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Structure-antioxidant capacity relationship of dihydrochalcone compounds in *Malus*

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ABSTRACT

The antioxidant capacity (AC) of six dihydrochalcone compounds was evaluated using DPPH and ABTS assays. In water-based solution 3-hydroxyphlorizin exhibited the highest AC among all dihydrochalcones. In acetone and acidic solutions (pH = 2.5 or 2.0), presence of an *o*-dihydroxyl at the B-ring increased AC, whereas glycosylation at the A-ring decreased AC of dihydrochalcones. By comparing the AC of dihydrochalcones with similar structures, it was found that the *o*-dihydroxyl at the B-ring and 2′-hydroxyl group at the A-ring were critical for maintaining the AC of dihydrochalcones by promoting hydrogen atom transfer or single electron transfer mechanism. Sequential proton-loss electron transfer commonly occurred during free radical scavenging in water-based solution. Moreover, we report a unique phenomenon in which glycosylation at the 2′-position enhanced the dissociation ability of the 4′-hydroxyl group and increased the AC of dihydrochalcones containing *o*-dihydroxyl. We speculate that this increase in AC might occur through intramolecular electron transfer.

1. Introduction

Free radicals are very reactive molecules that cause damage to the human body, as well as many chronic health problems (Forman, Davies, & Ursini, 2014; Pisoschi & Pop, 2015). Free radical scavenging by antioxidants is an important line of defense against free radical damage (Niki, 2014; Shahidi & Zhong, 2015). Flavonoids are widely found in fruits and vegetables and are considered excellent antioxidants (Bordenave, Hamaker, & Ferruzzi, 2014; Gomes de Moura & Ribeiro, 2017). The antioxidant capacity (AC) is used to evaluate the antioxidant potency of flavonoids and is defined as the amount of free radicals scavenged by antioxidant compounds (Ghiselli, Serafini, Natella, & Scaccini, 2000; Prior, Wu, & Schaich, 2005). Therefore the AC reflects the chemical equilibrium of reaction between antioxidants and free radicals. The AC of flavonoids is dependent upon the presence of hydroxyl groups at specific positions on the flavonoid skeleton (Amic et al., 2014; Mazzone, Galano, Alvarez-Idaboy, & Russo, 2016). Various action mechanisms are involved in the process of quenching free radicals by flavonoids (Galano et al., 2016), and three of them are considered as the primary antioxidant actions (Amic et al., 2014, 2017; Mazzone, Malaj, Galano, Russo & Toscano, 2015). One of these is hydrogen atom transfer (HAT), which is a one-step reaction governed by the O−H bond dissociation enthalpy (BDE). The other two pathways are single electron transfer followed by proton transfer (SET-PT) and sequential proton-loss electron transfer (SPLET). SET-PT and SPLET are two-step reaction processes, with the first step being governed by the ionization potential (IP) and proton affinity (PA), respectively (Scheme 1; Stepanić, Trošelj, Lučić, Marković & Amić, 2013; Vagánek, Rimarčík, Dropková, Lengyel & Klein, 2014). Moreover, HAT is preferred in non-polar solvents. For SET-PT pathway it is more convenient to occur in polar aqueous than non-polar solvents (Stepanić et al., 2013), while SPLET occurs in ionizing solvents (Litwinienko & Ingold, 2003; Amić et al., 2017). These free radical scavenging mechanisms can occur simultaneously, with the radical adduct formation being also possible (Shadnia & Wright, 2008). The total AC may include various mechanisms (Klein, Rimarčík, Senajová, Vagánek & Lengyel, 2016). Reaction conditions, free radical type, and flavonoid chemical structure determine which mechanisms predominant.

Dihydrochalcones (DHCs) are an important subgroup of flavonoids in apple fruit (Tsao, Yang, Young & Zhu, 2003; Lin, Hsu, Chen, Chern & Lee, 2007; Chen, Zhang, Wang, Li & Ma, 2012) and have good antioxidant potencies (Xiao et al., 2017). DHCs have a basic C_6-C_3-C_6 skeleton chemical structure. The A-ring and B-ring are not conjugated together like most flavonoids, but are instead linked with a flexible C_3 chain. Natural DHCs often have phenolic hydroxyl (−OH) groups, with some −OH groups at specific locations substituted with glycosides. The effect of chemical structure, −OH group position, and glycosylation on antioxidant potency of DHCs is still poorly understood.

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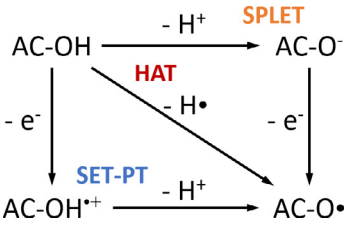

Scheme 1. Mechanisms of antioxidant action.

In this study, the ACs of two DHC aglycones and four glycosylated derivatives were measured under various conditions using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The antioxidant mechanisms of DHC molecules and the effect of glycosylation were determined. In addition, a unique phenomenon that occurred during free radical scavenging by DHCs was observed.

2. Materials and methods

2.1. Chemicals and reagents

Phlorizin (phloretin-2′-*O*-glucoside, P2G), trilobatin (phloretin-4′-*O*-glucoside, P4G), 3-hydroxyphlorizin (3-hydroxyphloretin-2′-*O*-glucoside, HP2G), and sieboldin (3-hydroxyphloretin-4′-*O*-glucoside, HP4G) were extracted and purified from crabapple fruits (*Malus* 'Red Splendor'). Phloretin (P) and 3-hydroxyphloretin (HP) were obtained by the hydrolysis of phlorizin and sieboldin, respectively, as described by Xiao, et al. (2017). Potassium peroxodisulfate ($K_2S_2O_8$), phenol, *p*-cresol, 4-ethylphenol, pyrocatechol, 4-methylcatechol, 4-ethylcatechol, phloroglucinol, phloroacetophenone, phloropropiophenone, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), deuterium oxide (D_2O), sodium deuteroxide (NaOD, 40 wt% solution in D_2O), hydrochloric acid, and potassium hydroxide were purchased from J&K Scientific (Beijing, China). Ultra-pure water was prepared using a Millipore Milli-Q system (Darmstadt, Germany). All water used was ultrapure, unless otherwise noted. Methanol and acetone were purchased from Guanghua Sci-Tech Co., Ltd. (Guangdong, China). All solvents were degassed with dry nitrogen to remove dissolved O_2 and CO_2 before use.

2.2. Antioxidant capacity evaluation

The DPPH assay was performed according to the method of Sousa, et al., (2016) with some modifications. It was prepared at 87 μM in solution with 40% methanol-water solution, acetone, or 40% methanol-phosphate buffer (50 mM) with a pH of 2.5, 4.0, 6.0, or 8.0 respectively and used for AC measurement. The pH was monitored using a pH meter (Mettler Toledo, Columbus, Ohio, USA). After adding 20 μL of 5 μM antioxidant compound to 180 μL of DPPH solution, absorbance was measured at 517 nm for acetone mixtures and at 529 nm for methanol-water solutions using an Infinite® 200 Pro (Tecan, Männedorf, Switzerland).

The ABTS assay was performed according to a published method (Re, et al., 1999) with some modifications. Briefly, 7 mM ABTS solution and 2.5 mM potassium peroxodisulfate solution were mixed to produce an ABTS radical cation ($ABTS^{\cdot+}$). This reaction mixture was kept in the dark for 14 h at room temperature before use. The $ABTS^{\cdot+}$ solution was diluted with water, acetone, or phosphate buffer (50 mM) with different pH values. The final absorbance of the $ABTS^{\cdot+}$ solution was 0.90 ± 0.05 at 734 nm, and it was used immediately for AC measurement. After the addition of 100 μL of 5 μM antioxidant compound to 0.9 mL of diluted $ABTS^{\cdot+}$ solution, the mixture was placed in the dark for 1 h. The absorbance was then measured at 723 nm for acetone mixtures and at 734 nm for all other mixtures using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan).

A preliminary study determined that the two free radicals were in excess when reacted with 5 μM of antioxidant compound, which guaranteed the full oxidation of DHC compounds by free radicals in the reaction volume.

2.3. Calculation of DHC dissociation constant

The pK_a values for all DHCs in water were determined as described by Ramešová, et al. (2012) with some modifications. Briefly, 50 μM of each DHC compound solution was acidulated to pH 3.0 with hydrochloric acid, then titrated with 0.1 M potassium hydroxide. The titration process was monitored with a precision pH meter at 25 ± 0.5 °C, with the titration flask being purged by nitrogen. The absorbance at 280 nm was recorded from pH 3.0 to pH 10.0. The pK_a was calculated from the pH and measured absorbance values by applying Eq. (1).

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{(A - A_{min})}{(A_{max} - A)} \quad (1)$$

The A_{max} and A_{min} are the maximum absorbance values measured at the maximum and minimum pH values of the curve, respectively. Plots of $\log[(A - A_{min})/(A_{max} - A)]$ against pH are linear with the intercept equal to pK_a.

The species distribution diagram for DHCs was calculated using Eq. (2). In this equation, $c(DHC^-)$ is the concentration of DHC-anions, K_a is the DHC dissociation constant, $c(H^+)$ is the concentration of hydrogen ions in solution, and$c(DHC)_0$ is the initial concentration of DHC (50 μM).

$$c(DHC^-) = \frac{K_a\, c(DHC)_0}{c(H^+) + K_a} \quad (2)$$

2.4. Nuclear magnetic resonance (NMR) spectroscopy

NMR analysis was performed using a Bruker-500 (Bruker Corporation, Germany) at 500 MHz for ^{1}H NMR spectra. The reference compound tetramethylsilane (TMS) was used as the internal standard, and all samples were dissolved in dimethyl sulfoxide-d_6 (DMSO-d_6).

2.5. Statistical analysis

All data are presented as means ± SE (n = 5). Significant differences were detected by *t*-tests using SPSS 16.0 software (IBM, New York, USA) with $P < 0.05$.

3. Results and discussion

3.1. Antioxidant capacity of DHCs

Six DHC compounds were used in this study, phloretin (P), phlorizin (P2G), trilobatin (P4G), 3-hydroxyphloretin (HP), 3-hydroxyphlorizin (HP2G), and sieboldin (HP4G) (Fig. 1). The AC of all DHCs was evaluated using the DPPH assay in methanol-water solution and ABTS assay in water (Fig. 2A and C). DPPH analysis of the aglycones HP and P revealed that the AC of HP, which has an *o*-dihydroxyl group at the B-ring, was higher than that of P, which has only one −OH group at the B-ring. However, the AC of HP was lower than that of P as determined by ABTS analysis. Glycosylation at the 2′-position significantly reduced the AC of P, but increased that of HP in both assays. Glycosylation at the 4′-position decreased the AC of both P and HP. Generally, the AC of HP2G was highest among all six DHCs in both assays. The AC of P2G was the lowest measured by DPPH assay, whereas the AC of HP4G was the lowest measured by ABTS assay.

It is generally accepted that the *o*-dihydroxyl group chemical structure is conducive to the antioxidant potency of flavonoid (Leopoldini, Russo & Toscano, 2011). Interestingly, the ACs of HP and