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Stabilizing Effect of Ascorbic Acid on Flavan-3-ols and Dimeric Procyanidins from Cocoa

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Cocoa flavanols and procyanidins have numerous biological activities. It is known that (−)-epicatechin, (+)-catechin, epicatechin-(4β-8)-epicatechin (dimer B2), and epicatechin-(4β-6)-epicatechin (dimer B5) are unstable at physiologic pH, degrading almost completely within several hours, whereas they are relatively stable at pH 5.0. The present study investigated the effects of ascorbic and citric acid on the stability of monomers and dimers in simulated intestinal juice (pH 8.5) and in sodium phosphate buffer (pH 7.4). The addition of ascorbic acid to the incubation mixture significantly increased the stability of the monomers and dimers, whereas the addition of citric acid provided no protective effects. LC-MS showed that with the degradation of dimer B2 and dimer B5, doubly linked A-type dimers were formed. The present results, although not directly transferable to in vivo conditions, suggest that ascorbic acid may stabilize cocoa flavanols and procyanidins in the intestine where the pH is neutral, or alkaline, before absorption.

KEYWORDS: Cocoa; procyanidin; (+)-catechin; (−)-epicatechin; (−)-epicatechin-(4β-8)-epicatechin; (−)-epicatechin-(4β-6)-epicatechin; flavonoid; flavanol

INTRODUCTION

Cocoa flavonoids have been reported to have a wide range of biological properties, including modulating eicosanoid synthesis, increasing nitric oxide synthesis, lowering the rate of low-density lipoprotein (LDL) oxidation, inhibiting platelet activation, stimulating the production of anti-inflammatory cytokines, and inhibiting the production of certain proinflammatory cytokines (*1*−*8*). These diverse biological activities are thought to be attributable to a group of polyphenol compounds present in cocoa, including the flavan-3-ol monomers (−)-epicatechin and (+)-catechin and several procyanidin oligomers built upon these monomeric units (**Figure 1**).

In a previous study we demonstrated that the stability of monomers and dimers is pH dependent (*9*). (−)-Epicatechin, (+)-catechin, epicatechin-(4β-8)-epicatechin (dimer B2), and epicatechin-(4β-6)-epicatechin (dimer B5) (**Figure 1**) are unstable at pH >6 and degrade almost completely within a few hours, whereas at pH 5.0, they are relatively stable. In the present work we studied the major decomposition products of dimer B2 and dimer B5 at physiologic pH, and we examined the effects of two commonly used organic acids (ascorbic acid and citric acid) on the stability of the monomers and dimers under various conditions.

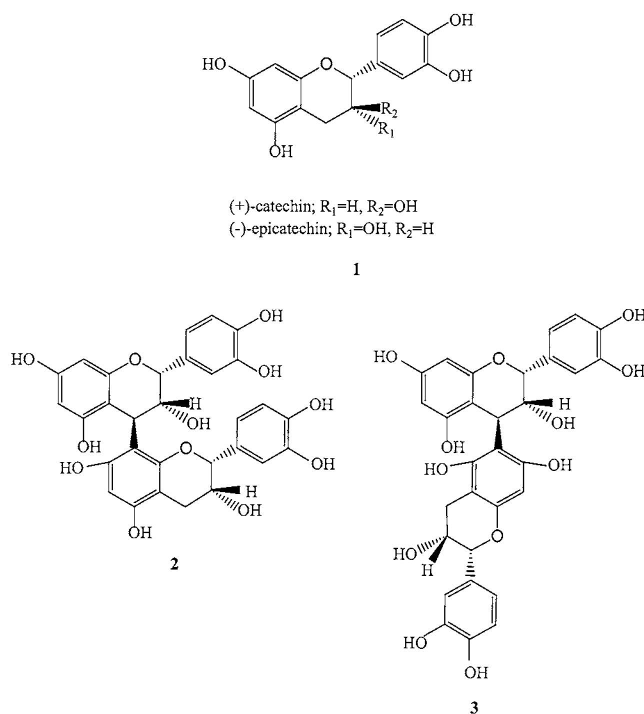

Figure 1. Structures of (**1**) (+)-catechin and (−)-epicatechin; (**2**) dimer B2, epicatechin-(4β-8)-epicatechin; and (**3**) dimer B5, epicatechin-(4β-6)-epicatechin.

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MATERIALS AND METHODS

Isolation and Purification of Two Dimers from the Dimer Fraction. To obtain this fraction, the method described by Adamson et al. (*9*) was used to initially extract the procyanidins from cocoa, followed by gel permeation chromatography (GPC) and preparative high-performance liquid chromatography (HPLC) to yield the purified procyanidin dimer fraction. The dimer fraction was then separated using a semipreparative HPLC column to yield two isomers, and the structures of the two dimers were confirmed as previously described (*10*).

Effects of Citric and Ascorbic Acid on the Stability of Monomers and Dimers in Simulated Intestinal Juice and Sodium Phosphate Buffer. The stability of the two monomers, (−)-epicatechin and (+)-catechin (Sigma Chemical Co., St. Louis, MO), and the two dimers, dimer B2 and dimer B5, was assessed in simulated intestinal juice (1.5% sodium hydrogen carbonate solution, pH 8.5) (*11*) or sodium phosphate buffer (60 mM), in the absence, or presence, of citric acid or ascorbic acid. Stock solutions of monomers and dimers (1 mM) were prepared in water. All solutions were stored at −70 °C. One hundred microliters of the stock solution was mixed with 0.8 mL of sodium phosphate buffer or simulated intestinal juice and 100 μL of 10 mM citric acid or ascorbic acid followed by incubation at 37 °C. An aliquot of the incubation solution (100 μL) was periodically sampled and analyzed by HPLC.

Analysis of Monomers and Dimers by Reversed-Phase HPLC. Chromatography was carried out according to a modified method of Hammerstone et al. (*12*) using a Hewlett-Packard model 1100 HPLC system with an autoinjector, an autosampler, and a fluorescence detector linked to a Hewlett-Packard Chem Station. In brief, 30 μL of the sample was injected onto a 250 × 4.6 mm i.d., 5 μm, Hypersil ODS column (Alltech, Deerfield, IL). The binary mobile phase consisted of (A) 0.5% acetic acid in water and (B) 0.5% acetic acid in acetonitrile. Separations were effected by a series of linear gradients of B into A at a flow rate of 0.7 mL/min as follows: elution starting with 2−25% B in A, 0−25 min; 25−50% B in A, 25−35 min; 50−95% B in A, 35−50 min; 95−2% B in A, 50−60 min; 2% B in A, 60−65 min. Changes in the concentrations of the monomers and dimers were monitored using a fluorescence detector with excitation at 230 nm and emission at 310 nm. Identification of each peak was confirmed by comparison of retention time and coelution with authentic standards of (−)-epicatechin, (+)-catechin, dimer B2, and dimer B5. Quantitative determination of monomers and dimers was based on the external standard method. Standard curves were constructed using standard solutions of monomers and dimers using the same HPLC protocol.

Determination of the Decomposition Products of Dimers. HPLC-MS analyses were performed on an Agilent 1100 HPLC/ion trap MS system equipped with a binary pump, an autoinjector, a column heater, a diode array detector, and an ion trap mass spectrometer equipped with electrospray ionization. Data were collected simultaneously using the Agilent workstation and Bruker Daltonik software. Separations were achieved on 30 μL injections using a 200 × 2.1 mm i.d., 5 μm, Hypersil ODS column at a column temperature of 40 °C, a binary mobile phase of (A) 1% acetic acid in water and (B) 1% acetic acid in acetonitrile, and a flow rate of 0.4 mL/min. Separations were effected by a series of linear gradients of B into A to maximize resolution as follows: elution starting with 2% B in A; 0−25 min, 2−25% B in A; 25−35 min, 25−50% B in A; 35−50 min, 50−95%. UV data were collected at 280 ± 8 nm in addition to UV spectroscopic data from 200 to 400 nm. Mass spectrometric data were collected in the negative ion mode using the following conditions: dryer gas, 10 L/min N_2; dryer gas temperature, 350 °C; capillary voltage, 3.0 kV; capillary exit, −83.5 V; trap drive, 39.1; scan range, m/z 150−1200 scanned at 13000 (m/z)/s. Prior to the mass spectrometer, 0.4 mL/min of methanol was fed into the HPLC effluent to aid ionization. MS/MS analysis was conducted on separate injections of samples using identical HPLC conditions but adjusting the MS-trap conditions as follows: isolation mass, fixed at m/z 576 ± 4; cutoff at m/z 164; fragmentation voltage, 1.0 V. Fragmentation was conducted in a background of helium gas.

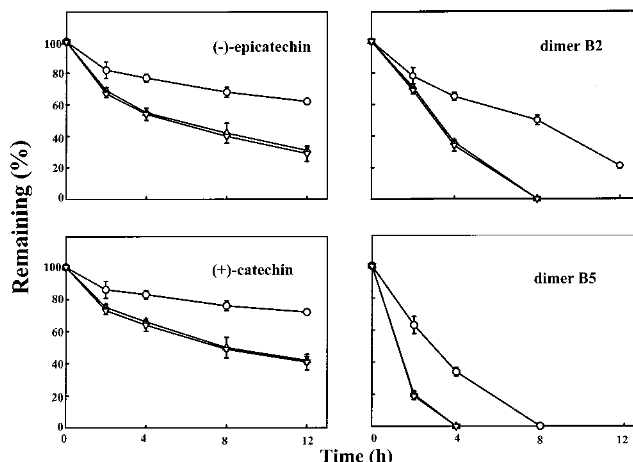

Figure 2. Effect of citric acid and ascorbic acid on the stability of (−)-epicatechin, (+)-catechin, dimer B2, and dimer B5 in simulated intestinal juice (pH 8.5): (△) 1.0 mM citric acid; (○) 1.0 mM ascorbic acid; (▽) control. Data are expressed as mean ± SD of $n = 5$ samples.

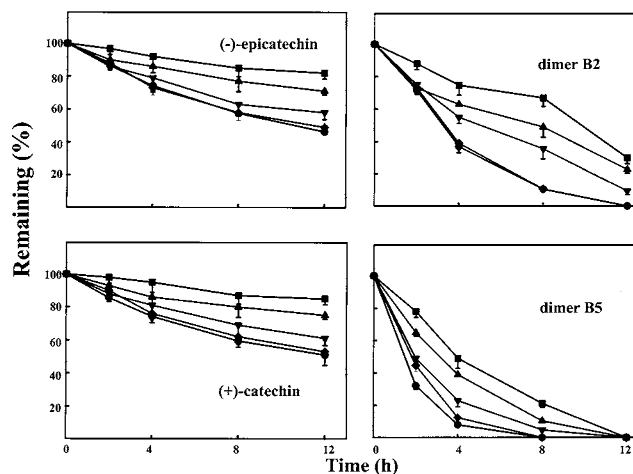

Figure 3. Dose-dependent effect of ascorbic acid (AA) on the stability of (−)-epicatechin, (+)-catechin, dimer B2, and dimer B5 (0.1 mM) in sodium phosphate buffer (pH 7.4): (■) 1.0 mM AA; (▲) 0.5 mM AA; (▼) 0.25 mM AA; (◆) 0.025 mM AA. Data are expressed as mean ± SD of $n = 5$ samples.

RESULTS

Effects of Ascorbic Acid on the Stability of Monomers and Dimers. The two monomers and the two dimers were unstable in simulated intestinal juice (pH 8.5). As depicted in **Figure 2**, ∼50% of the monomers and dimers were degraded within 12 h. The dimers were less stable than the monomers. The addition of citric acid did not provide any protective effects. In contrast, the addition of 1 mM ascorbic acid significantly improved the stability of both the monomers and dimers (**Figure 2**). The stability of monomers and dimers in the presence of ascorbic acid was also examined in sodium phosphate buffer (pH 7.4). We observed that ascorbic acid afforded a similar protection level under this condition, although the degradation rates in the two solutions were different. Monomers and dimers degraded more rapidly in the simulated intestinal juice than in the sodium phosphate buffer (**Figures 2** and **3**). This may be attributed to the higher pH value in the former solution (*10*).

To test whether ascorbic acid could regenerate the monomers and dimers, ascorbic acid was added to the incubation mixture 5 min after the reaction was started. Although the addition of

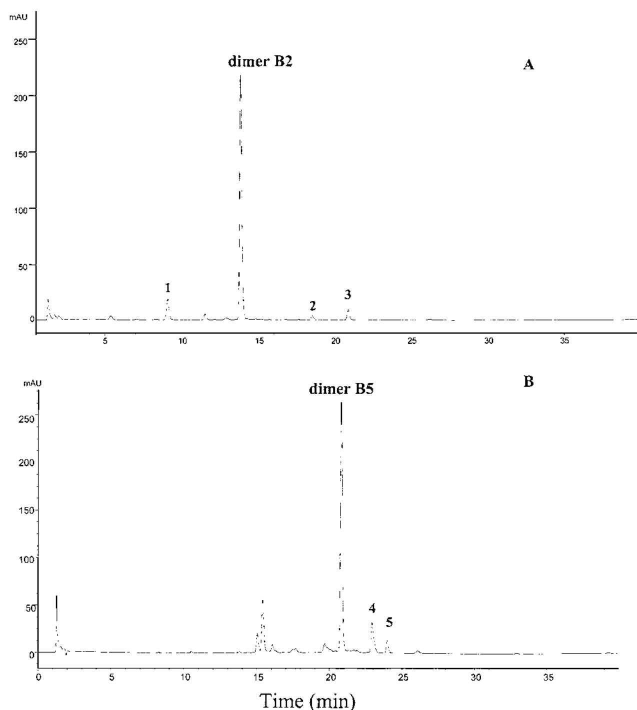

Figure 4. HPLC profile of (A) dimer B2 and (B) dimer B5 15 min after incubation in sodium phosphate buffer (pH 7.4).

ascorbic acid did not regenerate the flavonoids, it reduced the rate of their degradation (data not shown).

Dose-Dependent Effect of Ascorbic Acid on the Stability of Monomers and Dimers in Sodium Phosphate Buffer. As depicted in **Figure 3**, the addition of 0.025 mM ascorbic acid did not provide any protection to the monomers and dimers; the addition of 0.25 mM ascorbic acid slightly improved the stability of the monomers and dimers under the same conditions. When ascorbic acid increased to 0.5−1.0 mM, the stability of monomers and dimers was significantly improved relative to the control sample.

Determination of the Decomposition Products of Dimers. As observed previously (*10*), epimerization was observed with the degradation of the two monomers in the alkaline solution. Catechin was formed from (−)-epicatechin, and epicatechin was formed from (+)-catechin. As depicted in **Figure 4**, when dimer B2 was incubated in sodium phosphate buffer (pH 7.4), HPLC analysis showed that there was no interconversion to dimer B5, nor was there breakdown to epicatechin or catechin. Similarly, incubation of dimer B5 did not result in the formation of dimer B2, epicatechin, or catechin. With the degradation of dimer B2 and dimer B5, several interesting peaks (peaks 1−5) were formed (**Figure 4**). Mass spectrometric data of the B2 and B5 dimers are consistent with previous reports, as both show a molecular ion at m/z 576.8, retro-Diels−Alder fragments at m/z 424.7, and a cleavage product at m/z 288.8 (*13*−*15*). Furthermore, the fragment at m/z 406.7 is attributed to the loss of water from the retro-Diels−Alder product. The final fragment observed in the spectrum at m/z 450.7 has previously been observed by Karchesy et al. (*14*). MS/MS experiments gave three additional fragments not observed in the MS experiments. The first was the result of loss of water from the parent compound to give a fragment at m/z 558.7. Additionally, a retro-Diels−Alder product from the cleaved monomer (m/z 288.6) gave a fragment at m/z 244.7 (data not shown).

As numbered in the HPLC trace, peaks 1−5 (**Figure 4**) gave mass spectrometric data similar to that observed for the precursor dimers (dimer B2 and dimer B5) with minor differences. The LC-MS spectra as well as the MS/MS spectra of all these peaks were essentially the same; therefore, only a representative spectrum of peak 3 is shown. A typical molecular ion was observed at m/z 574.7 in addition to the retro-Diels−Alder product at m/z 422.6 (**Figure 5**). These observations are consistent with the work of Jacques and Haslam (*14*), who elucidated the structure of a number of A-type dimers. Thus, peaks 1−5 are products of the B-type dimer (**Figure 1**) conversion to the doubly linked A-type dimers (**Figure 5**). More interestingly, the putative ion that corresponds to the loss of water from the retro-Diels−Alder product does not have the expected signal at m/z 405.0, but rather at m/z of 406.6, which is identical to the fragment observed for the B2 and B5 dimers. Additionally, fragments corresponding to the loss of one and two water molecules were observed at m/z 556.7 and 538.7, whereas a pseudo cleavage product was observed in a cluster of ions around m/z 284.6 (**Figure 5**).

DISCUSSION

Cocoa flavanols and procyanidins have been shown to possess a variety of physiological functions. Several studies have indicated that the antioxidative activities of the procyanidin oligomers are higher than those of the monomers (*17*−*20*). It has been shown that cocoa dimers can provide protection against red blood cell hemolysis and that dimers can be detected in rat (*21*) and in human (*22*) plasma following the consumption of a flavanoid-rich cocoa. We have previously examined the stability of the monomers and dimers from cocoa in different solutions with various pH values from 5 to 9 (*10*). In that work, it was shown that the two dimers were especially susceptible to degradation at alkaline pH and that they degrade almost completely within several hours. Given the above, it is important to find ways to protect the flavonoids from degradation in order to keep their strong antioxidative activities. The organic acids used in the current study were chosen due to their common occurrence in beverages. When 1 mM citric acid was added to the sodium phosphate buffer solution containing 100 μM monomers or dimers, the pH of the incubation solution was reduced from 7.40 to 7.30. HPLC analysis showed that citric acid did not afford any protection to either the monomers or the dimers (**Figure 2**). In contrast, when the same amount of ascorbic acid was added, the stability of the monomers and dimers was significantly improved, even though the pH of the incubation mixture was reduced only to 7.35 (**Figures 2** and **3**). A similar protective effect of ascorbic acid on the functional constituents of tea flavonoids has been reported (*23*, *24*). The protective effect observed for ascorbic acid, but not for citric acid, suggests that the stabilizing activity of ascorbic acid on the cocoa monomers and dimers cannot be attributed to the drop in the pH value of the incubation mixture. It is known that ascorbic acid can function as an antioxidant and that it can regenerate α-tocopherol by converting the α-tocopherol free radical to the reduced form in vivo, thus sparing this lipophilic antioxidant (*25*). Given the above, we suggest that, when incubated together with cocoa flavonoids, ascorbic acid serves as a reductant that protects the cocoa flavonoids from degradation.

It has been reported that the antioxidative activity of procyanidin A-dimers is less than that of the B-dimers (*26*). Thus, there is interest in finding ways to reduce the conversion of the B-dimer to the A-dimer. Our present work shows that ascorbic acid can significantly increase the stability of the two monomers and the two dimers, dimer B2 and dimer B5. As

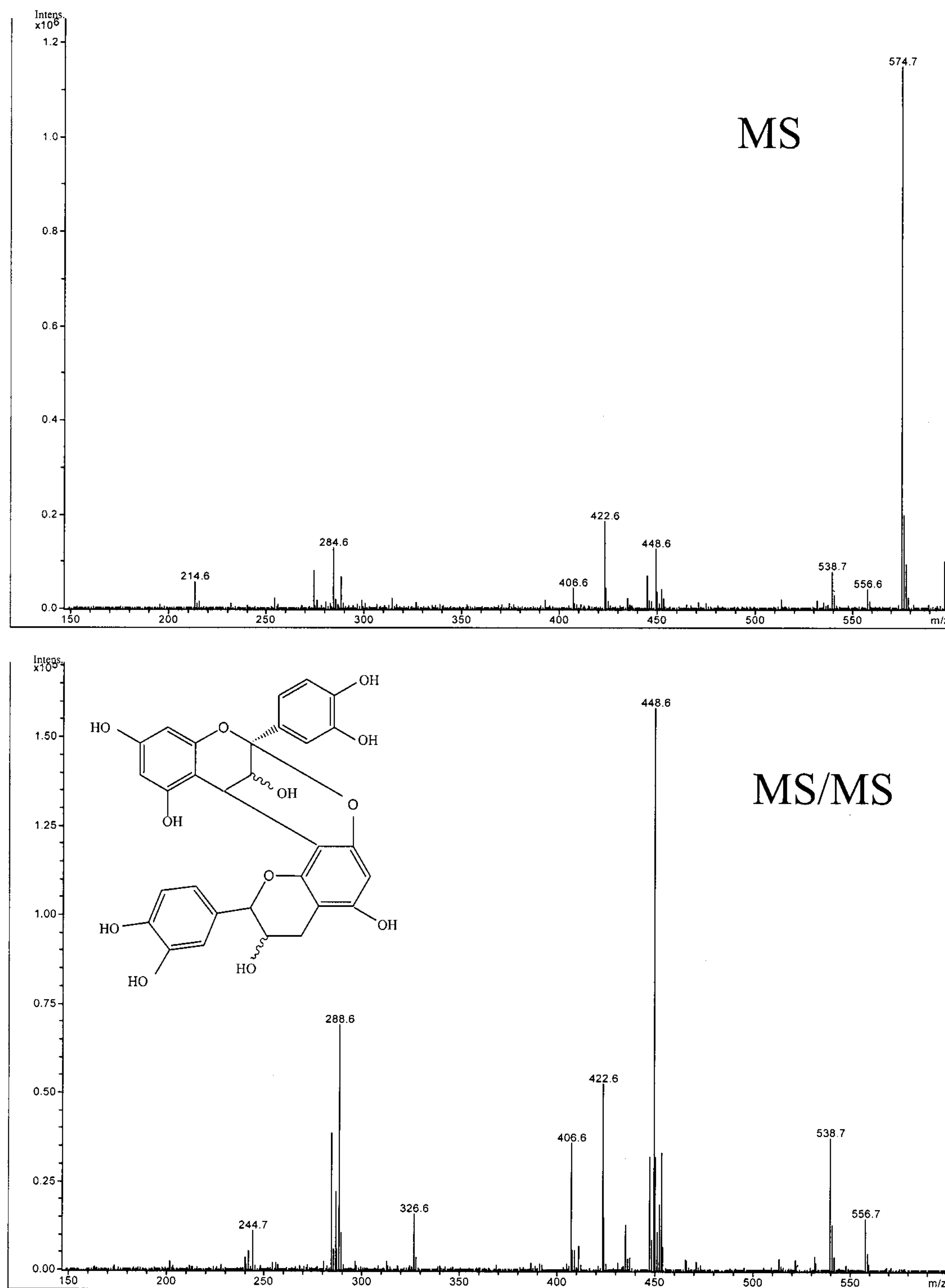

Figure 5. (A) Mass spectra and (B) MS/MS spectra of peak 3 in **Figure 4**.

depicted in **Figure 2**, dimer B2 and dimer B5 were almost completely degraded after incubation in sodium phosphate buffer for 4 and 8 h, respectively. However, when dimer B2 and dimer B5 were incubated for similar time periods in the buffer with 0.2 mg/mL ascorbic acid, only 30% of dimer B2 and 50% of dimer B5 were degraded. Similarly, ascorbic acid increased significantly the stability of (−)-epicatechin and (+)-catechin. The present study is in agreement with our previous study (*10*) and the report by Kondo et al. (*28*), who observed that dimer A1 was immediately produced by reacting dimer B1 with DPPH radicals under neutral conditions. In our work, using LC-MS, we observed that in neutral and alkaline solution (pH >5), A-type dimers could be immediately produced from dimer B2 and dimer B5 even in the absence of added radicals (**Figure 4**), whereas the addition of ascorbic acid significantly reduced the rate of conversion of B-type dimer to A-type dimer.

Cocoa flavanols and procyanidins can be absorbed by both rats (*20*, *28*) and humans (*5*, *22*, *30*−*33*). Plasma concentrations of (−)-epicatechin in human subjects can reach 355 nmol/L after the consumption of 80 g of chocolate containing 136 mg (∼469 μmol) of (−)-epicatechin (*31*, *33*). Our group has shown that in addition to (−)-epicatechin, (+)-catechin, dimer B2, and dimer B5 can be detected in the plasma of rats given an oral dose of flavanol-rich cocoa extract (*21*) and that dimer B2 can

be detected in the plasma of humans given a meal of 23 g of a flavanol-rich cocoa powder (*22*). We have obtained data that suggest that there is a selective absorption of epicatechin over catechin and a selective absorption of dimer B2 over dimer B5 (*21*). In another study (*10*), it was found that cocoa monomers and dimers exhibited poor stability at pH >5. As the intestinal pH is ∼8.5, the present results provide one explanation why the concentration of dimers in rat plasma was much lower than that of monomers (110 vs 2400 ng/mL), despite similar concentrations in the cocoa extract of 8 and 11.6%, respectively. The current results also provide one explanation why the concentration of dimer B5 (55.4 nM) is much lower than the concentration of dimer B2 (248.2 nM) in rat plasma, whereas the ratio of dimer B2 to dimer B5 in the cocoa extract is ∼2 (*21*). In addition, dimer B2, but not dimer B5, has been detected in human plasma (*22*), and it was speculated that this was due to the instability of dimer B5, as compared with dimer B2, at alkaline pH. Given that dimer B5 may exhibit stronger antioxidative activity than dimer B2 (*35*), the present study suggests a mechanism of protection of dimer B5 from degradation in neutral pH by ascorbic acid.

The present results, although not directly transferable to in vivo conditions, may have implications for the absorption of cocoa flavonoids. Given that the intestinal pH is neutral to slightly alkaline, it is reasonable to speculate that the concurrent presence of ascorbic acid may partially prevent the degradation or epimerization of flavonoids in the intestine before absorption. The influence of dietary ascorbic acid on the absorption of cocoa flavonoids deserves further study. The present study also suggests that the stability and profile of the flavonoids in foods and beverages containing cocoa can be significantly influenced by the type of acid used in the beverage and food products. Finally, reductants, other than ascorbic acid, might be used to protect the cocoa flavonoids from decomposition, and the relationship between oxygen concentration and the decomposition rate should be addressed in future studies.

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