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Inhibition of Key Digestive Enzymes by Cocoa Extracts and Procyanidins

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ABSTRACT: This study determined the in vitro inhibitory effects of cocoa extracts and procyanidins against pancreatic α-amylase (PA), pancreatic lipase (PL), and secreted phospholipase A_2 (PLA_2) and characterized the kinetics of such inhibition. Lavado, regular, and Dutch-processed cocoa extracts as well as cocoa procyanidins (degree of polymerization (DP) = 2−10) were examined. Cocoa extracts and procyanidins dose-dependently inhibited PA, PL, and PLA_2. Lavado cocoa extract was the most potent inhibitor (IC_{50} = 8.5−47 μg/mL). An inverse correlation between log IC_{50} and DP ($R^2 > 0.93$) was observed. Kinetic analysis suggested that regular cocoa extract, the pentamer, and decamer inhibited PL activity in a mixed mode. The pentamer and decamer noncompetitively inhibited PLA_2 activity, whereas regular cocoa extract inhibited PLA_2 competitively. This study demonstrates that cocoa polyphenols can inhibit digestive enzymes in vitro and may, in conjunction with a low-calorie diet, play a role in body weight management.

KEYWORDS: cocoa, *Theobroma cacao*, procyanidins, phospholipase A_2, pancreatic lipase, α-amylase

INTRODUCTION

Chronic imbalance between energy intake and energy expenditure is the major cause of weight gain and development of obesity (body mass index (BMI) ≥ 30).[1] Today, >60% of Americans are overweight, and if the current trajectory continues, the rate will reach 86% by 2030.[2,3] Elevated BMI is attributable to a global shift in diet toward increased intake of energy-dense foods that are high in fat and carbohydrates but low in vitamins, minerals, and other micronutrients as well as the increased prevalence of a sedentary lifestyle.[3] Elevated BMI is also a major risk factor in the development of heart disease, fatty liver disease, cancer, and type II diabetes.[4] One strategy for the prevention of overweight and obesity-related disease is the use of agents that interfere with the hydrolysis and absorption of dietary carbohydrates and lipids. Pancreatic α-amylase (PA), pancreatic lipase (PL), and pancreatic phospholipase A_2 (PLA_2), which are delivered into the intestinal lumen as constituents of pancreatic juices, are the major enzymes involved in the hydrolysis of dietary starch and fat.[5] PA is an endoglucosidase that catalyzes the hydrolysis of starch to maltose and maltotriose.[6] PL is a key enzyme for absorption of dietary triglycerides and rapidly converts a triglyceride to a 2-monoglycerol and two free fatty acids.[5] Orlistat (marketed over-the-counter as Alli in the United States), a potent competitive inhibitor of PL, is available as an antiobesity drug. It has been reported that orlistat promoted both short-term and long-term weight loss and minimized weight regain in overweight or obese subjects.[7] PLA_2 serves in the initial digestion of phospholipids to free fatty acids and lysolipids. Considerable evidence from cell and animal studies suggests the importance of PLA_2 in facilitating the digestion and absorption of lipids.[8] Given the key role these three enzymes play in starch and lipid digestion, they represent attractive targets for the prevention of excessive body weight gain and obesity-related diseases including diabetes.

A growing literature has suggested polyphenols from teas, berries, and other plants can inhibit some digestive enzymes in vitro and in vivo. For example, Horigome et al. reported that proanthocyanidins from various plants (i.e., black locust, bush clover, wistaria, and Japanese knotgrass) have inhibitory effects on lipase, α-amylase, and trypsin.[9] Studies have shown green tea catechins can inhibit the intestinal absorption of lipids in vivo.[8,10] This was associated with in vitro inhibitory activities of tea catechins against PLA_2. Among the green tea catechins, EGCG is the most potent inhibitor, and it inhibited PLA_2 in vitro by 64.9% at 2 mM. Harach et al. reported rosemary leaf extract, containing 5−10% phenolic compounds, induced a significant reduction of weight and fat mass gain associated with an increase of fecal lipid excretion in high fat-fed mice, and this effect was related to the inhibition of PL activity by the extract.[11]

Cocoa (*Theobroma cacao*) is a rich source of polyphenols with levels reaching 12−18% by dry weight.[12] Cocoa polyphenols are primarily composed of monomeric flavanols ((−)-epicatechin and, to a lesser degree, (+)-catechin) and oligomeric and polymeric C4β−C8 linked B-2 type procyanidins (Figure 1). The monomers account for only about 10% of the total with the oligomeric and polymeric procyanidins accounting for about 90% of the flavanol content.[13] Procyanidins with a degree of polymerization (DP) up to decamer have been identified in cocoa.[14] Evidence from the literature indicates that cocoa processing dramatically affects the polyphenol and flavanol content. As cocoa beans are processed on the farm, they are often fermented for 2−>6 days, and substantial flavanol loss occurs. Unfermented cocoa that has been immediately water

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washed and dried is referred to as lavado cocoa and contains the highest amount of polyphenols. Once fermented and dried, the nib of the cocoa bean is roasted and ground, resulting in the cocoa liquor or separated into cocoa powder and cocoa butter, which are the basis for chocolate manufacture. Dutch-processing (or alkalization) can also be applied to change the color and develop the flavor of cocoa products. Fermentation and Dutch-processing have been reported to result in the loss of as much as 90% of the cocoa flavanols.[15]

Studies on the health benefits of cocoa have primarily focused on the effects on the risk of cardiovascular disease.[16] Recently, a few studies on the antiobesity and antidiabetic potential of cocoa have been conducted in animal models. Matsui et al. showed that cocoa supplementation for 3 weeks significantly decreased weight gain in high fat-fed rats compared to high fat-fed controls.[17] Another study by Ruzaidi et al. also showed that dietary cocoa extract (1−3% w/w) dose-dependently reduced body weight gain, serum glucose levels, and total triglycerides in diabetic rats compared to control-fed animals.[18] Tomaru et al. reported that a diet containing 0.5 or 1.0% cocoa procyanidins decreased the levels of blood glucose and fructosamine in diabetic obese mice compared with the control treatment.[19] Jalil et al. found that short-term (acute) supplementation of cocoa extracts significantly reduced the plasma glucose level in obese−diabetic rats at 60 and 90 min compared with untreated.[20] Given recent animal model studies showing that dietary intake of cocoa might be beneficial in preventing the onset of obesity and type II diabetes, as well as cocoa being a rich source of procyanidins, studies of inhibition of key digestion enzymes by cocoa polyphenols were warranted.

The purpose of the present study was to determine the in vitro inhibitory effects of a series of cocoa extracts, ranging from high total flavanols (lavado) to low flavanols (Dutch-processed), and isolated cocoa procyanidins against PA, PL, and PLA_2 and to characterize the kinetics of such inhibition.

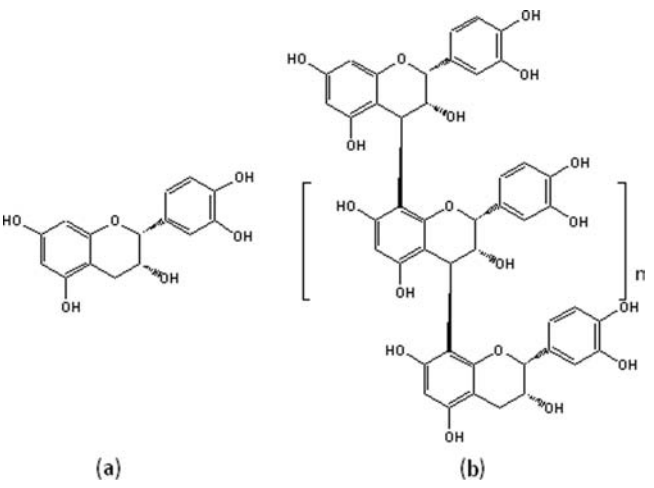

Figure 1. Structures of (a) (−)-epicatechin and (b) procyanidin oligomers with C4β−C8 linkage repeating units.

MATERIALS AND METHODS

Materials. Cocoa procyanidins (DP = 2−10, B type) and three cocoa extracts (from regular, lavado, and Dutch-processed cocoa powder) were provided by The Hershey Co. (Hershey, PA). The purity of all cocoa procyanidins was >85% by HPLC-MS. The polyphenol levels in the three extracts were assessed using the Folin−Ciocalteu reagent (Sigma Aldrich). (−)-Epicatechin (EC), orlistat and lipase from porcine pancreas (type II), and 4-nitrophenyl butyrate (4-NPB, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared in dimethyl sulfoxide (EMD Chemicals Inc.) and stored at −80 °C. α-Amylase from porcine pancreas and Red-starch were purchased from Megazyme (Wicklow, Ireland). An EnzChek Phospholipase A_2 Assay Kit was purchased from Invitrogen (Carlsbad, CA). All other reagents were of the highest grade commercially available.

Pancreatic α-Amylase Inhibition Assay in Vitro. Inhibition of PA by cocoa extracts and procyanidins was examined using a modification of the chromogenic Red-starch method (Megazyme, IR). PA (0.3 U/mL) in 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride and Red-starch (7 mg/mL in 0.5 M potassium chloride) were combined with cocoa procyanidins (0−100 μM) or cocoa extracts (0−200 μg/mL). After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 95% ethanol. After equilibration to room temperature, the solution was centrifuged at 1000*g* for 10 min, and the absorbance of the supernatant was measured at 510 nm using a Beckman DU 650 spectrophotometer.

Pancreatic Lipase Inhibition Assay in Vitro. Inhibition of PL by cocoa polyphenols was tested by monitoring the cleavage of 4-NPB to release 4-nitrophenol. Cocoa procyanidins (0−20 μM) or cocoa extracts

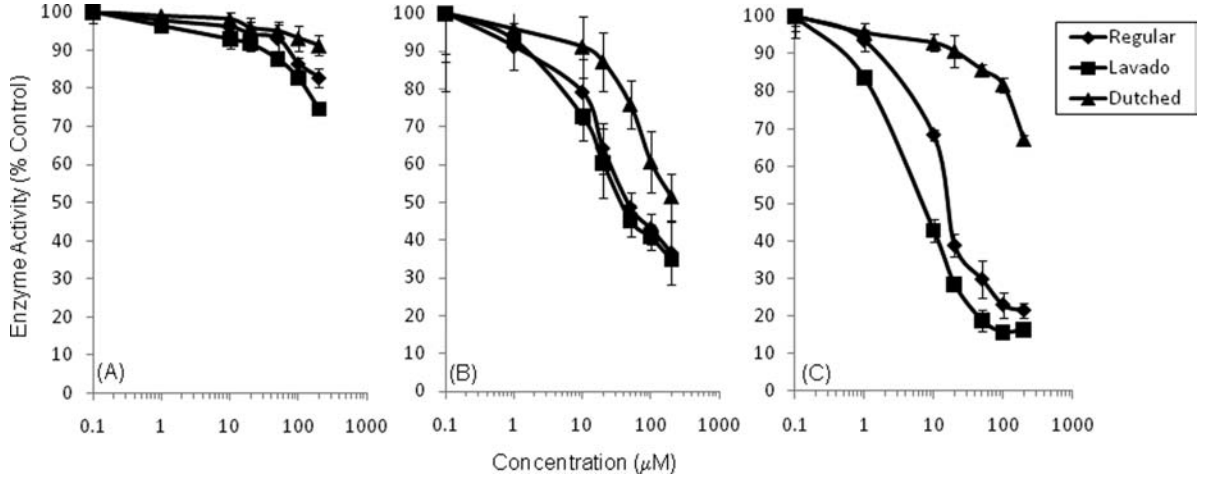

Figure 2. Inhibition of (A) PA, (B) PL, and (C) PLA_2 activity by cocoa extracts (regular, lavado, and Dutch-processed). Values are normalized to vehicle-treated controls and expressed as the mean ± SD of at least three independent experiments.

(0−200 μg/mL) were combined with PL (100 μg/mL) in 0.1 M Tris-HCl buffer (pH 8), and 4-NPB (0.2 mM) was added to start the reaction. Following incubation at room temperature for 10 min, absorbance was read at 400 nm. Orlistat was used as a positive control.

Phospholipase A_2 Inhibition Assay in Vitro. Inhibition of PLA_2 was examined using a commercially available fluorometric method (Invitrogen). Buffered PLA_2 solution (1 U/mL, pH 8.9) and cocoa procyanidins (0−100 μM) or cocoa extracts (0−200 μg/mL) were combined in a 96-well plate. A fluorogenic PLA_2 substrate (Red/Green BODIPY PC-A2, 1.67 μM) was dispensed to each well to start the reaction. After incubation at room temperature in the dark for 10 min, fluorescence was determined at λ_{ex} = 485 nm and λ_{em} = 538 nm (Fluoroskan Ascent FL, ThermoFisher Scientific Inc.).

Kinetic Analysis. Cocoa procyanidin pentamer and decamer as well as regular cocoa extract were selected for kinetic analysis of inhibition against PL and PLA_2. Reaction conditions were analogous to those above with the following modification. Cocoa procyanidins or cocoa extracts were held at constant concentrations and incubated in the presence of increasing concentrations of substrates (50−400 μM PL substrate; 0.5−4 μM PLA_2 substrate) together with enzymes and buffer solutions.

Data Analysis. The median inhibitory concentration (IC_{50}) of each cocoa procyanidin and extract was determined by interpolation or extrapolation of a dose−response curve using GraphPad Prism software (San Diego, CA). For kinetic analysis, Michaelis−Menten plots were generated using GraphPad Prism, and the maximum velocity (V_{max}), Michaelis−Menten constant (K_m), and mode of inhibition were determined from those plots

Data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. V_{max} and K_m values were compared by one-way ANOVA or Student's t test as appropriate. p values <0.05 were considered as statistically significant.

Table 1. Phenol Contents of the Lavado, Regular, and Dutch-Processed Cocoa Extracts[a]

cocoa extract	phenol content (mg/g)
lavado	481.4 ± 9.2 a
regular	271.0 ± 5.8 b
Dutch-processed	128.4 ± 4.0 c

[a] Phenol content expressed as gallic acid equivalents. Values not sharing a common letter are significantly different (p < 0.05).

RESULTS

Inhibition of Digestive Enzymes by Cocoa Extracts in Vitro. The inhibitory effects of cocoa extracts against PA, PL, and PLA_2 were dose-dependent (Figure 2). Lavado, regular, and Dutch-processed cocoa extracts inhibited PA by 25, 20, and 10%, respectively, at 200 μg/mL. PL was more sensitive to all three of the cocoa extracts with IC_{50} = 47.0, 57.7, and 172 0.4 μg/mL for lavado, regular, and Dutch-processed cocoa extract, respectively. PLA_2 was the most sensitive to cocoa extract, with the lavado and regular cocoa extracts showing IC_{50} = 8.5 and 19.7 μg/mL, respectively. The Dutch-processed cocoa extract was not as potent and inhibited PLA_2 only by 30% at 200 μg/mL. To determine if the inhibition of digestive enzymes correlated with the phenol content of the extracts, we examined the levels of these compounds in the cocoa extracts using the Folin−-Ciocalteu reagent (Table 1). We found, as expected, that the lavado extract had the highest levels of phenols, followed by regular cocoa, and last Dutch-processed cocoa.

Inhibition of Digestive Enzymes by Cocoa Procyanidins in Vitro. Dose−response curves shown in Figure 3 summarize the inhibitory activity of cocoa procyanidins (DP = 2−10) as well as (−)-epicatechin (EC) against PA, PL, and PLA_2. Lower molecular weight compounds with DP < 5 showed <15% inhibition against PA at a concentration of 100 μM, whereas the higher molecular weight procyanidins (DP = 5−10) inhibited PA by 17−45.5% at 100 μM. Cocoa procyanidins generally showed much stronger inhibitory activity against PL. EC and the dimer showed only 15% inhibition at 20 μM, whereas the trimer and tetramer caused 25% inhibition at 20 μM. Compounds with DP ≥ 5 inhibited PA by 37−53% at 20 μM. Orlistat was used as a positive control and inhibited PL by 72% at 10 μM. Although EC only inhibited PLA_2 by 4.5% at a concentration up to 100 μM, the cocoa procyanidins were particularly effective in inhibition of PLA_2. Cocoa procyanidins with DP = 2−5 inhibited PLA_2 by 46−74% at 100 μM. For higher DP procyanidins (DP = 6−10), approximately 90% of total enzyme activity was inhibited at 50 μM, and the IC_{50} values for these compounds were <5 μM.

Correlations between DP of Cocoa Procyanidins and Their IC_{50} Values. On the basis of the dose−response curves for the pure procyanidins against PL and PLA_2, DP appears to be an

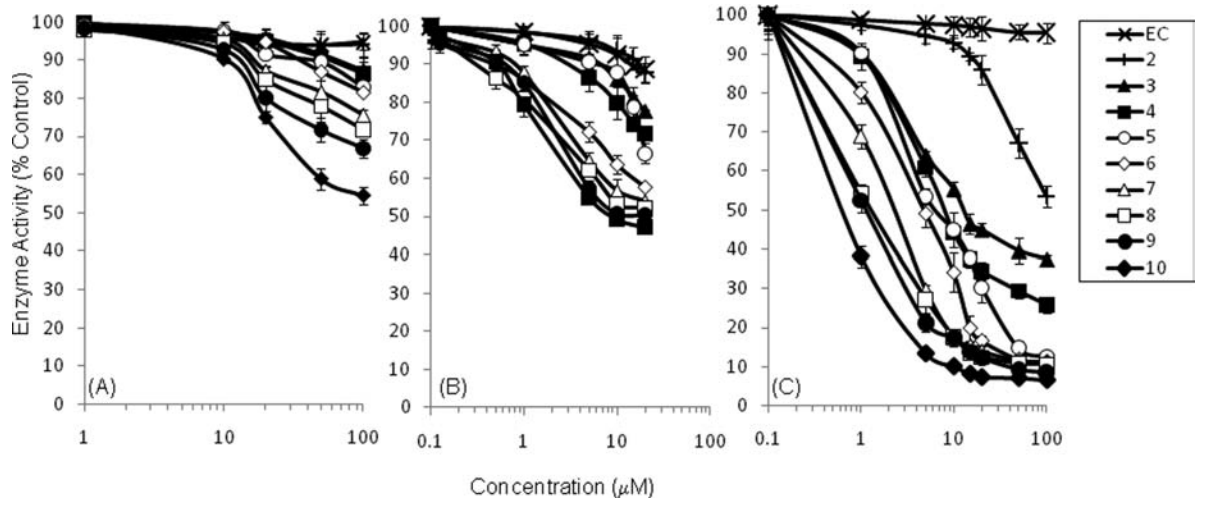

Figure 3. Inhibition of PA (A), PL (B), and PLA_2 (C) activity by EC and cocoa procyanidins (DP = 2−10). Values are normalized to vehicle-treated controls and expressed as the mean ± SD of at least three independent experiments.

important factor determining the potency of compound. By regression analysis, we observed a strong inverse relationship between log IC_{50} and DP ($R^2 > 0.93$, Figure 4). Similar analysis comparing log IC_{50} and hydrophobicity (log P) showed no significant correlation (data not shown). Because the procyanidins did not approach the IC_{50} of PA, a similar analysis could not be conducted.

Kinetic Analysis of PL and PLA_2 Inhibition. Because PL and PLA_2 were more sensitive to inhibition by cocoa extracts and procyanidins, we selected these enzymes for further kinetic analysis to determine the mode of inhibition. The procyanidin pentamer and decamer as well as the regular cocoa extract were selected as test inhibitors. All three test substances reduced the V_{max} and increased K_m of PL (Figure.5; Table 2). These results suggest a mixed-type inhibition with respect to substrate concentration.

On the other hand, Michealis−Menten plots of PLA_2 inhibition by the procyanidin pentamer and decamer showed decreased V_{max} values but no effect on K_m, indicating a noncompetitive inhibition with respect to substrate concentration (Figure 6; Table 3). By contrast, the regular cocoa extract increased K_m but had no effect on V_{max}. These results suggest a competitive mode of inhibition against PLA_2 with respect to substrate concentration (Figure 6; Table 3).

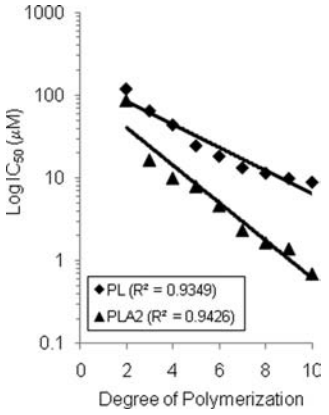

Figure 4. Relationship between degree of polymerization (DP = 2−10) of cocoa procyanidins and the IC_{50} against PL and PLA_2. Regression analysis was performed using Graph Pad Prism software (San Diego, CA). R^2 values are shown in the figure key.

DISCUSSION

In this study, the in vitro inhibitory effects of cocoa extracts and cocoa procyanidins against PA, PL, and PLA_2 were investigated. Kinetic analysis was performed to determine the mode of inhibition by regular cocoa extracts and the procyanidin pentamer and decamer with respect to substrate concentration. To our knowledge, this is the first detailed study to report the in vitro inhibition of key digestive enzymes by cocoa extracts and cocoa procyanidins. Additionally, this is the first report on the kinetics of inhibition of PL and PLA_2 by procyanidins from any source. This study extends previous work by Gonçalves et al., showing that these compounds can affect digestive proteases.[21,22]

It is increasingly recognized that polyphenols can regulate carbohydrate and lipid metabolism by affecting the activity of digestive enzyme. Inhibition of α-amylase in vitro by cocoa phenolic extracts has been noted earlier in Quesada et al.[20] In our study, all three cocoa extracts demonstrated inhibitory activities in vitro. Among three cocoa extracts, lavado (meaning "washed" in Spanish) cocoa undergoes the least processing (without fermentation or Dutch-processing), and this extract exerted the highest inhibitory activity against all three digestive enzymes. By contrast, the Dutch-processed or alkali-treated cocoa, which is the most highly processed, showed the least inhibitory effect against the enzymes tested. Because it is expected that the lavado cocoa extract is the highest in polyphenols and flavanols, followed by the regular cocoa extract, and the least would be found in the Dutch-processed cocoa extract, these results suggest that the inhibitory effects of cocoa extracts are related to their polyphenol content. In this study, the lavado cocoa extract was a potent enzyme inhibitor, and our results are comparable or superior to some recent studies with other polyphenol-rich extracts. Moreno et al. found that grape seed extract at a concentration of 1 mg/mL resulted in 80% inhibition against PL, and they also suggested that the inhibitory effect may be caused by a synergistic action of several phenolic compounds including procyanidins within the extracts.[23] Polyphenol-rich berry fruits such as strawberry and raspberry have been shown to inhibit PA, but the effects were relatively weak (e.g., 25 mg/mL strawberry extract inhibited PA by 14.7% in vitro).[24,25]

In general, the cocoa procyanidins (DP = 2−10) showed greater inhibitory activity against PLA_2 than against PL and PA.

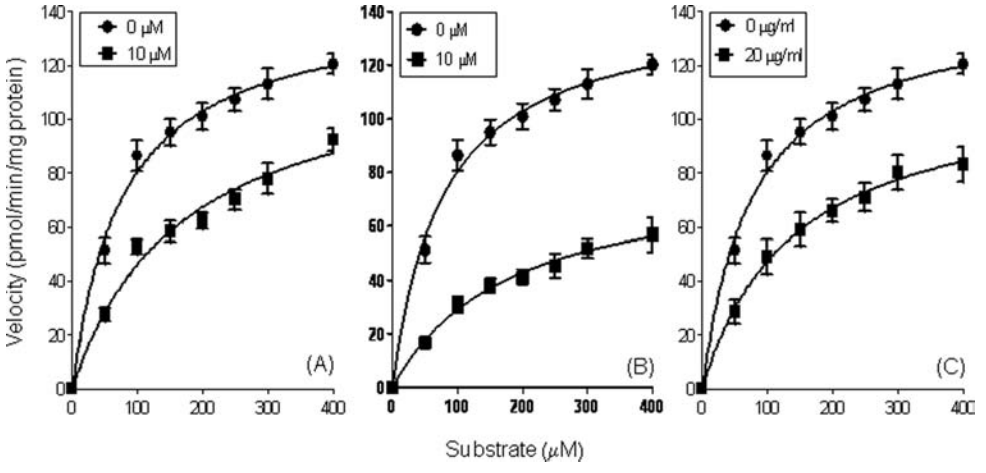

Figure 5. Inhibitory kinetics of cocoa procyanidin pentamer (A), decamer (B), and regular cocoa extract (C) on PL were determined using Michaelis−Menten analysis. Values are expressed as the mean ± SD of at least three independent experiments.

Table 2. Effects of Cocoa Procyanidin Pentamer, Decamer, and Regular Cocoa Extracts on V_{max} and K_m Values of PL[a]

	pentamer (μM)		decamer (μM)		regular cocoa extract (μg/mL)	
	0	10	0	10	0	20
V_{max}	142.8 a	123.8 b	142.8 a	81.38 b	142.8 a	113.5 b
K_m	77.91 a	168.3 b	77.91 a	180.5 b	77.91 a	140.2 b
inhibition type	mixed		mixed		mixed	

[a] For each inhibitor, values in the same row not sharing a common letter are significantly different ($p < 0.05$).

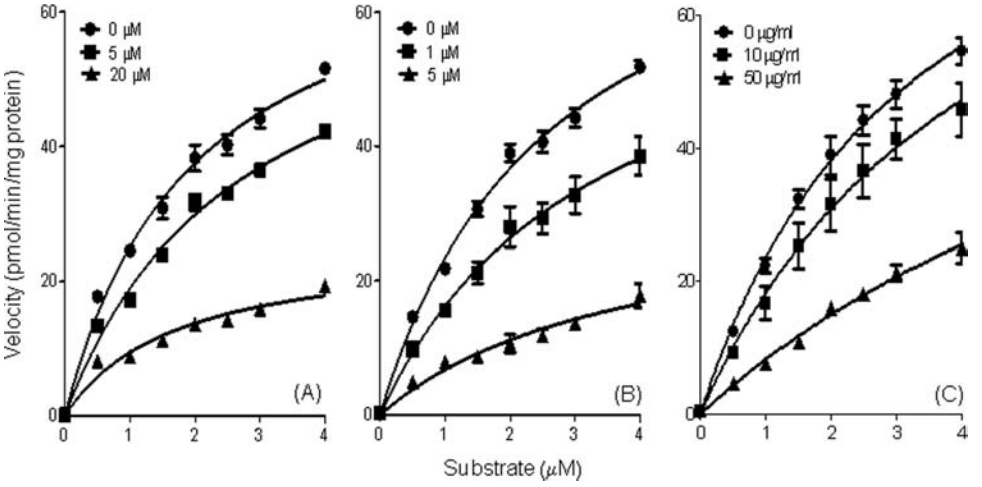

Figure 6. Inhibitory kinetics of cocoa procyanidin pentamer (A), decamer (B), and regular cocoa extract (C) on PLA_2 were determined using Michaelis−Menten analysis. Values are expressed as the mean ± SD of at least three independent experiments.

Table 3. Effects of Cocoa Procyanidin Pentamer, Decamer, and Regular Cocoa Extract on V_{max} and K_m Values of PLA_2[a]

	pentamer (μM)			decamer (μM)			regular cocoa extract (μg/mL)		
	0	5	20	0	1	5	0	10	50
V_{max}	74.66 a	69.97 a	25.53 b	85.14 a	69.53 b	32.37 c	53.14 a	53.36 a	50.91 a
K_m	1.981 a	2.691 a	1.71 a	2.637 a	3.283 a	3.793 a	3.361 a	4.685 a	11.55 b
inhibition type	noncompetitive			noncompetitive			competitive		

[a] For each inhibitor, values in the same row not sharing a common superscript are significantly different ($p < 0.05$).

These results mirror the results of our study on cocoa extracts and suggest that procyanidins are the components in cocoa responsible for the inhibition of these digestive enzymes. The inhibitory potency of the cocoa procyanidins is increased as a function of DP. These results are in agreement with some recent studies. Sugiyama et al. reported that the oligomeric procyanidins in apples significantly decreased the plasma triglyceride levels in both mice and humans and inhibited PL activity in vitro. They also suggested that DP was an important factor in determining the inhibitory potency, and a strong inverse correlation was observed.[24] Another study found that procyandins from persimmon peel showed strong inhibitory activity against α-amylase in vitro (IC_{50} < 100 μg/mL), and the inhibition of α-amylase activity was dependent on the DP.[26]

The results of kinetic analysis suggested that regular cocoa extracts and the procyanidin pentamer and decamer inhibited PL activity in a mixed mode. By contrast, the procyanidin pentamer and decamer noncompetitively inhibited PLA_2 activity, whereas the regular cocoa extract inhibited PLA_2 in a competitive fashion. These results demonstrate the diversity of potential interactions between the procyanidins, the enzyme surface, and/or the substrate, and such interactions need further study by in silico or crystallographic methods. These results suggest that other compounds in cocoa beyond the procyanidins might also contribute to the inhibitory potency of the extract. In addition to the flavanols, cocoa is also rich in methylxanthines (caffeine, theobromine, and theophylline), which have been shown to have thermogenic, diuretic, and appetite-suppressing properties that may aid in obesity and diabetes prevention.[27] However, scientific data in relation to the in vitro inhibition of digestive enzymes by methylxanthines are still limited.

The biological properties of cocoa polyphenols are modulated by their bioavailability. One proposed limitation of cocoa procyanidins is their low systemic bioavailability. Studies have shown that monomers and dimers in cocoa can be absorbed, and they began to appear in plasma within 30−60 min after consumption.[28,29] Despite their presence in cocoa in high amounts, procyanidin oligomers larger than dimers have not been detected

in human plasma following the consumption of cocoa products.[30] However, because our studies are focused on the small intestine lumen as the site of action, we believe the bioavailability is not a limiting factor. Previous studies have shown that these compounds are stable in the stomach and small intestinal milieu and are expected to be present in the small intestinal lumen at relatively high concentrations following consumption of cocoa products, particularly those with high polyphenol content (e.g., dark chocolate).[31,32] We believe that the effective concentrations in our enzyme inhibition assays are physiologically achievable in this situation, although further studies are needed to confirm the in vivo activity and small intestinal bioavailability of these compounds.

In summary, the present study provides the first evidence that cocoa extracts and cocoa procyanidins are potent inhibitors of key enzymes in the digestion of carbohydrates and lipids in vitro, and these inhibitory activities are related to polyphenol content in cocoa extracts and the degree of polymerization of cocoa procyanidins. Further in vivo studies are needed to examine whether cocoa extracts and/or cocoa procyanidins can inhibit digestive enzymes in vivo and related downstream pathways such as aberrant eicosanoid metabolism at dose levels achievable in the diet.

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ABBREVIATIONS USED

BMI, body mass index; DP, degree of polymerization; EC, (−)-epicatechin; IC_{50}, median inhibitory concentration; K_m, lis−Menten constant; PA, pancreatic α-amylase; PL, pancreatic lipase; PLA_2, secreted phospholipase A_2; V_{max}, maximum velocity.

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