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Review

Analysis and biological activities of anthocyanins

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Abstract

Anthocyanins are naturally occurring compounds that impart color to fruits, vegetables, and plants. They are probably the most important group of visible plant pigments besides chlorophyll. Apart from imparting color to plants, anthocyanins also have an array of health-promoting benefits, as they can protect against a variety of oxidants through a various number of mechanisms. However, anthocyanins have received less attention than other flavonoids, despite this. This article reviews their biological functions and pre-clinical studies, as well as the most recent analytical techniques concerning anthocyanin isolation and identification.

Keywords: Anthocyanin; Biological activity

Contents

1. Introduction

Anthocyanins (in Greek *anthos* means flower, and *kyanos* means blue) are the more important plant pigments visible to the human eye. They belong to the widespread class of phenolic compounds collectively named flavonoids. They are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts (Fig. 1).

The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of this attachment, and the nature and number of aliphatic or

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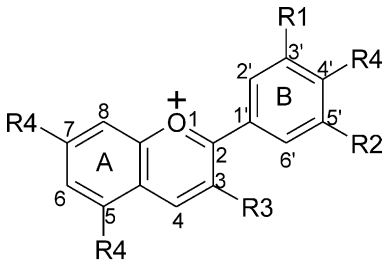

Fig. 1. The flavylium cation. R1 and R2 are H, OH, or OCH_3; R3 is a glycosyl or H; and R4 is OH or a glycosyl.

aromatic acids attached to sugars in the molecule. To date, there are 17 known naturally occurring anthocyanidins or aglycones which are listed in Tables 1. The role of anthocyanidins in plants is summarized in Tables 2.

Only six anthocyanidins are common in higher plants—pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp). The glycosides of the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most widespread in nature, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers. The distribution of the six most

Table 1
Naturally occurring anthocyanidins

		Substitution pattern							
Name	Abbreviation	3	5	6	7	3′	4′	5′	Color
Apigeninidin	Ap	H	OH	H	OH	H	OH	H	Orange
Aurantinidin	Au	OH	OH	OH	OH	H	OH	H	Orange
Capensinidin	Cp	OH	OMe	H	OH	OMe	OH	OMe	Bluish-red
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH	Bluish-red
Europinidin	Eu	OH	OMe	H	OH	OMe	OH	OH	Bluish-red
Hirsutidin	Hs	OH	OH	H	OMe	OMe	OH	OMe	Bluish-red
6-Hydroxycyanidin	6OHCy	OH	OH	OH	OH	OH	OH	H	Red
Luteolinidin	Lt	H	OH	H	OH	OH	OH	H	Orange
Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe	Bluish-red
5-Methylcyanidin	5-MCy	OH	OMe	H	OH	OH	OH	H	Orange-red
Pelargonidin	Pg	OH	OH	H	OH	H	OH	H	Orange
Peonidin	Pn	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH	Bluish-red
Pulchellidin	Pl	OH	OMe	H	OH	OH	OH	OH	Bluish-red
Rosinidin	Rs	OH	OH	H	OMe	OMe	OH	H	Red
Tricetinidin	Tr	H	OH	H	OH	OH	OH	OH	Red

Table 2
The role of anthocyanins and 3-deoxyanthocyanidins in plants

Plant	Compound	Origin	Function
Angiosperms			
Senecio cruentus	Cinerarin	Petals	Pollination
Sorghum	Apigeninidin	Leaf sheath	Phytoalexin anti-microbial antioxidants
Gymnosperms			
Abies concolor	Petunidin-3-glucoside Cyanidin-3-glucoside	Cone	?
Pinus contorta	Anthocyanin	Leaves	Cold tolerance
Pinus banksiana	?	Seedlings	Photoinhibibition tolerance
Ferns			
Davallia divaricata	Pelargonidin-3-*p*-coumaryl-glc-5-glc(monardein)	Young leaves	?
Ferns species	Apigenidin	Leaves	?
Mosses			
Bryum, Splachunm	Luteolinidin-5-glc	Leaves	?
Liverwort			
Cephaloziella exilifolia	Anthocyanin-like	Thallus	?

Excerpted from Cooper-Driver et al. (1998).

common anthocyanidins in the edible parts of plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%). The following four classes of anthocyanidin glycosides are common: 3-monosides, 3-biosides, 3,5-diglycosides and 3,7-diglycosides. 3-glycosides occur about two and half times more frequently than 3,5-diglycosides. So, the most widespread anthocyanin is cyanidin 3-glucoside.

Based on several reviews to date, it is estimated that more than 400 anthocyanins have been found in nature. In the book entitled "*Anthocyanins in Fruits, Vegetables, and Grains*" (Mazza and Miniah, 1993), 258 anthocyanins are listed; and according to the reviews of Harborne and Williams (1998, 2001), from January 1995 to December 1997, 85 new anthocyanins were recorded, whilst from January 1998 to December 2000, some 50 new anthocyanin pigments were found in plants.

2. Recent advances in anthocyanin analysis and identification

Anthocyanins are soluble in polar solvents, and they are normally extracted from plant materials by using methanol that contains small amounts of hydrochloric acid or formic acid. The acid lowers the solution's pH value and prevents the degradation of the non-acylated anthocyanin pigments. However, as hydrochloric acid or formic acid is concentrated during the evaporation of the methanol-hydrochloric acid or methanol-formic acid solvent, pigment degradation occurs (e.g. in the extract of Azalea cv. Alice Erauw, the cyanidin-3-monosides are converted into unstable aglycone). Small amounts of acid may also cause partial or total hydrolysis of the acyl moieties of acylated anthocyanins that are present in some plants. One report compared various techniques for the extraction of anthocyanins from red grapes and demonstrated that solvents containing up to 0.12 mol/l hydrochloric acid can cause partial hydrolysis of acylated anthocyanins (Revilla et al., 1998). Acetone has also been used to extract anthocyanins from several plant sources (Giusti et al., 1998, Garcia-Viguera et al., 1998). In comparison to acidified methanol, this technique allows an efficient and more reproducible extraction, avoids problems with pectins, and permits a much lower temperature for sample concentration (Garcia-Viguera et al., 1998). Solid-phase extraction (SPE) on C_{18} (SPE) cartridges or Sephadex is commonly used for the initial purification of the crude anthocyanin extracts. The anthocyanins are bound strongly to these adsorbents through their unsubstituted hydroxyl groups and are separated from unrelated compounds by using a series of solvents of increasing polarity.

The characterization of a mixture of anthocyanins usually involves the separation and collection of each compound, and subsequent analysis by nuclear magnetic resonance (NMR) and fast atom bombardment mass spectroscopy (FAB-MS). For the separation and structural analysis, the use of liquid chromatography–mass spectrometry (LC–MS) technique, which combines the separation of LC with the selectivity and sensitivity of the MS detector, permits the identification of individual compounds in a mixture of compounds. Glässgen et al. (1992) described the use of liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) for the identification of anthocyanins in the plant tissue and cell cultures of Indian black carrot (*Daucus carota* L. ssp. *sativus*). Several cyanidin derivatives were identified, including acylated and non-acylated compounds. Recently liquid chromatography-electron impact ionization mass spectrometry (LC-EI-MS) was also used to identify the anthocyanins of *Catharanthus roseus* extracts (Piovan et al., 1998). Baldi et al. (1995) used LC–MS with an atmospheric pressure-ionization ion-spray interface to analyze the anthocyanins contained in the grape skins (*Vitis vinifera* L.). Nineteen derivatives of cyanidin, delphinidin, petunidin, malvidin and peonidin were identified by this ionization technique. The individual mass spectra showed peaks for the molecular ions, together with a fragment corresponding to aglycone; when acylation was present, an additional fragment was detected at mass/charge values corresponding to the loss of acyl moiety from the molecular ion. Since Saito et al. (1983) employed FAB-MS in the structural analysis of acylated anthocyanins violanin and platyconin, many new acylated anthocyanins have been found with the help of this technique.

Atmospheric-pressure ionization (API) techniques have several advantages over other MS detection methods. In API-MS the ion source is located outside the MS, the ions are formed at atmospheric pressure, and then sampled into the mass spectrometer. These are soft ionization techniques (only the molecular ion is formed), although the application of a potential at the entrance of the mass spectrometer (fragment voltage) creates suitable conditions for CID, and the production of fragment ions. Two API interfaces are available commercially, namely, the atmospheric pressure chemical ionization interface (APCI) and the ESI interface. Revilla et al. (1999) analyzed the anthocyanins present in extracts of grape skins and red wine with a LC–MS system equipped with an ESI interface. In the same year, da Costa et al. (1998) used a LC–MS system that was equipped with an APCI interface for the analysis of anthocyanins (3-glucosides and 3-rutinosides of cyanidin and delphinidin) from blackcurrant fruit (*Ribes nigrum*). The molecular ion $[M^+]$, and the mass fragments corresponding to successive loss of the sugar residues, $[M^+\text{-}146]$ and $[M^+\text{-}146\text{-}162]$ were detected under appropriate conditions. Wang and Sporns (1999), Wang et al. (2000) used respectively matrix-assisted

laser desorption/ionization mass spectrometry (MALDI–MS) to perform both qualitative and quantitative analyses of anthocyanins in wine and fruit juice, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) to analyze the content of anthocyanins in various foods.

Another technique recently used for anthocyanin analysis is capillary electrophoresis (CE) which has excellent mass sensitivity, high resolution, low sample consumption and minimal generation of solvent waste. Bridle et al. (1997) reported the separation of a mixture of standards, as well as strawberry and elderberry anthocyanins, by capillary zone electrophoresis (CZE). da Costa et al. (1998) published a CZE method to separate blackcurrant anthocyanins in a fused-silica capillary using acidic phosphate buffers [pH 1.50-acetonitrile (2/1 v/v)]. The interaction of the anthocyanins with the capillary wall was evaluated by comparing the separation of anthocyanins on a fused-silica capillary column to that on a linear polyacrylamide (PLA) coated one. The results showed that there was almost no interaction at pH 1.8 between the silanols on the uncoated capillary and the anthocyanins. The four anthocyanins present in the blackcurrant juice, namely cyanidin 3-glucosides and 3-rutinosides, delphinidin 3-glucosides and 3-rutinosides, were separated within the time range expected for an LC analysis, while consuming much less sample and solvent. Watanabe et al. (1998) analyzed elderberry pigments (*Sambucus nigra* L.) in commercial food samples (candy, juice, and jelly) using micellar electrokinetic chromatography (MEKC). The anthocyanins, all cyanidin derivatives, were separated in less than 10 min. Other important progress in anthocyanin analysis has been reported (Bicard et al., 1999). It concerns the analysis of anthocyanins by CZE in acidic media.

Together with the above techniques, NMR, in both one and two dimensions, is a very useful technique for structural elucidation of anthocyanins. Some examples for its applications are given here. Giusti et al. (1998) reported the separation of anthocyanins from red radish (*Raphanus sativus*) and their structural elucidation by one- and two-dimensional NMR. Four anthocyanins were obtained: pelargonidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside] 5-*O*-(6-*O*-malonyl-β-glucopyranoside); pelargonidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-feruloyl)-β-glucopyranoside] 5-*O*-(6-*O*-malonyl-β-glucopyranoside); pelargonidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside] 5-*O*-(β-glucopyranoside) and pelargonidin 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-feruloyl)-β-gluco-pyranoside] 5-*O*-(β-glucopyranoside). They also investigated the three-dimensional conformation of the molecule by using NOESY techniques, which showed proximity between the hydrogen from the cinnamic acid acylating group and the C-4 of the pelargonidin. By using NMR and other techniques, Cabrita et al. (2000) identified the anthocyanin trisaccharides of *Vaccinium padifolium*. Similarly, Terahara et al. (2001) isolated and identified delphinidin, cyanidin 3-*O*-β-D-galactosides and delphinidin 3-*O*-β-D-(6-(*E*)-*p*-coumaryl)galactopyranoside from *Camellia sinensis*. Tanaka et al. (2001) isolated a malonylated anthocyanin from the blue flowers of *Meconopsis*, and then identified it as cyanidin 3-*O*-[(6-*O*-malonyl-2-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside using NMR spectroscopy. Toki et al. (2001) isolated three acylated anthocyanins from the scarlet flowers of *Anemone coronaria*, which all belong to pelargonidin-type anthocyanins. The first pigment was pelargonidin 3-*O*-[2-(β-D-xylopyranosyl)-6-*O*-(malonyl)-β-D-galactopyranoside], the second pigment was pelargonidin 3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(methyl-malonyl)-β-D-galactopyrano-side] and the third one was (6″-*O*-(pelargonidin 3-*O*-[2″-*O*-(β-D-xylopyranosyl)-β-D-galactopyranosyl] [(4-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-*O*-tartaryl] malonate.

3. Functions of anthocyanins

The most significant function of anthocyanins is their ability to impart color to the plants or plant products in which they occur. They play a definite role in the attraction of animals for pollination and seed dispersal, and hence they are of considerable value in the co-evolution of these plant-animal interactions. Anthocyanins and 3-deoxyanthocyanidins however have roles in flowering plants other than as attractants. They can act as antioxidants, phytoalexins or as antibacterial agents.

Anthocyanins may be important factors along with other flavonoids in the resistance of plants to insect attack (Harborne, 1988). For example, cyanidin 3-glucoside was shown to protect cotton leaves against the tabacco budworm (Hedin et al., 1983).

In addition to their functions in plants, anthocyanins have many other uses. For example, their important function in cognitive decline and neural dysfunction has been investigated. Joseph et al. (1999) found that fruit extracts including anthocyanins were effective in reversing age-related deficits in several neural and behavioral parameters, e.g. oxotremorine enhancement of a K1-evoked release of dopamine from striatal slices, carbachol-stimulated GTPase activity, striatal Ca buffering in striatal synaptosomes, motor behavioral performance on the rod walking and accelerod tasks, and Morris water maze performance.

4. Biological activities of anthocyanins

Anthocyanins also possess known pharmacological properties and are used by humans for therapeutic

purposes. Following the recognition that pigment extracts are more effective than *O*-(β-hydroxyethyl) rutin in decreasing capillary permeability and fragility and in their anti-inflammatory and anti-oedema activities (Wagner, 1985), it is possible that anthocyanins may replace rutin and its derivatives in the treatment of illnesses involving tissue inflammation or capillary fragility. The crude anthocyanin extracts of *Vaccinium myrtillus* have been given orally and by intravenal or intramuscular injection to reduce capillary permeability and fragility.

In a study on testing the effect of anthocyanins on tumors, some anthocyanins were not effective in suppressing tumor growth (Ghiselli et al., 1998). However, an antioxidant activity study of anthocyanin fractions from Italian red wine showed that the anthocyanin fraction was the most effective both in scavenging reactive oxygen species and in inhibiting lipoprotein oxidation and platelet aggregation (Ghiselli et al., 1998). This result suggests that anthocyanins could be the key component in red wine that protects against cardiovascular disease. Another report on the anti-tumor activity of anthocyanins was published by Kamei et al. (1998). They found that the anthocyanin fraction from red wine suppressed the growth of HCT-15 cells, which are derived from human colon cancer or AGS cells from human gastric cancer. The suppression rate by the anthocyanin fraction was significantly higher than that of the other fractions.

Obi et al. (1998) examined the ability of anthocyanin obtained from the petals of *H. rosasinensis* to prevent carbon tetrachloride-induced acute liver damage in rats. The results showed that those rats treated with anthocyanin and carbon tetrachloride had significantly less hepatotoxicity ($P<0.05$) than those given carbon tetrachloride alone. This was assessed by measuring the levels of serum aspartate and alanine aminotransferase activities 18 hours after carbon tetrachloride was given. This result suggested that *H. rosasinensis* anthocyanin may be protective against carbon tetrachloride-induced liver injury.

Yoshimoto et al. (1999) studied the antimutagenicity of water extracts prepared from the storage roots of four varieties of sweet potato with different flesh colors, using *Salmonella typhimurium* TA 98. They found that two anthocyanin pigments purified from the purple-colored sweet potato, 3-(6,6′-caffeylferulylsophoroside)-5-glucoside of cyanidin (YGM-3) and peonidin (YGM-6), effectively inhibited the reverse mutation induced by heterocyclic amines-mutagen, Trp-P-1, Trp-P-2, and IQ in the presence of rat liver microsomal activation systems.

Jankowski et al. (2000a,b) reported that the administration of anthocyanin dyes from *Aronia melanocarpa* to rats before the intraperitoneal injections of Platelet-Activating Factor (PAF) and ceruleine had a beneficial effect on the development of acute experimental pancreatitis in rats. It was revealed that this was due to the reduction of pancreatic swelling and a decrease in lipid peroxidation and adenosine deaminase activity. They also examined the effect of anthocyanins from Cabernet red wine on the course and intensity of symptoms of experimental diabetes in rats (Jankowski et al. 2000a,b). The results showed that a simultaneous daily administration of anthocyanins obtained from Cabernet red wine and streptozotocin substantially decreased sugar concentrations in the urine and blood serum. These anthocyanins also inhibited the loss of body mass caused by the injection of streptozotocin. Simultaneously, the anthocyanin pigment prevented the generation of free oxygen radicals, and decreased the peroxidation of lipids.

Pawlowicz et al. (2000) attempted to determine the influence of anthocyanins from chokeberries on the generation of autoantibodies to oxidize low density lipoproteins (oLAB) in pregnancies complicated by intrauterine growth retardation (IUGR). An experiment was conducted with a study group of 105 pregnant women (on the turn of trimester two according to LMP) with IUGR (sonographic examination results below the 5th percentile for real gestational age) who were randomly divided into 2 groups. Fifty women were administered anthocyanins and 55 women were given a placebo. There was a control group of 60 healthy pregnant women. They then examined the level of oxidative stress measured by the serum concentration of autoantibodies required to oxidize low density lipoproteins (oLAB). In the anthocyanin group, the oLAB titres decreased from 1104 ± 41 mU/ml before treatment to 752 ± 36 mU/ml in the first month and 726 ± 35 mU/ml in the second month, at $P<0.01$. In the placebo group, the oLAB titres showed a slightly increasing trend: 1089 ± 37 mU/ml before treatment, 1092 ± 42 mU/ml in the first month and 1115 ± 43 mU/ml in the second month, at $P>0.05$. The oLAB titres in the control group were 601 ± 49 mU/ml before treatment, 606 ± 45 mU/ml in the first month, and 614 ± 43 mU/ml in the second month, at $P>0.05$. The results indicated that natural antioxidants (anthocyanins) can be useful in controlling oxidative stress during pregnancies complicated by IUGR.

Hibiscus anthocyanins (HAs), a group of natural pigments occurring in the dried flowers of *Hibiscus sabdariffa* L., are used in soft drinks and herbal medicines. Their antioxidant bioactivity has been studied and it appears that HAs can significantly decrease the leakage of lactate dehydrogenase and the formation of malondialdehyde induced by a treatment of *tert*-butyl hydroperoxide (*t*-BHP). The in vivo investigation showed that the oral pretreatment of HAs before a single dose of *t*-BHP significantly lowered the serum levels of hepatic enzyme markers (alanine and aspartate aminotransferase) and reduced oxidative liver damage. The

histopathological evaluation of the liver revealed that hibiscus pigments reduce the incidence of liver lesions including inflammation, leucocyte infiltration, and necrosis induced by *t*-BHP in rats (Wang et al., 2000).

In 2001, Meiers et al. found that the aglycones of the most abundant anthocyanins in food, cyanidin (Cy) and delphinidin (Del), possess the ability to inhibit the growth of human tumor cells in vitro in the micromolar range. However, malvidin (Mv), an anthocyanidin typically found in grapes, was less active. The aglycones preferentially inhibited the growth of the human vulva carcinoma cell line A431, overexpressing the epidermal growth-factor receptor (EGFR). The glycosides cyanidin-3-beta-D-galactoside (Cy-3-gal, idaein) and malvidin-3-beta-D-glucoside (Mv-3-glc, oenin) did not affect tumor cell growth up to 100 μM. The tyrosine kinase activity of the EGFR, isolated from A431 cells, was potently inhibited by Cy and Del. However, Mv and the glycosides Cy-3-gal and Mv-3-glc were inactive up to 100 μM. Using intact cells, they also investigated the influence of anthocyanin treatment on downstream signaling cascades by measuring the phosphorylation of the transcription factor Elk-1. They observed that A431 cells were transiently transfected with a luciferase reporter gene construct whose expression is controlled by a MAP kinase pathway dependent phosphorylation of a GAL4-Elk-1 fusion protein. Cy and Del inhibited the activation of the GAL4-Elk-1 fusion protein in the concentration range where growth inhibition was observed. Thus, they concluded that the anthocyanidins Cy and Del are potent inhibitors of the EGFR, shutting off downstream signaling cascades.

Nitric oxide (NO) is a diatomic free radical produced from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues. It is believed that some chronic inflammatory diseases are associated with NO. Wang and Mazza (2002) for the first time reported that anthocyanins had strong inhibitory effects on NO production after detailed study. There are also reports of anthocyanins possessing antiulcer activity (Cristoni and Magistretti, 1987) and providing protection from UV radiation (Sharma, 2001).

5. Antioxidant properties and free radical scavenging properties

In 1994, Tsuda et al. reported the antioxidant activity of the anthocyanin pigments cyanidin 3-*O*-β-glucoside (C3G) and cyanidin (Cy), which was examined by using linoleic acid autoxidation, liposome, rabbit erythrocyte membranes, and rat liver microsomal systems. C3G and Cy had antioxidative activity in all systems. Cy had a stronger activity than C3G and the same activity as α-tocopherol in the liposome and rabbit erythrocyte membrane systems. In the rat liver microsomal system, Cy and C3G exhibited stronger activity than α-tocopherol. These data suggested that the pigments may play an important role in the prevention of lipid peroxidation of cell membranes induced by active oxygen radicals in living systems as they become dietary antioxidants after ingestion.

In 1996, Tsuda et al. investigated the antioxidative, radical scavenging, and inhibitory effects on lipid peroxidation of UV light irradiation of three anthocyanin pigments: pelargonidin 3-*O*-beta-D-glucoside (P3G), cyanidin 3-*O*-beta-D-glucoside (C3G), and delphinidin 3-*O*-beta-D-glucoside (D3G), isolated from the *Phaseolus vulgaris L.* seed coat, and their aglycones, pelargonidin chloride (Pel), cyanidin chloride (Cy), and delphinidin chloride (Del). They found that all the pigments had strong antioxidative activity in a liposomal system and reduced the formation of malondialdehyde from UVB (320–290 nm) irradiation. On the other hand, the extent of antioxidative activity in a rat liver microsomal system and the scavenging effect of hydroxyl radicals (·OH) and superoxide anion radicals ($\cdot O_2^-$) were influenced by their own structures. In the same year, Koide et al. (1996) found that hydrolyzed anthocyanidins contained in grape rinds and red rice gave an elevation of S phase, which suggested a block in the step from S-phase to G2-phase in HCT-15 cells. It seemed that the anthocyanidins contained in the grape rinds and red rice were effective in the suppression of cell growth.

Koide et al. (1997) also reported the anti-tumor effects in vitro and in vivo of extracts from red soybeans, which were composed of mostly cyanin conjugated with glucose and rhamnose. Meanwhile, Gracia et al. (1997) reported that anthocyanins acted as antioxidants on human low-density lipoprotein (LDL) and lecithin-liposome systems in vitro. They found that the inhibition of oxidation increased with the concentration of the antioxidant. In the LDL system, when the oxidation was catalyzed with 10 μM copper, malvidin was the best oxidation inhibitor, followed by delphinidin, cyanidin, and pelargonidin. When the oxidation was catalyzed with 80 μM copper, the order of antioxidant activity changed and decreased in the following order at all concentrations tested: delphinidin, cyanidin, malvidin, and pelargonidin. In the liposome system, catalyzed with either 3 or 10 μM copper, malvidin was the best inhibitor of both conjugated diene and hexanal formation. At 3 μM copper, delphinidin, cyanidin, and pelargonidin showed prooxidant activity. At 10 μM copper, pelargonidin followed malvidin in antioxidant potency, and cyanidin and delphinidin were prooxidants. All the effects of the anthocyanins listed above can be explained by several antioxidant mechanisms, including hydrogen donation, metal chelation, and protein binding.

Anthocyanins can also prevent the oxidation of ascorbic acid caused by metal ions through chelating the metal ions, and forming ascorbic(copigment)-metal-anthocyanin complex (Sarma et al., 1997). Anthocyanins can also scavenge $\cdot O_2^-$. Noda et al. (1998) reported that the anthocyanin delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside (nasunin), which was isolated as purple colored crystals from eggplant skins, *Solanum melongena* L. 'Chouja', can directly scavenge $\cdot O_2^-$ with a potency of 143±8 SOD-equivalent units/mg, and inhibit the formation of DMPO-OH (0.65±0.07 EPC-K1 μmol/mg). Their analysis involved using an electron spin resonance spectrometer and a 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) spin trapping method. Hydroxyl (·OH) or superoxide anion radicals ($\cdot O_2^-$) were generated by a Fenton reaction or hypoxanthine-xanthine oxidase system. L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetra-methyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6yl-hydrogen phosphate] potassium salt (EPC-K1) and bovine erythrocyte superoxide dismutase (SOD) were used as standards for OH and $\cdot O_2^-$ respectively. The spectrophotometric study showed that nasunin formed an iron complex with a molar ratio of nasunin:Fe^{3+} of 2:1, which indicates that hydroxyl radical scavenging by nasunin is not due to direct radical scavenging but through the inhibition of ·OH generation by chelating iron. Nasunin (1 μM) significantly protected against lipid peroxidation of brain homogenates ($P<0.001$) as measured by malonaldehyde and 4-hydroxyalkenals. These findings demonstrate that nasunin is a potent $\cdot O_2^-$ scavenger and iron chelator which can protect against lipid peroxidation.

A further study of this anthocyanin was conducted by the same authors (Noda et al., 2000) using the same method. By changing the concentration of DMPO to vary the trapping rate of ·OH, they studied the presence of a competitive reaction between nasunin and ·OH. The 50% inhibition dose (ID_{50}) obtained from the inhibition curve did not change, indicating ·OH scavenging of nasunin is not due to direct scavenging, but through the inhibition of the ·OH generating system by chelating ferrous ion and other effects. This confirms the conclusion of the previous study.

On the generation of thiobarbituric acid reactive substances (TBARS) during serum formation ex vivo, Tsuda et al. (1998) studied the susceptibility of serum to further lipid peroxidation of cyanidin 3-*O*-beta-D-glucoside (C3G) in rats. They found that the serum from the C3G-fed group showed a significantly lower susceptibility to further lipid peroxidation provoked by 2,2′-azobis(2-amidinopropane) hydrochloride or copper (II) ion than that of the control group. Concentrations of endogenous antioxidants remaining in the serum after blood coagulation were not affected by the C3G feeding. The results show that feeding rats C3G increases the ex vivo oxidation resistance of the serum without affecting serum endogenous antioxidant levels, and reduces the TBARS generated during serum formation without changing the concentrations of serum lipids.

Zobel et al. (1999) studied the possible anthocyanin protective mechanisms in mammalian cells. They compared their extracellular and intracellular antioxidative potential in vitro and in human colon tumor cells. The anthocyanins they used were *Aronia melanocarpa* Elliot anthocyanin (AA) concentrates, and fractions thereof, concentrates from elderberry, Macqui, and Tintorera fruits, as well as pure compounds. The antioxidative properties of the samples were studied in vitro with the ferric reducing/antioxidant power (FRAP) assay, which is an iron reducing assay for antioxidants. As a measure of intracellular oxidative/antioxidative effects, hydrogen peroxide-induced strand breaks as well as oxidized DNA bases were determined in human tumor HT29 clone 19A cells using a microgel electrophoresis assay (comet test). The results showed that isolated compounds (aglycones and glycosides) and complex plant samples are powerful antioxidants in vitro. Their activities exceeded those of Trolox and vitamin C in the FRAP assay. Also, hydrogen peroxide-induced DNA strand breaks were reduced in cells treated with the complex plant extracts. In contrast, the endogenous generation of oxidized DNA bases was not prevented. The findings indicated that the intracellular steady state of oxidized DNA bases is not altered by anthocyanins or anthocyanidins. Extracellularly, however, the compounds are potent antioxidants. This points to their potential for providing systemic protection in vivo, e.g., by scavenging oxidants in the blood stream and in the colon. Notably, both aglycones and glycosides have equally strong antioxidant activity.

The antioxidative activity of three anthocyanin pigments extracted from the fruits of the chokeberry, honeysuckle and sloe were studied by Gabrielska et al. (1999). They found that the antioxidant efficiency of these compounds follows this sequence: chokeberry > sloe > honeysuckle by using a thiobarbituric acid-reactive substance assay and an UV radiation-induced lipid oxidation in the liposome membrane. The antioxidative potency of anthocyanin extracts is concentration-dependent.

Similarly, Tsuda et al. (1999) found that cyanidin 3-*O*-beta-D-glucoside (C3G), a typical anthocyanin pigment, can lower the serum thiobarbituric acid-reactive substance (TBARS) concentration and increase the oxidation resistance of the serum to lipid peroxidation in rats. Their results suggested that C3G acts as a potent antioxidant in vivo when acute oxidative stress is encountered. Narayan et al. (1999) found that in vitro enzymatic and non-enzymatic polyunsaturated fatty acid peroxidation was significantly inhibited in a dose dependent manner by purified anthocyanin, a deep-red color pigment from carrot cell cultures.

6. The interaction with DNA

A study of the inter-reaction of anthocyanins and DNA was carried out by Sarma and Sharma (1999). They found that calf thymus DNA (ctDNA) and cyanidin can form a cyanidin–DNA copigmentation complex, which displayed a 15–20 nm bathochromic shift when cyanin is mixed with calf thymus DNA (ctDNA). Exposure of either cyanidin or ctDNA individually to hydroxyl radicals (OH) obtained in a Fenton reaction caused severe oxidative damage. However, formation of the cyanin-DNA complex prior to exposure to OH protected both the cyanidin and ctDNA from oxidative damage. Based on the above results, they suggested that cyanidin-DNA copigmentation may be a possible defense mechanism against the oxidative damage of DNA and may have in vivo physiological functions attributable to the antioxidant ability of anthocyanins. It has been suggested that anthocyanins have the ability to stabilize DNA triple-helical complexes (Mas et al., 2000) (Fig. 2).

Espin et al. (2000) found that the anthocyanin extracts from black chokeberry, black-thorn, and strawberry possess an antiradical capacity. Deguchi et al. (2000a,b) also found that a novel purple pigment called hordeumin, a type of anthocyanin-tannin pigment, produced from barley bran-fermented broth, has radical scavenging activity. The hordeumin scavenged superoxide radicals in a concentration-dependent manner. A further study on hordeumin found that it has antimutagenicity effect. This effect of hordeumin was investigated according to the Ames method, an indication of the safety of food, using *Salmonella typhimurium* TA98. Furthermore, hordeumin also decreased the reverse mutation of dimethyl sulfoxide extracts from grilled beef (Deguchi et al., 2000a,b).

Another study designed to investigate the ability of endothelial cells (EC) to incorporate anthocyanins and to examine their potential benefits against various oxidative stressors proved that the enrichment of EC with

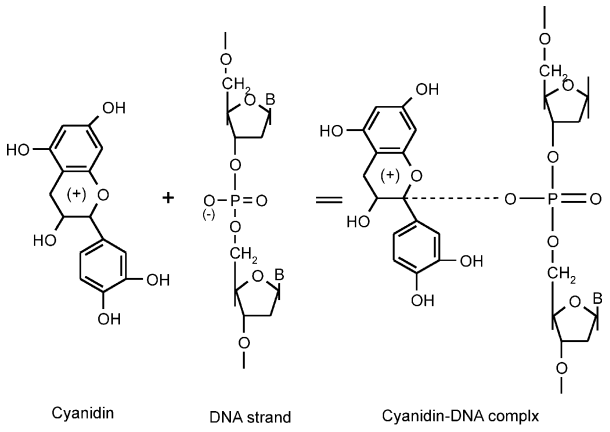

Fig. 2. Proposed mechanism for cyanidin–DNA interation which leads to the formation of cyanidin–DNA copigmentation complex.

elderberry anthocyanins confers significant protective effects in endothelial cells against the following oxidative stressors: hydrogen peroxide, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and iron(II) sulphate/ascorbic acid (AA) (Youdim et al., 2000). The antioxidant activity in fruits and leaves from different cultivars of the thornless blackberry (*Rubus* sp.), red raspberry (*Rubus* idaeus L.), black raspberry (*Rubus occidentalis* L.), and strawberry (*Fragaria*×*ananassa* D.) was reported by Wang and Lin (2000). The results showed a linear correlation between the total phenolic content and ORAC activity for the fruits and leaves. For ripe berries, a linear relationship existed between ORAC values and the anthocyanin content.

As early as 1933, Horwitt (1933) observed that the urine of rabbits fed 500 mg of anthocyanin pigment from grapes became highly pigmented. He concluded that small quantities of the grape anthocyanins or anthocyanidins were absorbed and passed through to the circulation. This conclusion was supported by the HPLC analysis of the plasma and urine samples of rats that consumed *Vaccinium myrtillus* anthocyanins (Morazzoni et al., 1991). In a study to determine whether anthocyanins can be absorbed as glycosides and to evaluate their pharmacokinetics in humans, Cao et al. (2001) found that anthocyanins were absorbed in their unchanged glycated forms in elderly women.

Hagiwara et al. (2001) studied the potential of purple corn color (PCC), a natural anthocyanin, to modify colorectal carcinogenesis in male F344/DuCrj rats, initially treated with 1,2-dimethylhydrazine (DMH). They found that under their experimental conditions, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) clearly exerts promoting effects on DMH-induced colorectal carcinogenesis, and that this can be reduced by adding 5.0% PCC to their diet.

Matsui et al. (2001a,b) examined the alpha-glucosidase (AGH) inhibitory effect of natural anthocyanin extracts. They used a free AGH assay system and found that 12 anthocyanin extracts had a potent AGH inhibitory activity. In particular, *Pharbitis nil* (SOA) extract showed the strongest maltase inhibitory activity, with an IC_{50} value of 0.35 mg/ml, as great as that of *Ipomoea batatas* (YGM) extract ($IC_{50}=0.36$ mg/ml). However, neither extract inhibited the sucrase activity at all. For the immobilized assay system, which may reflect the pharmacokinetics of AGH in the small intestine, SOA and YGM extracts gave more potent maltase inhibitory activities than those of the free AGH assay, with IC_{50} values of 0.17 and 0.26 mg/ml, respectively. Both extracts also inhibited alpha-amylase action, indicating that anthocyanins have a potential function in suppressing the increase of postprandial glucose levels from starch.

They also examined (Matsui et al., 2001a,b) another four kinds of diacylated pelargonidin (Pg: SOA-4 and SOA-6), cyanidin (Cy: YGM-3), and peonidin (Pn:

YGM-6) 3-sophoroside-5-glucosides isolated from the red flowers of the morning glory, *Pharbitis nil* cv. Scarlett O'Hara (SOA), and the storage roots of purple sweet potato, *Ipomoea batatas* cv. Ayamurasaki (YGM). These were subjected to the immobilized AGH (iAGH) system to mimic the membrane-bound AGH in the small intestine. The results showed that the acylated anthocyanins had strong maltase inhibitory activities with IC_{50} values of <200 μM, whereas no sucrase inhibition was observed. Of these, SOA-4 [Pg 3-*O*-(2-*O*-(6-*O*-(*E*-3-*O*-(β-D-glucopyranosyl)caffeyl)-β-D-glucopyranosyl)-6-*O*-*E*-caffeyl-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside] possessed the most potent maltase inhibitory activity ($IC_{50}=60$ μM). As a result of a marked reduction of iAGH inhibitory activity by deacylating the anthocyanins, that is, Pg (or Cy or Pn) sophoroside-5-glucoside, acylation of anthocyanin with caffeic (Caf) or ferulic (Fer) acid was found to be important in the inhibition of iAGH (maltase) expression. In addition, the fact that Pg-based anthocyanins showed the most potent maltase inhibition, with an IC_{50} value of 4.6 mM, and the effect being in the descending order of potency of Pg > Pn/Cy strongly suggested that no replacement at the 3′(5′)-position of the aglycone B-ring may be required for inhibiting iAGH action.

7. Conclusions

Anthocyanins represent a class of important antioxidants, as they are so common in human foods. In recent years, many papers have been published on the in vitro antioxidant activity of anthocyanins and their other functions, as well as studies assessing the correlation between their antioxidant capacity and chemical structure. However, there are still fewer studies compared to studies of other flavonoids. The antioxidant efficacy in vivo of anthocyanins has also been less thoroughly documented, possibly due to the limited knowledge of their pharmacokinetics. Based on these facts, the authors of this review hope that this paper highlights the importance of anthocyanins in order to promote further research in this field.

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