthocyanin extract was purified by Sephadex LH-20 chromatography using 50% aqueous methanol containing 1% TFA as eluent. The individual anthocyanins were separated using preparative HPLC (Gilson 305/306 pump equipped with a UV 6000 LP detector) equipped with an ODS Hypersil column (25 × 2.2 cm; i.d.; 5 µm). Two solvents were used for elution: A = formic acid–water (1:9; v/v) and B = formic acid–water–methanol (1:4:5; v/v) (Andersen, 1987). The elution profile consisted of a linear gradient from 10 to 100% B for 30 min, isocratic elution (100% B) for the next 12 min, followed by a linear gradient from 100 to 10% B for 2 min. The flow rate was 14 ml/min for 44 min, and aliquots of 500 µL were injected.

Co-chromatography (TLC, on-line HPLC)

(Co-chromatography included TLC and on-line HPLC. TLC was carried out on microcrystalline cellulose (F 5556, Merck) with the solvent FHW (HCO_2H-conc HCl-H_2O; 1:1:2 v/v). The analytical HPLC instrument (HP-1050 module, system, Hewlett-Packard) was equipped with an ODS Hypersil column (25 × 0.46 cm, 5 µm). Two solvents; C, (water with 0.5 % trifluoroacetic acid) and D, (acetonitrile with 0.5% trifluoroacetic acid) were used for elution. The elution profile for HPLC consisted of initial conditions with 90% C and 10% D followed by linear elution for 10 min (14% D), isocratic elution 10-14 min, and the subsequent linear conditions; 18 min (16 % D), 22 min (18 % D), 26 min (23 % D), 31 min (28 % D) and 32 min (40 % D), isocratic elution 32-40 min, and final linear elution 40 - 41 min (10% D). Aliquots of 15 µL were injected and the flow rate was 1 ml / min. Prior to injection, all samples were filtered through a 0.45 µm Millipore membrane filter. This method was a modifiaction of the techniques earlier reported (Andersen and Francis, 2004; Takeoka et al., 2002).

Spectroscopy

UV–VIS absorption spectra were recorded on-line during HPLC analysis, and the spectral measurements were made over the wavelength range 200–600 nm in steps of 2 nm. The NMR experiments were obtained at 600.13 and 150.92 MHz for ^1H and ^{13}C, respectively, on a Bruker DRX–600 instrument at 25°C. The deuterio-methyl ^{13}C signal and the residual ^1H signal of the solvent, CF_3COOD–CD_3OD (95:5; v/v), were used as secondary references (d 49.0 and d 3.4 ppm from tetramethylsilane for ^1H and ^{13}C, respectively (Andersen and Fossen, 2003). The 1D ^1H NMR and the 2D HMBC, HSQC and DQF-COSY experiments were obtained with the 5 mm TB1 probe.

Quantitative determination

The quantitative determination of the anthocyanin content in the leaf stalks of cassava was related to a standard curve based on pure amounts of cyanidin-3-galactoside, isolated from *Aronia melanocarpa*, black chokeberry, (Chandra et al., 2001). Integration data recorded during on-line HPLC analysis detected at 520 ± 20 nm were directly correlated with a regression equation based on seven different concentrations of cyanidin-3-galactoside recorded by on-line HPLC under similar conditions (Table 1).

RESULTS AND DISCUSSION

The HPLC chromatogram of the weakly acidified methanolic extract of leaf stalks of cassava detected in the visible spectral region revealed one major 1 (92%) and one minor 2 (4%) anthocyanins (Table 2). The pigments were purified by partition against ethyl acetate and Amberlite XAD-7 column chromatography, and separated by Sephadex LH-20 column chromatography and preparative HPLC. The pure anthocyanins were checked for their homogeneity by analytical HPLC (Table 2).

The UV–VIS spectra of 1 recorded on-line during HPLC analysis showed visible maxima around 520 nm, and $A_{440}/A_{VIS\text{-}Max}$ was around 31%, indicating cyanidin or peonidin -3-glycosides (Andersen, 1987) (Table 2). The downfield part of the 1D ^1H NMR spectrum of 1 showed a singlet at 9.03 ppm (H-4), a 3H AMX system at 8.36 ppm (dd, 8.7 Hz, 2.3 Hz; H-6´), 8.13 ppm (d, 2.3 Hz; H-2´) and

Table 2. Chromatographic and spectral data of the anthocyanins in cassava leaf stalks. See Figure 1 for structures.

Compound	R_f (TLC), FHW	t_R (HPLC) (min)	Absorption maxima (nm)	$A_{440}/A_{VIS\text{-}max}$ (%)
1	0.48	21.9	520	31
2	0.35	17.4	530	25
1[a]	0.48	21.9	520	31
2[b]	0.35	17.4	530	25

[a]Cyanidin 3-rutinoside and [b] delphinidin 3-rutinoside from blackcurrant (*Ribes nigrum*) (Frøytlog et al., 1998).

Table 3. ^{1}H and ^{13}C NMR spectra data for cyanidin 3-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (1) and delpinidin 3-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (2) in CD_3OD:CF_3COOD (95:5, v/v) at 25°C.

	^{1}H, δ (ppm), multiplicity & J_{HH} (Hz)		^{13}C (δ) ppm	
aglycone	**1**	**2**	**1**	**2**
2			162.98	164.50
3			144.25	145.62
4	9.03 *s*	8.98 *s*	135.02	135.21
5			157.45	159.39
6	6.76 *d* 2.0	6.76 *d* 2.0	102.52	103.26
7			169.21	170.22
8	6.98 *dd* 2.0, 0.82	6.96 *d* 2.0	95.25	95.01
9			156.36	157.65
10			111.66	112.87
1’			119.89	119.58
2′	8.13 *d* 2.3	7.87 s	116.88	112.57
3’			145.92	147.44
4’			154.27	144.90
5’	7.11 d 8.7		116.10	147.57
6’	8.36 dd 8.7, 2.3	7.87 s	126.95	112.71
glucopyranoside				
1″	5.37 d 7.8	5.39 d 7.8	102.53	103.15
2″	3.76 d 7.8	3.76 d	73.45	74.60
3″	3.63 t 9.0	3.65 t	76.72	77.85
4″	3.48 dd 9.0, 9.3	3.53a	69.76	71.19
5″	3.80 m	3.82a	75.87	77.27
6A″	4.15 dd 11.1, 1.6	4.01a	66.24	67.65
6B″	3.68 m	3.79a		
6″-α rhamnopyranosyl				
1‴	4.74 d 1.5	4.73 d 1.2	101.92	102.08
2‴	3.89 dd 1.5, 3.2	3.90 m	70.53	71.90
3‴	3.72 m	3.74 m	71.26	72.31
4‴	3.41 m	3.42 m	72.54	73.78
5‴	3.64 m	3.64 m	68.28	69.72
6‴	1.25 d 6.3	1.26 d 6.4	16.91	16.85

[a]determined from HSQC spectrum.

and 7.11 ppm (d, 8.7 Hz; H-5′) and an unresolved 2H AB system at 6.98 ppm (H-8) and 6.76 ppm (H-6), respectively (Table 3), in accordance with the anthocyanin, cyanidin. After the chemical shifts of the protons of 1 were assigned, the chemical shifts of the corresponding carbons (Table 3) were assigned from the HSQC experi-

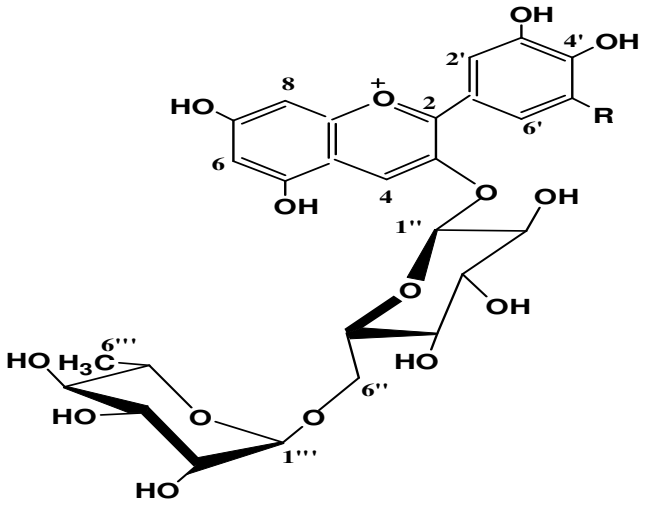

Figure 1. The structure of the anthocyanins identified in *Manihot esculenta*. R = H: cyanidin 3-*O*-(6''-*O*-α-rhamnopyranosyl-β-glucopyranoside) (1), and R =OH: delphinidin 3-*O*-(6''-*O*-α-rhamnopyranosyl-β-glucopyranoside) (2).

ment. The remaining quaternary C-atoms were assigned using the HMBC spectrum, which was optimized for $^{2}J_{CH}$ and $^{3}J_{CH}$ couplings (Table 3). The two anomeric cross peaks at 5.37/102.53 and 4.74/101.92 ppm in the HSQC spectrum of 1 indicated two monosaccharides. Starting from the doublet at 5.37 ppm (J=7.8 Hz, H-1´´), the observed cross peak with the signal at 3.76 ppm in the DQF-COSY spectrum permitted the assignment of H-2´´. The chain of coupled protons H-2´´, H-3´´, H-4´´, H-5´´ and H-6A´´ and H-6B´´ was thereafter assigned (Table 3) from cross peaks in the same spectrum. Subsequently, the chemical shifts of the corresponding carbon atoms (Table 3) were assigned from the HSQC spectrum, which together with ^{1}H–^{1}H coupling constants were in agreement with a β-linked glucopyranose. Similarly, the proton and carbon chemical shifts (Table 3) of the other monosaccharide having the anomeric proton at 4.74 ppm (J=1.5 Hz) were in accordance with α-rhamnopyranosyl moiety. The cross peak in the HMBC experiment at 5.37/144.25 ppm between the anomeric glucoside proton and C-3 of the aglycone, showed that the sugar moiety was linked to the aglycone 3-position. The linkage point between the two sugar units was indicated to be at C-6´´ by the cross peak between the anomeric rhamnosyl proton and C-6´´ at 4.74/66.24 ppm. Thus, the identity of 1 was determined to be cyanidin 3-(6´´-O-α-rhamnopyranosyl-β-glucopyranoside) (Figure 1).

The minor anthocyanin (2) was in minute quantities and because of this, column chromatography with sephadex LH-20 was unable to isolate it completely from the major anthocyanin (1); it was isolated by preparative HPLC. The R_f (TLC), t_R (HPLC) and UV-Vis spectral data indicated that 2 was delphidin-3-rutinoside when com-pared with data from anthocyanin of Black currant (*Ribes nigrum*) (Frøytlog et al., 1998), run under the same conditions (Table 2). The structure was confirmed with 1D ^{1}H-NMR. The 1D ^{1}H-NMR of the aromatic region of 2 showed that this pigment contained a different aglycone compared to 1. The singlet at 8.98 ppm (H-4), the two meta coupled AX system at 6.76 ppm (H-6) and 6.96 ppm (H-8), together with another singlet at 7.87 ppm integrating for two protons (H-6´and 2´) (Table 3), in the 1D ^{1}H-NMR were in accordance with a delphinidin aglycone. The protons in the sugar region indicated typical two peaks for a β-glycoside and α-rhamnosyl moiety by their anomeric protons at 5.39 ppm (H-1´´, J= 7.8 Hz) and 4.73 ppm (H-1´´´, J= 1.2 Hz) respectively (Table 3). Thus the identity of 2 was confirmed as delphinidin-3-*O*-(6''-*O*-α-rhamnopyranosyl-β-glucopyranoside) (Figure 1).

DISCUSSION

Cyanidin-3-rutinoside has been detected as a minor anthocyanin in several berries and fruits such as strawberry (Lopes-Da-Silva et al*.,* 2002), blackberry (Fan-Chiang et al*.,* 2005), blueberry (Lohachoompol et al*.,* 2004), boysenberry (Cooney et al., 2004), berry of *Rhamnus alaternus* (Longo et al., 2005a), berry of *Smilax aspera* (Longo et al., 2006), olive fruits (Vinha et al., 2004), sweet cherry (Mozetic et al., 2004), blue honeysuckle (Chaovanalikit et al., 2004), apple skin (Gomez-Cordoves et al., 1996) and banana bract (Pazmino-Duran et al*.,* 2001). It has also been detected in litchi (*Litchi chinensis)*; (Rivera-Lopez et al., 1999; Sarni-Manchado et al., 2000), black currant (Rubinskiene et al., 2005), bay berry (Longo et al., 2005 b) and fresh zuiki (Terasawa et al., 2007) as the major anthocyanin. Lich peel contained about 30 mg / 100 g (Lopez et al., 1999; Sarni-Manchado et al., 2000) while fresh zuiki contained about 47mg/ 100g of cyanidin 3-rutinoside.

Cyanidin-3-rutinoside is known to show various biological activities such as antioxidant activities (Kaehkoenen et al*.,* 2003; Gabrielska et al*.,* 2005; Oki et al*.,* 2006), R-glucosidase inhibition (Adisakwattana et al*.,* 2004), inhibition of cancer migration and invasion (Chen et al*.,* 2006).

In this paper, cyanidin-3-rutinoside accounted for 92% of the anthocyanins in the leaf stalk of cassava and the absolute amount was 124 mg/100 g. [This value is higher than the highest amount previously reported in fresh Zuiki (*Colocasia esculenta*) by Terasawa et al. (2007)]. It is also interesting to note that cassava has been recognised as one of the most important food crops in Uganda, second to bananas in terms of area cultivated, total production and per capita consumption (Bua et al*.,* 1997). The leaf stalks of cassava are thrown away after harvesting the tubers. This therefore is a good source of cyanidin-3-rutinoside considering the high absolute amount available in leaf stalks of cassava. At the same time, the leaf stalks, which are thrown away as waste can be turned into a resource when it is used in the production of cyanidin-3-rutinoside.

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