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Calf Thymus DNA-Binding Ability Study of Anthocyanins from Purple Sweet Potatoes (*Ipomoea batatas* L.)

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ABSTRACT: A total of 10 anthocyanin compounds were identified from five purple sweet potato (*Ipomoea batatas* L.) varieties, Qunzi, Zishu038, Ji18, Jingshu6, and Ziluolan, by high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS) to assess their calf thymus DNA-binding ability *in vitro*. The interaction between anthocyanins and calf thymus DNA in Tris-HCl buffer solution (pH 6.9) was evaluated by fluorescence spectroscopy. Using ethidium bromide (EB) as a fluorescence probe, fluorescence quenching of the emission peak was seen in the DNA−EB system when anthocyanins were added, indicating that the anthocyanins bound with DNA. The acylated groups influenced the ability of the interaction with DNA. Anthocyanins from purple sweet potato with more acylated groups in sorphorose have a stronger binding ability with DNA.

KEYWORDS: Anthocyanin, sweet potato, calf thymus DNA, fluorescence spectroscopy

INTRODUCTION

Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of flavylium salts, which are a group of naturally occurring phenolic compounds. They have shown antioxidative, antimutagenic, antidiabetic, and anticarcinogenic acitivities *in vitro*.[1] Anthocyanins and some polyphenols, such as catechin, could protect DNA from OH radical-induced strand breaks and base damage.[2,3] One of the possible defense mechanisms against oxidative damage of anthocyanins is by forming complexes with other molecules, which not only protects partner compounds but also avoids oxidative damages of anthocyanins. It was presumed that association between anthocyanin from rice and calf thymus DNA protects both DNA and anthocyanin from the damage caused by the OH• radical; therefore, whether anthocyanins interact with DNA need further confirmation.[2,3] Moreover, anthocyanins with different chemical structures exhibit different extents of physiological functionality.[4−9] However, no research was found for the DNA-binding ability comparison of different anthocyanins to our knowledge.

To accomplish the evaluation of the DNA-binding ability of anthocyanins, five Chinese purple sweet potato (*Ipomoea batatas* L.) varieties were chosen as anthocyanin materials because of their bioactivity and anthocyanin composition. Anthocyanins from purple sweet potato have biological activity, exhibiting antimutagenicity, radical-scavenging activity, α-glucosidase inhibitory activity and anti-inflammatory activity, and cognition deficit amelioration activity in the animal model.[8−14] Moreover, the anthocyanin composition of purple sweet potato was diverse, and their DNA-binding ability was not evaluated previously.[15]

Therefore, the aim of this paper was to study the DNA-binding ability of anthocyanin from purple sweet potatoes. Anthocyanin compositions were identified from five varieties, Qunzi, Zishu038, Ji18, Jingshu6, and Ziluolan, by high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS). Furthermore, the DNA-binding ability of anthocyanins was investigated by fluorescence spectroscopy. The relationship between the structural diversity and the interaction extent *in vitro* was discussed. Results would give useful information for further studying the protecting mechanism of anthocyanins to DNA.

MATERIALS AND METHODS

Chemicals. Ethanol anhydrous, methanol, and acetontrile were purchased from Dima Technologies (Beijing, China). Formic acid (≥98%) was purchased from Sigma-Aldrich (Chemie GmbH, Germany). These solvents/chemicals used were chromatography-grade. Analytical-grade hydrochloric acid and Tris (≥99.8%) were obtained from Beijing Chemistry Co. (Beijing, China). Calf thymus DNA and ethidium bromide (EB) were purchased from Sigma Chemical Co. (Ontario, CA). The Sep-Pak C_{18} solid-phase extraction (SPE) cartridge (12 mL, 2000 mg) was obtained from Waters (Milford, MA). Syringe filter units (0.22 μm) were supplied by Hercules (Beijing, China). Distilled water was used throughout.

Sweet Potato Varieties and Anthocyanin Extraction. Five varieties of purple sweet potatoes, Qunzi, Zishu038, Ji18, Jingshu6, and Ziluolan, were supplied by Beijing Academy of Agriculture and Forestry Sciences. Qunzi, Zishu038, and Ziluolan were grown at Daxing sweet potato breeding farm (Beijing, China). Ji18 was from Jinan experimental field (Shandong, China). Jingshu6 was obtained from Beijing Agricultural College (Beijing, China). Duplicate samples were taken for each of the five varieties, thoroughly washed with tap water, and air-dried at room temperature. The roots were homogenized in a blender. Anthocyanin extraction conditions were as follows: volume ratio of homogenized purple sweet potato and extracted solution, 1:15; temperature, 50 °C; pH, 3; and extraction time, 1 h. All samples were extracted with 60% ethanol. The resultant extraction solution was concentrated with a rotary evaporator (BUCHI R-215, Switzerland) and then filtered by a 0.22 μm syringe filter.

Fractionation by Sep-Pak C_{18} SPE Cartridge. The Sep-Pak C_{18} SPE cartridge was conditioned consecutively with 8 mL of methanol

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and 8 mL of water. An aliquot of 1 mL of resultant solution was loaded on the SPE cartridge. Subsequently, water, ethyl acetate, and methanol were consecutively applied to the activated column. Eluting with water and ethyl acetate removed sugars, organic acids, phenolic acids, and other flavonoids, while methanol wash led to anthocyanin recovery, as previously described.[16] Methanol eluent was collected and concentrated with a rotary evaporator and dried through nitrogen. The dried product was further resolved in appropriate solution and filtered through a 0.22 μm syringe filter for liquid chromatography−mass spectrometry (LC−MS) analysis and DNA experiments.

LC−MS Analysis. The LC−MS analysis of anthocyanins purfied by Sep-Pak C_{18} SPE were performed by an Agilent 1200 series liquid chromatograph containing an autosampler coupled with a 6300 series ion-trap mass spectrometer (Agilent, Santa Clara, CA). An aliquot of 3 μL of the sample was injected onto an analytical-scale Zorbax SB-AO column (particle size, 1.8 μm; 100 × 3.0 mm; Agilent, Santa Clara, CA) maintained at 25 °C. The elution mode was a linear acetonitrile gradient (10−60%, 30 min) containing 0.5% formic acid at a flow rate of 0.3 mL/min.

Diode-array detector (DAD) detection with 280 and 520 nm as detection wavelengths was performed. Anthocyanins and phenolic acids were detected using an ion trap in the positive-ion mode. Used MS parameters were as follows: nebulizing pressure, 30 psi; source temperature, 110 °C; desolvation temperature, 350 °C; desolvation gas flow, 11 L/min nitrogen; scan ranger, m/z 100−1500; smart parameter setting; compound stability, 20%; trap drive level, 100%.

Fluorescence Spectroscopy Analysis. To each 10 mL volumetric flask, 0.025 mL of DNA (1 mg/mL) and 0.2 mL of EB (1 mg/mL) were added. Then, anthocyanins (20 μg/mL) eluted from the Sep-Pak C_{18} cartridge were added to the flask, which reach four different concentrations (10, 20, 30, and 40 μg/mL). The flask without adding anthocyanin was set as the control. Flasks were filled with Tris-HCl buffer (pH 6.9) to 10 mL. These solutions were equilibrated for 10 min at 25 °C in the dark. The fluorescence emission spectra were measured by a F-3010 Hitachi fluorescence spectrophotometer (Hitachi, Japan) in the emissive wavelength range of 510−800 nm with an exciting wavelength at 485 nm.

DNA-Binding Ability Comparison of Anthocyanins. Both 0.5 mL of anthocyanins (0.25 mg/mL) from Qunzi and 0.5 mL of DNA (0.5 mg/mL) in Tris-HCl buffer (pH 6.9) were mixed, followed by standing at room temperature (23−25 °C) for 5 min, while the treatment of 0.5 mL of Tris-HCl buffer substituted DNA as the control. Then, 0.5 mL of buffer solution (pH 1.0) was added to the mixer. The resulting mixture was allowed to stand for 10 min, followed by filtering through a 0.22 μm syringe filter for LC analysis. The decreasing rate of different anthocyanin varieties in the presence of DNA was calculated from the following equation: decreasing rate (%) = $[(A_0 - A_1)/A_0] \times 100\%$, where A_0 is the peak area of the control and A_1 is the peak area of the anthocyanins in the presence of DNA.

RESULTS AND DISCUSSION

Five varieties of purple sweet potato were extracted with 60% ethanol. The Sep-Pak C_{18} SPE cartridge was chosen to separate extraction. Sugars and acids have no affinity for the column, thus, can be washed away by water. The less polar compounds, such as phenolic acids and flavonoids, could be removed from the column with ethyl acetate. Anthocyanins, our target compounds, were subsequently recovered with methanol. A visible red fraction can only be obtained through methanol eluting, confirming the existence of anthocyanin, which will be further purified using HPLC.

The HPLC profile of the anthocyanin fractions with 520 nm as a detection wavelength was shown in Figure 1. All five anthocyanin fractions were complex mixtures consisting of several anthocyanin components, which is in accordance with previous

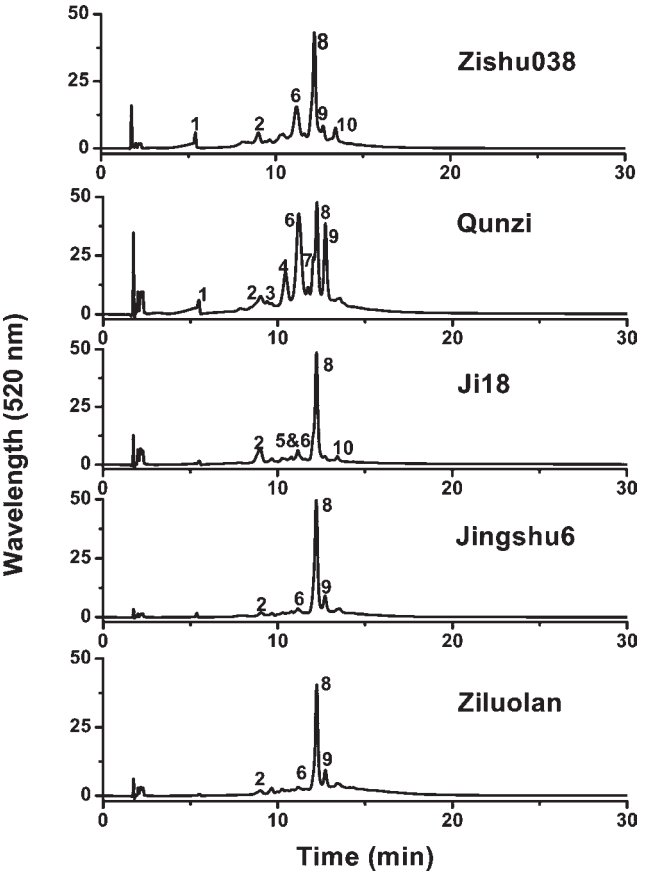

Figure 1. HPLC chromatograms of the anthocyanin fraction upon Sep-Pak C_{18} SPE cartridge cleanup from five purple sweet potato varieties.

reports.[17,18] A total of 10 corresponding peaks of anthocyanins were separated from the extracts of the five varieties analyzed. Specifically, Qunzi contained eight kinds of anthocyanins, which had the highest variety in anthocyanin composition, while Jingshu6 and Ziluolan only contained four varieties. For Zishu038, six compounds with peaks of 1, 2, 6, 8, 9, and 10 were shown in the HPLC profile. Ji18 had five anthocyanin conmounds with peaks of 2, 5, 6, 8, and 10 detected.

Furthermore, MS was used to determine molecular mass and identify the structures of these compounds. First, peak 1 was detected in the positive-ion mode and performed in a wider mass range scan (m/z 100−1500) with lower compound stability (20%). Using the current method, all components can be detected as their protonated adducts.[19] A single prominent protonated molecular ion peak was shown at m/z 787 $[M + H]^+$, indicating that only one compound existed in peak 1 (Figure 2A).

To further identify its structure, peak 1 was analyzed with MS/MS. The result was shown in Figure 2B. It produced three fragment ions at m/z 625, 463, and 301 in the spectrum. The identification was based on the parent ion $[M + H]^+$ and their fragment ions associated with the loss of known moieties attached to an aglycon of purple sweet potato anthocyanins. Peak 1 had the mother ion m/z 787 $[M + H]^+$ and daughter ions at m/z 625, 463, and 301. The daughter ion at m/z 625 was produced through losing one glucose group having a molecular mass as 787 − 625 = 162. m/z 463 occurred by elimination of one molecule of sophorose $[787 - 2 \times 162]^+$, furnishing the peonidin aglycon with m/z 301 (Figure 2B). Peak 1 was

identified as peonidin 3-sophoroside-5-glucoside according to the mass spectra and previous research.[17,20]

Other peaks were identified similar to peak 1. The identified structures of 10 peaks were summarized in Table 1. As reported in previous papers, the anthocyanins from purple sweet potato belonging to either peonidin or cyanidin aglycon were bound with glucose and sophorose, with sophorose and glucose acylated with caffeic, ferulic, and *p*-hydroxybenzoic acids.[17] The relative amounts of anthocyanin compounds calculated by their peak area from five varieties were displayed in Table 2. It was clear that the major anthocyanins identified were penonidin-type, except that Qunzi and J18 contained a small amount of anthocyanins with cyanidin type.

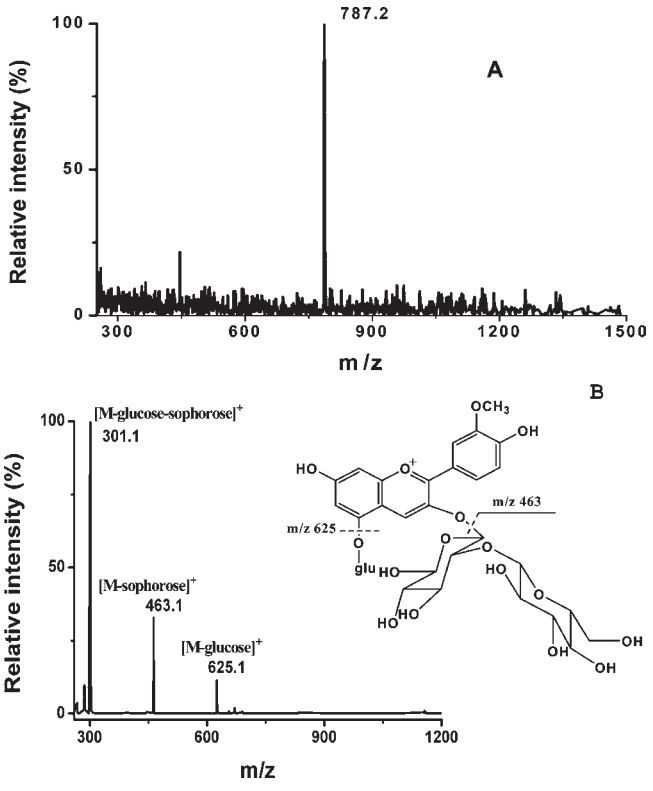

Figure 2. (A) MS spectrum of peonidin 3-sophoroside-5-glucoside. (B) MS/MS spectrum of peonidin 3-sophoroside-5-glucoside.

Whether the DNA molecule can make a complex with anthocyanins from purple sweet potato was examined by fluorescence spectra. EB is a commonly used spectral probe for establishing the binding mode of double-helical DNA to small molecules, which increases the emission peak because of intercalation.[21] If anthocyanin intercalates into the helix of DNA, it would compete with EB, leading to a decrease in the fluorescence intensity.[22]

The emission spectrum of the DNA−EB system in the absence and presence of anthocyanin from Ziluolan at different concentrations was given in Figure 3A. It was observed that there was a significant maximum absorption peak at 595 nm in the DNA−EB complex. Remarkable fluorescence decreases of the DNA−EB complex were observed, when anthocyanin from Ziluolan were added until it reached four different concentrations (10, 20, 30, and 40 μg/mL). A dose−response phenomenon was shown with an increasing anthocyanin concentration. Consequently, anthocyanin substituted EB and bound with DNA. Anthocyanins from the other four varieties also caused the fluorescence value to decrease (data not shown). However, the polyphenols from the ethyl acetate fraction by the Sep-Pak C_{18} SPE cartridge caused the lower fluorescence value to decrease, indicating that polyphenols exhibited a lower DNA-binding ability compared to anthocyanins (data not shown). Furthermore, although EB, an intercalating agent for DNA, is a potent mutagen, anthocyanins were reported to be involved in antimutagenic activity.[23,24]

In general, molecules have three distinct modes of interaction with DNA: intercalation into the base pairs, in the grooves, and outside the helix by electrostatic interactions through π-stacking, attractive van der Waals, hydrogen-bonding, and hydrophobic interactions.[25] Because anthocyanins mainly exist as flavylium cations in aqueous solutions at lower pH, the formation mechanism of the anthocyanin−DNA complex is presumed that anthocyanins bind to DNA outside the helix because of electrostatic affinity with negatively charged DNA first. Then, anthocyanin, as quercetin and its derivative dihydroquercetin, may penetrate the DNA helix and arrange its planar structure more or less parallel to the adjacent planes of the nitrogenous bases.[26]

The DNA-binding ability depends upon the characterization of molecules (stereostructures, electric charge of molecules, etc.);[25] therefore, dissimilar compounds should have different DNA-binding ability. Because the type and relative amount of anthocyanins from different varieties of sweet potato were different, the

Table 1. Mass Spectrometric Data and Identification of Anthocyanin Compounds in Five Purple Sweet Potato Varieties

	m/z			
peak label[a]	MH^+	aglycon	other fragment ions	compound identity
1	787	301	463, 625	peonidin 3-sophoroside-5-glucoside
2	907	301	463, 745	*p*-hydroxybenzoylated (peonidin 3-sophoroside-5-glucoside)
3	949	287	449, 787	feruloylated (cyanidin 3-sophoroside-5-glucoside)
4	963	301	463, 671, 801	feruloylated (peonidin 3-sophoroside-5-glucoside)
5	1055	287	449, 893	cyanidin 3-(6,6′-caffeoyl-*p*-hydroxybenzoylsophoroside)-5-glucoside
6	949	301	463, 787	peonidin 3-(6-caffeoylsophoroside)-5-glucoside
7	1111	287	449, 787, 841, 949	cyanidin 3-(6,6′-caffeoylferuloylsophoroside)-5-glucoside
8	1069	301	463, 907	peonidin 3-(6,6′-caffeoyl-*p*-hydroxybenzoylsophoroside)-5-glucoside
9	1125	301	463, 825, 963	peonidin 3-(6,6′-caffeoylferuloylsophoroside)-5-glucoside
10	1083	301	463, 921	peonidin 3-(feruloylated-*p*-hydroxybenzoylsophoroside)-5-glucoside

[a] Refer to Figure 1.

Table 2. Relative Amounts of Anthocyanin Compounds in Five Purple Sweet Potato Varieties

	percentage of anthocyanin calculated from the peak area (%)[a]				
peak label[b]	Zishu038	Qunzi	Ji18	Jingshu6	Ziluolan
1	6.46 ± 0.13 a	1.29 ± 0.14 b	–	–	–
2	6.73 ± 0.39 b	5.83 ± 0.46 b	15.08 ± 1.00 a	2.95 ± 0.14 c	3.64 ± 0.20 c
3	–	5.75	–	–	–
4	–	11.54 ± 0.19 a	–	1.72 ± 0.09 c	3.71 ± 0.12 b
5	–	–	7.12	–	–
6	25.32 ± 1.07 a	36.91 ± 1.57 b	–	6.76 ± 0.08 c	4.36 ± 0.13 d
7	–	3.49	–	–	–
8	47.21 ± 1.75 b	21.4 ± 0.97 c	74.55 ± 1.60 a	75.46 ± 1.16 a	73.43 ± 1.39 a
9	6.75 ± 0.11 c	17.28 ± 1.20 a	–	13.11 ± 1.22 b	14.86 ± 1.00 b
10	7.53 ± 0.12 a	–	3.25 ± 0.13 b	–	–

[a] The values within each row followed by the same letter are not significantly different ($p < 0.05$) by Duncan's multiple range test. – means no anthocyanin detected in this variety. [b] Refer to Figure 1. Each value is the mean of three replications ± standard deviation.

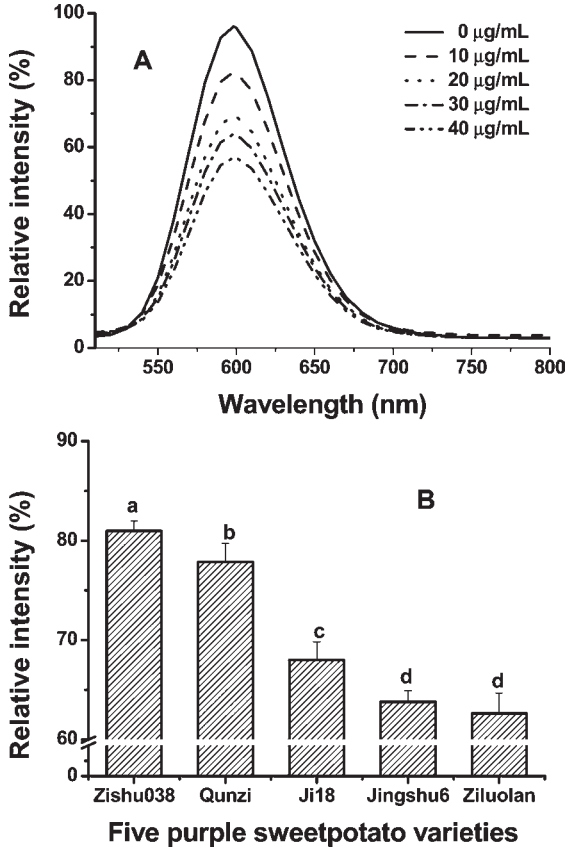

Figure 3. (A) Fluorescence spectra of the DNA–EB system in the presence of anthocyanins from Ziluolan at different concentrations. (B) Fluorescence data of the DNA–EB system in the presence of anthocyanins from five purple sweet potato varieties at the same concentration (30 μg/mL).

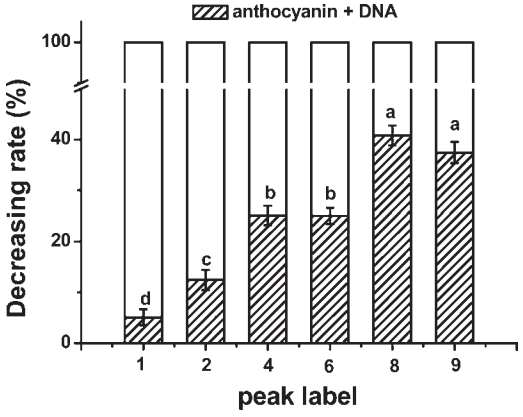

Figure 4. Decreasing rate of anthocyanin from Qunzi calculated from the peak area in the presence of DNA. The peak label refers to Figure 1. Bars in the figure represent standard deviations of four replications. Different letters that represent the values of the decreasing rate are significantly different ($p < 0.05$) by Duncan's multiple range test.

comparison of their DNA-binding abilities was studied. Fluorescence data of the DNA–EB system in the presence of anthocyanin from five varieties at the same concentration (30 μg/mL) were shown in Figure 3B. Ziluolan and Jingshu6 had similar ability in quenching the emission intensity of the DNA–EB complex with higher reduction than the other three varieties, indicating they had the stronger binding ability with calf thymus DNA. Ji18 and Qunzi exhibited less strong binding ability, whereas Zishu038 possessed the lowest activity.

Qunzi was chosen to further evaluate the effect of anthocyanin structures on the DNA-binding ability because of its highest variety in anthocyanin composition. A red deposit occurred after adding buffer solution (pH 1.0) for 10 min, which was the anthocyanin–DNA complex. Anthocyanins that interacted with DNA were taken off through filtering the deposit; therefore, a higher decreasing rate indicated that this kind of anthocyanin exhibited a stronger binding ability with DNA. The comparison of anthocyanins of penonidin type from Qunzi was mainly discussed in this paper. The decreasing rate of anthocyanins with penonidin type from Qunzi in the presence of DNA was shown in Figure 4. These anthocyanins were different from their acylated moiety, which influence their interaction ability with DNA. They were differentiated into two types, acylated in the glucose moiety (peaks 2 and 4) or acylated in the sophorose moiety (peaks 6, 8, and 9), while peak 1 had not acylated group. Peak 4 exhibited a higher decreasing rate than peak 2, indicating that acylated with the feruloyl group had the stronger ability of

interaction with DNA than the *p*-hydroxybenzoyl group. The decreasing rate of peak 8 was similar to peak 9 while stronger than peak 6. It is indicated that the strongest binding ability with DNA was anthocyanins acylated with the caffeoyl-*p*-hydroxybenzoyl group and the caffeoyl-feruloyl group, and acylated with the caffeoyl group exhibited a weaker ability than them, while peak 1 without the acylated group had the weakest interaction ability. There were two acylated groups in peaks 8 and 9, only one acylated group in peak 6, and zero acylated groups in peak 1. It concluded that anthocyanins with more acylated groups in sorphorose have a stronger binding ability with DNA. These results correspond to the fact that more acylation in anthocyanin was responsible for its enhanced stability, which increase the added value of acylated anthocyanins.[27] How the acylated groups in anthocyanin affect the interaction extent with DNA and whether these DNA−anthocyanin complexes exhibit different abilities of protecting both DNA and anthocyanin required further studies in the future.

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