Pol. J. Environ. Stud. Vol. 21, No. 4 (2012), 837-844

Original Research

Antioxidative and Chelating Properties of Anthocyanins in *Azolla imbricata* Induced by Cadmium

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Received: 3 September 2011
Accepted: 8 December 2011

Abstract

To investigate the biological activities of anthocyanins, which are induced by cadmium in *A. imbricata*, the antioxidant properties of anthocyanins were investigated using various antioxidant assays, namely 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, reducing power, and β-carotene bleaching assay. Results showed that anthocyanins exhibited excellent antioxidant activities in all assays and the EC_{50} values of DPPH radicals scavenging, ABTS radicals scavenging, reducing power and β-carotene bleaching assay were 19.08, 10.69, 40.93, and 44.19 $\mu g \cdot mL^{-1}$, respectively. The Cd^{2+} chelation potency of anthocyanins was also investigated *in vitro*. Under given conditions, Cd^{2+} chelating ability of anthocyanins increased significantly with increase in contact time, anthocyanins concentration in dialysis tubing and Cd^{2+} concentration in solution. Based on these results, anthocyanins inducibly synthesized by Cd^{2+} treatment was a powerful antioxidant, as well as Cd^{2+} chelator, might play a role in detoxification of Cd in *A. imbricata*.

Keywords: Cd, anthocyanins, antioxidation, chelation, *A. imbricata*

Introduction

Due to its high toxicity to plants as well as animals, and its high solubility in water, cadmium (Cd) is considered to be one of the most problematic heavy metal pollutants [1]. Cd is a non-redox metal unable to participate in Fenton-type reactions, but it leads to the formation of reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH)[1, 2]. Although ROS may function as important signaling molecules that control and regulate biological processes such as growth, cell cycle, hormone signaling, biotic and abiotic stress responses and development, they can be extremely harmful to organisms at high concentrations [2-7]. ROS can oxidize proteins, membrane lipids, and nucleic acids [1, 5, 6], and execute programmed cell death [4, 7].

To eliminate or reduce Cd toxicity, plants have evolved various protective mechanisms. One of them is the antioxidant system, which is composed of antioxidant materials such as ascorbate, glutathione, α-tocopherol, etc., and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX), etc. [1, 2, 5, 8]. SOD acts as the first line of defense, converting $O_2^{\cdot -}$ to H_2O_2, which is then reduced to H_2O and O_2 either by APX in ascorbate-glutathione cycle or by GPX cycle and CAT in cytoplasm and

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other cellular compartments [2-8]. Another significant mechanism in plants is inducing the biosynthesis of metallothioneins (MTs), low-molecular-weight, gene-encoded, cysteine-rich polypeptides, and the phytochelatins (PCs), which, in contrast, are enzymatically synthesized, cysteine-rich peptides [1, 8, 9]. Their functions are attributed to heavy metal detoxification through the chelation of metal ions and, in some cases, their subsequent vacuolar sequestration, thus preventing them from interfering with cellular metabolism [1, 9, 10].

Anthocyanins are a group of flavonoid compounds that are responsible for the colors (red, blue, or purple) of many flowers, vegetables, and fruits [11]. Anthocyanins have been suggested to play a variety of roles in plant tissues. In flower petals, fruit skins, and seed coats, anthocyanins are essential for plants to recruit pollinators and attract animals to disperse seeds [12, 13]. These compounds may also contribute to protection from photoinhibition [14, 15], scavenging of ROS [16-18], chelation of metallic pollutants [19, 20], and symbiosis of plant – microorganism [21].

Anthocyanin accumulation can be induced by biotic and abiotic stress, such as pathogen attack, UV-B radiation, low temperature, heavy metal contamination, and nutrient stress such as phosphorus (Pi) limitation in many plants [12, 14, 15, 17, 19, 22]. Our previous work showed that Cd^{2+} induced anthocyanins accumulation in *A. imbricata* [23]. However, the physiological significance of anthocyanin accumulation under heavy metal stress remains largely unknown. Among the possible roles of anthocyanins, we suggested that inductive synthesis of anthocyanins could be regarded as a biochemical detoxification strategy, thus strengthening *A. imbricata* against Cd stress. To test this hypothesis, we used an *in vitro* model to investigate the antioxidant activity of anthocyanins using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) radical scavenging, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, reducing power and β-carotene bleaching assay, in comparison with known antioxidants α-tocopherol and butylated hydroxytoluene (BHT). Meanwhile, the Cd^{2+} chelating activity of anthocyanins was also evaluated. The results obtained will help us better understand the detoxification mechanisms of plants against heavy metal toxicity.

Experimental Procedures

Plant Material and Growth Conditions

Azolla imbricata (Roxb.) Nakai were collected from Sanyang everglade, Wenzhou city, China. The growth conditions were as described previously [23]. Briefly, the fronds of *A. imbricata* were placed in containers filled with 2/5-fold Hoagland' nutrient solution, in which nitrates were replaced by chlorides, because *Azolla* spp possesses the ability to utilize atmospheric N_2 because of a symbiosis with the blue-green alga *Anabaena azollae*, which grows in the cavities of *Azolla* leaflets [24]. Modified complete Hoagland's solution comprises (in $mg \cdot L^{-1}$): KCl, 1118.2; KH_2PO_4, 680.0; $CaCl_2 \cdot 2H_2O$, 735.0; $MgSO_4 \cdot 7H_2O$, 492.0; $ZnSO_4 \cdot 7H_2O$, 0.22; H_3BO_3, 2.85; $Na_2MoO_4 \cdot 2H_2O$, 0.12; $CuSO_4 \cdot 5H_2O$, 0.08; $MnCl_2 \cdot 4H_2O$, 3.62; $FeCl_3 \cdot 6H_2O$, 5.4; tartaric acid, 3.0. The fronds were cultured in a growth chamber (25±3ºC, 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 7 days.

Induction of Anthocyanins

The experiment was performed in plastic pots kept inside a growth chamber at 25ºC with a 16/8 h photoperiod under cool white fluorescent light at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. At the start of the experiment, 100 g (fresh mass) healthy and green-colored fronds and 10 L of N-free 2/5-fold Hoagland's nutrient solution were added to a plastic pot. Cd^{2+} treatment concentration was 0.1 $mg \cdot L^{-1}$ (as $CdCl_2$), which strongly induced anthocyanins synthesis but could not be lethal for fronds of *A. imbricata* [23]. The solution was renewed daily to minimize changes in concentrations of Cd^{2+} and nutrients. Samples were collected for analysis on the 5th day.

Extraction and Purification of Anthocyanins

The extraction and purification of anthocyanins were accomplished according to the modified method of Zhang et al. [25]. 100 g above collected fronds were extracted in the dark by stirring with 50 mL of 0.1% HCl (v/v) in methanol for 20 h at room temperature. The samples were filtered through Whatman No. 1 paper and the solid residues were taken back and extracted again in the same conditions. The anthocyanins extracts of the twice-extraction were mixed and concentrated using a rotary evaporator at 40ºC. The concentrated crude extracts were loaded onto an Amberlite XAD-7 resin column (2.0×50 cm) until the resin bed became red due to the absorbed anthocyanins. Anthocyanins were absorbed onto the resin column while sugars, acids, and other water-soluble compounds were removed by washing with ultrapure water. The pigments were eluted by adding methanol containing 0.1% HCl until the resin returned to its original color. The purified methanol fraction was dried with a rotary evaporator at 40ºC under vacuum until the methanol was evaporated.

Antioxidant Capability Assays

The residue was re-dissolved in 0.1% HCl (v/v) methanol solution at a concentration of 1 $mg \cdot mL^{-1}$ and then diluted to prepare the series concentrations for antioxidant assays. Synthetic antioxidant compounds, BHT, natural antioxidants, and α-tocopherol were used for comparison in all assays.

$DPPH^{\cdot}$ scavenging activity was performed following the procedure described by Shimada et al. [26], with minor modifications. Briefly, 0.1 mM solution of $DPPH\cdot$ in ethanol was prepared and 1mL of this solution was added to 3 mL of sample solution in methanol at different concentrations (5, 10, 20, and 40 $\mu g \cdot mL^{-1}$). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a UV-vis

spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Inhibition of DPPH$^{\cdot}$ in percent (I%) was calculated as follows:

$$I\% = [(A_{control}–A_{sample})/A_{control}]\times100$$

EC_{50} value was the effective concentration that could scavenge 50% of the DPPH radicals.

$ABTS^{\cdot+}$ scavenging activity assay followed the method of Re et al. [27] with some modifications. The $ABTS^{\cdot+}$ was generated by the reaction between 7 mM ABTS solution and 2.45 mM potassium persulphate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 16 h. Before use, the absorbance at 734 nm was adjusted to 0.700 (±0.020) by dilution with ethanol. 2.97 mL of the $ABTS^{\cdot+}$ solution was mixed with 30 μL of the samples at varying concentrations (5, 10, 15, and 20 μg·mL^{-1}) and incubated for 6 min. The final absorbance was determined at 734 nm by a UV-vis spectrophotometer. Inhibition of $ABTS^{\cdot+}$ in percent (I%) was calculated as follows:

$$I\%= [(A_{control}–A_{sample})/A_{control}]\times100$$

EC_{50} value was the effective concentration that could scavenge 50% of the $ABTS^{\cdot+}$.

Reducing power assay was determined according to the method of Oyaizu [28]. The different concentrations of sample (25, 50, 100, and 200 μg·mL^{-1}) in 1.0 mL methanol was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50ºC for 20 min. 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 1000×g. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a UV-vis spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power. EC_{50} value was the effective concentration at which the absorbance was 0.5 for reducing power.

β-Carotene bleaching assay was performed as given by Shyu et al. [29] and modified slightly. 2.0 mg of β-Carotene was dissolved in 10 mL chloroform ($CHCl_3$). 1 mL of the chloroform solution was mixed with 20 μL linoleic acid and 200 mg Tween-40. The chloroform was evaporated under vacuum at 45ºC, then 50 mL distilled water was added, and the mixture was vigorously shaken. The β-carotene-linoleic acid emulsion obtained was freshly prepared before each experiment. 30 μL of the samples at varying concentrations (10, 25, 50, and 100 μg·mL^{-1}) was mixed with 250 μL of the emulsion, and then the absorbance was determined at 470 nm at 50ºC for 2 h. β-Carotene bleaching inhibition was estimated as the following equation:

$$\text{Bleaching inhibition (\%)} = (A_{\beta\text{-carotene content after 2 h of assay}}/A_{\text{initial }\beta\text{-carotene content}}) \times100$$

EC_{50} value was the effective concentration that could give 50% antioxidant ability.

Cd^{2+} Shelating Ability Assay

The chelating ability of anthocyanins with Cd^{2+} was examined by dialysis assay, as described by Lavid et al. [30]. In the equilibrium dialysis assay, two chambers are separated by dialysis (as a semi-permeable membrane). A known concentration of receptor is placed in one of the chambers and a known ligand concentration in the other. An appropriate membrane is selected such that the ligand is free to pass from one chamber to the other, but the receptor is retained in one chamber. As the ligand diffuses, some of it will bind to the receptor and some will remain free in the solution. If the affinity for the receptor is high, a large concentration of the ligand will remain bound to the receptor. When equilibrium is reached, the total concentration of ligand will be higher in the receptor chamber. In our experiment, 5 mL of anthocyanins (1~5 mg·mL^{-1}) were placed in dialysis tubing (2-18/32", London, UK). The tube was stirred in 100 mL of Cd^{2+} (25~300 mg·L^{-1}) with a magnetic stirrer at 60 rpm. After 3~48 h, the entire contents in each chamber were collected, digested in an HNO_3:$HClO_4$ (3:1, v/v) mixture at 80ºC, and Cd concentration measured by a Hitachi Z-5000 Polarized Zeeman atomic absorption spectrophotometer.

Statistical Analysis

The data were presented as mean±standard deviation (n=3). Statistical analyses were performed using a one-way analysis of variance (ANOVA), and the significance of the difference between means was determined by Duncan's multiple range test. Differences at $p<0.05$ were considered significant. EC_{50} values were obtained by interpolation from linear regression analysis.

Results

Antioxidant Activities of Anthocyanins and Standards

Fig. 1A showed the DPPH$^{\cdot}$ scavenging activity of anthocyanins and standards. We used BHT and α-Tocopherol as standard radical scavengers. The anthocyanins and standards significantly inhibited the activity of DPPH$^{\cdot}$ in a concentration-dependent manner ($p<0.05$). At 10 μg·mL^{-1}, the scavenging effects were 37.55, 32.67, and 19.91% for anthocyanins, BHT and α-Tocopherol, respectively. At 40 μg·mL^{-1}, the scavenging effects could be increased to 91.70, 77.73, and 58.71% for anthocyanins, BHT, and α-Tocopherol, respectively. The EC_{50} values of scavenging DPPH$^{\cdot}$ for anthocyanins, BHT, and α-Tocopherol were 19.08, 23.43, and 33.19 μg·mL^{-1}, respectively (Table 1). So, Anthocyanins had the highest scavenging activity, followed by BHT and α-Tocopherol.

Fig. 1B illustrated the $ABTS^{\cdot+}$ scavenging ability of 5, 10, 15, and 20 μg·mL^{-1} of anthocyanins and comparison with the same concentrations of BHT and α-Tocopherol.

Table 1. EC_{50} in antioxidant activities of anthocyanins, BHT, and α-Tocopherol.

	EC_{50} (μg·mL^{-1})			
Sample	Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals	Scavenging ability on 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) cation radical	Reducing power	β-Carotene bleaching inhibition
Anthocyanins from *A. imbricata*	19.08±0.75^{a}	10.69±0.38^{b}	40.93±1.95^{a}	44.19±5.83^{a}
Butylated hydroxytoluene	23.43±0.38^{b}	8.89±0.10^{a}	65.47±0.53^{b}	40.55±1.89^{a}
α-Tocopherol	33.19± 2.72^{c}	20.19± 0.28^{c}	90.75± 3.24^{c}	73.22± 6.30^{b}

EC_{50} means the effective concentration at which 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) cation radical were scavenged by 50%, the absorbance was 0.5 for reducing power and β-Carotene bleaching was inhibited by 50%. EC_{50} value was obtained by interpolation from linear regression analysis.
Values (mean±SD, n=3) in the same column followed by a different letter (a, b, c) are significantly different ($p<0.05$).

Anthocyanins, BHT, and α-Tocopherol showed a concentration-dependent scavenging of $ABTS^{\cdot+}$ ($p< 0.05$) and had an EC_{50} value of 8.89, 10.69, and 20.19 μg·mL^{-1}, respectively (Table 1). At the concentrations of 10 and 20 μg·mL^{-1}, the anthocyanins exhibited 43.55% and 86.46% antioxidant activity, respectively, which was higher than those of the positive controls α-Tocopherol (23.40% and 50.89%, respectively), and lower than that of BHT (58.76% and 96.27%, respectively).

Fig. 1C showed the reducing power of anthocyanins compared to α-Tocopherol and BHT as standards. Like DPPH· scavenging activity, the reducing power of anthocyanins and standards were in a concentration-dependent manner ($p<0.05$). The reducing power of anthocyanins, BHT and α-Tocopherol increased from 0.69, 0.56, and 0.31 at 50 μg·mL^{-1}, respectively to 1.61, 1.19, and 0.96 at 200 μg·mL^{-1}, respectively. As shown in Table 1, the EC_{50} values for anthocyanins, BHT, and α-Tocopherol were 40.93, 65.47, and

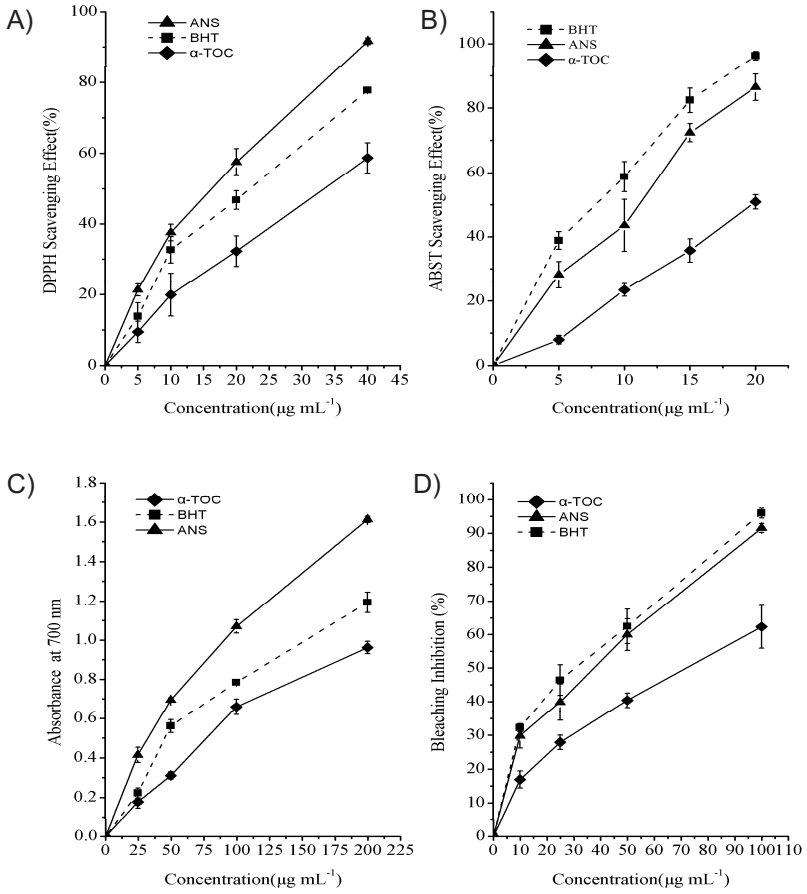

Fig. 1. Antioxidant activities of ANS, BHT and α-Toc: (A) scavenging DPPH radical, (B) scavenging ABTS cation radical, (C) reducing power, and (D) β-carotene bleaching inhibition.
ANS: anthocyanins from *A. imbricata*, α-Toc: α-tocopherol, BHT: butylated hydroxytoluene.

90.75 μg·mL^{-1}, respectively, and the reducing power of samples were in the order of Anthocyanins >BHT >α-Tocopherol.

Fig. 1D showed the antioxidant activities of anthocyanins, as well as the positive controls BHT and α-Tocopherol, as measured by β-carotene bleaching method. The anthocyanins and standards significantly inhibited the β-carotene bleaching in a concentration-dependent manner ($p<0.05$). At concentrations of 25 μg·mL^{-1}, the inhibition was 39.87, 46.42, and 27.93% for anthocyanins, BHT, and α-Tocopherol, respectively. At 100 μg·mL^{-1}, the inhibition was increased to 91.83, 96.02, and 62.34% for anthocyanins, BHT, and α-Tocopherol, respectively. As can be seen from Table 1, the EC_{50} values for anthocyanins (44.19 μg·mL^{-1}) showed higher activities than α-tocopherol (73.22 μg·mL^{-1}), and the difference was statistically significant ($p<0.05$). The EC_{50} values for anthocyanins was lower than BHT (40.55 μg·mL^{-1}), but the difference was statistically insignificant ($p>0.05$). The β-carotene bleaching inhibition of anthocyanins and standard compounds followed the order: BHT≈anthocyanins >α-Tocopherol.

Cd^{2+} Chetation Ability of Anthocyanins

Fig. 2A showed the time-course of the chelation process. Cd^{2+} concentration in dialysis tubing increased significantly with an increase in contact time while Cd^{2+} concentration in solution decreased significantly with increasing time ($p<0.05$). The results also indicated that the chelation process occurred mainly during the first 24 h of interaction between Cd^{2+} and anthocyanins.

A correlation between anthocyanin concentrations and Cd^{2+} chelating to the anthocyanins was shown in Fig 2B. Cd^{2+} chetation exhibited a good correlation with anthocyanin concentrations ($p<0.05$). The concentration of Cd^{2+} in dialysis tubing was 143.79 mg·L^{-1} for 1 mg·mL^{-1} of anthocyanins, while it was greatly increased to 843.04 mg·L^{-1} for 5 mg·mL^{-1} of anthocyanins. Meanwhile, higher anthocyanin concentrations in dialysis tubing resulted in lower Cd^{2+} concentration in solution ($p<0.05$). This was expected because more chelating sites for Cd^{2+} were available at higher concentrations of anthocyanins.

Fig. 2C showed the effect of initial Cd^{2+} concentration on anthocyanins' chelating ability. Increased Cd^{2+} concentration in solution resulted in greater Cd^{2+}-chelating by the anthocyanins when Cd^{2+} concentration < 200 g·L^{-1} ($p<0.05$). However, there was only a slow change in the extent of Cd^{2+} chelation when Cd^{2+} concentration reached 200 g·L^{-1} ($p > 0.05$). This indicated that Cd^{2+} in the solution would interact with the chelating sites and thus facilitated chelatation under lower concentrations. At higher Cd^{2+} concentrations (≥200 g·L^{-1}), more Cd^{2+} were left un-chelated in solution due to the saturation of chelating sites.

Discussion

Cd could enter the fronds of *A. imbricata* very quickly and accumulate to high concentration [23]. In *Azolla* spp, Cd exposure induces various symptoms of phytotoxicity, e.g. a decrease of chlorophyll content [23, 31], loss of essential nutrient [32], inhibition of nitrogenase activity, disorder of nitrogen metabolism, reduced accumulation of nitrogen [33], and inhibition of growth [34]. On the other hand, *Azolla* spp have evolved a suite of detoxification mechanisms to eliminate or reduce Cd toxicity. Under 4 mg·L^{-1} Cd^{2+} treatment, the activities of SOD and CAT in *A. imbricata* increasing up to 46.43% and 63.1%, respectively [31]. The expression of AzMT2 (MTs gene isolated from

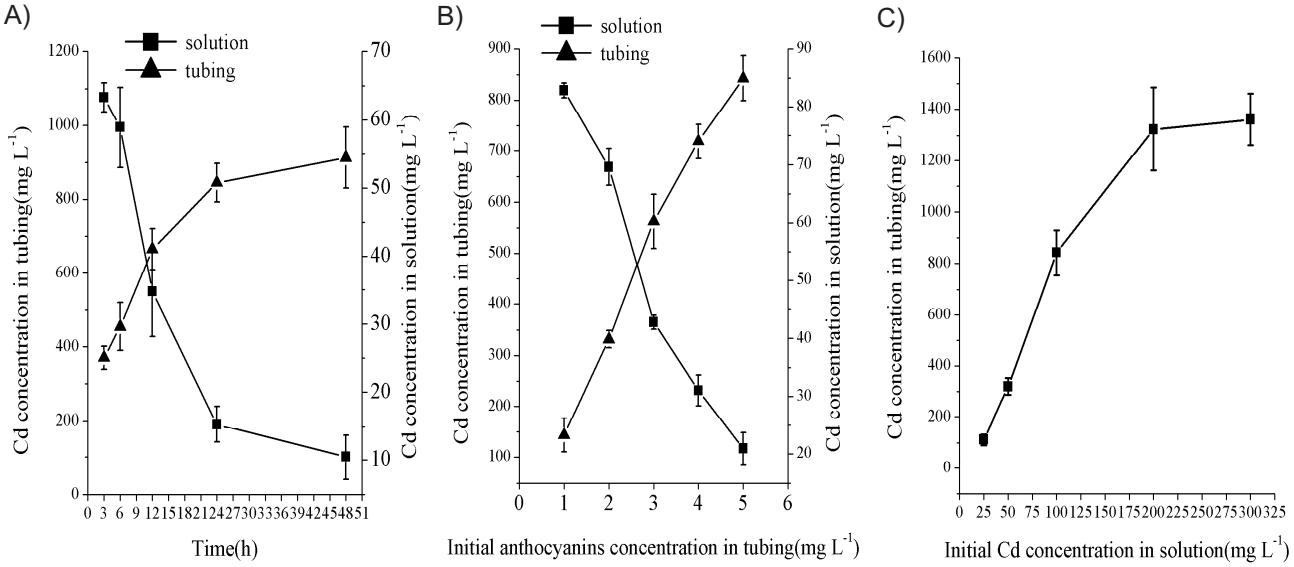

Fig. 2. Chelating ability of anthocyanins: (A) Effect of time on Cd^{2+} chelating by anthocyanins. Initial anthocyanins concentration in dialysis tubing was 5 mg·mL^{-1} and initial Cd^{2+} concentration in solution was 100 mg·L^{-1}, (B) Effect of anthocyanin concentrations on Cd^{2+}-chelating (24 h). Initial Cd^{2+} concentration in solution was 100 mg·L^{-1}, and (C) Effect of Cd^{2+} concentration on chelating to anthocyanins (24 h). Initial anthocyanin concentrations in dialysis tubing was 5 mg·mL^{-1}.
"Tubing" refers to Cd^{2+} that was chelated by anthocyanins and consequently trapped within the tubing. "Solution" refers to Cd^{2+} that was not chelated by anthocyanins and is consequently free in the outer solution.

Azolla filiculoides) increased dramatically in response to Cd^{2+}, suggesting that AzMT2 may play an important role in detoxification of Cd^{2+} by *A. filiculoides* [35].

To provide reliable results, the antioxidant activity of anthocyanins was measured by different methods, as it was known that the determination of this parameter was influenced by many variables, such as the oxidation substrate, the oxidation mechanism, and the reaction medium [36]. Of these, scavenging DPPH radical, scavenging ABTS cation radical, reducing power and β-carotene bleaching inhibition assay are most commonly used for the evaluation of an antioxidant molecule.

Consistent with the hypothesis, the results of this study clearly indicated that anthocyanins had powerful antioxidant capacity against various antioxidant systems and the capacity was concentration-dependent (Fig. 1). Kong et al. [37] reported that anthocyanins had been shown to be highly effective scavengers of most types of oxidizing molecules such as various free radicals. Furthermore, their activities exceeded the well-known antioxidants α-tocopherol in all assays (Fig. 1, Table 1). This observation also agrees with other investigations [38] that have reported that anthocyanins and anthocyanidins have shown higher antioxidant activity than α-tocopherol and vitamin C. Likewise, individual anthocyanin cyanidin and delphinidin had 4-fold higher antioxidant activity than α-tocopherol and vitamin C [39]. When compared to the antioxidative potentials of BHT, anthocyanins were found to have different levels of antioxidant properties in the test models used. Anthocyanins exerted strong antioxidant activity, which was almost equal to BHT in the β-carotene bleaching assay, only slightly lower than the antioxidant power of BHT in the ABTS cation radical scavenging activity assay and clearly surpass that shown by BHT in the DPPH radical scavenging activity and reducing power assay (Fig. 1, Table 1). Although the mechanism for different levels of antioxidant properties in four assays may appear difficult to explain, it should be noted that anthocyanins from litchi pericarp exhibited a higher DPPH radicals scavenging activity and reducing power than BHT [40]. Though evidence for a direct *in vivo* antioxidative function in intact plants is still lacking, the *in vivo* antioxidant activity of anthocyanins was assessed by measuring PSII photochemical efficiency, through *in vivo* chlorophyll fluorescence [41] and using fluorochromes dichlorofluorescein and scopoletin, in conjunction with epifluorescence microscopy [17]. These experimental results also suggested that anthocyanins could act as antioxidants *in planta*, leading to increased tolerance to oxidative stress.

Chemical structures of anthocyanins have been proposed to account for the antioxidant ability of anthocyanins [8]. They are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts [37]. The high antioxidant activities of anthocyanins have been ascribed to oxonium ion in the C ring. The aglycone moiety, the oxidation state of the C ring, the hydroxylation and methylation pattern, as well as the acylation by phenolic acids are considered crucial factors for the expression of antioxidant effects [42]. Du et al. [43] reported that the hydroxyl groups on A and B rings contributed equal antioxidant activities, and the glucose and galactose groups had the same effect on antioxidant activity. In conclusion, these structural factors modulate the ability of anthocyanins to act as free radical scavengers.

However, it is still a matter of debate how anthocyanins actually carry out their antioxidant functions in plant cells, because of their predominant vacuolar distribution in most species [17, 18, 41]. Vacuolar anthocyanins are unlikely to encounter superoxide radicals originating directly from cytoplasmic organelles, since superoxide radicals cannot readily permeate the tonoplast membrane [17, 18]. However, superoxide radicals in cytosol are rapidly protonated to form the hydroperoxyl radical, or else converted by superoxide dismutase to H_2O_2, both of which can freely diffuse from cellular organelles into the vacuole (even in epidermal cells) and be scavenged by anthocyanins [16]. Moreover, anthocyanins have been shown to occur in cytosolic spaces in *Lactuca sativa* and *Pseudowintera colorata* prior to their transport into the vacuole, which could effectively scavenge superoxide radicals [18] and H_2O_2 [17], respectively. To sum up, the antioxidant function of anthocyanins is feasible in both cytoplasmic and vacuolar spaces in living plant cells, supporting the putative direct roles of anthocyanins as primary ROS scavengers. Therefore, we suggest that anthocyanins may stimulate antioxidative defenses, thus strengthening *A. imbricata* against the Cd-induced oxidative stress.

The putative chelating function of anthocyanins could also alleviate Cd-mediated toxicity in *A. imbricata*. Under the conditions of this experiment, Cd^{2+} concentration in dialysis increased significantly with an increase in contact time (Fig. 2A), initial anthocyanin concentrations in dialysis tubing (Fig. 2B) and initial Cd^{2+} concentrations in solution (Fig. 2C). The data obtained in the present study clearly indicated that Cd^{2+} could direct chelation to anthocyanins. A lot of studies have shown that anthocyanins were able to form strong ligand complexes with metal ions, such as Ni, Sn, Mg, Fe, Zn, Cu, and Mo [19, 20, 44, 45]. Kondo et al. [46] also obtained Cd-commelinin, in which the complexation metal Mg^{2+} was replaced with Cd^{2+}. Although the mechanism of their chelating properties is fragmentary, one of the main characteristics of anthocyanins with o-dihydroxyl groups in the B ring is their ability to form metal-anthocyanin complexes [47].

The shoots of *Azolla* plants grown in the presence of cadmium showed high content of cadmium only in the ventral lobe submerged in the growth medium, suggesting that cadmium was absorbed directly from the medium and primarily located in epidermal cells [48], and the intracellular localization of anthocyanins was apparent in the reddish vacuoles of epidermal cells in *Azolla* spp [21]. In other studies, epidermal accumulation was also reported for both metals [49, 50] and anthocyanins [51]. Therefore, it is proposed that Cd^{2+} could be complexed by anthocyanins and sequestered in the vacuole of epidermal cells in *A. imbricata*. Moreover, Chloroplasts are the major ROS generators

under various environmental stresses [52], while epidermal cells have less chloroplast than other tissues. Therefore, sequestration of excess metals in the epidermal cell layers may serve as a means to sequester metals in tissues where the metals do less harm. In addition, Hale et al. [19] suggested that the complexation of Mo-anthocyanin facilitated vacuolar sequestration of Mo, thereby allowing plants to separate Mo from vital biochemical processes in other cell compartments. Glińka et al. [53] also confirmed the anthocyanins-rich extract from red cabbage leaves were responsible for lowering the number of nuclei with Pb deposits in *A. cepa* root meristem. Such results observed in this study (and also reported in the literature) suggest that the synergistic induction of anthocyanins may afford a detoxifying sink for Cd^{2+} when the cell wall and extracellular exudates, the first barrier against Cd stress [1, 8, 10], are surpassed.

Conclusions

Based on the data obtained from this study, anthocyanins might act in concert with other biochemical and physiological mechanisms such as MTs, SOD, and CAT activities, to maintain normal physiological functions under Cd stress, both through their antioxidant activity and metal chelation potential. Further studies are needed to investigate the chemical composition of anthocyanins in order to elucidate the structure-activity relationships of anthocyanins.

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (30800159) and Zhejiang Provincial Natural Science Foundation of China (LY12C03007).

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