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Decomposition of Cocoa Procyanidins in the Gastric Milieu

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There is considerable interest in the bioavailability of flavonoids and phenolic components of the diet and their bioactivity *in vivo.* However, little is known of pre-absorption events in the gastric lumen. The effects of the acidic environment, as found in the gastric milieu, on procyanidin oligomers of catechin polyphenols has been investigated. The results show that under these conditions the procyanidin oligomers (trimer to hexamer) are hydrolysed to mixtures of epicatechin monomer and dimer, thus enhancing the potential for their absorption in the small intestine.

Key Words: **procyanidin; epicatechin; gastric juice; absorption; glucuronide; jejunum; ileum.**

Procyanidins are constituents of cocoa, apples, grapes, tea, wine, and strawberries. Chemically they exist as individual flavan-3-ol monomer units or, more commonly, oligomeric chains of catechins and epicatechins or procyanidins (Fig. 1) (1). Cocoa beans (*theobroma cacao*) are extremely rich in polyphenols, in the forms of procyanidin oligomers of epicatechin flavanols, comprising 12–18% of dry weight of the whole bean (2). Epicatechin and polyphenolic extracts of cocoa display potent antioxidant properties *in vitro* (3, 4), inhibit lipid peroxidation (5–7) and suppress peroxynitrite-induced nitration of tyrosine *in vitro* (8, 9). Chocolate polyphenols have also been suggested to display immunoregulatory effects (10).

Early studies had demonstrated that catechin ingested at doses of 8–80 mg/kg body weight circulates as conjugates of glucuronic acid and sulphate (11). More recent reports in humans have detected epicatechin at levels of 0.7 μM in plasma 2 to 3 h after consumption of 80 g black chocolate (12). In addition, plateau levels of 1 μM total catechins were observed after consumption of 5 cups of green tea (13) and 75 nM total catechins in plasma after consumption of 120 ml of red wine (14). Further understanding of the metabolism of catechin has been gained from oral administration to rats, indicating circulating 5-glucuronide conjugates of catechin and 3′-O-methyl metabolites (15, 16).

However, little is known about the absorption and uptake of the oligomeric procyanidins. We are interested in whether such polyphenol complexes are stable in the acidic environment of the stomach after consumption, prior to absorption. We have examined the effects of mimicking such an environment on the procyanidin oligomers ranging from a dimer to hexamer isolated from *Theobroma cacao*, as well as on the monomer epicatechin, and the implications for the stability of the procyanidins in the acidic milieu of the stomach. The stability of the larger oligomers at low pH will ultimately control the forms of such compounds which will be presented to the jejunum and ileum of the small intestine. This in turn will have important implications for the nature of the potentially bioactive components of procyanidins *in vivo.*

MATERIALS AND METHODS

Materials. Procyanidin oligomers purified from Cocoapro (17, 18) were kindly supplied by Mars, Inc. (Hackettstown, NJ). The reported oligomeric compositions are: dimer 99% (with 1% monomer), trimer 94.8% (with 4% tetramer), tetramer 95.4% (with 4.1% trimer), pentamer 92% (with 5.8% tetramer), and hexamer 86.2% (with 7.2% pentamer, 2.9% tetramer and smaller amounts of all other oligomers up to nonamer). The procyanidin monomer (−)-epicatechin was obtained from the Sigma-Aldrich Chemical Co. Ltd. (Dorset, UK). The simulated gastric juice (without pepsin) (pH 2) was supplied by Sigma-Aldrich Chemical Co. Ltd. (Dorset, UK). HPLC grade dichloromethane, acetic acid, and methanol were purchased from Rathburn Limited (Walkerburn, UK) and Elgastat UHP double distilled water (18.2 MΩ grade) was used throughout the study. HPLC columns for reverse phase were purchased from Waters (Watford, Herts, UK). For normal phase HPLC a lichrosphere column (Phe-

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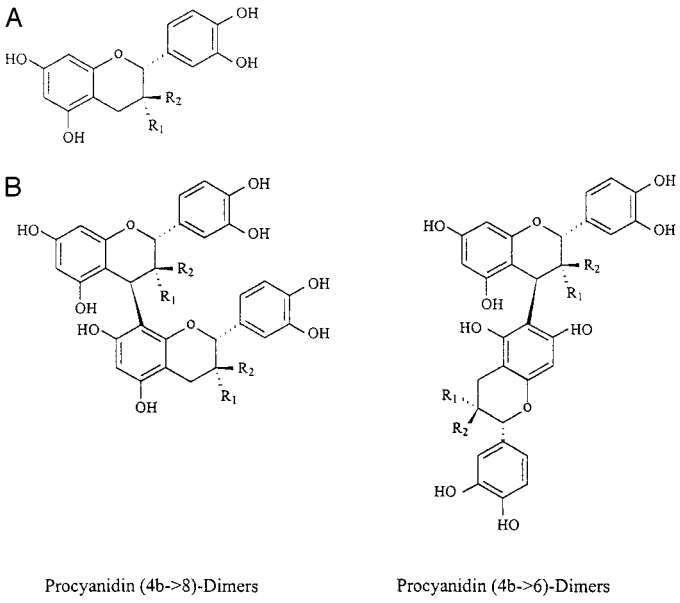

FIG. 1. Structure of epicatechin and procyanidin oligomers. R_1 = H, R_2 = OH (+)-catechin. R_1 = OH, R_2 = H = (−)-epicatechin.

nomenex, Cheshire, UK) was utilised. All other reagents used were of the analytical grade and obtained from Sigma Chemical Company (Poole, Dorset, UK). All other reagents used were of analytical grade.

Incubation of procyanidins at low pH (2.0). Stock solutions of procyanidin oligomers (monomer to hexamer; 1 mM) were prepared in sodium phosphate buffer (50 mM). All solutions were stored at −70°C and made up freshly before experiments. Procyanidin oligomers (100 μM) were incubated in phosphate buffer (adjusted to pH 2 with 5 M HCl acid for 0, 0.2, 1.5, 2.5, and 3.5 h at 37°C. Standard solutions of the oligomers (1, 5, 10, 50, 100, and 500 μM) were run using the same HPLC protocol and standard curves were constructed. Both the acidified procyanidin samples (monomer-hexamer) and the standards were run on the normal phase HPLC method for up to 3.5 h. In addition, the monomer, dimer, and trimer incubations were also followed using a reverse phase HPLC protocol. Oligomers, tetramer to hexamer, could not be well resolved using this protocol.

Incubation of procyanidins in simulated gastric juice. Stock solutions of the monomer to hexamer procyanidin oligomers (1 mM) were made up in phosphate buffer (10 mM). Simulated gastric juice had a pH of ~2.0. 100 μl of the oligomer stock solutions were diluted with 0.9 ml with gastric juice and inculcated for 0.2, 1.5, 2.5, and 3.5 h. The pH of the 100 μM oligomer mixtures was not significantly different from that of the simulated gastric juice alone. Samples were analysed by both normal and reverse phase HPLC as detailed below.

Analysis of procyanidins by reverse phase HPLC. Reverse phase analysis was employed for analysis of (−)-epicatechin, dimer and trimer procyanidins. Since the larger oligomers were not fully separated by this method, normal phase HPLC was applied for the latter. Salicylic acid (20 μM final concentration) was added to the samples as internal standard. Thirty μl of the sample was injected onto a Nova-Pak C_{18}, 4.6 × 250 mm column, with 4 μm particle size, and analysis was carried out using a Waters 626 pump and 600 controller system with an autoinjector 717 and a photodiode array detector 996 linked to the Millenium Software system. Full analysis was as previously described (19).

Analysis of procyanidins by normal phase HPLC. The normal phase method used to separate the epicatechin chocolate oligomers is based on the method of Adamson and Lazarus *et al.* (18), with modification in fluorescent detector wavelengths to excitation at 230 nm and emission at 310 nm. Five μl of the sample was injected onto a Lichrosphere silica 100 column, with particle size 5 μm, and analysis was carried out using a Hewlett Packard Model 1100 HPLC system with an auto-injector, auto-sampler, and fluorescence detector linked to a Chem-system station. The ternary mobile phase consisted of three phases: phase A: 82% dichloromethane, phase B: 14% methanol and phase C: 4% of acetic acid and water in a 1:1 (v/v). The separations were effected by a series of linear gradients of B into A with a constant 4% of C at a flow rate of 1 ml/min as follows: elution starting with 14% B in A: 14–28.4% B in A, 0–30 min: 28.4–39.2% B in A, 30–45 min: 39.2–86% B in A, 45–60 min.

RESULTS

Procyanidin oligomers (100 μM) were incubated in acid (pH 2.0) and in simulated gastric juice (pH 2.0) at 37°C for up to 3.5 h and the products analysed and identified by HPLC. In acid, there is a time-dependent decomposition of each oligomer (trimer to hexamer) with the progressive appearance of dimer and monomer over the period of interaction (Figs. 2B–2E). Decomposition of the oligomers during the first 90 min is more pronounced (60–80%) than during the subsequent equivalent period and virtually complete by 3.5 h. The dimer is least susceptible to acid-dependent degradation decaying progressively, almost linearly, to monomer such that 15% is decomposed after 2.5 h interaction (Fig. 2A). The trimer, consisting initially of two trimeric components, was modified more rapidly than the dimer with loss of approximately 70% after 2.5 h, with the appearance mainly of dimer and the monomer epicatechin (Fig. 2B). After 2.5 h the two major tetrameric components are cleaved to the extent of 70–80% with the formation mainly of dimer and some monomer (Fig. 2C). Similar findings were observed for the pentamer and hexamer (Figs. 2D and 2E). A representative HPLC chromatogram for the incubation of the pentamer in acid at pH 2.0 is shown in Fig. 3A. All oligomers produced minor proportions of other oligomeric components during decomposition which varied in concentration. Incubation of the monomer epicatechin at pH 2 for up to 3.5 h had no effect on its concentration.

Similar observations applied to the interaction of the oligomers (100 μM) with gastric juice producing in the main to monomeric and dimeric products after decomposition (Fig. 4). A representative HPLC chromatogram for the incubation of the pentamer in simulated gastric juice is shown in Fig. 3B. In general, the dimer was the predominant form of degradation product derived from the oligomers compared to the monomer. Interaction in gastric juice for up to 3.5 h produced about 40 μM of dimer with a smaller yield of monomer for the oligomers trimer-hexamer (Figs. 4B–4F). The dimer decomposed by approximately 30 μM in gastric juice with only a 20 μM yield of monomer (Fig. 4A). This may be due to the formation of larger oligomers from the monomer under to acidic conditions. The

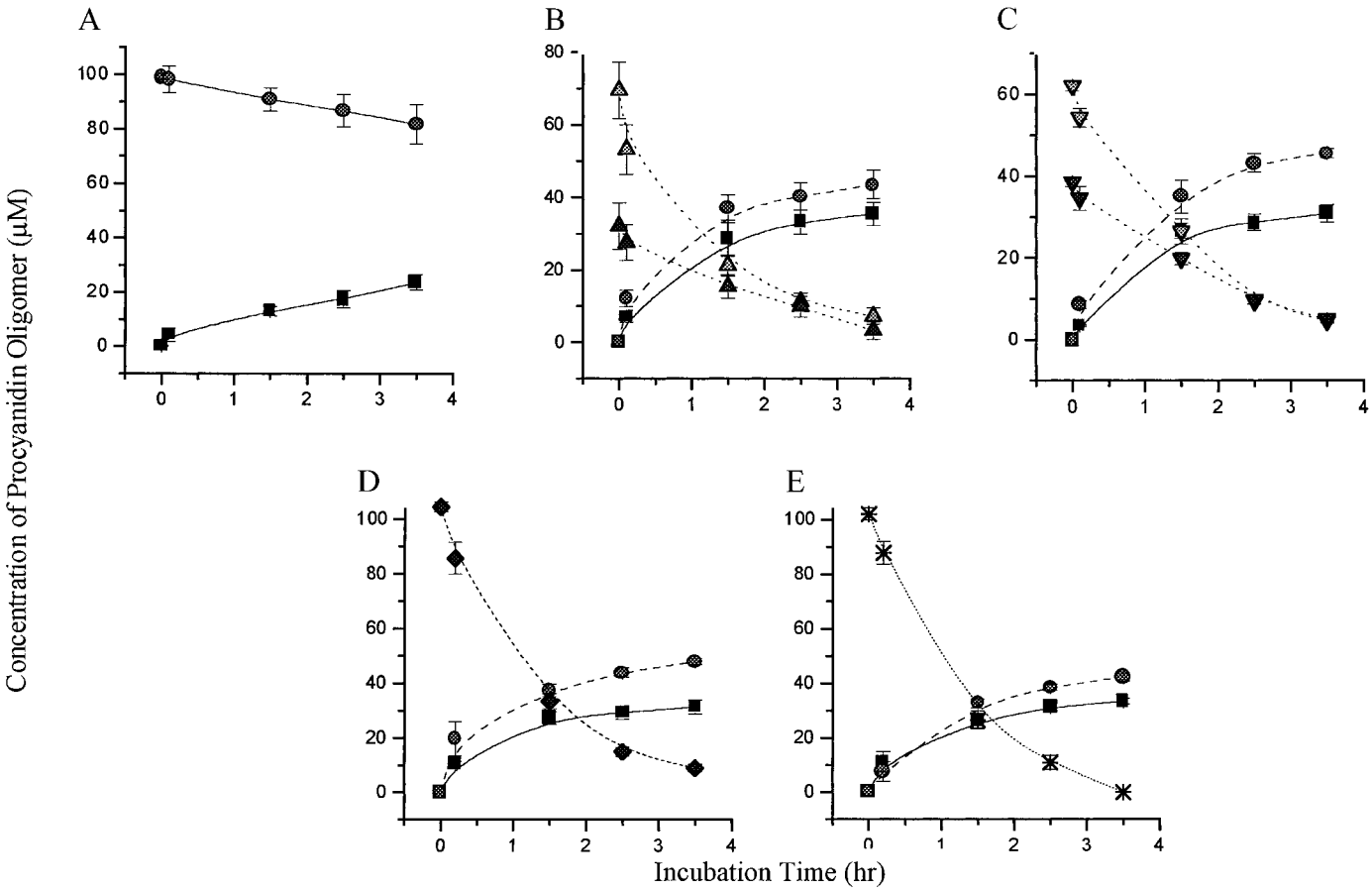

FIG. 2. Decomposition of procyanidin oligomers (100 μM) during incubation in acid (pH 2.0) for up to 3.5 h. (A) dimer, (B) trimer, (C) tetramer, (D) pentamer, and (E) hexamer. Experiments were conducted as described under Materials and Methods. Data are plotted as mean ± SD of three separate experiments each analysed twice by HPLC. (■) monomer (epicatechin); (●) dimer; (▲) trimer; (▼) tetramer; (◆) pentamer; (✳) hexamer.

yields of monomer and dimer were similar to that seen after acid incubation suggesting that it is the acidic component of gastric juice which potentiates the cleaving of the oligomers. This is further confirmed by treatment of oligomers with simulated gastric juice adjusted to pH 7.4. Minimal decomposition was observed, with approximately 8% modification of the native pentamer after 4 h of incubation. This is probably attributed to oxidation of the procyanidins under these conditions (data not shown).

DISCUSSION

An understanding of the absorption, distribution, and metabolism of polyphenols is essential for determining their significance and bioactivities *in vivo*. There are many factors that influence the extent and rate of absorption of ingested compounds by the small intestine (20). These include, physiochemical factors such as molecular size, lipophilicity, solubility, pKa, and biological factors including gastric and intestinal transit time, lumen pH, membrane permeability and first pass metabolism (21, 22). Thus procyanidin oligomers are unlikely to be absorbed at the level of the small intestine in their native forms nor would their size allow paracellular transport.

It is clear from the studies described here that procyanidin oligomers are unstable under conditions of the acidic environment of the gastric milieu and decompose essentially to epicatechin monomeric and dimeric units. Furthermore, the higher the polymerisation index of the monomer, the more readily the components are cleaved (Figs. 3 and 4). Thus absorption of flavonoids from chocolate or cocoa consumption are likely to be influenced by pre-absorption events in the gastric lumen within the residence time, such that the decomposition products in the forms of procyanidin dimers and monomers are the major components for consideration for absorption via the small intestine or entering the colon.

Previous reports of the absorption of monomeric flavonoids and their glycosides across the small intestine using jejunum and ileum isolated from the rat have shown that phase I and phase II metabolism in terms

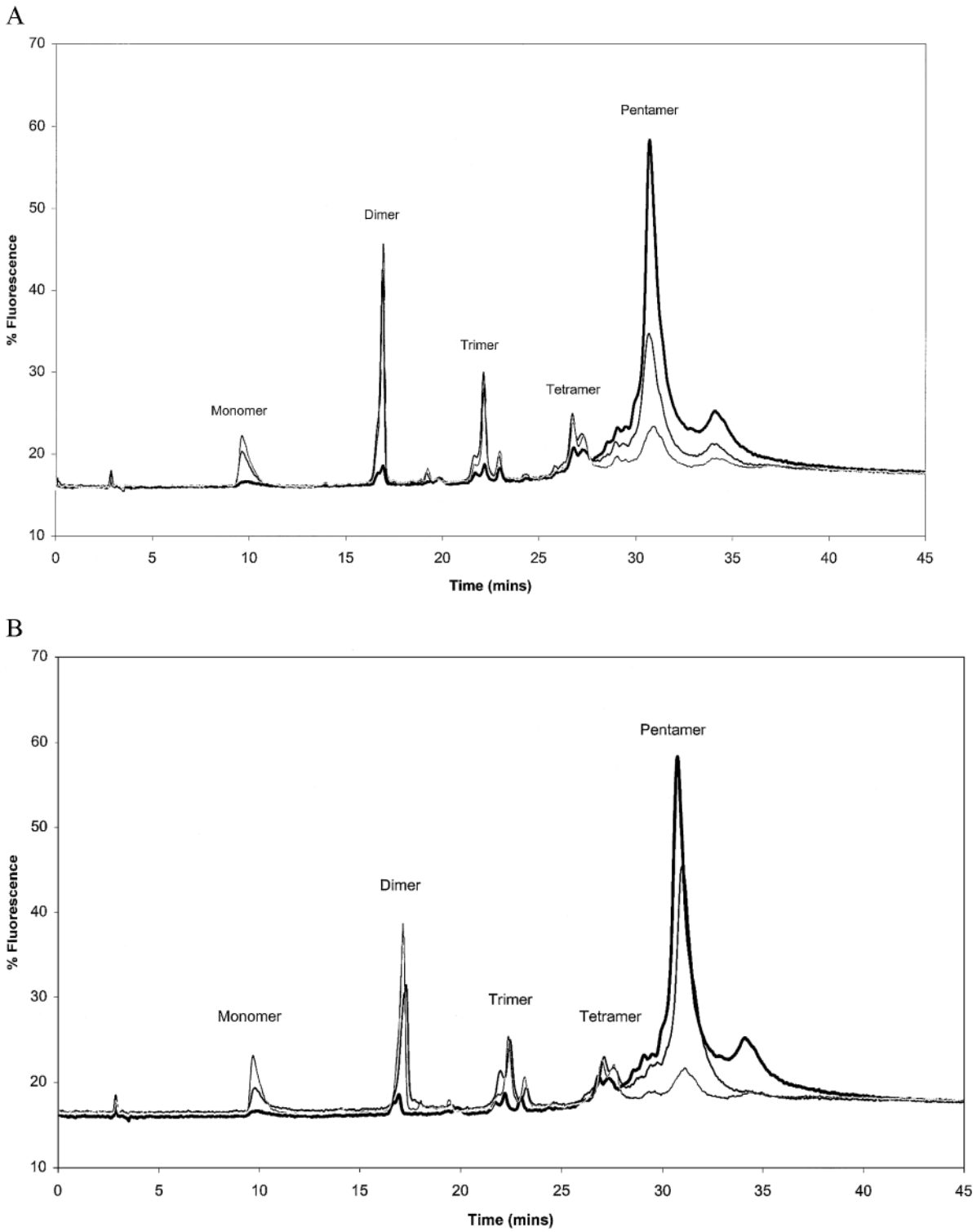

FIG. 3. Representative HPLC chromatograms of the decomposition of the pentamer over time during incubation in (A) acid; pH 2.0 and (B) simulated gastric juice; pH 2.0. Each line represents a time point of incubation: 0, 1.5, and 3.5 h. The pentamer peak is observed to decrease in area over time whereas the monomer and dimer peaks increase. —: 0 h; – –: 1.5 h; · · ·: 3.5 h.

of hydrolysis of glycosides to the aglycone and glucuronidation and of the aglycone respectively, can occur at the level of the intestinal mucosa (19). As oligomeric procyanidins are observed to liberate monomer and dimer units on incubation with acidic gastric contents, this would suggest that even though oligomers are not

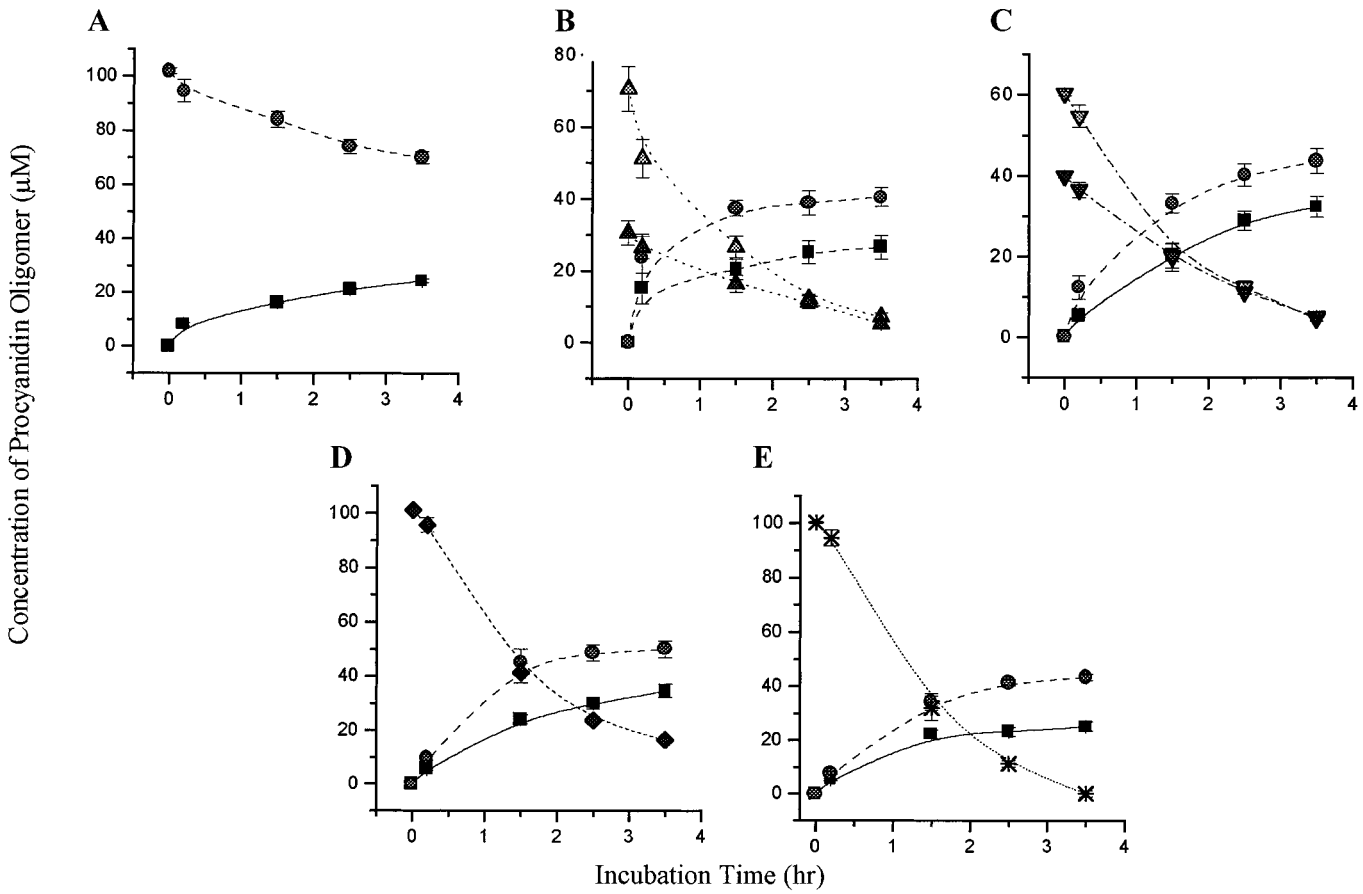

FIG. 4. Decomposition of procyanidin oligomers (100 μM) during incubation simulated gastric juice (pH 2.0) for up to 3.5 h. (A) dimer, (B) trimer, (C) tetramer, (D) pentamer, and (E) hexamer. Experiments were conducted as described under Materials and Methods. Data are plotted as mean ± SD of three separate experiments each analysed twice by HPLC. (■) monomer (epicatechin); (●) dimer; (▲) trimer; (▼) tetramer; (◆) pentamer; (✳) hexamer.

likely to cross the enterocytes of the small intestine, they may release large quantities of epicatechin which can be absorbed (12, 14–16). This has important implications for the action of procyanidins *in vivo*.

Studies have shown that these polyphenols are powerful scavengers of reactive oxygen and reactive nitrogen species *in vitro* (3–9) although it is not clear how the extent and positions of conjugation, whether glucuronidation, sulphation or O-methylation, might influence the antioxidant activities. Arteel and Sies (8) demonstrate that epicatechin oligomers found in cocoa and chocolate powder, especially the tetramer, are effective inhibitors of peroxynitrite-induced tyrosine nitration implying their role as potent dietary sources for defence against reactive nitrogen species. Our studies suggest that the bioactivities of these polyphenols *in vivo* will be independent of their oligomeric forms *in vitro* and that metabolism of their monomeric and dimeric units is the significant feature. This is the first report of the modifying effects of the acidic gastric lumen on dietary phenols and may have implications for the absorption of procyanidins from apples, edible plants (such as pinebark extract), and for other forms of larger molecules derived from catechins such as the theaflavins and thearubigins, chemically formed aggregates of catechin flavanols, in black tea.

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