Features and Functions of Purple Pigment Compound in Halophytic Plant *Suaeda japonica* : Antioxidant/Anticancer Activities and Osmolyte Function in Halotolerance

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Abstract - *Suaeda japonica* is a halophytic plant that lives in mudflat at intertidal zone of western and southern coastal areas of Korea. The seawater-living plants showed a purple color during their whole life. In contrast, freshwater-living plants displayed a green color in leaves. When seawater-living plants were transferred to potting soil, the purple color was gradually changed to green in the leaves. The extracted purple pigment compound exhibited typical characteristics of betacyanin that were represented by water solubility, pH- and temperature-dependent color changes, sensitivity to light, UV-Vis spectra, and gel electrophoretic migration pattern. The LC-MS analysis of the extracted pigment compound showed the presence of two major protonated molecular ions ($[M+H]^+$) at *m/z* 651.1 and *m/z* 827.1. Antioxidant activity of the pigment compound was determined using stable free radical DPPH assay. It was found to have an antioxidant activity that is linearly increased in proportion to the reaction time for up to 30 min, and the activity was comparable to that of control BHA at 9.0 ㎎/㎖. The anticancer activity against several tumor cell lines was also examined following the MTT assay. The significant growth inhibitory effect was observed on two tumor cell lines, SW-156 (human kidney carcinoma) and HEC-1B (human endometrial adenocarcinoma). Probably, the pigment compound may function as an osmolyte to uphold halotolerant physiological processes in saline environment.

Key words ‒ Antioxidant/anticancer activities, Betacyanin, Halotolerance, Osmolyte, Purple pigment, *Suaeda japonica*

Introduction

The halophytic plants (halophytes) are characterized by the high adaptability against high-level ionic concentration and are classified into facultative and obligatory halophytes (Flowers *et al.*, 1986). Facultative halophytes (e.g. many species of Poaceae, Cyperaceae, Juncaceae; *Glaux maritima, Plantago maritima, Aster tripolium*) live in a slightly saline soil, but their growth is easily inhibited by the elevated salt levels. Obligatory halophytes (euhalophytes, e.g. *Salicornia, Salsola, Suaeda, Halocnemum*) are confined to saline habitats, and their growth is promoted by the uptake of moderate amounts of salt; only when the salt reaches to a very high level is growth impaired with the signs of stress, such as the production of pigments or the breakdown of chlorophyll (Larcher, 1995). However, it was reported that these plants can spontaneously or artificially grow in low-saline soils (Barbour and Michael, 1970; Barbour *et al.*, 1987). Plants growing in saline habitats cannot evade the effects of salt and should therefore develop at least some degree of salt tolerance either by keeping excessive amounts of salt from protoplasm or by eliminating the toxic and osmotic effects caused by the high ionic concentrations (Flowers and Colmer, 2008). Halophytes found in the species of *Salicornia, Suaeda*, and *Atriplex* compensate for the high osmotic potentials by accumulating salt in the cell sap. When these plants absorb salt, they intake water and become chubby, thereby decreasing salt concentration. This condition induces development of succulence that is generally shown in halophytic plants. Some other halophytic plants have a different mechanism of salt tolerance. For example, the cells of root endodermis make up wax layer and do not pass salt (NaCl) (Glenn *et al.*, 1998). Some dicotyledonous halophytes, such as species of *Salicornia* and *Suaeda*, have succulent leaves composed of enlarged cells in which the vacuoles occupy most of the cell volume. A few compatible

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solutes are known to be glycine betaine, proline, polyols and cyclitols, some of which are not only benign but also protective to enzymes (Pollard and Wyn Jones, 1979).

Many halophytic *Suaeda* species have been reported as utilizable plants. The *S. salsa* was utilized as food, medicine, forage and bioenergy, and this plant plays an important role in the restoration of salinized or contaminated saline land (Song and Wang, 2015). The *S. australis* extract showed marked antibacterial activities (Kim *et al*., 2016). The *S. maritima* extract showed the anti-*Toxoplasma gondii* effect (Hong *et al*., 2014) and had excellent antioxidant effects (Kim *et al*., 2016). The *S. japonica* has been used as a functional food and a medicinal plant due to its beneficial effects including *α*-glucosidase inhibition and antioxidant and anti-diabetic activities (Kang *et al*., 2017). The *S. japonica* belongs to the family Chenopodiaceae. As an annual herbage it is a representative obligatory halophyte that can grow in high salt concentration. These plants live in the mudflat at intertidal zone of western and southern coastal areas of Korea and exhibit a characteristic purple color in leaf and stem. They remain submerged in seawater during the most of their life. However, it was found that a few of the same species also grow in terrestrial soil nearby, but not in mudflat, without being soaked in seawater. They appear to be produced by the seeds transferred from mudflat during the process of embankment construction. These plants no longer contact with seawater and grow in freshwater soils, although their habitats are limited to a particular area. These plants may have a natural ability to survive in both saline and non-saline environments (Barbour and Michael, 1970).

Studies on *Suaeda* species may give a new insight into a mechanism of the salt tolerance as well as the saline adaptation (Ayarpadikannan, 2012; Alhdad *et al*., 2013; Flowers and Colmer, 2015; Xu *et al*., 2017). Although some mechanisms such as the accumulation of salts in leaf vacuoles have been shown in halophytic plants, little is known about importance of pigment compound that is associated with the halotolerance in *Suaeda* species. As one of the steps toward understanding how the plant is well adapted to strongly saline habitats, the morphological differences between seawater-living and freshwater-living *S. japonica* plants were compared. The role of peculiar purple pigment compound as a probable osmolyte in the saline environment was also discussed. Besides, the pigment compound

that is highly accumulated in leaves was extracted and examined its basic characteristics such as the water solubility, pH- and temperature-dependent color changes, sensitivity to light, UV-Vis spectra, gel electrophoretic migration pattern, LC-MS pattern, antioxidant activity, and anticancer activity against several tumor cell lines in this study.

Materials and Methods

Plant collection and pigment extraction

The plants (*Suaeda japonica* Makino) were collected in mudflat (seawater-living plants) and terrestrial soil nearby (freshwater-living plants) at Ha-Ri, Samsan-Myeon, Gangwha-Gun, Incheon, Korea. They were washed in distilled deionized water, blotted onto paper towels, and immediately used for pigment extraction or stored at -70℃ until use. For adaptation to terrestrial environment, some seawater-living plants were transferred to the potting soil and watered with freshwater.

The pigment compound was extracted in 5 ㎖ water per gram fresh leaves as described essentially in Chung and Byun (2009). Briefly, after grinding in mortar with pestle, they were centrifuged at 15,000 rpm for 10 min and the supernatant was transferred to the fresh vials. The water-extracted pigment compound was further treated with ethyl acetate, ethyl ether, and chloroform successively to remove other organic substances. The extracted pigment solution could be further concentrated to the solid state by butanol extraction and air dried, and finally used as the sample of all experiments in this research.

Analyses of pigment compound

To observe the absorption spectrum of the extracted purple pigment, wavelength (200 to 700 ㎚) was scanned by UV-Vis spectrophotometer. The effects of pH, temperature, and light on pigment stability were performed with pigment samples that were dissolved in H_2O (0.5 ㎎/㎖). For the stability studies on pH, the pigment samples were kept at different pH values (ranging from pH 1, pH 2, pH 3, pH 6, pH 7, pH 8, pH 9, pH 10, and pH 11) at 25℃ for 12 h and observed. For the stability studies on temperature, the pigment samples (pH 7) were kept at different temperature conditions (ranging from 25℃, 45℃, 65℃, and 85℃) for 12 h and observed. The color change of pigment sample (pH 7) was also observed under the exposure to

daylight during two weeks.

To clarify whether the purple pigment belongs to anthocyanin or betacyanin that are two major representative water-soluble pigment compounds in plants, the extracted pigment sample was migrated on a horizontal slab gel electrophoretic apparatus (Chung and Byun, 2009). Electrophoresis of purple pigment was carried out in a citrate buffer (10 mM citric acid anhydrous, 3 mM sodium citrate dihydrate, pH 3.0) on 3.0% agarose gel. Already well known two kinds of water-soluble plant pigments (anthocyanins and betacyanins) were used as standard references. Standard anthocyanin pigments were extracted from leaf of maple (*Acer palmatum,* Fossen and Andersen, 1999), fruit of blueberry (*Vaccinium corymbosum,* Lohachoompol *et al*., 2008), flowers of morning glory (*Pharbitis nil,* Saito *et al*., 1996), balloon flower (*Platycodon grandiflorum,* Goto *et al*., 1983), and leaf of begonia (*Begonia semperflorens,* Chirol and Jay, 1995). Standard betacyanin pigments were extracted from root of red beet (*Beta vulgaris,* Strack *et al*., 2003), flowers of cockscomb (*Celosia cristata,* Schliemann *et al*., 2001), flowers of four o'clock (*Mirabilis jalapa,* Gandía-Herrero *et al*., 2005), and fruit of pokeweed (*Phytolacca americana,* Schliemann *et al*., 1996).

LC-MS (liquid chromatography-mass spectrometry) analysis of the extracted pigment compound was performed at the Korea Basic Science Institute (Daejeon, Korea) using a Instrument [LCQ DECA XPplus (Thermo Finnigan, UK ; Ionization, Micro flow-ESI (+) ; Column, C18 ; Mobile phase, 100% water (0.5% acetic acid / 0.02% formic acid) / 80% acetonitrile (0.5% acetic acid / 0.02% formic acid) ; Sample solvent, 50% methanol].

Antioxidant assay

The free radical scavenging activity of the pigment compound was assayed using a stable DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical, following standard method (Blois, 1958; Kim *et al*., 1981). The DPPH solution (0.08 ㎎/㎖) was prepared in 50% ethanol. The pigment sample solutions dissolved in 50% ethanol were prepared in different concentrations (0.3 to 9 ㎎/㎖). The reaction mixture contained 1.5 ㎖ of DPPH solution and 0.3 ㎖ of sample solution. The absorbance was read at 528 ㎚. The readings were measured at different time intervals for a total duration of 30 min. The DPPH, which forms a deep violet color in solution, reacts with antioxidants and color loss at 528 ㎚ correlates to antioxidant content. BHA (butylated hydroxyanisole) was used as control. Each test was carried out twice and the absorption value was averaged.

Assay for anticancer activity

The anticancer activity and the resulting data analysis were performed essentially as described by Choi *et al*. (2006) following the MTT [3-(4,5-dimethyl-thiazol-2-yl) -2,5-diphenyl-tetrazolium bromide] assay (Rubinstein *et al*., 1990). The human tumor cell lines used were A549 (Korean Cell Line Bank No. 10185, lung carcinoma), HEC-1B (ATCC No. HTB-113, uterus adenocarcinoma), SW-156 (ATCC No. CRL-2175, kidney hypernephroma), and T98G (Korean Cell Line Bank No. 21690, brain glioblastoma multiformae). Cells undergoing exponential growth were suspended in a fresh PBS (phosphate buffered saline) medium at a concentration of 2×10^5 cells/㎖, dispensed in a 96-well flat plate in a volume of 0.1 ㎖ per well, and stabilized by incubation at 37℃ in a 5% CO_2 for 24 h. Then, 100 ㎕ each sample was added to the wells. After incubation at 37℃ for 44 h, 50 ㎕ of MTT (0.5 ㎎/㎖ PBS) was added to each well, and the resulting 250 ㎕ mixture was further incubated for 4 h at 37℃. The formazan crystals formed in each well were dissolved in 100 ㎕ of dimethylsulfoxide solution by mixing with plate shaker. The optical density of the colored reaction sample was measured by reading at 570 ㎚ on a multi-well scanning spectrophotometer.

Results

Plant morphology in different environmental conditions

The *S. japonica* is an annual herbaceous dicotyledonous plant and the height of a mature plant is approximately 30 ㎝. The seawater-living plants showed collectively purple color in leaves that are distinctively succulent and extended (Fig. 1 A and B), whereas the freshwater-living plants showed collectively green color in leaves that are less succulent and somewhat more extended (Fig. 1 C and D). When seawater-living plants were transferred to potting soil and watered with freshwater for adaptation to the terrestrial environment, the purple color was gradually changed to green color in the leaves (Fig. 1E). They were morphologically very similar to the freshwater-living
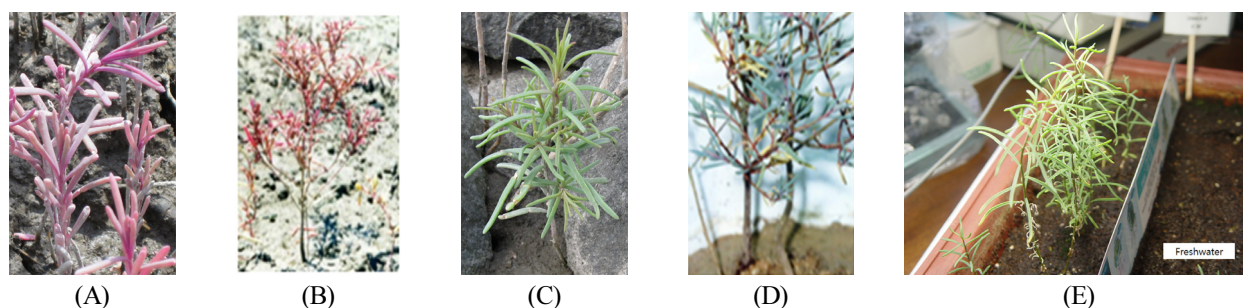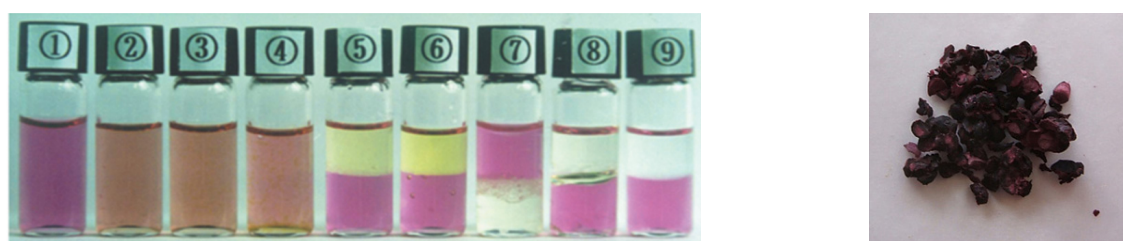
Fig. 1. Morphologies of *S. japonica* in saline and non-saline environments. A and B, Young and fully grown seawater-living plants at the mudflat. C and D, Young and fully grown freshwater-living plants at the terrestrial soil nearby. E, Freshwater-adapted seawater-living plants after transfer to the potting soil.

(A) 1, water; 2, methanol; 3, ethanol; 4, acetone;5, ethyl acetate; 6, ethyl ether; 7, chloroform; 8, benzene; 9, hexane   (B) Concentrated solid state of pigment

Fig. 2. Water-extracted purple pigment compound after blending with various solvents.

plants and grew slightly faster than seawater-living plants. The seawater-living plants showed a peculiar purple color during their whole life. In contrast, both freshwater-living plants and freshwater-adapted seawater-living plants lost the purple color almost completely and displayed a green color in leaves like the glycophytes (Fig. 1 C, D, and E).

Extraction of pigment compound

Various solvents were attempted to extract the purple pigment compound from leaf tissues. The purple pigment was found to be best extracted in water. No purple pigment was virtually extracted from freshwater-living plants. Fig. 2 shows the water-extracted pigments after blending with various solvents. The purple pigment was completely distinguished from the photosynthetic green pigment, chlorophyll, as can be seen in ethyl acetate or ethyl ether solvents in Fig. 2A. The purple pigment compound was insoluble in ethyl acetate, ethyl ether, chloroform, benzene, and hexane (Fig. 2A). The extracted pigment solution could be further concentrated to the solid state (Fig. 2B).

UV-Vis spectra

To observe the absorption spectrum of the extracted purple pigment, it was measured in both UV and visible light ranges (200 ㎚ to 700 ㎚). As shown in Fig. 3A, the absorbance maxima occurred in the UV range and in the visible range at near 540 ㎚. However, the absorption maximum at 540 ㎚ of purple pigment was disappeared under alkaline condition (Fig. 3B).

Stability studies on pH, temperature, and light

The variation of color and stability of the compound was observed when pH or temperature was changed (Tables 1 and 2). The pigment color was purple in acidic conditions, but became yellowish in alkaline conditions. As the pH of acidic purple pigment solution was raised, the color was slightly changed to reddish purple up to pH 7, but became yellowish at above pH 8. This result indicates that the pigment is relatively stable up to pH 7. The pigment compound was stable (reddish purple) at room temperature (25℃) under the pH 7 condition, but was slightly unstable (pale purple) at 45℃ to 85℃. When the purple color of water-extracted pigment compound was
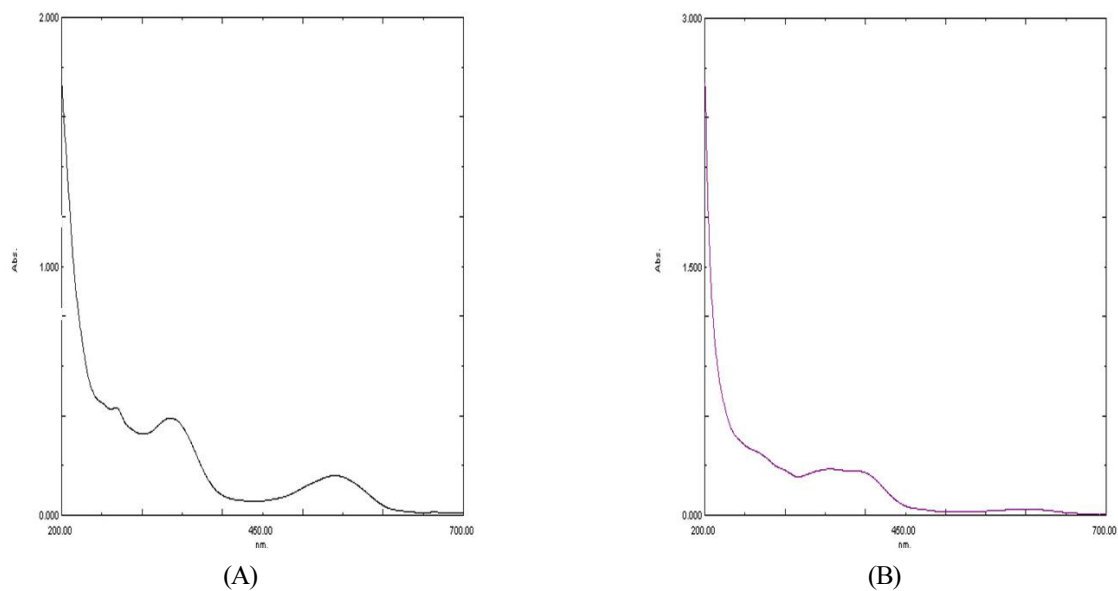
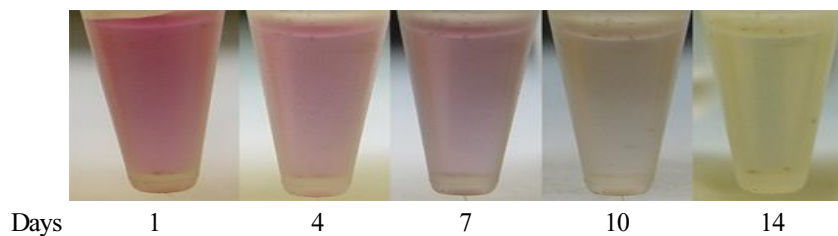Fig. 3. The UV-Vis spectra of water-extracted purple pigment before (A) and after (B) alkali treatment.

| Days | 1 | 4 | 7 | 10 | 14 |

Fig. 4. Color changes of pigment compound under exposure to daylight.

Table 1. pH-dependent color change and stability of pigment at 25℃

pH	Color	Stability[z]
1	Deep Purple	+++[y]
2~3	Purple	++
6~7	Reddish Purple	+
8~9	Colorless/Pale Yellow	-
9~10	Pale Yellow	--
10~11	Yellow (ppt)[x]	---

[z]Stability was determined by the degree of color fading from the initial color.

[y]+ and - denote stability and unstability, respectively.

[x]ppt = precipitated.

exposed to daylight, it was gradually faded and became yellowish after 14 days (Fig. 4).

Gel electrophoretic migration pattern

When the standard anthocyanin references (maple, blueberry, morning glory, balloon flower, and begonia) and the standard betacyanin references (red beet, cockscomb, four o'clock, and pokeweed) were separated together on an agarose gel at the same time, they were migrated to opposite directions; the anthocyanins to cathode while the betacyanins to anode (Fig. 5). The purple pigment of *S. japonica* (lane 10) was moved to anode along with the standard betacyanin references (lanes 2, 4,

Table 2. Temperature-dependent color change and stability of pigment at pH 7

Temp	Color	Stability[z]
25℃	Reddish Purple	+[y]
45℃	Pale Purple	-
65℃	Pale Purple	-
85℃	Pale Purple	-

[z]Stability was determined by the degree of color fading from the initial color.
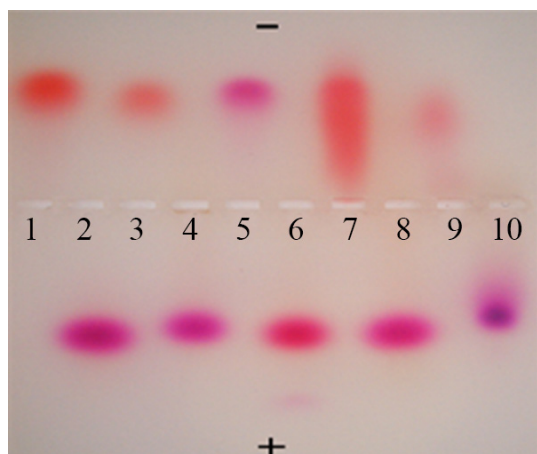[y]+ and - denote stability and unstability, respectively.

Fig. 5. Gel electrophoretic migration patterns of anthocyanins and betacyanins in citrate buffer.

1. Begonia (*Begonia semperflorens*)　　anthocyanin
2. Pokeweed (*Phytolacca americana*)　　betacyanin
3. Maple (*Acer palmatum*)　　anthocyanin
4. Cockscomb (*Celosia cristata*)　　betacyanin
5. Balloon flower (*Platycodon grandiflorum*)　　anthocyanin
6. Red beet (*Beta vulgaris*)　　betacyanin
7. Morning glory (*Pharbitis nil*)　　anthocyanin
8. Four o'clock (*Mirabilis jalapa*)　　betacyanin
9. Blueberry (*Vaccinium corymbosum*)　　anthocyanin
10. *Suaeda japonica*　　this study

6, and 8) as can be seen in Fig. 5, indicating that this pigment belongs to betacyanin.

LC-MS pattern

LC-MS analysis of the extracted pigment compound was performed. As shown in Fig. 6, total ion chromatogram showed two major peaks at 21.32 min and 28.41 min of the retention time (A). The extracted ion chromatogram of each peak showed the protonated molecular ions at m/z 651.1 (B) and 827.1 (C), respectively.

Antioxidant activity

Antioxidant activity of the extracted pigment compound was determined by DPPH assay (Table 3). The pigment compound was found to have an antioxidant activity that is linearly increased in proportion to the reaction time for up to 30 min, and the activity was comparable to that of control BHA at 9.0 ㎎/㎖.

Anticancer activity

The anticancer activity against several tumor cell lines was also examined following the MTT assay (Table 4). The significant growth inhibitory effect was observed on two tumor cell lines SW-156 (human kidney hypernephroma) and HEC-1B (human endometrial adenocarcinoma).

Discussion

Influenced by external conditions of habitat, halophytic plants generally have a characteristic morphology that distinguishes from glycophytic plants. The *S. japonica* plant used in this study has a characteristic purple colored succulent leaves during the life cycle in saline environment. Among the treated solvents to extract the purple pigment, the water was the best. This result was consistent with that of the betacyanin extraction from *Opuntia* fruit (Castellar *et al*., 2003). The extracted pigment compound color was gradually faded out and became yellowish after exposure to 14-daylight (Fig. 4), indicating that the pigment compound is unstable and destroyed by light once isolated from the plant. It has been reported that light causes betacyanin degradation (Woo *et al*., 2011). The pigment color was purple in acidic conditions, but became yellowish in alkaline conditions. According to the report of Jackman and Smith (1996), the betacyanins keep their
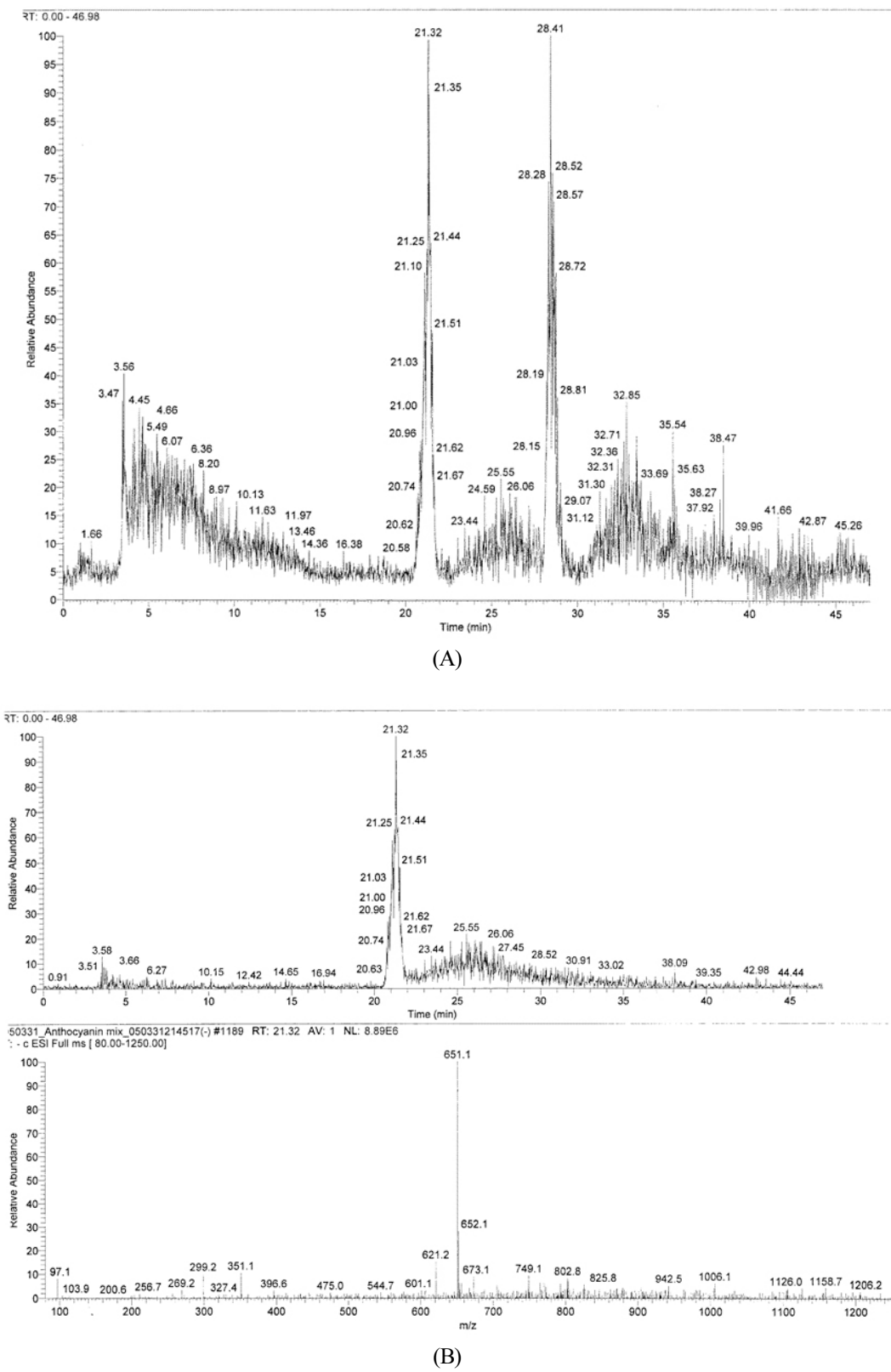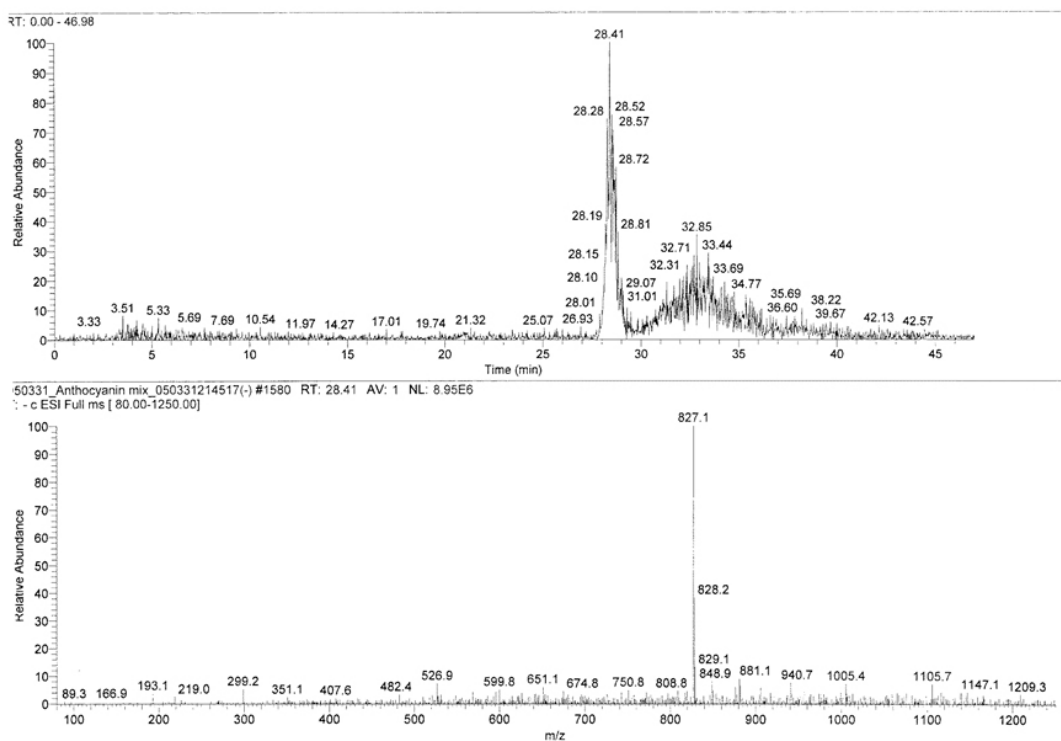
(A)

(B)

Fig. 6. LC-MS chromatogram (A), Total ion chromatogram showing two major peaks at 21.32 min and 28.41 min. (B) and (C), Extracted ion chromatogram of each peak showing the protonated molecular ions at m/z 651.1 and 827.1, respectively.

(C)

Fig. 6. Continued.

Table 3. Antioxidant activity of the pigment compound (unit: OD at 528 ㎚)

Conc. (mg/㎖)	Reaction Time (min)				
	2.5	5	10	20	30
DPPH Soln.			2.1825		
0.036 BHA	1.0919	0.8286	0.7250	0.6345	0.5829
0.3	1.8034	1.7957	1.7707	1.7437	1.7299
0.7	1.7594	1.7113	1.7065	1.6748	1.6581
1.0	1.6901	1.6397	1.6218	1.5813	1.5594
1.5	1.6640	1.5241	1.5216	1.4818	1.4525
2.0	1.5988	1.4484	1.4406	1.3716	1.3036
2.5	1.5517	1.3806	1.3771	1.3085	1.2708
3.0	1.5495	1.3702	1.3531	1.2934	1.2438
5.0	1.4851	1.2513	1.1546	1.0665	1.0169
9.0	1.2053	0.9110	0.7527	0.5934	0.5017

appearance over the broad pH range from 3 to 7, but degrade at above pH 9. In the present study, the purple pigment color of *S. japonica* was changed to yellow at above pH 8 (Table 1). The pigment color was relatively stable at the pH range from 1 (deep purple) to 7 (reddish purple), but unstable at alkaline conditions (yellowish). When the pigment samples at pH 7 (reddish purple) were raised to the various levels of temperature with 20℃ intervals, the colors were slightly changed to pale purple at 45 to 85℃, indicating that the pigment is relatively stable at high temperatures (Table 2). Betacyanins are known to be light-sensitive, pH-dependent of color change, and easily hydrolyzed at alkaline pH (Jackman and Smith, 1996; Stintzing

Table 4. Growth inhibitory effects on tumor cell lines

Conc. (mg/mℓ)	Cell Lines[z]			
	A549	HEC-1B	SW-156	T98G
10.0	48.0[y]	58.4	70.5	18.5
5.0	23.7	29.5	66.6	-[x]
2.5	26.1	22.9	35.2	2.4
1.0	15.7	-	-	-

[z]A549, human lung carcinoma; HEC-1B, human endometrial adenocarcinoma; SW-156, human kidney carcinoma; T98G, human brain carcinoma.

[y]Numbers in each column indicate the percentage of cell growth inhibition.

[x]- denotes no data.

and Carle, 2004). Therefore, the color change from purple to yellow (Table 1) indicates that the pigment of *S. japonica* is betacyanin, because betacyanins are degraded under alkaline conditions while the another water-soluble anthocyanins are known to blue under alkaline conditions (Cabrita *et al*., 2000; Jackman and Smith, 1996). Since the extracted purple pigment was most stable at acidic pH, this pigment might be stored in acidic vacuoles (pH 2.7 to pH 7.7) where pigments are known to be located (Nelson and Harvey, 1999).

The Fig. 3 displays the typical UV-Vis spectra of betacyanin when compared with those of other reports (Cai *et al*., 1998; Woo *et al*., 2011). The absorption peak ranging from 530 to 545 ㎚ is a spectral characteristic of the betacyanins (Castellar *et al*., 2003; Allegra *et al*., 2005; Tsai *et al*., 2010). The absorption maximum at 540 ㎚ of purple pigment was disappeared as shown in figure 3B, suggesting that betacyanins are degraded under alkaline conditions. This result is consistent with that of the color changes under alkaline pH conditions (Table 1). The betacyanins are difficult to separate from protein. According to the report of Cai *et al*. (1998), the betacyanins have a special relationship with protein. When the UV-Vis spectral determination of diverse *Amaranthus* betacyanins was conducted, they observed that even the purified samples had also abnormally strong absorbance at the range of UV light. The purified betacyanins using the procedures by $(NH_4)_2SO_4$ salting-out, HCl-treatment, centrifugal separation, and isolation by Sephadex G-25 and G-50 columns to remove proteins still contained protein. They concluded that the betacycnins could not readily be completely isolated from the protein. Likewise, the UV spectral peaks of purple pigment as shown in Fig. 3 seem to be due to the typical UV absorbance spectra of

unseparated protein or other contaminated components. Betacyanins in plants usually are in the form of glycosylated betanidin derivatives. They are also well known to display two absorption maxima, one in the UV-range due to the *cyclo*-DOPA (*cyclo*-3,4-dihydroxyphenylalanine) structure and a second one in the visible range. Besides, acylated betacyanins display an additional absorption in the UV region of about 300 to 330 ㎚, where the absorption of nonacylated betacyanins is very weak (Cai *et al*., 2001; Stintzing and Carle, 2004). In this study, the purple pigment compound of *S. japonica* displayed an extra absorption maximum in the UV range at near 330 ㎚, suggesting that the pigment compound is probably acylated. The absorption maximum values of the betacyanins can be slightly changed due to the structural differences. Generally, the red and violet colors in betalain-bearing plants are produced by different substitution patterns of betacyanins. For example, betacyanins are formed of a betalamic acid unit linked to a molecule of *cyclo*-DOPA. The latter highly aromatic structure is responsible for the deep violet color for the pigment and this aromatic structure also induces a strong bathochromic shift of 60-70 ㎚ (absorption max. 534-554 ㎚) (Wyler and Dreiding, 1961; Strack *et al*., 2003). The absorption maximum of purple pigment in the present study was 540 ㎚ in the visible range and the characteristic purple color may be also due to the betacyanin-specific *cyclo*-DOPA structure.

To clarify furthermore if this purple pigment of *S. japonica* belongs to anthocyanin or betacyanin, an another novel method by agarose gel electrophoresis (Chung and Byun, 2009) was additionally applied. At acidic pH 1 to 3, anthocyanins exist chiefly in the form of the flavylium cation (Goto *et al*., 1976; Tanaka *et al*., 2008) and occur in the most intense reddish

colors. As the pH increases, deprotonation occurs so that at neutral pH, anionic forms of the quinonoidal base predominate (Cabrita *et al*., 2000; Jang *et al*., 2005). On the other hand, the charge of betacyanin becomes positive at pH less than 1.5, negative at pH between 2.5 and 9.5, and neutral (zero charge), an isoelectric point, at pH between 1.5 and 2.5 (Yang *et al*., 2003). Therefore, using the citrate buffer (pH 3.0) in this study, most of the anthocyanin molecules will be in the positively charged flavylium forms, while the betacyanin molecules will be in the negatively charged forms (Chung and Byun, 2009). With oppositely charged ions they will migrate to the opposite directions. Indeed, the extracted standard pigment samples (anthocyanins and betacyanins) from various plant species in the present study were moved to the opposite directions on an agarose gel in citrate buffer (pH 3.0); anthocyanins to cathode and betacyanins to anode (Fig. 5). The purple pigment of *S. japonica* was moved to anode along with other standard betalcyanins extracted from red beet, four o'clock, pokeweed, and cockscomb. This result also strongly suggests that the purple pigment of *S. japonica* is betacyanin, but not anthocyanin.

In addition, betacyanins have been exclusively found in the family Chenopodiaceae. Moreover, anthocyanins and betacyanins have never been found together in the same plant and intermolecular copigmentation phenomena with betacyanins have not been reported so far (Stafford, 1994; Stintzing and Carle, 2004; Tanaka *et al*., 2008). Their structural difference is based on divergent biochemical pathways on the arogenate level, the precursor of tyrosine (betacyanins) and phenylalanine (anthocyanins), respectively (Steglich and Strack, 1990; Tanaka *et al*., 2008).

Betacyanins have been extracted from various plant sources. Among the betacyanins present in plants include betanin, isobetanin, probetanin, and neobetanin. Other important betacyanins are amaranthine and isoamaranthine, isolated from species of *Amaranthus*. The betacyanin isolated from Christmas cactus flowers (*Schlumbergera x buckleyi*) showed in LC-MS a protonated molecular ions ([M + H]$^+$) at *m/z* 637, 769, and 945 (Strack *et al*., 2003). In LC-MS, the protonated molecular ions of betacyanins were found at *m/z* 727 and 829 in fruit extracts of pokeberry (*Phytolacca americana*), at *m/z* 695 in pigment of vine cactus (*Hylocereus polyrhizus*), and at 727, 871, 872, and 903 in extracts of cockscomb (*Celosia argentea*) (Strack *et al*.,

2003). The betacyanin of Djulis (*Chenopodium formosanum*) exhibited the maximum absorbance at 530 ㎚ and showed four peaks including betanin (*m/z* 551.0), isobetanin (*m/z* 551.0), amaranthine (*m/z* 727.0), and isoamaranthine (*m/z* 727.0) (Tsai *et al*., 2010). The major mass difference of betacyanins is due to the presence or absence of additional sugar moiety (Strack *et al*., 2003). The LC-MS analysis (Fig. 6) of the *S. japonica* pigment compound showed two major peaks with the protonated molecular ions at *m/z* 651.1 and 827.1, indicating that the pigment compound may be composed of at least two major betacyanin components. Further analysis is required to find the unknown chemical structures of these two compounds. Antioxidant activity of the pigment compound was also identified. The activity was comparable to that of control BHA at 9.0 ㎎/㎖ (Table 3). If the pigment were more highly purified by applying another additional biochemical procedures, the activity would be increased much more at the lower concentrations. Betacyanins are present in a large number of plants, vegetables, flowers, and fruits and have been reported for their antioxidant activities (Betancourt *et al*., 2017). The natural betacyanins are increasing in importance for food industry applications and are replacing a number of synthetic colorants that have been questioned and dropped from the market due to safety issues. Betacyanins extracted from different vegetable sources are bioavailable and have been reported for their antioxidant protective activities that include inhibition of lipid peroxidation and heme decomposition, and inhibition of oxidative metabolism in neutrophils from obese individuals (Caldas-Cueva *et al*., 2015). As shown in Table 4, the pigment compound displayed inhibitory activity toward human tumor cell lines. Especially, the significant growth inhibitory effect was observed on SW-156 (human kidney carcinoma) and HEC-1B (human endometrial adenocarcinoma). Therefore, it seems that the *S. japonica* pigment compound could be also utilized for human health.

Taken together, the purple pigment compound of *S. japonica* exhibits typical characteristics of betacyanin that were represented by water solubility, pH- and temperature-dependent color changes, sensitivity to light, UV-Vis spectra, and gel electrophoretic migration pattern. The purple pigment might be also involved in the halotolerance, since it is specifically accumulated in high abundance in seawater-living plants (Fig.

1 A and B), but disappeared in freshwater-living plants (Fig.1 C and D) as well as in freshwater-adapted seawater-living plants (Fig. 1E). In another representative halophytic plant *Mesembryantheum crystallinum*, the production of betacyanin was usually associated with the salt (NaCl)-stressed state and the betacyanin was already regarded as an osmolyte (Adams *et al*., 1998). In *Salicornia europaea* L., the betacyanin synthesis was induced as a result of salt stress (Bothe, 1976), suggesting that betacyanins function as osmolytes to uphold physiological processes in the halophytic plant (Stintzing and Carle, 2004). Similarly, the purple pigment compound (betacyanin) of *S. japonica* may also function as an osmolyte to uphold halotolerant physiological processes in saline environment. In conclusion, this study reveals the presence of the purple betacyanin compound that may enable this halophytic plant to tolerate saline environment in which it can function as a probable osmolyte without any significant harmful effects on plant growth and development. The purple pigment shows also that it can function as antioxidant and anticancer agents.

Acknowledgment

References

Adams, P., D. Nelson, S. Yamada, W. Chmara, R.G. Jensrn, H.J. Bohnert and H. Griffiths. 1998. Growth and development of *Mesembryanthemum crystallinum* (Aizoaceae). New Phytol. 138:171-190.

Alhdad, G.M., C.E. Seal, M.J. Al-Azzawi and T.J. Flowers. 2013. The effect of combined salinity and waterlogging on the halophyte *Suaeda maritima*: The role of antioxidants. Environ. Exp. Bot. 87:120-125.

Allegra, M., P.G. Furtmüller, W. Jantschko, M. Zederbauer, L. Tesoriere, M. Livrea and C. Obinger. 2005. Mechanism of interaction of betanin and indicaxanthin with human myeloperoxidase and hypochlorous acid. Biochem. Biophys. Res. Comm. 332:837-844.

Ayarpadikannan, S., E.S., Chung, C.W. Cho, H.A. So, S.O. Kim, J.M. Jeon, M.H. Kwak, S.W. Lee and J.H. Lee. 2012. Exploration for the salt stress tolerance genes from a salt-treated halophyte, *Suaeda asparagoides*. Plant Cell Rep. 31:35-48.

Betancourt, C., M.J. Cejudo-Bastante, F.J. Heredia and N. Hurtado. 2017. Pigment composition and antioxidant capacity of betacyanins and betaxanthins fractions of *Opuntia dillenii* (Ker Gawl) haw cactus fruit. Food Res. Int. 101:173-179.

Barbour, M.G. and G. Michael. 1970. Is any angiosperm an obligate halophyte?. Amer. Midland Naturalist 84:106-119.

Barbour, M.G., J.H. Burk and W.D. Pitts. 1987. Terrestrial Plant Ecology. Second ed, Benjamin/Cummings Pub. Co., Menlo Park, CA (USA).

Blois, M.S. 1958. Antioxidant determinations by the use of a stable free radical. Nature 181:1199-2000.

Bothe, H. 1976. Salzresistenz bei Pflanzen. Biologie in unserer Zeit. 6:3-9.

Cabrita, L., T. Fossen and Ø.M. Andersen. 2000. Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. Food Chem. 68:101-107.

Cai, Y., M. Sun and H. Corke. 2001. Identification and distribution of simple and acylated betacyanins in Amaranthacea. J. Agric. Food Chem. 49:1971-1978.

Cai, Y., M. Sun, H. Wu, R. Huang and H. Corke. 1998. Characterization and quantification of betacyanin pigments from diverse *Amaranthus* species. J. Agric. Food Chem. 46:2063-2070.

Caldas-Cueva, J.P., P. Morales, F. Ludena, I. Betalleluz-Pallardel, R. Chirinos, G. Noratto and D. Campos. 2015. Stability of betacyanin pigments and antioxidants in ayrampo (*Opuntia soehrensii* Britton and Rose) seed extracts and as a yogurt natural colorant. J. Food Processing & Preservation 40:541-549.

Castellar, R., J.M., Obón, M. Alacid and J.A. Fernández-López. 2003. Color properties and stability of betacynins from *Opuntia* fruits. J. Agric. Food Chem. 51:2772-2776.

Chirol, N. and M. Jay. 1995. Acylated anthocyanins from flowers of *Begonia*. Phytochemistry 40:275-277.

Choi, J.H., S.K. Yoon, K.H. Lee, M.S. Seo, D.H. Kim, S.B. Hong, J.Y. Kim, H.D. Paik and C.H. Kim. 2006. Antitumor activity of cell suspension culture of green tea seed (*Camellia sinensis* L.). Biotechnol. Bioprocess Eng. 11:396-401.

Chung, S.H. and Y.H. Byun. 2009. Convenient methods for the extraction and discrimination of water-soluble plant pigments. J. Kor. Contents Association 9:353-360.

Flowers, T.J., M.A. Hajibaggheri and N.J.W. Clipson. 1986. Halophytes. Quart. Rev. Biol. 61:313-337.

Flowers, T.J. and T.D. Colmer. 2008. Salinity tolerance in halophytes. New Phytol. 179:945-963.

_____. 2015. Plant salt tolerance: adaptations in halophytes. Ann. Bot. 115:327-331.

Fossen, T. and Ø.M. Andersen. 1999. Cyanidin 3-(2",3"-digalloylglucoside) from red leaves of *Acer platanoides*. Phytochemistry 52:1697-1700.

Gandía-Herrero, F., F. García-Carmona and J. Escribano. 2005. Fluorescent pigments: new perspectives in betalain research and applications. Food Res. Intl. 38:879-884.

Glenn, E.P., J.J. Brown and J.W. O'Leary. 1998. Irrigating crops with seawater. Sci. Amer. August. pp.76-81.

Goto, T., T. Hoshino and M. Ohba. 1976. Stabilization effect of neutral salts on anthocyanins: flavylium salts, anhydrobases and genuine anthocyanins. Agr. Biol. Chem. 40:1593-1596.

Goto, T., T. Kondo, H. Tamura and K. Kawahori. 1983. Structure of platiconin, a diacylated anthocyanin isolated from the Chinese bell-flower *Platycodon grandiflorum*. Tetrahedron Letters 24:2181-2184.

Hong, S., Lee H.A., Y.S. Lee, D.W. Kim, J.H. Jeonh, T.W. Kim and O. Kim. 2014. Anti-toxoplasmosis effect of the halophyte *Suaeda maritima*. Korean J. Plant Res. 27:415-420.

Jackman, R.L. and J.L. Smith. 1996. Anthocyanins and betalains: *In* Hendry G.A.F. and J.D. Houghton (eds.), Natural Food Colorants (2nd ed.), Chapman & Hall, London, UK. pp. 245-309.

Jang, Y.P., J. Zhou, K. Nakanishi and J.R. Sparrow. 2005. Anthocyanins protect against A2E photooxidation and membrane permeabilization in retinal pigment epithelial cells. Photochem. Photobiol. 81:529-536.

Kang, K.Y., Y.H. Hwang, S.J. Lee, J.J. Kim, S.J. Nam and S.T. Yee. 2017. Verification of the antioxidant activity of a subterranean part of *Suaeda japonica* Makino. Industrial Crops & Products 109:836-842.

Kim, H.R., G.N. Park, B.K. Jung, W.J. Yoon, Y.H. Jung and K.S. Chang. 2016. Antibacterial activity of *Suaeda australis* in halophyte. J. Kor. Oil Chemist's Soc. 33:278-285.

Kim, S.D., J.H. Do and H.I. Oh. 1981. Antioxidant activity of *Panax ginseng* browning products. J. Kor. Agr. Chem. Soc. 24:161-166.

Larcher, W. 1995. Physiological Plant Ecology - Ecophysiology and Stress Physiology of Functional Groups. Third ed, Springer. pp. 396-409.

Lohachoompol, V., M. Mulholland, G. Srzednicki and J. Craske. 2008. Determination of anthocyanins in various cultivars of highbush and rabbit eye blueberries. Food Chem. 111: 249-254.

Nelson, N. and W. Harvey. 1999. Vacuolar and plasma membrane proton adenosinetriphosphatases. Physiol. Rev. 79:361-385.

Pollard, A. and R.G. Wyn Jones. 1979. Enzyme activities in concentrated solutions of glycine betaine and other solutes. Planta 144:291-298.

Rubinstein, L.V., R.H. Shoemaker, K.D. Paull, R.M. Simon, S. Tosini, P. Skehan, D.A. Scudiero, A. Monks and M.R. Boyd. 1990. Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J. Natl. Cancer Inst. 82:1113-1118.

Saito, N., F. Tatsuzawa, K. Kasahara, M. Yokoi, S. Iida, A. Shigihara and T. Honda. 1996. Acylated peonidin glycosides in the slate flowers of *Pharbitis nil*. Phytochemistry 41: 1607-1611.

Schliemann, W., R.W. Joy IV, A. Komamine, J.W. Metzger, M. Nimtz, V. Wray and D. Strack. 1996. Betacyanins from plants and cell cultures of *Phytolacca americana*. Phytochemistry 42:1039-1046.

Schliemann, W., Y. Cai, T. Degenkolb, J. Schmidt and H. Corke. 2001. Betalains of *Celosia argentea*. Phytochemistry 58:159-165.

Song, J. and B.S. Wang. 2015. Using euhalophytes to understand salt tolerance and to develop saline agriculture: *Suaeda salsa* as a promising model. Ann. Bot. 115:541-553.

Stafford, H.A. 1994. Anthocyanins and betalains: evolution of the mutually exclusive pathways. Plant Sci. 101:91-98.

Steglich, W. and D. Strack. Betalains. 1990: *In* Brossi A.A. (ed.), The Alkaloids - Chemistry and Pharmacology, Vol. 39, Academic Press, San Diego, USA. pp. 1-62.

Stintzing F.C. and R. Carle. 2004. Functional properties of anthocyanins and betalanins in plants, food, and in human nutrition. Trends Food Sci. Technol. 15:19-38.

Strack, D., T. Vogt and W. Schliemann. 2003. Recent advances in betalain research. Phytochemistry 62:247-269.

Tanaka, Y., N. Sasaki and A. Ohmiya. 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. The Plant J. 54:733-749.

Tsai, P.J., C.H. Sheu, P.H. Wu and Y.F. Sun. 2010. Thermal and pH stability of betacynin pigment of Djulis (*Chenopodium formosanum*) in Taiwan and their relation to antioxidant activity. J. Agric. Food Chem. 58:1020-1025.

Woo, K.K., F.H. Ngou, L.S., Ngo, W.K. Soong and P.Y. Tang.

2011. Stability of betalain pigment from red dragon fruit (*Hylocereus polyrhizus*). Amer. J. Food Technol. 6:140-148.

Wyler, H. and A.S. Dreiding. 1961. Über Betacyane, die Stickstoffhaltigen Farbstoffe der Centrospermen - Vorlaufige Mitteilung. Experientia 17:23-25.

Xu, Y., Y. Zhao, H. Duan, N.S. Fang and J. Song. 2017.

Transcriptomic profiling of genes in matured dimorphic seeds of euhalophyte *Suaeda salsa*. BMC Genomics 18:727-740.

Yang, R.Y.K., O. Bayraktar and H.T. Pu. 2003. Plant-cell bioreactors with simultaneous electropermeabilization and electrophoresis. J. Biotechnol. 100:13-22.