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Characterization of Anthocyanins from *Ajuga pyramidalis* Metallica Crispa Cell Cultures

D. L. Madhavi,[†] S. Juthangkoon,[†] K. Lewen,[‡] M. D. Berber-Jimenez,[‡] and M. A. L. Smith*,[†]

Department of Natural Resources and Environmental Sciences and Department of Food Science and Human Nutrition, University of Illinois, Urbana−Champaign, Illinois 61801

The anthocyanins from *Ajuga pyramidalis* Metallica Crispa cell cultures were characterized by a combination of HPLC, chemical hydrolysis, MS, and ^{1}H NMR. Chromatographic analysis of the in vivo and in vitro samples showed the same suite of anthocyanins and relative composition. Chemical hydrolysis and mass spectral analysis of the major pigment from suspension cultures showed the presence of cyanidin, three glucoses, two ferulic acids, and malonic acid. The structure of the major pigment in the suspension cultures was determined to be 3-*O*-(6-*O*(E)-ferulyl)-2-*O*-[(6-*O*(E)-ferulyl)-β-D-glucopyranosyl-β-D-glucopyranosyl]-5-*O*-(6-*O*-malonyl)-β-D-glucopyranosylcyanidin. The anthocyanins in the in vitro extract were more stable toward light compared to those in the in vivo extract. The initial degradation of the major pigment both from in vitro and in vivo sources was mainly due to demalonylation.

Keywords: *Ajuga; anthocyanins; cell culture; stability-anthocyanins*

INTRODUCTION

The wider utilization of anthocyanins as natural food colorants has been restricted because of their limited stability, particularly at beverage pH. Extensive studies have been carried out to identify stable acylated anthocyanins from fruits, vegetables, and ornamental plants (Markakis, 1982; Francis, 1989, 1992; Yoshida et al., 1991; Shi et al., 1992). In vitro production of anthocyanins has also been researched as a potential means for large-scale production of anthocyanins, and numerous papers have appeared on the production of anthocyanins from cell cultures (Ilker, 1987; Cormier and Do, 1993; Callebaut et al., 1993; Kobayashi et al., 1993; Mizukami, 1993; Yamamoto et al., 1989). In a few reported cases, the profile of in vitro produced pigment has proven to be similar to, or simpler than, pigments from the source plant, which implies that the stability of the in vitro pigment would be different from that of the in vivo source. Very little information is available in the literature on the stability of anthocyanins from in vitro sources. In only one case, in cell cultures of *Perilla frutescens*, has stability of the in vitro product been specifically examined and found to be comparable to that of the in vivo source (Koda et al., 1992).

Ajuga pyramidalis Metallica Crispa, a member of Labiatae, is an ornamental ground cover plant with purple foliage. *Ajuga* species are used as medicinal plants in European countries. In vitro systems for the production of anthocyanins have been scaled-up to the bioreactor level in *Ajuga reptans*, a closely related species (Callebaut et al., 1993). However, the characteristics of the anthocyanins in vitro have not been completely elucidated. No information is available on the stability of anthocyanins from cell cultures. This paper presents the structural and conformational characteristics of the major pigment in *A. pyramidalis*

suspension cultures. A comparative study of the stability of in vivo and in vitro derived anthocyanins with that of other previously characterized sources is presented.

MATERIALS AND METHODS

A. pyramidalis Metallica Crispa plants were maintained in the greenhouse and as shoot cultures in Woody Plant medium (Lloyd and McCown, 1980) supplemented with the growth regulator 6-(γ,γ-dimethylallylamino)purine (0.98 μM).

Callus Initiation and Pigment Production. Leaf disks (1.3 cm diameter) from shoot cultures were used for callus initiation. Explants were placed in the dark at 25 °C on a callus induction medium containing Woody Plant medium salts, supplemented with 100 μM Fe as FeNa$_2$EDTA, rose vitamins (Rogers and Smith, 1992), 0.1 g/L myoinositol, 0.15 g/L PVP-T (polyvinylpyrrolidone 10), 30 g/L sucrose, 0.05 g/L L-ascorbic acid, 2.26 μM 2,4-dichlorophenoxyacetic acid (2,4-D), and 3.49 μM kinetin. Subcultures were performed at 2 week intervals. Two weeks after the third subculture, callus colonies (approximately 0.25 g) were transferred to pigment production medium and held under a photosynthetic photon flux of 55 μmol m^{-2} s^{-1}. The pigment production medium was similar to the induction medium except for an increase in the carbohydrate level or composition. Effects of various carbohydrate sources on growth and anthocyanin production were studied by substituting sucrose with glucose, fructose, galactose, arabinose, or lactose at a 30 g/L level as the sole carbohydrate source. Effects of various growth regulators on growth and anthocyanin production were studied by substituting 2,4-D with naphthaleneacetic acid (NAA) or indoleacetic acid (IAA) and kinetin with zeatin or benzylaminopurine (BA). There were five replicates per treatment, and each treatment was evaluated 4 weeks after inoculation. Suspension cultures were established in pigment production medium containing 50 g/L sucrose, 2.26 μM IAA, and 3.49 μM zeatin under a photosynthetic photon flux of 150 μmol m^{-2} s^{-1}.

Extraction of Anthocyanins. For quantitative estimation of the anthocyanins, the cultures were homogenized in ice-cold acidified methanol (1% HCl) (1:10 w/v) and extracted overnight at 4 °C. For qualitative studies, such as HPLC analysis, stability studies, and isolation of the pigments by semipreparative HPLC, the anthocyanins were extracted in 3% aqueous trifluoroacetic acid (TFA) overnight at 4 °C.

Quantitative Estimation of Anthocyanins. The methanol−HCl extracts were filtered, and the absorbance of the solution was measured at 525 nm using a Beckman DU-65

* Author to whom correspondence should be addressed (e-mail imagemal@uiuc.edu).

† Department of Natural Resources and Environmental Sciences.

‡ Department of Food Science and Human Nutrition.

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spectrophotometer. The concentration of anthocyanins was calculated in terms of cyanidin 3,5-diglucoside using a molar absorption coefficient of 3.01×10^4 reported by Callebaut et al. (1990). The total anthocyanin yield was expressed as milligrams per 50 mL of medium in a 250 mL culture flask. The results are the means of five replicates in each treatment.

HPLC Analysis of Anthocyanins. The 3% TFA extract after filtration was extracted with ethyl acetate (1:3 v/v) three times. The aqueous fraction was evaporated to remove residual ethyl acetate and adsorbed onto an activated On-Guard-RP Sep-Pak cartridge (Dionex, CA). The anthocyanins were eluted with methanol, and the solvent was evaporated to obtain a dry residue. For HPLC analysis, the residue was dissolved in 10% formic acid and filtered through a 0.2 μm filter membrane (Phenomenex, Torrance, CA). HPLC was performed using a Hitachi L-6200A Intelligent Pump (Tokyo, Japan), a Hitachi diode array detector (Tokyo, Japan), and a Rheodyne (Cotati, CA) 7125 injector. The column was a YMC-Pack ODS-AM (250 $\times$ 4.6 mm, 5 μm) connected to a YMC-Pack S5 120A ODS-AM guard column. The solvents used were 10% formic acid (A) and methanol–tetrahydrofuran (1:1) (B) at a flow rate of 1 mL/min. Separation was obtained by an isocratic elution of 13% B in A. Absorbance was monitored at 520 nm.

Purification of the Major and Minor Anthocyanins from Cell Cultures. Semipreparative HPLC was performed to isolate the major and minor pigments from suspension cultures using a YMC-Pack ODS-AM (250 $\times$ 10 mm, 5 μm) column connected to a YMC-Guardpack ODS-AM (10 $\times$ 30 mm) guard column. The elution profile was the same as for analytical separation, and the flow rate was 4.7 mL/min. The isolated pigments were further purified by preparative paper chromatography with butanol–acetic acid–water (4:1:5) to remove trace impurities.

Hydrolysis of Anthocyanins. Anthocyanins from the leaves and suspension cultures and the isolated pigments were subjected to acid hydrolysis in 2 N HCl–methanol (1:1) for 1 h as described by Markham (1982). The aglycons were extracted with ethyl acetate, evaporated to dryness under N_2, and dissolved in methanol. The major and minor pigments were also subjected to alkaline hydrolysis in 2 N NaOH for 2 h at room temperature according to the method of Markham (1982). The acyl groups were extracted with ethyl ether after acidification of the hydrolysate. The ethyl ether portion was evaporated to dryness under N_2 and dissolved in methanol. The pigments were further subjected to acid hydrolysis, followed by the removal of the aglycons as before with ethyl acetate. The aqueous residue was used for carbohydrate analysis.

Anthocyanidins derived from the acid hydrolysis were analysed using a Whatman Partisil ODS-3 (250 $\times$ 4.6 mm, 5 μm) column connected to a YMC-Pack S5 120A ODS-AM guard column. The solvents used were 10% formic acid (A) and 100% acetonitrile (B). Separation was obtained by an isocratic elution of 20% B in A at a flow rate of 1 mL/min. Analysis of cinnamic acids in the major and minor pigments was performed using the same HPLC system at 10% acetonitrile. The aqueous residue was concentrated to dryness under vacuum, dissolved in water, and neutralized with sodium bicarbonate. Carbohydrate analysis was performed using a Dionex gradient pump, a Dionex pulse amperometric detector, and Hitachi Model D-6500 chromatography data station software for collection of data. Carbohydrates were separated with a Dionex CarboPac PA1 column (250 $\times$ 4 mm) using an isocratic elution with 15 mM NaOH at 1 mL/min. Identification of the anthocyanidins, cinnamic acids, and carbohydrates was based on retention times of external standards.

Mass Spectrometry. Low-resolution fast atom bombardment MS of the major and minor pigments was performed on a Fisons VG analytical ZAB-SE mass spectrometer in a dithiothreitol–dithioerythritol (3:1) matrix. Tandem MS was performed on a Fisons VG 70-SE-4F spectrometer employing FAB ionization. The collision gas used was argon. Low-resolution electrospray MS was performed using a VG Quattro mass spectrometer in a methanol, formic acid, and water matrix.

Table 1. Effects of Carbohydrate Source on Growth and Anthocyanin Production in *A. pyramidalis* Metallica Crispa Callus Cultures

treatment (30 g/L)	fresh wt[a] (g/treatment)	anthocyanins[a] (mg/treatment)
glucose	3.4 c	0.06 c
fructose	4.4 e	0.06 c
sucrose	2.2 b	0.06 c
arabinose	0.27 a	0.00 a
lactose	0.44 a	0.03 b
galactose	3.9 d	0.08 d
no sugar	0.48 a	0.01 a
DF	34	34
SEM	0.1	0.004

[a] Within columns, means followed by a different letter are significantly different at $p = 0.05$ (lsd test).

Table 2. Effects of Growth Regulators on Growth and Anthocyanin Production in *A. pyramidalis* Metallica Crispa Callus Cultures

treatment (2.26 μM auxin + 3.49 μM cytokinin)	fresh wt[a] (g/treatment)	anthocyanins[a] (mg/treatment)
2,4-D + kinetin	5.9 c	0.08 a
IAA + kinetin	2.7 a	0.14 a
IAA + BA	3.9 b	0.21 ab
IAA + zeatin	3.9 b	0.29 b
NAA + kinetin	2.9 a	0.14 a
NAA + BA	3.9 b	0.21 ab
NAA + zeatin	4.0 b	0.27 b
DF	34	34
SEM	0.26	0.039

[a] Within columns, means followed by a different letter are significantly different at $p = 0.05$ (lsd test).

^{1}H NMR Spectrometry. ^{1}H NMR (500 MHz) spectra were obtained on a General Electric GN500 spectrometer in a 3 mm tube (Wilmad, Buena, NJ) at 20 °C using CD_3OD + 10% TFA-*d* (Aldrich, Milwaukee, WI) as solvents. Chemical shifts were recorded as parts per million (ppm) downfield from CD_3OD as the reference. NOE and COSY (400 MHz) experiments were performed using a Varian Unity 400 spectrometer using the same solvents. NOE experiments were performed at 20 and 40 °C.

Stability of Anthocyanins. The stability of total anthocyanins was determined in a nonsugar system according to the method of Baublis et al. (1994). The anthocyanins, extracted in 3% aqueous TFA and dried as before, were dissolved in sodium citrate buffer (pH 3.5, 0.05 M). The solutions, diluted appropriately, were transferred to sterile screw-top test tubes to give 1 mL of extract per tube. The tubes were flushed with nitrogen, sealed with Teflon tape, and capped immediately to eliminate oxygen transfer to the tube. The tubes were transferred to a radiation exposure chamber that consists of a plexiglass water bath surrounded by fluorescent lamps providing a total irradiance of 140 μmol m^{-2} s^{-1} at the sample level. The sample tubes were supported vertically around the inside wall of the bath at a distance of about 3 cm from the lamps. Bath temperature was maintained at 27 $\pm$ 1 °C by a homemade solid-state proportional thermoregulator connected to a resistance heater (Spomer, 1982). Samples were withdrawn at regular intervals and diluted with 0.1 N HCl, and the absorbance was measured at 520 nm. Results are the average of duplicate experiments. Degradation of the major anthocyanin was followed by HPLC.

RESULTS AND DISCUSSION

Cultural Parameters. The effects of varying the carbohydrate source on growth and anthocyanin production of callus cultures are shown in Table 1. Fructose in the medium resulted in the highest fresh weight. Calli grown on medium containing arabinose turned brown and died within 1 week after inoculation, whereas
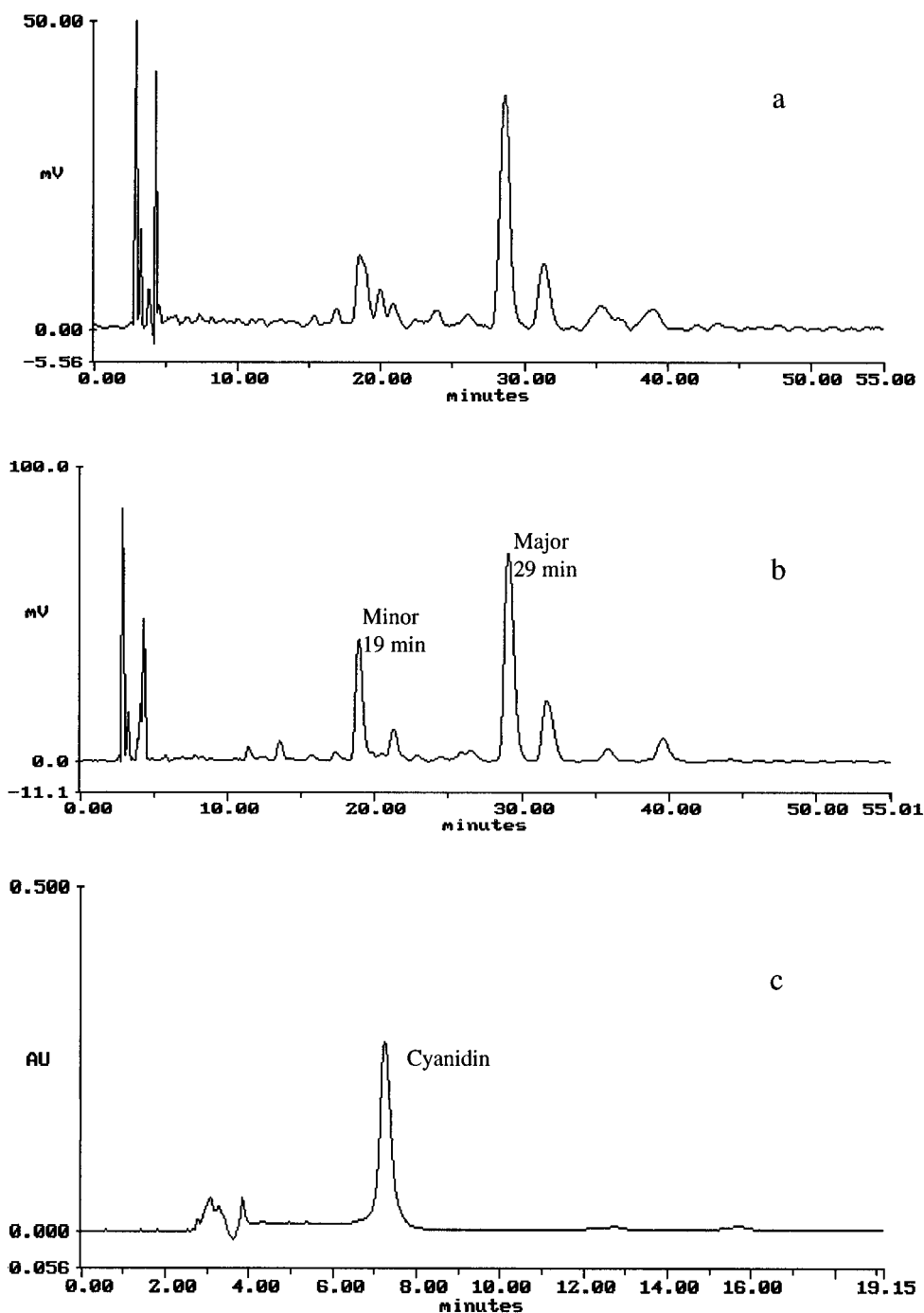
Figure 1. Reversed phase HPLC profile of anthocyanins from *A. pyramidalis* Metallica Crispa: (a) in vivo; (b) in vitro; (c) acid hydrolysate of the anthocyanins in vitro.

lactose did not support growth. Anthocyanin accumulation from treatments with sucrose, fructose, or glucose did not differ significantly ($p = 0.05$). Galactose enhanced anthocyanin accumulation. The results represent essentially the increase in fresh weight and corresponding yield of anthocyanins 4 weeks after inoculation, on the basis of the method of Callebaut et al. (1990). Table 2 presents the effects of various growth regulators on the growth and anthocyanin production in callus cultures. In general, 2,4-D plus kinetin resulted in higher growth rates and lower anthocyanin production compared to other treatments. A reduction in fresh weight was observed in all other treatments. Highest anthocyanin production was observed in combinations of IAA or NAA with zeatin. Suspension cultures had 41–42 mg of anthocyanins/100 g of fresh

weight as compared to 5–7 mg in callus cultures and 10–12 mg in leaf tissue.

The response of *A. pyramidalis* Metallica Crispa callus cultures to various cultural parameters was similar to those of other anthocyanin-producing cell culture systems. Studies on various carbohydrate sources indicated that the callus was unable to utilize lactose as a carbon source. This has also been reported in cell cultures of *A. reptans* (Callebaut et al., 1990) and *Populus* (Matsumoto et al., 1973). The higher biomass and anthocyanin production obtained in the presence of galactose have been reported in cell cultures of *Daucus carota* (Nagarajan et al., 1989). In contrast, galactose was poorly utilized in cell cultures of *Populus* (Matsumoto et al., 1973) and strawberry (Mori and Sakurai, 1994). The lower anthocyanin production in
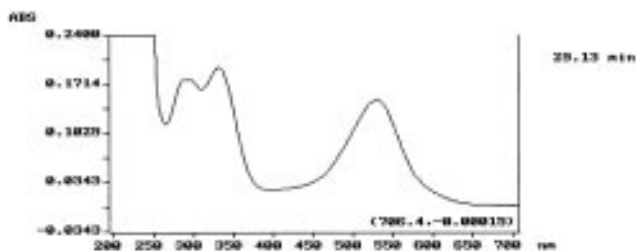
Figure 2. UV−visible absorption spectrum of the major anthocyanin from *A. pyramidalis* Metallica Crispa suspension cultures. Spectral information: $\lambda_{\text{vis max}}$ 528 nm; $\lambda_{\text{acyl max}}$ 330 nm.

the presence of 2,4-D has also been observed in *A. reptans* cell cultures (Callebaut et al., 1993), in addition to a number of other in vitro systems such as *Callistephus chinensis* (Rau and Forkmann, 1986) and *D. carota* (Ozeki and Komamine, 1985). Studies in carrot cell cultures have indicated that 2,4-D may regulate the expression of chalcone synthase, one of the key enzymes in the biosynthesis of anthocyanins (Ozeki and Komamine, 1985).

Total Anthocyanins. The anthocyanin profiles from leaves and suspension culture were comparable (Figure 1a,b). Cyanidin was the major aglycon in vivo and in vitro in the acid hydrolysate of the total anthocyanins. The major (Rt 29 min, Figure 1b) and minor pigment (Rt 19 min, Figure 1b) were isolated for further studies. Analysis of the acid hydrolysate indicated cyanidin as the aglycon. Ferulic acid was detected in the HPLC analysis of the alkaline hydrolysate. Carbohydrate analysis revealed glucose as the only carbohydrate. Figure 2 presents the UV−visible absorption spectrum of the major pigment generated by the diode array detector. The pigments had UV_{max} at 296 and 330 nm and vis_{max} at 528 nm, which indicated acylation. The ratio of E_{330}/E_{528} was 1.29, which indicated the presence of approximately two cinnamic acid residues according

to Harborne's rule (Harborne, 1958). The ratio of E_{440}/E_{528} was 20%, which indicated possible glycosylation at the C-5 position. The higher value we observed as compared to Harborne (1958) could be due to differences in the solvent.

Mass Spectrometry. The $[M]^+$ values for the major and minor pigments were 1211.4 and 1125.6, respectively. Tandem MS (MS/MS) was used to selectively fragment the molecular ion peak. Figures 3 and 4 present the MS/MS spectra of the major and minor pigments obtained when the molecular ions (1211.2 and 1125.6, respectively) were selectively ionized. Table 3 presents the molecular ion mass obtained by low-resolution FAB and electrospray ionization techniques and major fragments identified in MS/MS. The fragmentation pattern indicated that the pigments are structurally similar with the absence of malonic acid in the minor pigment. The difference in the molecular weights between the major and the minor pigments of 86 mass units and the presence of a fragment with m/z 449 (cyanidin plus glucose) in the minor pigment clearly indicated the absence of malonic acid in the minor pigment. Loss of malonylglucose $[M - 248]^+$ and the fragment with m/z 535 (cyanidin plus glucose plus malonic acid) observed in the major pigment has been reported in other members of Labiatae which contain acylated and malonylated cyanidin 3,5-diglucosides (Takeda et al., 1986). According to Takeda et al. (1986) such cyanidin-derived pigments are probably widespread in Labiatae. The fragmentation pattern clearly indicated the attachment of malonic acid to a glucose and the presence of a fragment with two glucose and two ferulic acid units. In *A. reptans* cell cultures, Callebaut et al. (1993) have reported the presence of glucose, *p*-coumaric acid, ferulic acid, and malonic acid in the major anthocyanin.

NMR Spectrometry. Figure 5 presents the ^{1}H NMR spectra of the major pigment. The peak assignments
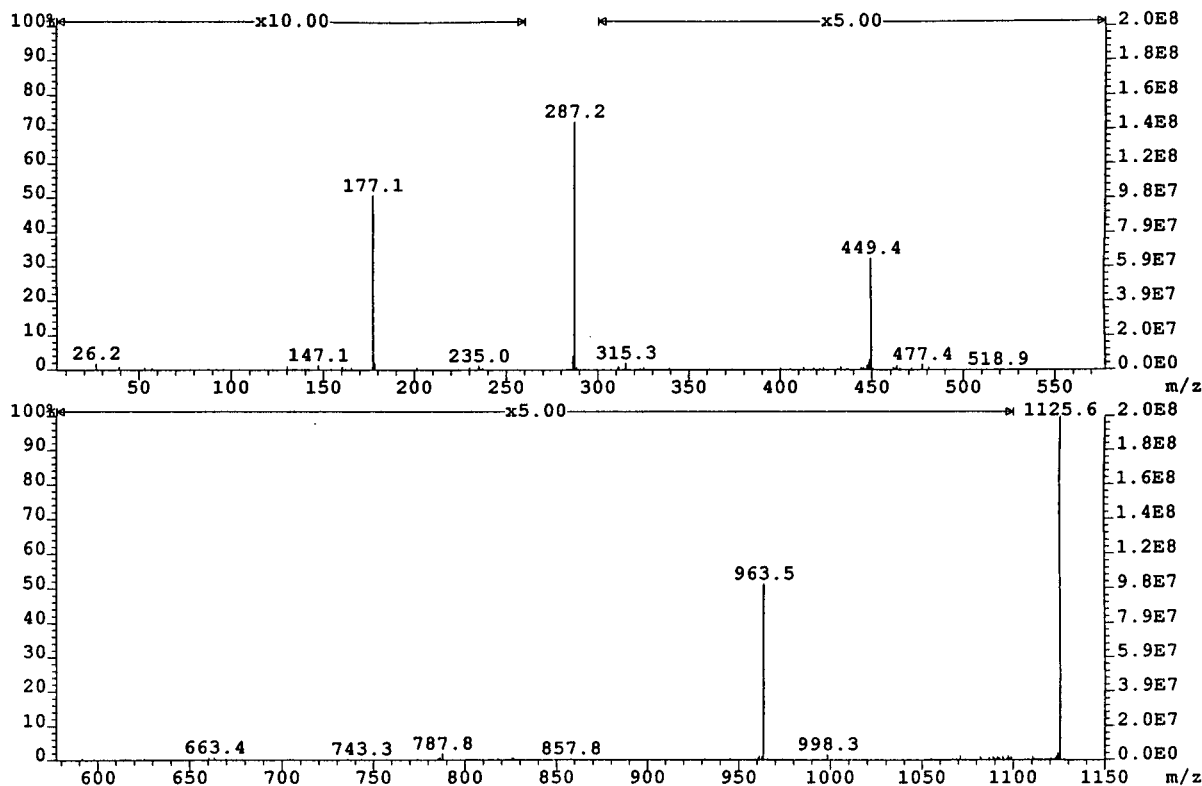

Figure 3. Tandem mass spectrum of the minor anthocyanin from *A. pyramidalis* Metallica Crispa suspension cultures.
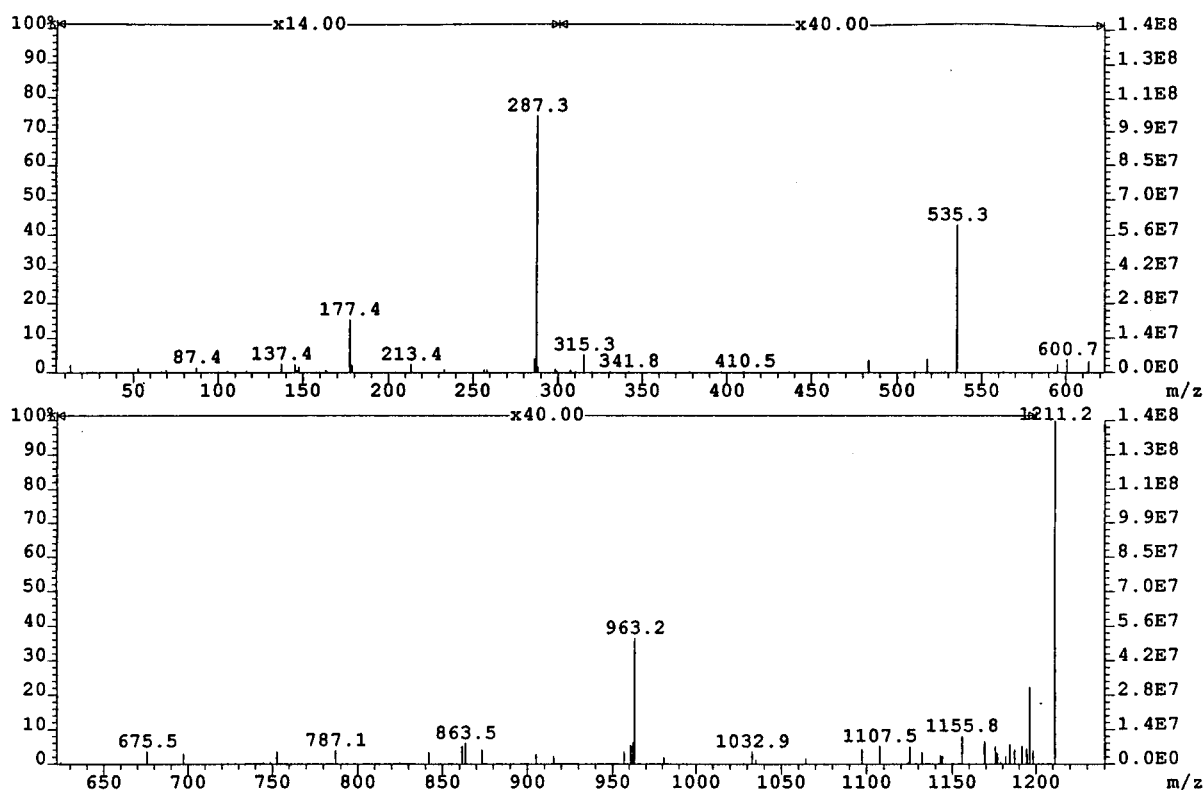
Figure 4. Tandem mass spectrum of the major anthocyanin from *A. pyramidalis* Metallica Crispa suspension cultures.

Table 3. Molecular Ions Obtained from Different Ionization Techniques and Fragments Identified in Tandem MS of the Major and Minor Pigments from *A. pyramidalis* Metallica Crispa Suspension Cultures

	$M^+[m/z]$	
ionization technique	major pigment	minor pigment
low-resolution FAB	1211.2	1125.6
electrospray	1211.6	1125.7
fragments in MS/MS		
ferulic acid	177.4	177.1
cyanidin	287.3	287.2
cyanidin + glucose		449.4
cyanidin + glucose + malonic acid	535.3	
cyanidin + 2 glucose + 2 ferulic acid	963.2	963.5

and attachment of the various molecules in the major pigment were based on chemical shifts, coupling constants, and $^1H-^1H$ COSY (Table 4). The aglycon was identified as cyanidin, confirming the MS and chemical hydrolysis data. The remaining signals in the aromatic region corresponded to two molecules of ferulic acid. The coupling constants between α and β protons were 16 Hz, which indicates that the acids have an *E* configuration. The presence of two ferulic acids was further confirmed by two singlets (OCH$_3$) at 3.7 and 3.8 ppm, respectively. In the carbohydrate area of the proton spectra, three anomeric protons were observed, indicating the presence of three carbohydrates.

The attachment of the carbohydrates to the aglycon was determined by NOE spectroscopy. Irradiation of anomeric protons A-1 (5.37 ppm) and B-1 (5.02 ppm) gave positive NOE signals at H-4 (4%) and H-6 (6%) of the aglycon, respectively. This indicated that carbohydrate A-1 was attached at the C-3 position and carbohydrate B-2 at the C-5 position of cyanidin. When the anomeric proton C-3 (4.75 ppm) was irradiated, an NOE was not observed in the aromatic region. The coupling constants of the anomeric protons were A-1 6.5 Hz for A-1, 7.6 Hz for B-1, and 6.6 Hz for C-1, indicating β

anomers. The coupling constants of the corresponding protons proved the hexoses to be glucoses in β-pyranoside form (7−9 Hz), confirming the chemical hydrolysis results. The attachment of glucose C was determined on the basis of the reported data on cyanidin 3,5-diglucoside (Yoshida et al., 1990) and red cabbage anthocyanins (Idaka et al., 1987). The A-2 proton showed a downfield chemical shift (3.97 ppm), which indicated that glucose C was glycosidically linked to glucose A at the 2-OH position.

Since the tandem MS data indicated the presence of a fragment with two glucose and two ferulic acid units, it was concluded that the ferulic acids are attached to glucoses A and C and malonic acid is attached to glucose B. The protons of ferulic acids 1 and 2 were further differentiated on the basis of the chemical shifts. The protons of ferulic acid 1 attached to glucose A are shifted downfield compared to those of ferulic acid 2 attached to glucose C. On the basis of the chemical shifts of methylene protons (CH$_2$O) and reported data of Yoshida et al. (1990) and Kondo et al. (1985), it was concluded that the ferulic acids and malonic acid were attached to the 6-OH positions of the glucoses. The attachments observed support the MS/MS fragmentation data (m/z 535 − cyanidin + glucose + malonic acid and m/z 963.2 − cyanidin + 2 glucose + 2 ferulic acid in the major pigment). Thus, the structure of the major pigment in *A. pyramidalis* Metallica Crispa suspension cultures was determined to be 3-*O*-(6-*O*(*E*)-ferulyl)-2-*O*-[(6-*O*(*E*)-ferulyl)-β-D-glucopyranosyl-β-D-glucopyranosyl]-5-*O*-(6-*O*-malonyl)-β-D-glucopyranosylcyanidin (Figure 5).

Stability of Anthocyanins. Figure 6 presents the stability of the total anthocyanins from various extracts. Anthocyanins from the in vitro extract were more stable compared to those from the in vivo extract under the conditions of the experiment. The absorbance of the anthocyanins at 520 nm from the in vivo extract was reduced to 20% of the original absorbance by 15 days of
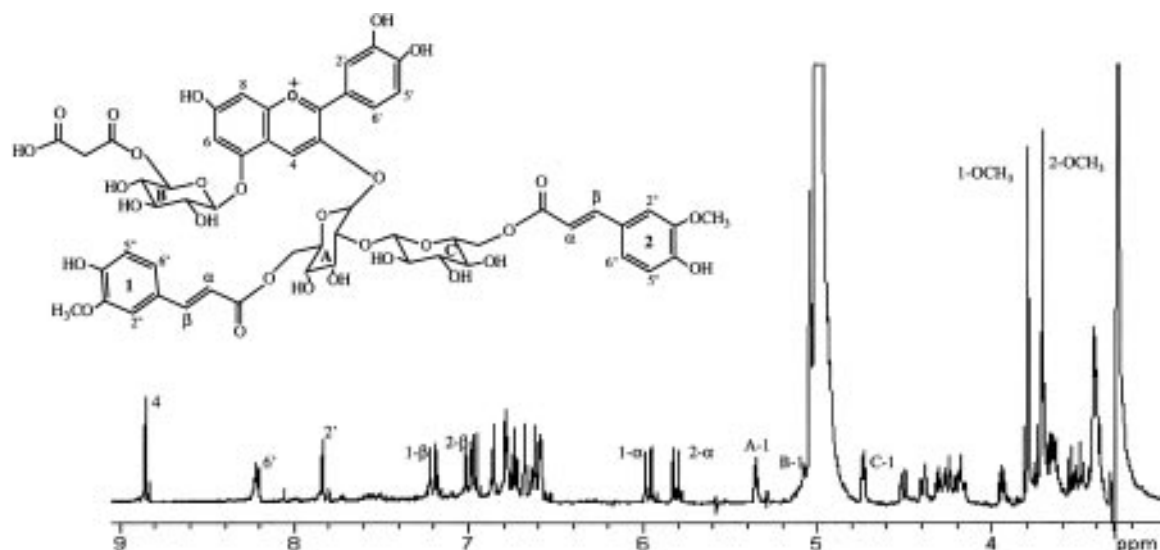
Figure 5. ^{1}H NMR spectrum of the major anthocyanin from *A. pyramidalis* Metallica Crispa suspension cultures (20 °C, CD$_3$OD + 10% TFA-*d*, 500 MHz).

Table 4. Assignments and Multiplicities of the Identified Protons from the ^{1}H NMR Spectra (20 °C, CD$_3$OD + 10% TFA-*d*, 500 MHz) of the Major Anthocyanin from *A. pyramidalis* Metallica Crispa Suspension Cultures

Aglycon Protons		
[1]H	δ (ppm)	*J* (Hz)
2′	7.85 d	2.2
6′	8.23 dd	8.6, 2.2
5′	6.98 d	8.9
4	8.87 s	
6	6.8 d	1.8
8	6.66 d	1.8

Ferulic Acid Protons

	1		**2**	
	δ (ppm)	*J* (Hz)	δ (ppm)	*J* (Hz)
2″	6.87 d	1.8	6.7 d	1.8
6″	6.79 dd	8.1, 1.8	6.61 dd	6.0, 1.8
5″	6.75 d	8.2	6.64 d	8.4
α	5.98 d	16	5.83 d	16
β	7.22 d	16	7.02 d	16
OCH$_3$	3.8 s		3.7 s	

Carbohydrate Protons

	A		**B**		**C**	
	δ (ppm)	*J* (Hz)	δ (ppm)	*J* (Hz)	δ (ppm)	*J* (Hz)
1	5.37 d	6.5	5.02 d	7.6	4.75 d	6.6
6$_a$	4.47 dd	12.1, 2.5	4.52 dd	11.8, 1.8	4.27 dd	11.7, 1.5
6$_b$	4.31 dd	12.0, 7.3	4.21 dd	12.2, 6.6	4.18 dd	11.9, 4.6

Figure 6. Comparison of the stability of anthocyanins from *A. pyramidalis* Metallica Crispa in vivo (◇) and in vitro (□) extracts with *Tradescantia pallida* (△) and cranberry (○).

Table 5. Degradation of the Major Anthocyanin from *A. pyramidalis* Metallica Crispa in Vivo and in Vitro Extracts

days after exposure	% degradation[a]	
	in vivo	in vitro
0	0	0
8	50	16
15	66	28

[a] Results are the average of duplicate measurements.

radiation exposure. The in vitro extract retained nearly 80% of the original absorbance even after 40 days of light exposure. The degradation of the major anthocyanin was followed by HPLC analysis, and the peak areas were used to follow the degradation (Table 5). In the in vivo extract, nearly 66% degradation of the major peak was observed, while the in vitro extract showed only 28% degradation of the major peak after 15 days of radiation exposure. Concomitant to the degradation of the major peak, an increase in the proportion of the minor peak (Rt 19 min, Figure 1b) was observed in both in vivo and in vitro extracts. The percent area of the minor peak increased from 8 to 51% in the in vivo extract and from 12 to 22% in the in vitro extract from 0 to 15 days of radiation exposure. This indicates that the initial degradation of the major pigment is mainly due to demalonylation.
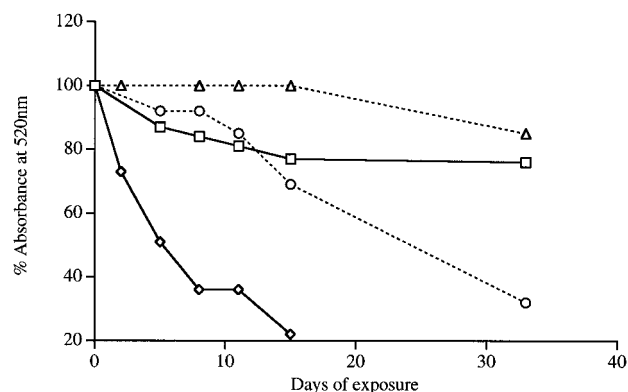
The stability of anthocyanins from cell cultures was greater than that of the anthocyanins from in vivo extract. The in vitro extract was also decisively more stable compared to cranberry anthocyanins (nonacylated, less stable) and slightly less stable compared to *Tradescantia* anthocyanins (acylated, highly stable). Structurally, *Ajuga* pigments are simpler compared to *Tradescantia* and do not have B-ring substitution. The anthocyanins from the in vitro extract were, however, unusually stable. The major pigment, which had similar retention times from in vivo and in vitro sources, showed different rates of degradation (Table 5). The reasons for these variations are not clear. Copigmentation effects in the in vitro extract could be one of the reasons for the enhanced stability of the anthocyanins. Cell cultures may overaccumulate known copigmenting agents such as flavonols, phenolic acids, and tannins (Markakis, 1982), which may be found at lower concen-

trations in the greenhouse-grown plant. Further work is underway with purified anthocyanins to resolve this question.

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