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Transformation of Proanthocyanidin A2 to Its Isomers under Different Physiological pH Conditions and Common Cell Culture Medium

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ABSTRACT: Proanthocyanidins constitute an important class of polyphenols ubiquitously found in plants. They have been extensively studied for their antioxidant capacity and bioactivity in vitro and in animal models. However, their stability under different pH conditions and in cell culture medium has not been well documented. In the present study, it was observed that proanthocyanidin A2 (PA2) was relatively more stable in acidic condition than in weak alkaline condition. PA2 was also quite unstable in basal-Dulbecco's Modified Eagle medium (b-DMEM medium) at 37 °C. The addition of PA2 to the cell culture medium accelerated its epimerization with a half-life of <15 min, and ethylenediaminetetraacetic acid (EDTA) could not stop the reaction. The results also demonstrated that the major isomers transformed in the weak alkaline condition or cell culture medium at 37 °C were identified as epicatechin-(4β→8; 2β→O→7)-*ent*-catechin (proanthocyanidin A4) and epicatechin-(4β→6; 2β→O→7)-*ent*-catechin. The rates of transformation were dependent on the pH or the components of the medium. Therefore, the results obtained for PA2 in the cell culture bioassays, which were usually carried out for 24 h, might not represent the true activity of the original PA2. The stability and transformation of PA2 should be considered when the bioactivity of PA2 is evaluated in a given cell culture system.

KEYWORDS: b-DMEM medium, isomers, proanthocyanidin A2, physiological pH conditions

INTRODUCTION

Proanthocyanidins (PAs) are an important class of polyphenols ubiquitously found in plants. PAs consist of more than one monomeric flavan-3-ol units linked mainly through C4→C8 or sometimes C4→C6 bonds. These linkages are called B-type linkages. When an additional ether linkage is formed between C2 and C7 (mainly), the compounds are called A-type PAs. Chemical and enzymatic conversions of proanthocyanidin B-type to A-type have been reported.[1,2]

In our previous studies, proanthocyanidin A2 (PA2) isolated from longan flower was found to possess strong in vitro antioxidative and anti-inflammatory activities.[3,4] We also found that the water extract of longan flower is able to improve the symptoms of metabolic syndrome in fructose-fed rats.[5] However, knowledge of the stability of PA2 in cell culture system as well as in the digestive system is lacking. It is known that certain bioactive compounds such as EGCG have poor stability in cell culture media.[6] To fully understand the biological effects and bioavailability of PA2, it is important to study its stability under common cell culture media. The purpose of this study was to examine the stability of PA2 under different physiological pH conditions and common cell culture medium.

MATERIALS AND METHODS

Chemicals and Cell Culture Medium. PA2 was purified in our laboratory from the acetone extract of male longan flowers, which were supplied by the Farmer's Association of Nanhua Township, Tainan, Taiwan. Basal-Dulbecco's Modified Eagle medium (b-DMEM) was purchased from Gibco (Grand Island, NY). Flavone (internal standard of HPLC analysis), superoxide dismutase (SOD), and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich (St. Louis, MO). Thin-layer chromatography (TLC) plates (silica gel 60 on aluminum sheet) were purchased from Merck (Darmstadt, Germany). Visualizing agent for TLC plate (anisaldehyde−sulfuric acid reagent) was prepared according to Scott's method.[7] High-performance liquid chromatography (HPLC) grade solvents and other reagent grade chemicals were purchased from Sigma-Aldrich. HPLC grade water (18 MΩ) was prepared using a Millipore Milli-Q purification system (Bedford, MA).

Preparation of PA2. Dried longan flowers were extracted with 80% acetone in water at room temperature for 3 days. The ratio of flower powders to solvent was 1:5 (w/v). The resulting slurries were filtered through a Whatman no. 1 filter paper. This extraction procedure was repeated twice for the residue, and the filtrates were combined. All of the filtrates were collected and concentrated under vacuum at 40 °C and freeze-dried (Freezone 18, Labconco Ltd., Kansas City, MO). The freeze-dried 80% acetone extract thus obtained was used directly for further partition and purification. The 80% acetone extract (1 g) was redissolved in 20 mL of water followed by sequential partition with *n*-hexane and ethyl acetate (20 mL each). The three fractions (water, ethyl acetate, and *n*-hexane) were evaporated to dryness under vacuum. The ethyl acetate (EA) fraction was then subjected to Sephadex LH-20 column chromatography with ethanol as eluting solvent. The PA2-rich subfractions were detected by TLC after elution in solvent system (ethyl acetate/toluene/formic acid = 6:3:1), collected, combined, and applied to MCI gel (75−150 μm, Mitsubishi Chemical Corp., Tokyo, Japan) column chromatography. The MCI gel column was eluted by a gradient of water/methanol from 0 to 100%. The PA2-rich subfractions were recrystallized under a water and methanol system to obtain the pure PA2. The structure of PA2 was confirmed by ^{1}H, ^{13}C, and two-dimensional (2D) nuclear magnetic resonance (NMR) as previously reported.[3]

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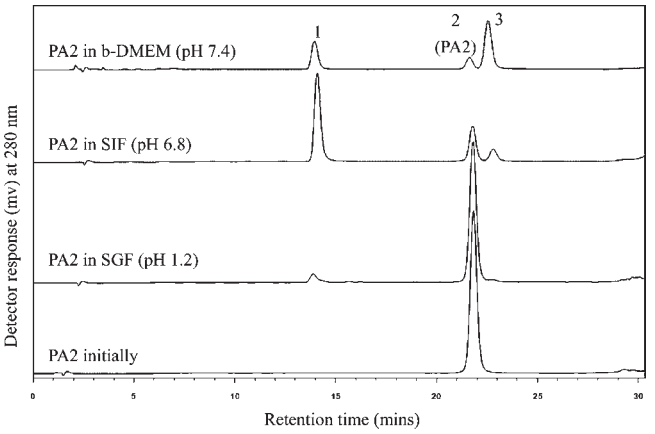

Figure 1. HPLC chromatograms of proanthocyanidin A2 (PA2) in simulated gastric fluid (SGF), simulated intestinal fluid (SIF), or b-DMEM. Peaks 1 and 3 are isomers of PA2; peak 2 is PA2.

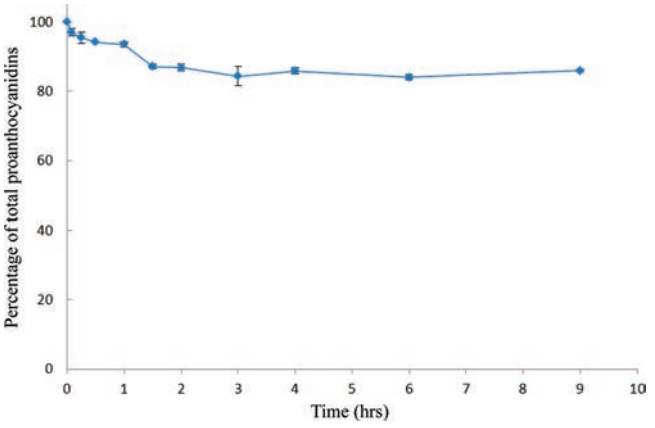

Figure 2. Time-dependent changes of PA2 in simulated gastric fluid (SGF). All data are expressed as the mean ± SD (n = 3).

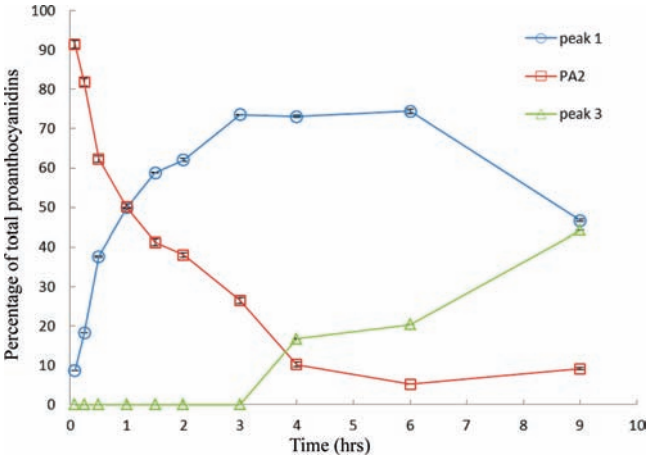

Figure 3. Time-dependent changes of PA2 in simulated intestinal fluid (SIF). All data are expressed as the mean ± SD (n = 3).

Stability of PA2 in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF). The SGF was prepared by adjusting 0.2% aqueous sodium chloride to pH 1.2 with concentrated hydrochloric acid. The SIF was 0.05 M sodium phosphate buffer solution (pH 6.8). PA2 (30 mg) was added to 100 mL of SGF or SIF and incubated at 37 °C for 9 h. At different time points, samples (1 mL) were taken and flavone was added as an internal standard. Samples were extracted twice with equal volumes of ethyl acetate. The EA extracts were evaporated to dryness with nitrogen gas. The residues were redissolved in methanol. These samples were immediately analyzed or stored at −80 °C before HPLC analysis. The quantifications of PA2 and its isomers were based on the standard curve of each compound. The results were shown as the percentage of total proanthocyanidins.

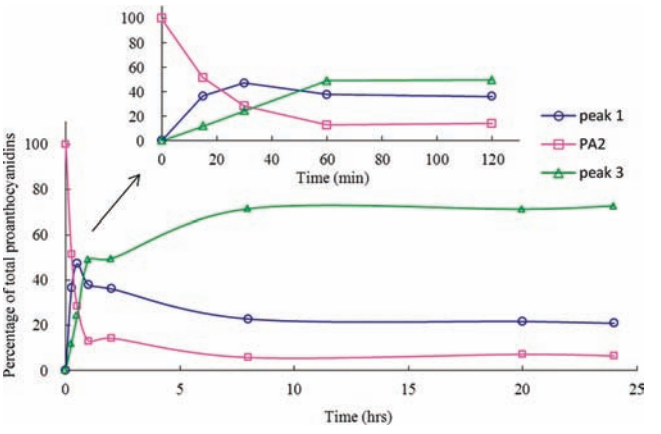

Figure 4. Time-dependent changes of PA2 to its isomers in b-DMEM at different incubation time points.

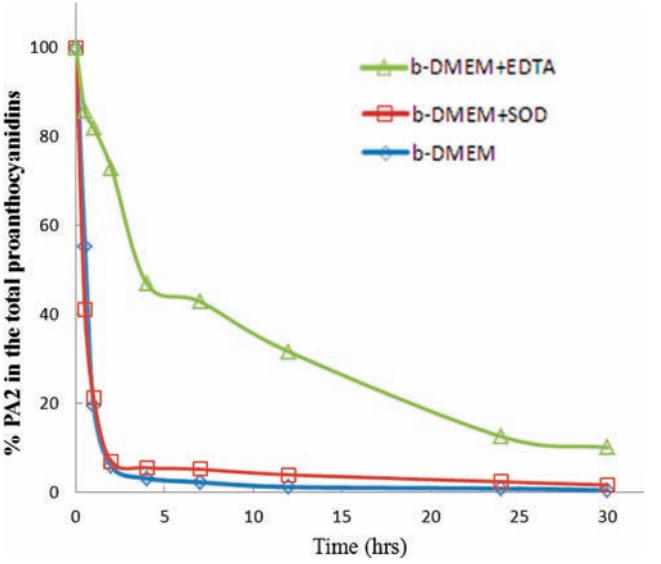

Figure 5. Effect of superoxide dismutase and ethylenediaminetetraacetic acid on the stability of PA2 in b-DMEM.

Stability of PA2 in b-DMEM. PA2 (50 μg/mL) was dissolved and incubated in b-DMEM, containing 4500 mg/L glucose, 3700 mg/L sodium bicarbonate, amino acids, vitamins, and other inorganic salts, kept at pH 7.4 and 37 °C for 24 h, and sampled at different time points. The samples (0.5 mL) were pretreated and analyzed as described above.

Effect of SOD and EDTA on the Stability of PA2 in b-DMEM. The stability of PA2 was assessed in b-DMEM in the absence or presence of SOD or EDTA. PA2 (50 μg/mL) was dissolved and incubated in b-DMEM with SOD (10 U/mL) or EDTA (14 mM) at

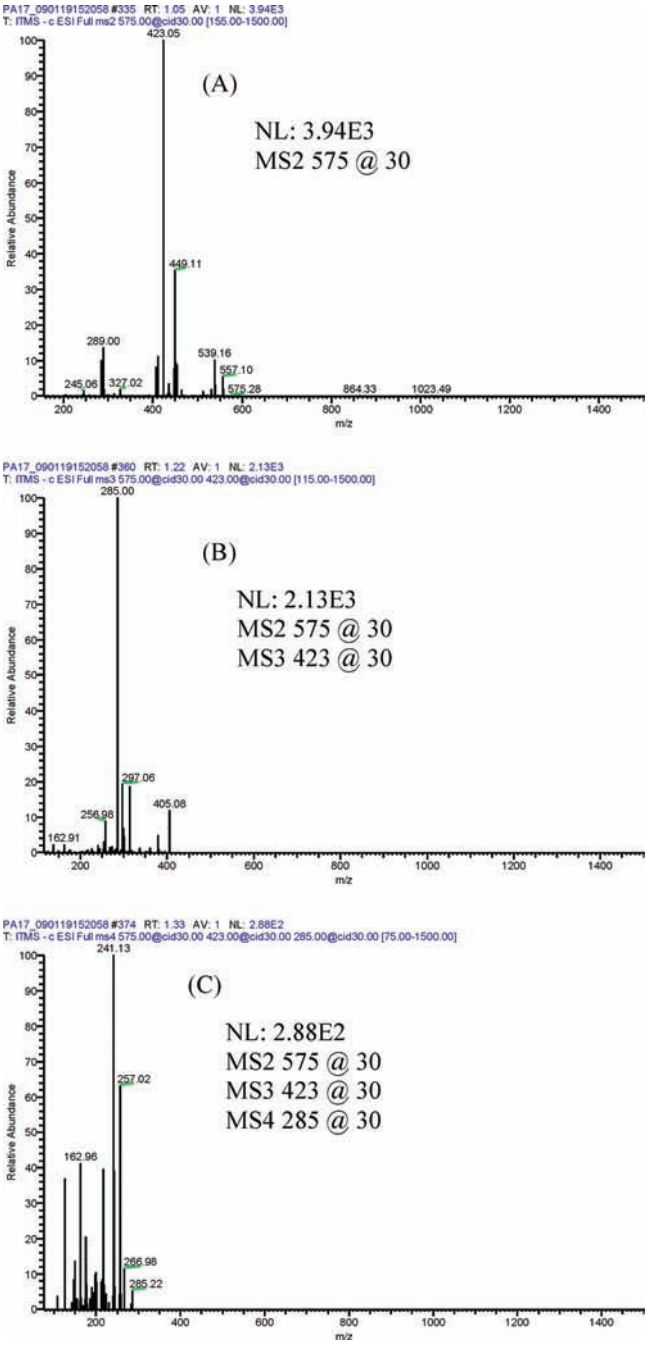

Figure 6. Negative ion ESI tandem mass spectra of peak 1: (A) MS^2 of $[M - H]^-$ (m/z 575); (B) MS^3 of m/z 423 ion; (C) MS^4 of m/z 285 ion.

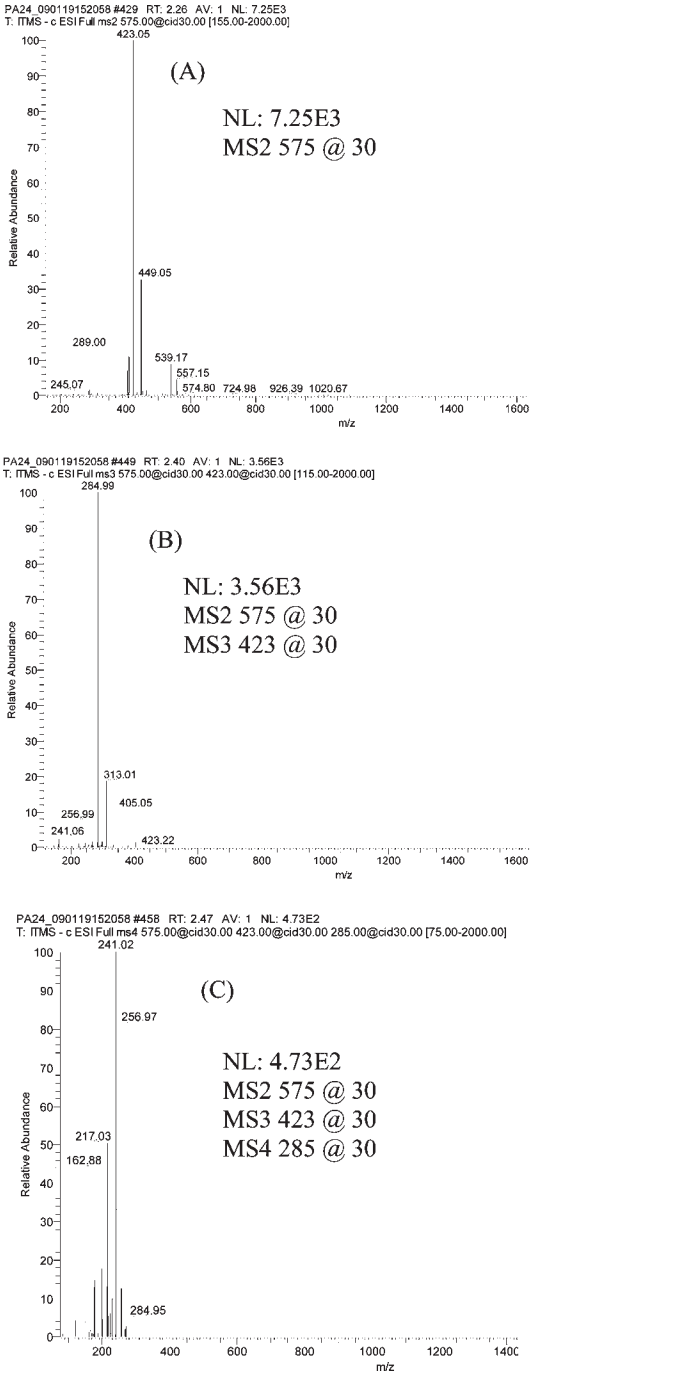

Figure 7. Negative ion ESI tandem mass spectra of PA2: (A) MS^2 of $[M - H]^-$ (m/z 575); (B) MS^3 of m/z 423 ion; (C) MS^4 of m/z 285 ion.

pH 7.4 and 37 °C for 30 h and periodically sampled at different time points. The samples were pretreated and analyzed as described above.

HPLC Analysis. PA2 and its isomers in the different media were extracted with ethyl acetate and then analyzed using a HPLC system, which consisted of a Schambeck SFD S5200 autosampler (Bad Honnef, Germany), a Jasco PU-2080 chromatographic pump (Tokyo, Japan), a Hitachi L-7420 UV−vis detector, and a Waters Atlantis T3 C18 reversed-phase column (150 mm × 4.6 mm i.d., 3 μm particle size; Milford, MA). The mobile phase was composed of two solvents: A (0.5% aqueous acetic acid solution) and B (methanol). Elution conditions were as follows: solvent B started at 20%, increased to 30% in 6 min, increased from 30 to 35% in 19 min, then increased from 35 to 95% in 10 min, and was kept at 95% for 10 min. The solvent flow rate was 0.8 mL/min, and the column temperature was set at 30 °C. UV 280 nm was used as the detecting wavelength.

Isolation and Identification of Isomers from PA2 Incubated in Sodium Bicarbonate Solution. PA2 (200 mg) was incubated in 200 mL of 0.15% aqueous sodium bicarbonate solution at 37 °C for 2 h. After extraction with ethyl acetate (3 × 200 mL) and evaporation of the solvent in vacuum, the residue was applied to a preparative column (Waters Atlantis T3 C18 column, 150 mm × 19 mm i.d.,

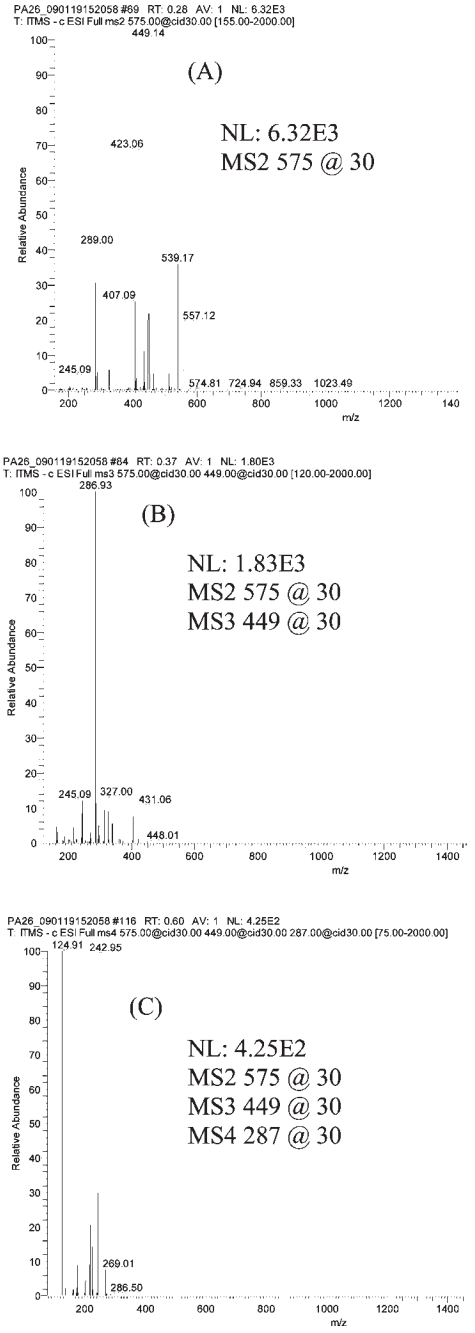

Figure 8. Negative ion ESI tandem mass spectra of peak 3: (A) MS^2 of $[M - H]^-$ (m/z 575); (B) MS^3 of m/z 449 ion; (C) MS^4 of m/z 287 ion.

5 μm particle size) with a compatible guard column. The mobile phase and elution conditions were the same as described above. The solvent flow rate was 4 mL/min, and the column temperature was kept at 30 °C. UV 280 nm was used as the detecting wavelength. Peaks were collected separately and dried under vacuum for MS and NMR analyses.

Mass Spectrometry and NMR Analyses. An LXQ Liner Ion mass spectrometer fitted with an ESI source (Thermo Finnigan, Waltham, MA) and operated in MS^n mode was used to obtain the mass spectra. MS operating conditions (negative ion) were optimized using standard PA2 dissolved in methanol with a collision energy of 35%, an ionization voltage of 4.5 kV, a capillary temperature of 200 °C, and a capillary voltage of 20 V. Nitrogen gas was used as the sheath gas at a flow rate of 30 arbitrary units (AU), and the auxiliary gas flow rate was 20 AU. For the NMR analysis, PA2 and its isomers were dissolved in deuterated methanol. ^1H, ^{13}C, and all 2D NMR spectra were acquired on a Bruker Avance-500 MHz FT-NMR (Rheinstetten, Germany).

RESULTS AND DISCUSSION

Stability of PA2 in SGF and SIF. The stability of PA2 was assessed in SGF and SIF. Digestive enzymes, such as pepsin in gastric juice and pancreatin in intestinal juice, were excluded from the study because of the ability of flavonoids to bind to proteins.[8] Figure 1 shows the HPLC chromatograms of PA2 incubated in SGF or SIF for 6 h. During the incubation in SIF, PA2 decreased and two new peaks were produced. This result suggested that PA2 may be transformed to other compounds in SIF (neutral) condition. The foregoing phenomenon was not observed when PA2 was incubated in SGF condition. It is evident that PA2 was relatively stable in SGF (acidic) condition. Only 17% of PA2 was lost in the 9 h of incubation in SGF condition (Figure 2). When PA2 was added into the SIF, the concentration of PA2 decreased rapidly with a half-life of about 1 h (Figure 3). After 9 h of incubation, only 5% of the added PA2 remained in the SIF. During the incubation, two major new peaks (peaks 1 and 3) appeared in the HPLC chromatogram (Figure 1). Peak 1 was produced from PA2 as soon as PA2 was added into the SIF (Figure 3). Peak 1 reached the maximum concentration at 3 h. On the other hand, peak 3 was produced after peak 1 reached its maximum concentration and started to decrease. Peak 3 increased slowly but steadily until 9 h.

Our results showed that PA2 was rather stable under acidic condition, whereas it was transformed into other compounds more easily under neutral condition.

Stability of PA2 in b-DMEM. We found that PA2 was also unstable in b-DMEM, which is the medium used to conduct RAW 264.7 cell experiments for anti-inflammation tests.[4] When PA2 was added into b-DMEM, its concentration decreased quite rapidly, with a half-life of only about 15 min (Figure 4). The HPLC chromatogram (Figure 1) of PA2 in b-DMEM after 2 h of incubation shows two additional peaks (peak 1 and 3). During the 24 h incubation period, the contents of the two new compounds increased but PA2 concentration decreased (Figure 4). After 24 h of incubation, only 6% of PA2 was left in the medium. PA2 transformed to peak 1 as soon as PA2 was added into b-DMEM. The concentration of peak 1 reached a maximum at 30 min and then decreased rapidly until 60 min. It then decreased slowly until 8 h and remained at the same level during 24 h of incubation time. Peak 1 accounted for 20% of total proanthocyanidins in the medium at the end of incubation. On the other hand, peak 3 was formed slightly more slowly than peak 1. Peak 3 reached a maximum concentration at 60 min and then became stable until 24 h. After 24 h of incubation, peak 3 was the major proanthocyanidin and occupied 73% of total proanthocyanidins in the medium at the end of incubation.

Effect of SOD and EDTA on the Stability of PA2 in b-DMEM. Recent studies indicated that several compounds may undergo degradation, autoxidation, or epimerization in cell culture media[6,9] or mild alkaline fluids.[10,11] (−)-Epigallocatechin-3-gallate (EGCG), the most abundant flavonoid in tea with strong biological activities, has been found to be unstable under most cell culture conditions.[6] Autoxidation and epimerization were demonstrated to be the two major reactions causing the instability of EGCG. To understand if similar reactions are also responsible for the conversion of PA2 in

Table 1. ^{1}H and ^{13}C NMR Data of Compounds 1−3 in CD_3OD

	1		2		3	
position	^{13}C	$^{1}H^{a}$	^{13}C	$^{1}H^{a}$	^{13}C	$^{1}H^{a}$
2	100.42		100.19		100.56	
3	67.66	4.12 (d, J = 3.5)	68.09	4.05 (d, J = 3.4)	67.63	4.09 (d, J = 3.6)
4	29.25	4.24 (d, J = 3.4)	29.27	4.40 (d, J = 3.4)	29.69	4.28 (d, J = 3.55)
4a	104.05		104.28		104.20	
5	156.66		157.01		155.33	
6	98.17	5.92 (d, J = 2.3)	98.32	5.99 (d, J = 2.3)	96.99	6.02 (d, J = 2.05)
7	158.16		158.14		158.21	
8	96.55	6.05 (d, J = 2.3)	96.64	6.06 (d, J = 2.3)	96.64	6.08 (d, J = 1.85)
8a	154.09		154.26		154.41	
9	132.23		132.47		132.22	
10	115.73	7.14 (d, J = 2.1)	115.95	7.13 (d, J = 2.1)	115.78	7.15 (d, J = 2.1)
11	145.65		145.66		145.66	
12	146.80		146.77		146.83	
13	115.64	6.81 (d, J = 8.3)	115.68	6.81 (d, J = 8.2)	115.64	6.81 (d, J = 8.3)
14	119.85	7.02 (dd, J = 8.3, 2.1)	119.79	7.01 (dd, J = 8.2, 2.1)	119.97	7.03 (dd, J = 8.35, 2.15)
2′	83.89	4.73 (d, J = 7.8)	81.78	4.92 (br s)	82.80	4.63 (d, J = 7.05)
3′	68.39	4.06 (m)	66.98	4.23 (br s)	68.53	3.98 (m)
4′ α	28.82	2.55 (dd, J = 16.3, 8.7)	29.90	2.75 (dd, J = 17.2, 2.1)	28.26	2.87 (dd, J = 16.35, 5.2)
4′ β		2.95 (dd, J = 16.3, 5.3)		2.94 (dd, J = 17.2, 4.9)		2.58 (dd, J = 16.35, 7.7)
4′a	102.83		102.44		103.53	
5′	156.15		156.61		155.33	
6′	96.60	6.07 (s)	96.52	6.09 (s)	108.69	
7′	152.21		152.31		152.01	
8′	106.56		107.23		96.50	6.06 (s)
8′a	150.83		152.14		152.43	
9′	130.95		131.20		132.14	
10′	115.44	6.95 (d, J = 1.5)	116.05	7.15 (d, J = 1.9)	114.96	6.77 (d, J = 1.75)
11′	146.41		146.00		146.21	
12′	146.75		146.31		146.21	
13′	116.36	6.83 (d, J = 8.2)	115.64	6.80 (d, J = 8.2)	116.13	6.73 (d, J = 8.1)
14′	120.32	6.86 (d, J = 8.2, 1.7)	120.39	6.97 (dd, J = 8.2, 1.9)	119.84	6.68 (dd, J = 8.25, 2.8)

[a] Coupling constants given (J, Hz) in parentheses.

b-DMEM, we added SOD or EDTA into the b-DMEM at 37 °C. As shown in Figure 5, the stability of PA2 could not be improved by adding SOD in b-DMEM. After 0.5 h of incubation, only 40% of PA2 remained in the medium regardless of the addition of SOD. The SODs constitute the first line of defense against reactive O_2 species (ROS) in cells. SODs could catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen.[12,13] The presence of SOD could not decrease the transformation of PA2, indicating that the instability of PA2 in the medium was not caused by the superoxide-mediated autoxidation. On the other hand, the addition of EDTA in the medium could increase the stability of PA2 efficiently (Figure 5). EDTA, a polyprotic acid, showed strong metal chelating activity and has been widely demonstrated as a chelating agent in the treatment of wastewater.[14] The effectiveness of EDTA in slowing the transformation of PA2 suggested that the instability of PA2 was possibly due to the metal ions in the medium rather than the superoxide-mediated autoxidation of PA2.

Zhu et al.[8] also reported that procyanidins B2 (PB2) and B5 (PB5) were less stable at alkaline condition than neutral condition. When PB2 and PB5 were incubated in SIF or at alkaline pH, they degraded almost completely within several hours.

Isolation and Identification of Isomers from PA2 Incubated in Sodium Bicarbonate Solution. b-DMEM consisted of 3700 mg/L sodium bicarbonate. It was observed that PA2 incubated in b-DMEM was rapidly converted to its isomers. It is possible that the transformation of PA2 may be induced by sodium bicarbonate. The PA2 isomers were prepared by incubating PA2 in 0.15% aqueous $NaHCO_3$ solution at 37 °C for 2 h to produce PA2 isomers. These two isomers were then isolated by preparative HPLC as described under Materials and Methods. Compound **1** (peak 1) was obtained as a white amorphous powder and compound **3** (peak 3) as an off-white amorphous powder. ESI-MS/MS analysis of these compounds yielded the same product ions (m/z 575) as PA2, indicating the isomeric nature of these three compounds. Characteristic product ion peaks in negative ion mode with m/z values of 423 and 449 for type A[15] were observed (Figures 6−8). All three compounds yielded an orange coloration upon reaction with anisaldehyde−sulfuric acid reagent, which is characteristic of proanthocyanidins.[16] The ^{1}H NMR (CD_3OD) spectra of all three compounds closely resembled one another (Table 1), and the presence of the isolated AB coupling system at δ 4.0−4.4 with $J_{3,4}$ = 3.4−3.6 Hz was

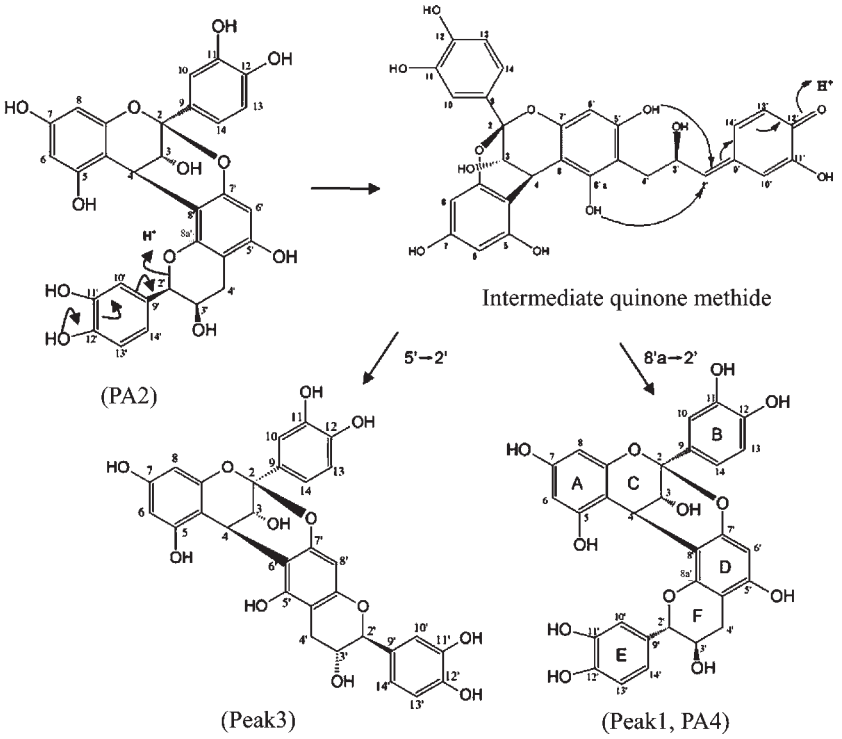

Figure 9. Proposed mechanism of conversion of proanthocyanidin A2 into its isomers.

ascribed as a diagnostic feature of the C-ring protons of A-type proanthocyanidins.[17] The meta-substitution pattern, as revealed by the two coupled doublets and a residual one-proton singlet at around δ 6.0, as well as the two AMX coupling systems in the aromatic region (δ 6.5−7.5) were indicative of a dimeric flavanol. These two A-type proanthocyanidins were also confirmed by the presence of 1 methylene, 13 methines, and 16 quarternary carbons in the ^{13}C NMR spectrum and the molecular weight of 576 amu for each compound, as established by ESI-MS/MS ([M − H]$^-$ at m/z 575).

The ^{1}H NMR spectrum of compound **1** almost resembled that of PA2. The presence of the AB coupling system at δ 4.12 (1H, d, J = 3.5 Hz, H-3) and δ 4.24 (1H, d, J = 3.4 Hz, H-4), the meta-coupled doublets at δ 5.92 and 6.05 (each 1H, d, J = 2.3 Hz, ring A), a residual one aromatic proton singlet at δ 6.07 (ring D), and two AMX systems in the aromatic region δ 6.5−7.5 due to rings B and E confirmed the A-type proanthocyanidin dimeric structure. This doubly linked dimeric structure was also demonstrated by the one acetal carbon at δ 100.42 in its ^{13}C NMR spectrum, which was assigned unequivocally by the 2D NMR (^{1}H−^{1}H COSY, NOESY, HSQC, and HMBC) and DEPT experiments. NMR spectral analysis of compound **1** shows very similar data for the upper unit but with slight changes in the lower unit, suggesting the epicatechin as the upper unit (similar to PA2). The presence of doublet at δ 4.73 with $J_{2',3'}$ coupling of 7.8 Hz clearly established the lower flavanoid unit as the 2′,3′-*trans* isomer, that is, catechin. As a rule, the (−)-epicatechin during epimerization through the quinone methide intermediate yields (−)-*ent*-catechin;[18] hence, compound **1** is identified as epicatechin-(4β→8;2β→O→7)-*ent*-catechin or proanthocyanidin A4 (PA4). This is further confirmed by the reported epimerization of PA2 into PA4 under mildly basic condition.[19]

On the other hand, compound **3** possesses data similar to those of compound **1**; careful examination of the ^{13}C NMR and 2D NMR (^{1}H−^{1}H COSY, NOESY, HSQC, and HMBC) data, however, revealed that there are highly diagnostic differences in C4′a, 6′, and 8′ chemical shifts that make it possible to distinguish between these two compounds. In compound **3** the chemical shifts of C-4a′, 6′, and 8′ are at δ 103.53 108.69, and 96.50, whereas in compound **1** the chemical shifts of these carbons are at about δ 102.83, 96.60, and 106.56, respectively, which makes it possible to identify the compound possessing the (4β→6;2β→O→7) double-linkage structure. The presence of a doublet at δ 4.63 with $J_{2',3'}$ coupling of 7.05 Hz clearly established the lower flavanoid unit as the 2′,3′-*trans* isomer, that is, catechin. Because compound **3** is formed from compound **1**, it is identified as epicatechin-(4β→6;2β→O→7)-*ent*-catechin. This was further confirmed by direct comparison of reported data.[20] The proposed mechanism of conversion is shown in Figure 9. Although the quinone methide intermediate is highly unstable and epimerized to yield compound **1**, it may be the bridge structure that will make it stable enough for the formation of compound **3**.

In conclusion, PA2 was found to be unstable in cell culture medium and could be converted to two isomers, PA4 (compound **1**) and epicatechin-(4β→6;2β→O→7)-*ent*-catechin (compound **3**). Therefore, the results obtained for PA2 in the cell culture bioassay would not represent the real activity of the original PA2. The interpretation of the bioassay results of PA2 performed in the cell culture system should be the combination effects of PA2 and its transformation products. A similar situation might also occur for other chemical compounds in the cell media; the stability of the compound in the cell media under investigation should be studied to avoid misinterpretation of the results.

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