JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Epigallocatechin-3-gallate Inhibits Lactase but Is Alleviated by Salivary Proline-Rich Proteins

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ABSTRACT: Lactase phlorizin hydrolase is a small intestinal brush border enzyme that catalyzes the hydrolysis of the milk sugar, lactose, and also many flavonoid glucosides. We demonstrate that epigallocatechin-3-gallate (EGCG), the principal flavonoid from green tea, inhibits *in vitro* hydrolysis of lactose by intestinal lactase. We then tested the hypothesis that salivary proline-rich proteins (PRPs) could modulate this inhibition and stabilize EGCG. Inhibition by EGCG of digestive enzymes (α-amylase > chymotrypsin > trypsin > lactase ≫ pepsin) was alleviated ∼2−6-fold by PRPs. Furthermore, PRPs appeared stable to proteolysis and also stabilized EGCG under digestive conditions *in vitro*. This is the first report on EGCG inhibition of lactase, and it quantifies the protective role of PRPs against EGCG inhibition of digestive enzymes.

KEYWORDS: Salivary proline-rich proteins, epigallocatechin gallate, amylase, lactase, protease, enzyme inhibition

INTRODUCTION

Polyphenols constitute one of the most numerous and ubiquitous groups of plant metabolites in all plant organs and, thus, are an integral part of both human and animal diets. Two of the reasons for the increasing interest in polyphenols are (i) their potential utility in reducing the risk of various chronic diseases, such as cardiovascular and neurodegenerative diseases,[1−3] and (ii) the effects caused by their ability to bind and precipitate macromolecules, such as digestive enzymes and also dietary proteins and carbohydrates, thereby modulating their digestibility.[4−9] For a given polyphenolic structure, the ability to bind and precipitate different proteins may vary considerably. The salivary proline-rich proteins (PRPs), which comprise about 70% of the protein content of saliva,[10] possess a very strong affinity for certain polyphenols.[10] This high relative affinity supports the formation of stable polyphenol−PRP complexes.[11,12]

Lactase is an important brush border digestive enzyme, which hydrolyses the milk sugar lactose but also plays a critical role in the absorption of many flavonoids by removing the sugar moiety from, for example, glucosides of quercetin and genistein.[13] Little is known about the ability of dietary components to inhibit lactase activity.

Epigallocatechin gallate (EGCG), the major catechin in green tea, possesses potent *in vitro* antioxidant activity.[14,15] The interactions of tea polyphenols or EGCG with the digestive enzymes α-amylase, pepsin, trypsin, and lipase has already been studied, with reference to their antinutritional effects[6] and potential control of obesity and diabetes.[4] Because much speculation exists regarding the role of PRPs and there is increasing data on the inhibition of digestive enzymes by polyphenols as playing a role in diabetes and metabolic syndrome,[4] there is an urgent need to establish whether PRPs really do modulate the inhibitory activity of polyphenols and to what extent.

MATERIALS AND METHODS

Materials. Human salivary α-amylase (EC 3.2.1.1), bovine pancreatic trypsin (EC 3.4.21.4), bovine pancreatic α-chymotrypsin (EC 3.4.21.1), pepsin from porcine stomach mucosa (EC 3.4.23.1), intestinal acetone powder from rat (as a source of lactase), glucose oxidase from *Aspergillus niger* (1.1.3.4), horseradish peroxidase (EC 1.11.1.7), bovine serum albumin (BSA), *N*-α-benzoyl-L-arginine ethyl ester (BAEE), *N*-acetyl-L-tyrosine ethyl ester (ATEE), dinitrosalicylic acid (DNS), soybean trypsin inhibitor, and EGCG were all purchased from Sigma Chemicals (Dorset, U.K.), while lactose and glucose were supplied by Fischer Scientific. PRPs were extracted from human saliva as trichloroacetic acid (TCA)-soluble proteins,[16] which were then dialyzed using dialysis tubing with a molecular weight cutoff of 12 kDa (Medicell International, Ltd.), as described previously.[17] All other chemicals and solvents were pure and of high analytical grade and used without any further purification.

Enzyme Activity Assays. For the activity measurement of salivary α-amylase, the procedure of Carmona et al. was used.[5] The activity of pepsin was monitored through the measurement of TCA-soluble peptides at 280 nm,[18] while the measurement of trypsin activity was based on spectral differences between BAEE and the carboxylate form of *N*-benzoyl-L-arginine.[19] For α-chymotrypsin activity, the change in absorbance at 237 nm was followed in a reaction mixture containing ATEE.[19]

For the lactase assay, 50 mg of acetone-extracted powder from rat intestine was weighed into a 1.5 mL tube, 1 mL of 1.5 mM sodium phosphate buffer (pH 6.8) was added, and the contents were vortexed and then centrifuged for 10 min at 17000*g*. The protein contents of the supernatant were determined using the Bradford assay.[20] The assay mixture contained 0.2 mL of supernatant containing enzyme, 0.2 mL of 50 mM lactose, and 0.1 mL of phosphate buffer at the indicated pH. Simultaneously, two blanks were prepared: blank 1 contained only the substrate (0.2 mL) and buffer (0.3 mL), whereas blank 2 contained only the enzyme (0.2 mL) and the buffer (0.3 mL). The reaction, started by adding the substrate, was carried out at 37 °C for 60 min and stopped by

Received: August 7, 2010
Accepted: February 4, 2011
Revised: January 25, 2011
Published: February 24, 2011

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adding 0.75 mL of Tris-HCl buffer. The amount of free glucose produced was measured using the glucose oxidase/peroxidase assay.[21] To avoid inhibition of glucose oxidase by EGCG during the assay, polyphenols were extracted from the assay solution after the reaction using Supelco [hydrophilic−lipophilic balance (HLB), 60 mg, 3 mL] solid-phase extraction (SPE) cartridges. The assay solution was passed through the cartridges preconditioned with 1 mL of methanol and 1 mL of phosphate buffer. The eluent containing no polyphenols was collected for the glucose oxidase/peroxidase assay. The assay mixture containing 0.25 mL of eluent from the SPE cartridge, 0.5 mL of the glucose oxidase/peroxidase reagent, and 0.25 mL of the phosphate buffer was incubated at 37 °C for 30 min in a water bath, and the reaction was stopped using 0.5 mL of 6 M sulfuric acid. The absorbance was read at 540 nm. The amount of glucose was determined by a comparison to a standard curve of 10−100 μg/mL glucose. The retention of EGCG by the HLB cartridges was checked by passing 0.25 mM aqueous solution of EGCG and determining the decrease in the absorbance of the eluent at 270 nm.

Inhibition of Enzymes by EGCG and the Effect of PRPs on Enzyme Inhibition. To judge the effect of EGCG upon digestive enzyme activity, EGCG (0.05−0.5 mM dissolved in buffers appropriate for the enzymes to be tested) was added to each enzyme assay. As a control, equal volumes of buffer without EGCG were added. The level of enzyme inhibition in the presence of EGCG was calculated as a percentage of the control. The effect of PRPs on the inhibition of each enzyme by EGCG was measured as described above, except that the dialyzate (obtained after dialysis of the TCA-soluble fraction of saliva and estimated to contain 0.7 mg/mL protein by the Bradford method) was added to the assay mixture to obtain a PRP/EGCG ratio of 3:1.

Kinetics of Enzyme Inhibition by EGCG. Kinetic parameters were determined by assaying the enzyme activity in both the absence and presence of 0.05, 0.1, and 0.2 mM EGCG at different substrate concentrations. The concentration of starch and lactose varied from 10 to 50 mM in the α-amylase and lactase assay, respectively. In the experiments with α-chymotrypsin, the ATEE concentration was between 0.1 and 0.7 mM, while with trypsin, BAEE was 0.03−0.2 mM. K_m and V_{max} values were calculated from straight lines obtained by plotting I/V versus $I/[S]$.[22]

Effect of PRPs on the Inhibition of α-Amylase by EGCG by Varying the Temperature and pH. The effect of PRPs on the inhibition of α-amylase was also studied at various pH values (5.0−9.0) and incubation temperatures (15−60 °C). All experiments were carried out in triplicate, and data were expressed as the mean ± standard error of the mean (SEM) of three replicates.

Stability of PRPs during *in Vitro* Digestion. Synthetic gastric and duodenal juices were prepared according to Oomen et al.[23] The pH values of the digestive juices were checked and, if necessary, adjusted with 1 M NaOH or HCl. A 5 mL aliquot of the EGCG−PRP mixture (preincubated for 30 min) was mixed with 10 mL of the gastric juice (pH 2.07, prepared by mixing 10 mg of pepsin, 10 mg of BSA, 47 mM NaCl, 2.2 mM NaH_2PO_4, 11 mM KCl, 3.6 mM $CaCl_2$, 5.6 mM NH_4Cl, 0.45 M HCl, 3.6 mM glucose, 1.4 mM urea, 50 μM glucuronic acid, and 1 mM glucosamine hydrochloride) and then incubated for 2 h at 37 °C. Another 5 mL aliquot of EGCG−PRP was mixed with 15 mL of duodenal juice (pH 7.8, comprising 15 mg of BSA, 45 mg of pancreatin, 7.5 mg of lipase, 80 mM NaCl, 40 mM $NaHCO_3$, 0.6 mM KH_2PO_4, 8 mM KCl, 0.5 mM $MgCl_2$, 0.45 M HCl, 1.6 mM urea, and 2 mM $CaCl_2$) and incubated in the same way. At the end of the incubation, both samples were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) to observe any degradation of the PRP.

SDS−PAGE. SDS−PAGE was performed using a Mini-Protean II electrophoresis cell (Bio-Rad, Hertfordshire, U.K.).[24] A 500 μL aliquot of each of the four samples (PRP and PRP−EGCG before digestion, PRP−EGCG after gastric digestion, and PRP−EGCG after duodenal digestion) were mixed with equal volumes of sample treatment buffer [made by mixing 2.5 mL of stacking gel buffer (0.5 M Tris-HCl at pH 6.0), 2.0 mL of glycerol, 4.0 mL of 10% SDS, and 0.1 mg of bromophenol blue to 10 mL of water]. The molecular-weight marker proteins (Bio-Rad, 161-0304) were diluted in 1:20 in buffer, heated for 5 min at 95 °C, and cooled, and 20 μL/well of each of the sample (containing 14 μg of protein) and marker proteins were loaded on a 12.5% precast gel (Bio-Rad). Electrophoresis was carried out at a constant voltage of 200 V, 75 mA, and 15 W for 40 min. Gels were stained with Coomassie Brilliant Blue staining solution for 1 h (1.0 g in 250 mL of 95% ethanol and 100 mL of glacial acetic acid, diluted to 1 L) and then destained for 3 h using destaining solution (made by mixing 250 mL of 95% ethanol and 100 mL of glacial acetic acid and diluting to 1 L with water).

Stability of EGCG during *in Vitro* Digestion. A 100 μL aliquot of EGCG in water (1 mg/mL) was combined with 430 μL of PRPs (0.7 mg/mL), then mixed with 370 μL of gastric or duodenal juices, checked for pH, and incubated for 2 h at 37 °C. As a control, EGCG−PRP was incubated without any digestive juice. To determine the effect of digestion upon EGCG degradation, 100 μL of EGCG was mixed with 370 μL of digestive juice, diluted to 900 μL, checked for pH, and then incubated as above. At the end of the incubation, 100 μL of taxifolin (0.664 mg/mL) was added to each sample as an internal standard. EGCG was extracted with ethylacetate from each of the samples, which was subsequently evaporated under vacuum using a Genevac (EZ-2 Series) and then reconstituted in 20% acetonitrile.

Samples were analyzed for EGCG in triplicate using high-performance liquid chromatography (HPLC), and the results were expressed as the mean ± standard deviation (SD). The HPLC system (Agilent Technologies 1200 Series) consisted of a solvent delivery system, autosampler, column oven, diode array detector, and Agilent ChemStation Software. Analysis was carried out using an isocratic mobile phase, consisting of 90:10 0.2% aqueous formic acid versus 0.2% formic acid in acetonitrile at 1.7 mL/min. A 10 μL portion of sample was injected onto a XDB-C18 column (4.6 × 50 mm, 1.8 μm; Agilent Technologies, Santa Clara, CA) and held at 35 °C. EGCG was quantified using a standard curve obtained by plotting the ratio of the EGCG to taxifolin peaks versus the concentration of EGCG.

Statistical Analyses. All of the experiments on inhibition, effect of PRPs, and inhibition of α-amylase at varying temperature and pH values were performed in triplicate, and the data were expressed as the mean ± SEM. IC_{50} values of EGCG in the presence and absence of PRPs and effect of temperature and pH on the activity of α-amylase, EGCG−α-amylase, and PRP−EGCG−α-amylase differences were analyzed for significant differences using one-way analysis of variation (ANOVA) followed by a Bonferroni test for multiple comparisons using Originlab 8.0.

RESULTS

As expected, EGCG inhibited α-amylase, trypsin, and chymotrypsin. It also inhibited lactase but not pepsin, and the extent and nature of inhibition varied from enzyme to enzyme (Table 1). The inhibition of lactase has not, to our knowledge, been reported before. Partially purified PRPs from saliva significantly decreased the inhibitory effect of EGCG, as indicated by IC_{50} values, for all of the enzymes investigated, from 1.7-fold (lactase) to 6.2-fold (α-amylase) (Table 1). Because the interaction between enzyme, PRP, and EGCG takes place in the gastrointestinal tract, we tested to see if EGCG and PRP were stable under digestive conditions. Several bands of proteins were resolved on SDS−PAGE (Figure 1). No change in the pattern of bands before and after digestion of PRP−EGCG was observed. The stability of EGCG was also tested in the presence and absence of PRP, under stomach and duodenal digestion conditions *in vitro*. EGCG was stable under gastric conditions, but more than 50% was lost under duodenal conditions. In the

Table 1. IC_{50} Values for the Inhibition of Digestive Enzymes by EGCG with and without the Addition of the PRP-Rich Fraction[a]

	IC_{50} (μM)	IC_{50} with PRP fraction (μM)
enzyme	mean ± SEM	mean ± SEM
α-amylase	28 ± 2.0 a	174 ± 3.0 b
chymotrypsin	46 ± 0.4 a	157 ± 4.0 b
trypsin	57 ± 3.0 a	143 ± 0.5 b
lactase	74 ± 2.0 a	126 ± 3.0 b

[a] Values within the same row (a and b) were significantly different at $p < 0.05$.

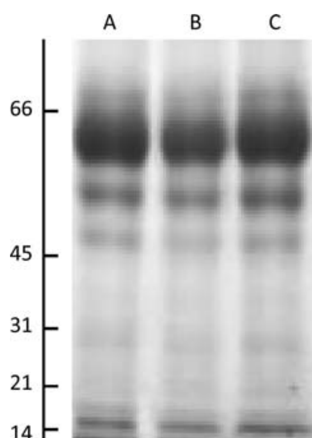

Figure 1. SDS−PAGE (12.5% polyacrylamide gel) of the PRP fraction from human saliva in the presence of EGCG under (A) control, (B) gastric, and (C) duodenal conditions *in vitro*. The numbers on the *y* axis represent the position of protein molecular-weight standards in kilodaltons.

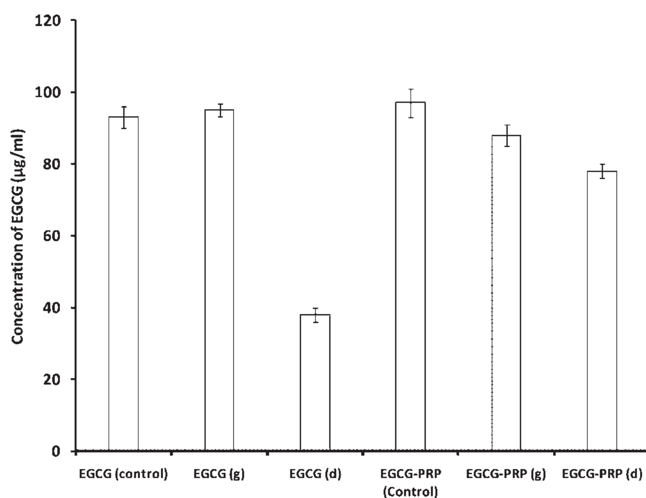

Figure 2. Amount of EGCG, as measured by HPLC, after incubation under control, gastric (g), or duodenal (d) conditions in the presence or absence of a PRP-rich fraction from human saliva.

presence of the PRP fraction, EGCG was stabilized under duodenal digestion conditions (Figure 2).

Because α-amylase was the enzyme most inhibited by EGCG and most affected by PRP, we examined this interaction further. As expected, the optimum pH for human salivary α-amylase was 5 (Figure 3). At this pH, EGCG at a concentration of 100 μM decreased the activity by ∼30%, which PRPs alleviated to ∼20%. At more acidic pH, the activity of the α-amylase was lower but EGCG reduced the activity proportionally more, e.g., by >60% at pH 2 (Figure 3). The inhibition of α-amylase by EGCG and the alleviation by PRP were also temperature-dependent. Below 35 °C, the behavior described above was seen. At higher temperatures, however, the PRP fraction and EGCG−PRP caused an increase in activity, presumably by complexing with the amylase, stabilizing it, and slowing denaturation, during the course of the reaction (Figure 4).

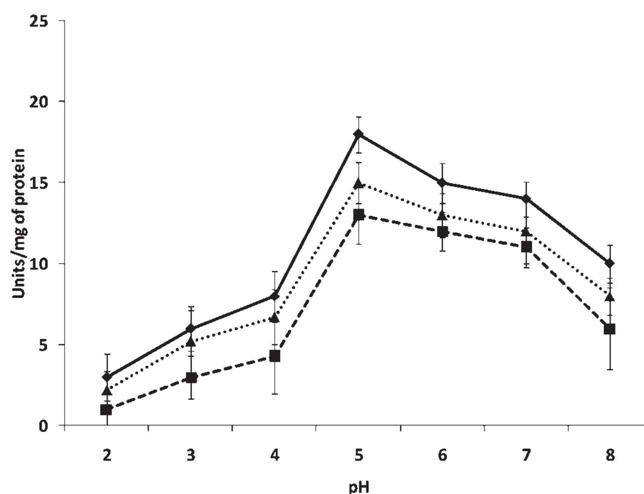

Figure 3. Effect of pH on the activity of α-amylase free (◆), with EGCG (100 μM) (■), or with EGCG (100 μM) and a PRP-rich fraction (138 g/ml) from human saliva (▲) at 37 °C. Values are the mean ± SEM.

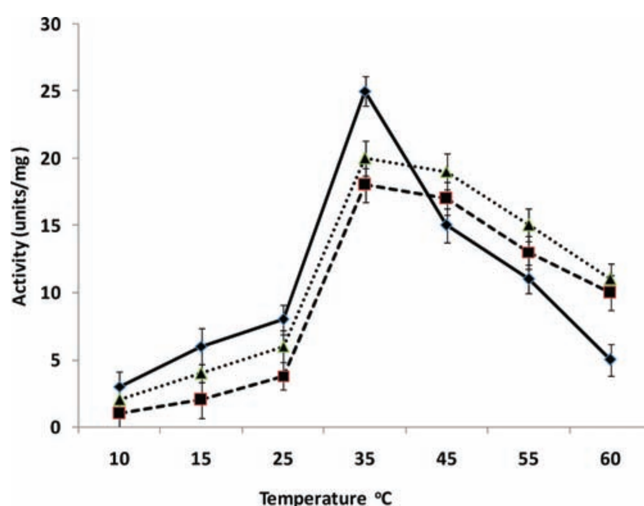

Figure 4. Effect of the temperature on the activity of α-amylase free (◆), with EGCG (100 μM) (■), or with EGCG (100 μM) and a PRP-rich fraction (300 μM) from human saliva (▲) at pH 5.0. Values are the mean ± SEM.

■ DISCUSSION

This is the first report showing that EGCG inhibits lactase, a brush border enzyme that hydrolyses the milk sugar, lactose. The concentration of EGCG required for significant inhibition is within the physiological range expected in the lumen of the

gastrointestinal tract after consumption of green tea.[25] This inhibition was alleviated by salivary PRPs, which are also stable enough to reach the site in the gut where lactase is active, and therefore, would protect lactase from EGCG inhibition. This interaction has not been reported before and is important when considering the nutritional effects of EGCG and carbohydrate digestion.

EGCG inhibits other digestive enzymes, such as human salivary α-amylase, by non-competitive inhibition with an IC_{50} value of 260 μM.[4] Trypsin was inhibited by a green tea polyphenolic extract (containing 44.8% EGCG).[26] EGCG inhibits proteasomal chymotrypsin-like activity[27] and has also been reported to inhibit pepsin,[6] whereas similar polyphenols activate this enzyme.[28] The inhibition of α-amylase by various flavonoids depends upon hydrogen bonds between the hydroxyl groups of the polyphenols and the catalytic residues of the binding site.[29]

PRPs are well-known polyphenol complexing proteins, owing to a high content of proline residues, and using nuclear magnetic resonance (NMR), the predominant mode of association was shown to be hydrophobic stacking of the polyphenol ring against the pro-S face of proline. The first proline residue of a Pro-Pro sequence was a particularly favored binding site.[30] This strength of binding derives from the fact that proline-rich polypeptides have highly restricted mobility (and, therefore, relatively low entropy) even before binding.[31] The binding leads to a smaller drop in entropy than it would for a normal, more flexible, peptide, and hence, a greater overall binding energy is achieved.

PRP in complex with EGCG was stable to both gastric and duodenal digestive conditions. This is in agreement with the fact that peptide bonds involving proline residues resist proteolytic breakdown,[32,33] and thus, PRPs and EGCG−PRP should survive exposure to gastrointestinal proteases in the stomach and the intestinal lumen *in vivo*. In addition, in comparison to pure EGCG, EGCG−PRP was much more stable under *in vitro* duodenal digestion conditions, consistent with a stabilizing effect of PRPs on polyphenols. This stabilization through complexation with PRPs may enable EGCG to partially resist degradation during digestion *in vivo*, with potential ramifications on the subsequent bioavailability of this molecule.

There have been several reports on the role of polyphenols as modulating digestion of sugars and the consequent effect on absorption of glucose into the bloodstream.[4−7,9] This effect is often considered a benefit because the slowed sugar digestion and absorption leads to a blunted glucose absorption curve, which is preferable to postprandial glucose "spikes".[34,35] However, the inhibitory effect of many polyphenols on several digestive enzymes has never considered the role of PRPs in modulating this effect. This could explain why the inhibition by green tea of crude amylase, as present in unpurified saliva, was unpredictable.[36]

In view of the results, it can be concluded that PRPs play a significant role in preventing digestive enzymes from being inhibited by EGCG and that this effect persists even under gastric and duodenal digestive conditions *in vitro*.

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Funding Sources

This study was supported by a British Commonwealth Scholarship/Fellowship to Shahina Naz.

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