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Stability of the Flavan-3-ols Epicatechin and Catechin and Related Dimeric Procyanidins Derived from Cocoa

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Cocoa flavanols and procyanidins possess wide-ranging biological activities. The present study investigated the stability of the cocoa monomers, (−)-epicatechin and (+)-catechin, and the dimers, epicatechin-(4β-8)-epicatechin (Dimer B2) and epicatechin-(4β- 6)-epicatechin (Dimer B5), in simulated gastric and intestinal juice and at different pH values. The dimers were less stable than the monomers at both acidic and alkaline pH. Incubation of Dimer B2 and Dimer B5 in simulated gastric juice (pH 1.8) or acidic pH resulted in degradation to epicatechin and isomerization to Dimer B5 and Dimer B2, respectively. When incubated in simulated intestinal juice or at alkaline pH, all four compounds degraded almost completely within several hours. These results suggest that the amount, and type, of flavanols and procyanidins in the gastrointestinal tract following the consumption of cocoa can be influenced by the stability of these compounds in both acidic and alkaline environments.

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

INTRODUCTION

Flavonoids are widely distributed in the human diet and occur primarily in plant-derived foods and beverages. During the past decade, numerous investigators have reported the antioxidative and anticarcinogenic properties of these nutrients (*1*−*3* and references therein). Cocoa and chocolate are rich sources of flavonoids, especially the flavan-3-ol monomers, (−)-epicatechin and (+)-catechin, and several procyanidin oligomers built upon these monomeric units (**Figure 1**). The flavanols, as well as the procyanidin oligomers present in cocoa and chocolate, have been reported to have potent biological properties that contribute to cardiovascular health, including the positive modulation of eicosanoid synthesis, increases in nitric oxide synthesis, reductions in the rate of LDL oxidation, and suppression of platelet activation (*4*−*8*). In addition, in cell culture models, cocoa flavanols and procyanidins can stimulate the production of antiinflammatory cytokines and inhibit the production of certain proinflammatory cytokines (*9*, *10*). Research has also shown that cocoa flavanols and procyanidins have the potential to protect against peroxynitrite-dependent oxidation of dihydrorhodamine 123 and nitration of tyrosine (*11*).

The biological effects of flavonoids predicted from in vitro studies may be difficult to extrapolate to the in vivo situation without knowing the absorption of the compounds from the gastrointestinal tract and their metabolism in the body. Several studies have suggested that cocoa flavanols and procyanidins are only partially absorbed both in rats (*12*) and in humans (*13*−

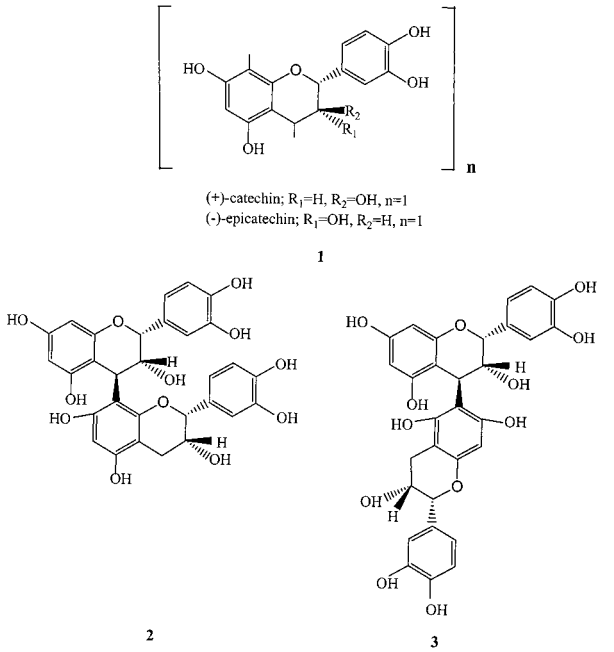

Figure 1. Chemical structures of (1) monomer and procyanidin oligomers, $n = 1$ (monomer) to 10 (decamer); (2) Dimer B2, epicatechin-(4β-8)-epicatechin; and (3) Dimer B5, epicatechin-(4β-6)-epicatechin.

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16). Previous studies have shown that the plasma concentration of (−)-epicatechin in human subjects can reach 355 nmol/L after the consumption of 80 g of chocolate, which contains 136 mg (∼469 μmol) (−)-epicatechin (*14*, *15*). Our laboratory has found that (−)-epicatechin, (+)-catechin, (−)-epicatechin-(4β-8)-epicatechin (Dimer B2), and (−)-epicatechin-(4β-6)-epicatechin (Dimer B5) were detected in the plasma of rats given an oral dose of flavanol-rich cocoa extract (unpublished data). Moreover, we have also found evidence of selective absorption of epicatechin over catechin (*13*) and of Dimer B2 over Dimer B5 (*13*). However, the mechanisms underlying the selective absorption of certain cocoa flavanols and procyanidin oligomers are unknown. Significantly, plant flavonoids are known to be easily degraded in alkaline solutions (*17*, *18*). In general, the plasma, bile fluids, and intestinal and pancreatic juices in mammals are mildly alkaline. For example, typical pH values of human plasma and bile are 7.4−7.5 and 7.1−8.5, respectively. Typical pH values of human intestinal and pancreatic juices are 8.3 and 7.0−8.5, respectively. Spencer et al. (*19*, *20*) have suggested that cocoa procyanidins largely decompose to monomers and dimers in simulated gastric milieu, which they estimated to be pH 2.0. The above work led us to conduct a series of experiments in which we examined the stability of cocoa monomers and dimers under various pH conditions.

MATERIALS AND METHODS

Isolation and Purification of Two Dimers from the Dimer Fraction. The purified procyanidin dimer fraction was provided by Mars Incorporated. To obtain this fraction, the method described by Adamson et al. (*21*) was used to initially extract the procyanidins from cocoa, followed by gel permeation chromatography and preparative high-performance liquid chromatography (HPLC) (*21*) to yield the purified procyanidin dimer fraction.

The dimer fraction was then purified to yield two isomers. The two dimer isomers were separated using a preparative 5 μm Hypersil ODS, 250 mm × 21.1 mm i.d. column (Phenomenex, Inc., Torrance, CA). In brief, 25 mg of the dimer fraction in 1.5 mL of H_2O was loaded onto the column during several injections (300 μL each). The binary mobile phase consisted of (A) 0.5% acetic acid in water and (B) 0.5% acetic acid in acetonitrile. The following gradient was employed at a flow rate of 9 mL/min: 86% A in B, 0−8 min; 86−75% A in B, 8−27 min. The separations were monitored using a diode array detector at 280 nm. The two major dimer peaks were manually collected, and peaks from several injections were combined, rotary evaporated, and freeze-dried.

The two peaks were identified as dimers by HPLC/MS (mass spectroscopy) analysis using the method of Hammerstone et al. (*22*). The structural configuration of the dimers was determined by ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR on a Brucker AC 500 instrument. Samples (5 mg) were dissolved in 0.6 mL of deuterated methanol, and chemical shifts were referenced to the ^{1}H or ^{13}C chemical shifts of the deuterated solvent. The two dimers were also analyzed by thin-layer chromatography (TLC) on Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO) cellulose plates, which were developed with 6% aqueous acetic acid solution. The plates were visualized by spraying with a 5% vanillin solution in 10% hydrochloric acid in ethanol (v/v), followed by heating the plate with a hot air blower as described by Foo and Lu (*23*).

Stability of Monomers and Dimers in Different pH. 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 different pH conditions. 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 0.9 mL of simulated gastric juice (0.24% hydrochloric acid−0.2% sodium chloride solution, pH 1.8), simulated intestinal juice (1.5% sodium hydrogen carbonate solution, pH 8.5) (*18*), sodium citrate buffer (60 mM) of pH values 2.0, 3.0, and 4.0, or sodium phosphate buffer (60 mM) of pH values 5.0, 6.0, 7.4, and 9.0, 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. (*22*) using a Hewlett-Packard model 1100 HPLC system with an autoinjector, autosampler, and fluorescence detector linked to a Hewlett-Packard Chem Station. In brief, 30 μL of the sample was injected onto the column [Hypersil ODS, 250 mm × 4.6 mm i.d., 5 μm (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 8% B in A, 0−5 min; 8−40% B in A, 5−60 min; 40−100% B in A, 60−65 min; 100% B in A, 65−70 min; 100−8% B in A, 70−75 min; 8% B in A, 75−80 min. The changes 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.

RESULTS AND DISCUSSION

While cocoa and chocolate contain significant quantities of the monomeric flavanols and their related dimeric procyanidins, there is limited information available on their metabolism and absorption. To enhance our understanding of flavanol and procyanidin metabolism and absorption, we investigated the stability of cocoa monomers and dimers in a variety of pH conditions.

We were able to isolate two peaks by semipreparative reversed-phase HPLC from the dimer fraction from cocoa. The peaks were identified by HPLC/MS analysis (*22*) and were determined to be dimers based on the electrospray MS data, which gave an $[M - H]^-$ peak at *m*/*z* 577 in both instances. The structures of the two dimers were determined by ^{13}C NMR on a Brucker AC 500 instrument as previously reported (*23*−*25*). The R_f of Dimer B2 on TLC was 0.63, and the R_f of Dimer B5 on TLC was 0.43.

Next, we performed a series of experiments examining the stability of (−)-epicatechin and (+)-catechin and Dimer B2 and B5 in simulated gastric and intestinal juices and in buffers of varying pH. Digestive enzymes, such as pepsin in gastric juice and pancreatin in intestinal juice, were excluded from these studies because of the ability of flavonoids to bind to proteins (*18*, *26*). Subsequent HPLC analysis demonstrated that (−)-epicatechin and (+)-catechin were stable in simulated gastric juice (0.24% hydrochloric acid−0.2% sodium chloride solution, pH 1.8) (data not shown). In contrast, Dimer B2 and Dimer B5 were unstable in simulated gastric juice (**Figure 2**), producing epicatechin; at the same time, isomerization was observed. After the dimer was incubated for 30 min in the simulated gastric juice, Dimer B2 was degraded and epicatechin and Dimer B5 could be detected as products (**Figure 2A**). Similarly, epicatechin and Dimer B2 could be detected as degradation products of Dimer B5 (**Figure 2B**). Time courses for the degradation of the dimers into epicatechin and their isomers are depicted in **Figure 3**.

When the two dimers or monomers were dissolved in simulated intestinal juice, the mixtures were initially light brown and rapidly became dark brown. HPLC analysis showed that the two monomers and two dimers degraded almost completely within several hours in medium. Dimer B2 and Dimer B5 were

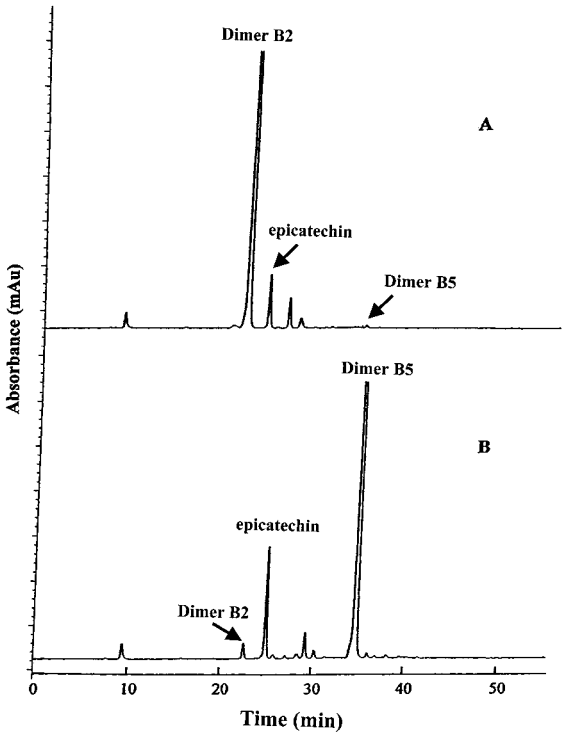

Figure 2. (**A**) HPLC profile of Dimer B2 30 min after incubation in simulated gastric juice (pH 1.8). (**B**) HPLC profile of Dimer B5 30 min after incubation in simulated gastric juice (pH 1.8).

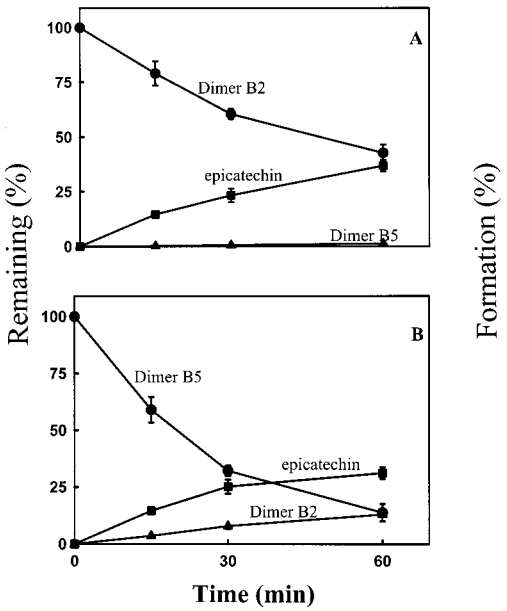

Figure 3. (**A**) Time courses for the stability of Dimer B2 in simulated gastric juice (pH 1.8) and the formation of epicatechin and Dimer B5. (**B**) Time courses for the stability of Dimer B5 in simulated gastric juice (pH 1.8) and the formation of epicatechin and Dimer B2. Data are expressed as mean ± SD of $n = 5$ samples.

especially susceptible to degradation in the simulated intestinal juice, with complete degradation occurring within 2 h. However, the degradation products formed from the dimers after incubation in this medium remain unknown. When Dimer B2 was incubated in simulated intestinal juice, HPLC analysis showed that there was no interconversion to Dimer B5 nor breakdown to epicatechin or catechin. Similarly, incubation of Dimer B5 did not result in the formation of Dimer B2, epicatechin, or catechin.

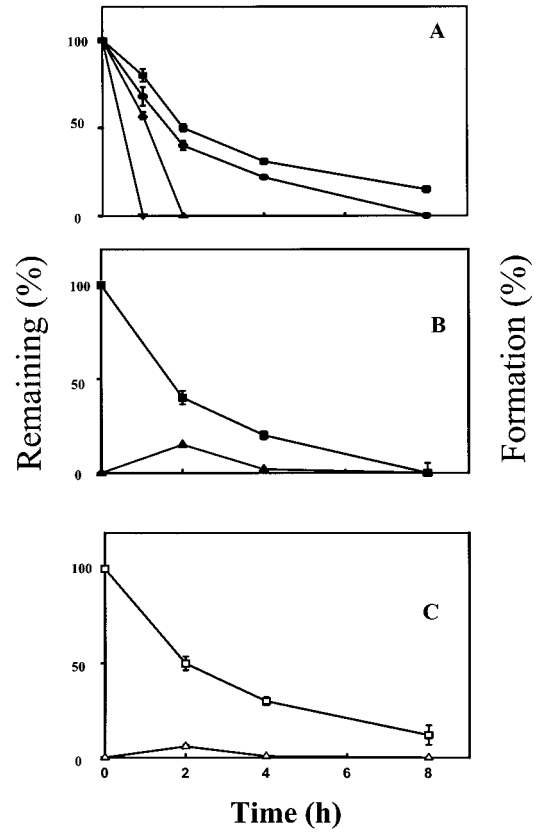

Figure 4. (**A**) Stability of (+)-catechin (■–■), (–)-epicatechin (●–●), Dimer B2 (▲–▲), and Dimer B5 (▼–▼) in simulated intestinal juice (pH 8.5). (**B**) Stability of (–)-epicatechin in simulated intestinal juice (■–■) and the formation of catechin (▲–▲). (**C**) Stability of (+)-catechin in simulated intestinal juice (□–□) and the formation of epicatechin (△–△). Data are expressed as mean ± SD of $n = 5$ samples.

In contrast, the two monomers in the simulated intestinal juice degraded almost completely by 8 h, and epimerization was observed (**Figure 4**). Catechin was formed from (−)-epicatechin and epicatechin from (+)-catechin. Time courses for the degradation and the formation of the epimers are depicted in **Figure 4**. Previous reports have shown that (−)-epicatechin and (+)-catechin undergo epimerization at the 2-position to yield (−)-catechin and (+)-epicatechin (*29*, *30*). Thus, it is important to note that with our chromatographic conditions, (+)-epicatechin and (−)-epicatechin and (+)-catechin and (−)-catechin have the same retention time and same molar absorbance on the HPLC chromatogram; therefore, we cannot definitively say whether the degradation product of (−)-epicatechin was (+)-catechin or (−)-catechin or both. Similarly, the degradation product of (+)-catechin cannot be distinguished between (−)-epicatechin and (+)-epicatechin.

When monomers and dimers were incubated in sodium citrate buffer of pH varying from 2.0 to 4.0, (−)−epicatechin and (+)−catechin were stable (data not shown), while the two dimers decomposed over time (**Figures 5** and **6**). Between pH 2.0 and pH 4.0, the stability of the two dimers was also pH-dependent, being less stable at lower pH. With the decomposition of Dimer B2, both epicatechin and Dimer B5 were formed, and the formation was pH-dependent (**Figure 5**). Similarly, when Dimer B5 degraded, epicatechin and Dimer B2 were formed, and the formation was also pH-dependent (**Figure 6**). Recently, Spencer et al. demonstrated that cocoa dimers degrade to monomers in an acidic gastric milieu (*20*). In the current study, we observed that Dimer B2 and Dimer B5 can degrade to epicatechin in either

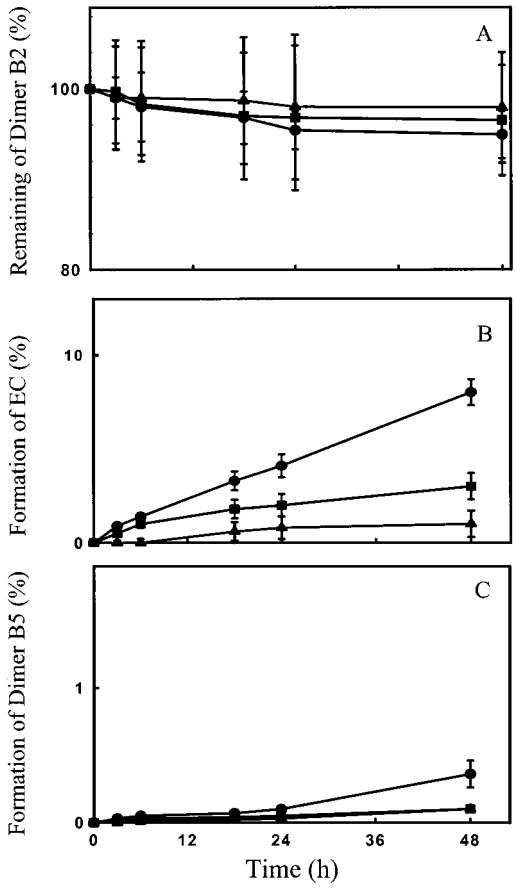

Figure 5. Stability of Dimer B2 in sodium citrate buffer at various pH values [pH 2.0 (●–●), pH 3.0 (■–■), and pH 4.0 (▲–▲)]. (**B**) Formation of epicatechin (EC) when Dimer B2 was incubated at these same pH values. (**C**) Formation of Dimer B5 when Dimer B2 was incubated at these same pH values. Data are expressed as mean ± SD of $n = 5$ samples.

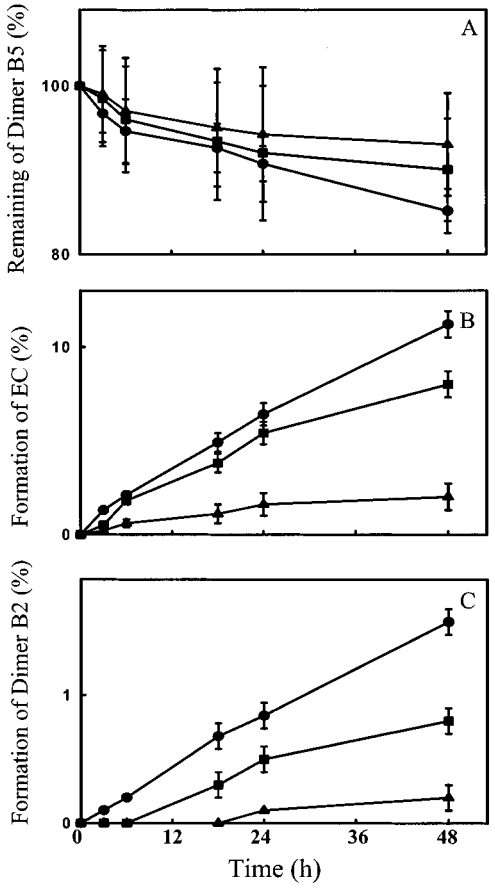

Figure 6. (**A**) Stability of Dimer B5 in sodium citrate buffer at various pH values [pH 2.0 (●–●), pH 3.0 (■–■), and pH 4.0 (▲–▲)]. (**B**) Formation of epicatechin (EC) when Dimer B5 was incubated at these same pH values. (**C**) Formation of Dimer B2 when Dimer B5 was incubated at these same pH values. Data are expressed as mean ± SD of $n = 5$ samples.

acidic buffer or simulated gastric juice (pH 1.8). In addition, we have extended the observations of Spencer et al. to show that the two dimers exhibit conversion of Dimer B2 to Dimer B5 and vice versa (**Figures 2**, **3**, **5**, and **6**).

The monomers and dimers were rapidly degraded at a pH greater than 9.0 within several minutes (data not shown). Between pH 5.0 and pH 9.0, we tested a series of sodium phosphate buffers (60 mM), which clearly showed that the stability of all four compounds was pH-dependent (**Figure 7**), with lower stability at higher pH. Thus, our results demonstrate that there is an inverse relationship between the stability of the procyanidin dimers and the monomers and pH. This is consistent with previous reports that flavonoids are not stable in neutral or mildly alkaline solutions (*17*, *18*). All of the monomers and dimers were relatively stable at pH 5.0. In pH 7.4, the monomers were more stable than the dimers. After 24 h incubation, 20% of the monomers remained, while after 2 h, Dimer B5 and Dimer B2 degraded by 60 and 30%, respectively. Therefore, the stability of the monomers and dimers at a pH between 5.0 and 9.0 can be ranked in the following order: (+)-catechin > (−)-epicatechin > Dimer B2 > Dimer B5 (**Figure 7**). Similar to incubation in simulated intestinal juice, no Dimer B2, Dimer B5, or epicatechin and catechin were formed after the dimers were incubated in neutral or alkaline pH (**Figure 7**).

In all conditions tested, the decomposition rate was different for the two dimers, with Dimer B5 being less stable than Dimer B2. The reason for the difference in stability between Dimers

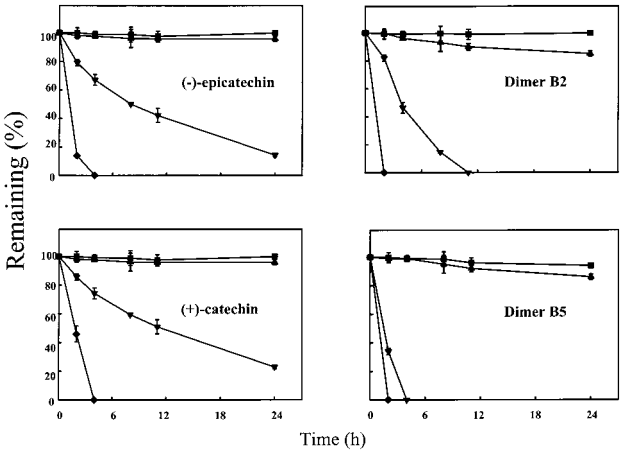

Figure 7. Stability of (–)-epicatechin, (+)-catechin, Dimer B2, and Dimer B5 in sodium phosphate buffer at various pH values [pH 9.0 (◆–◆), pH 7.4 (▼–▼), pH 6.0 (●–●), and pH 5.0 (■–■)]. Data are expressed as mean ± SD of $n = 5$ samples.

B2 and B5 is currently unknown. Dimer B2 and Dimer B5 have a similar backbone except for the difference in the interflavan bonds (**Figure 1**). Perhaps, because the two monomeric units are parallel to each other in Dimer B2 as compared to Dimer B5, it is easier for Dimer B2 to form hydrogen bonds, and as a consequence, Dimer B2 is more stable than Dimer B5. Another

reason might be that Dimer B5 is more likely to facilitate C-ring isomerization and to form A-type dimer (*27*, *28*).

Dimers B5 and B2 were especially sensitive in conditions with a pH between 7.4 and 9.0, with Dimer B5 degrading at a faster rate than Dimer B2 and resulting in several unknown products. While one could predict that the two dimers may have degraded into the monomers or experienced intraflavan bond conversion to their isomers (e.g., Dimer B2 to Dimer B5 and vice versa), we did not observe this in either the simulated intestinal juice or at a pH between 7.4 and 9.0. This may be due to the lability of the interflavan bond linking the monomeric units in procyanidins at an alkaline pH (*27*, *28*). Furthermore, under these conditions, we predict that one likely decomposition product would be the A-type dimer (*27*, *28*). Accordingly, we are currently investigating the breakdown pathways of Dimer B2 and Dimer B5 in simulated intestinal juice and their degradation products.

When flavonoid-rich cocoa or chocolate is fed to humans or experimental animals, such as rats, monomers and dimers can be detected in the plasma (*12*−*16*). However, while Dimer B2 has been detected in human plasma at quantifiable levels, Dimer B5 has not (*13*). Our results suggest that part of the mechanism responsible for this observation may be due to the fact that Dimer B5 is less stable in gastric juice, where the pH is 1.8, as well as in intestine and blood where the pH is neutral or slightly alkaline. The instability of dimers may also explain why the ratio of epicatechin to catechin (30:1) in both human and rat plasma is significantly different from the profile of the two monomers in cocoa extract (2:1) (*12*, *13*). This may also explain why the concentration of dimers (110 ng/mL) in rats is much lower than monomers (2400 ng/mL), while the percent concentrations of monomers and dimers in the cocoa extract are 11.6 and 8%, respectively.

In conclusion, Dimer B2 and Dimer B5 are quite vulnerable to degradation in both acidic and alkaline solutions. The two monomers, (−)-epicatechin and (+)-catechin, are very stable in acidic pH, but they are unstable in alkaline pH, with (−)-epicatechin being less stable than (+)-catechin. In alkaline pH, (−)-epicatechin can decompose and epimerize to catechin, while (+)-catechin epimerizes to epicatechin. In acidic pH, Dimer B2 decomposes to epicatechin and Dimer B5, while Dimer B5 decomposes to epicatechin and Dimer B2. Dimer B5 is less stable than Dimer B2 in both acidic and alkaline pH. The potential degradation of dimers in both the stomach and the intestine helps us to explain their low concentrations in plasma.

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