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Inhibitory Effect of Four Types of Tea on the \textit{in vitro} Digestion of Starch

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Abstract

Phenolic compounds have been shown to decrease the rate of starch hydrolysis by inhibiting digestive enzymes for starch. Tea, rich in phenolic compounds, has been widely reported for its beneficial health effects. This study explored the inhibitory abilities of four types of tea, i.e., green tea (GT), oolong tea (OT), black tea (BT), and white tea (WT), on the in vitro enzymatic digestion of potato starch (PS) and high-amylose maize starch (HAMS), respectively, using two in vitro digestion methods. All teas significantly inhibited the digestion of both starches, with a few exceptions at some time points. The resistant starch content was increased by all four teas in HAMS, but only by BT and WT in PS. The total phenolic contents in the teas could not predict the inhibititotry ability of the teas, possibly due to the structural diversity of phenolic compounds and the presence of other compounds that could also alter enzymatic activity. Our results suggested that tea, regardless of its type, can slow down the enzymatic digestion of starch and therefore imply a potential benefit for controlling postprandial hyperglycemia.

Keywords

Starch; in vitro digestion; resistant starch; tea; total phenolic content
1. Introduction

Starch is a polysaccharide comprising of a large number of glucose units linked by α-glycosidic bonds. Abundant in staple foods, e.g., wheat, maize (corn), rice, and potatoes, starch is the most common dietary carbohydrate and serves as a major source of energy in human diets. In the gastrointestinal tract (GIT), starch is digested by a series of enzymes into glucose for absorption. The kinetics of starch digestion and that of glucose release/absorption as the energy source for the body determine the nutritional quality of starch and starch-containing foods (Zhang & Hamaker, 2009). Rapid starch digestion may cause postprandial hyperglycemia and hyperinsulinemia cycle, which is involved in a variety of metabolic diseases including metabolic syndrome, obesity, type 2 diabetes, and cardiovascular diseases (Byrnes et al., 1995; Ludwig, 2002). Starch in certain foods, especially heavily processed foods (e.g., refined flour), can be rapidly digested and results in the rapid elevation of blood glucose levels. On the contrary, certain starch portions are slowly digested and even indigestible, and therefore cause a slower and steadier rise in blood glucose concentration.

While the structure, processing, and storage conditions of starch greatly dictates its rate of digestion (Jayawardena et al., 2017; Tamura et al., 2019), another critical factor is the activity of starch digestive enzymes in the GIT. α-Amylase is the major enzyme responsible for starch digestion in the intraluminal phase. It is present in the mouth as salivary α-amylase and in the small intestine as pancreatic α-amylase. Recent studies have demonstrated the ability of phenolic compounds, a large group of plant secondary metabolites, to inhibit the activity of α-amylase and thus slow the rate of starch digestion (Sales et al., 2012; Xiao et al., 2013). A natural and abundant source of phenolic compounds is tea, which is also known to confer other health benefits such as antioxidant, anti-inflammatory, and anti-tumor properties (Pan et al., 2013; Su et
al., 2016; Yen-Chen et al., 2018). Yet, previous studies that examined the effect of tea polyphenols on starch digestion showed inconsistent results. For instance, Sun et al. (2018) showed that tea polyphenols inhibited pancreatic α-amylase activity, and Kwon et al. (2008) reported that black tea (BT), green tea (GT), and oolong tea (OT) had a mild inhibitory effect (ranging from 35 to 40 percentage inhibition) on pancreatic α-amylase. On the contrary, Yang and Kong (2016) found the same teas to enhance the enzymatic activity. This same study, however, also found that high concentrations of isolated tea polyphenols mildly inhibited α-amylase activity. Such findings indicate that while polyphenols inhibit α-amylase activity, another component of tea may instead enhance it.

Despite the invaluable foundation set by previous studies, discrepancies clearly exist regarding the effect of tea and tea polyphenols on α-amylase activity and starch digestion. Therefore, this study further investigated the topic via a multifaceted approach to assess the effects of different types of tea, including GT, BT, OT, and white tea (WT), on the digestion of two types of starch, i.e., potato starch (PS) and high-amylose maize starch (HAMS). Two simulated in vitro digestion methods that have been widely adopted by different researchers were utilized. One measured the total reducing sugar contents at 1 h and 2 h upon digestion using a method outlined by Flores et al. (2014), while the other measured solely the glucose contents at 20 min and 120 min using a method described by Englyst et al. (1992). The former method aims to obtain the percent inhibition by the inhibitors, i.e., the teas. The latter method established the starch digestibility profiles, where the glucose content at 20 min represents rapidly digestible starch (RDS), that at 120 min is slowly digestible starch (SDS), and the remaining starch is regarded as resistant starch (RS) (Englyst et al., 1992). The total phenolic content in the teas were determined to evaluate a potential correlation with their inhibitory effects. The two
starches, with varied in their amylose contents (approximately 25% and 80% (w/w) in PS and HAMS, respectively), were utilized to assess the potential outcomes of differing amylose content in starch.

2. Materials and Methods

2.1 Materials

High amylose maize starch (HAMS; Gelose 80) was obtained from Ingredion (Bridgewater, NJ, USA). Potato starch (PS, S2004), amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, A7095, 300 U/mL), porcine pancreatin (EC 232-468-9, P7545, 8 x USP), invertase from baker’s yeast (EC 3.2.1.26, I4504, 300 U/mg), guar gum (G4129), sodium acetate trihydrate (S7670), maltose (M5885), and 3,5-dinitrosalicylic acid (128848), urea (U5378), lipase (L3126, EC 3.1.1.3), and porcine bile extract (B8631) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Potassium sodium tartrate tetrahydrate (AAA10163-30), sodium phosphate monobasic (97061-942), sodium phosphate dibasic (97061-588), sodium chloride (97061-904), sodium bicarbonate (BDH9280) were purchased from VWR chemicals, LLC (VWR chemicals, Solon, OH, USA). English breakfast black tea (BT) and green tea (GT) (Twinings®) were purchased from a local grocery store. White tea (WT) and oolong tea (OT) were purchased from grocery stores in Anxi and Anji (Fujian, China), respectively. D-glucose assay kit (GOPOD format, K-GLUC) was purchased from Megazyme (Wicklow, Ireland).

2.2 Brewing of tea samples

Four types of tea leaves (1.0 g) were brewed with 40.0 mL of boiling deionized (DI) water for 20 min, cooled to the room temperature (20 °C), filtered with Whiteman No. 4 filter paper, and labeled as GT, BT, OT, and WT, respectively.
2.3 Determination of total phenolic content

The fast blue salts BB assay was adopted to determine the total phenolic content in the brewed tea samples according to Lester et al. (2012). In detail, 1.0 mL of 100-fold diluted tea samples was mixed with 1.0 mL of 0.1% (w/v) Fast blue BB for 30 s, followed by adding 0.1 mL of 5.0% (w/v) NaOH. The mixture was stirred with a vortex mixer and incubated for 90 min under light at room temperature (20 °C). After that, absorbance was measured at 420 nm using a Mettler Toledo UV5Bio UV-vis spectrophotometer (Mettler Toledo, Columbus, OH, USA). Gallic acid standard solutions were used to establish the standard curve and results were expressed as milligram of gallic acid equivalents (GAE) per liter of tea.

2.4 In vitro digestion – Flores method

In the first in vitro digestion study, two types of raw starch, PS and HAMS, were subject to in vitro digestion with and without the presence of brewed teas according to the method outlined by Flores et al. (2014). Specifically, 1.0 mL of each tea sample was added with 0.5 g of starch sample in a 50 mL test tube, followed by adding 6.0 mL of simulated intestinal fluid and 3.0 mL of bile fluid. The compositions of simulated digestive juices were shown in Table 1. The pH of the simulated digestive juices was adjusted with 1 M HCl or 1 M NaOH. As a control, 1.0 mL of DI water was added instead of brewed tea. In order to account for the interference in absorbance measurements by particles other than sugar (e.g., digestive fluid components and tea residuals), a blank that contained neither starch nor tea was used. The digestion trials were conducted in an orbital shaking water bath at 37 °C with a shaking speed of 160 rpm for 2 h. At 1 h and 2h of digestion, 0.5 mL aliquots were collected and immediately placed in a boiling water bath for 10 min to deactivate the enzymes and halt the reaction.
Table 1. Compositions of simulated digestive juices.

<table>
<thead>
<tr>
<th>Simulated intestinal juice</th>
<th>Bile juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mL DI water</td>
<td>500 mL DI water</td>
</tr>
<tr>
<td>7.012 g NaCl</td>
<td>5.259 g NaCl</td>
</tr>
<tr>
<td>0.564 g KCl</td>
<td>0.376 g KCl</td>
</tr>
<tr>
<td>3.388 g NaHCO₃</td>
<td>5.785 g NaHCO₃</td>
</tr>
<tr>
<td>80.0 mg KH₂PO₄</td>
<td>0.25 g Urea</td>
</tr>
<tr>
<td>50.0 mg MgCl₂</td>
<td>15.0 g Bile salts</td>
</tr>
<tr>
<td>0.1 g Urea</td>
<td></td>
</tr>
<tr>
<td>9.0 g Pancreatin</td>
<td></td>
</tr>
<tr>
<td>1.5 g Lipase</td>
<td></td>
</tr>
</tbody>
</table>

**pH**

8.1 ± 0.2 6.9 ± 0.1

After the reaction was terminated, each sample was centrifuged at 2700 g for 3 min and the supernatant was then diluted to a proper concentration. The reducing sugar content was then determined using the 3, 5-dinitrosalicylic (DNS) colorimetric assay. For the assay, 0.2 mL of the diluted samples was combined with 0.1 mL of DNS reagent. This solution was heated in a boiling water bath for 8 min and allowed to cool to room temperature (20 °C). DI water (0.9 mL) was then added to each sample for further dilution. Absorbance was measured at 540 nm using a Mettler Toledo UV5Bio UV-vis spectrophotometer. Absorbance readings were subtracted by the reading of the blank sample to eliminate the interference of foreign particles on light scattering. The standard curve was established using maltose standard solutions and the results were reported as maltose equivalent (mM).

2.5. *In vitro digestion – Englyst method*

The second *in vitro* starch digestion study was administered using the method reported by Englyst et al. (1992) with a few modifications. Raw starch (80 mg) alone or raw starch mixed with 1 mL of brewed tea were added into 20 mL round-bottom test tubes. A test tube with no starch and inhibitors was used as the blank. Also mixed into the test tubes were 10 mg guar gum,
6 mm glass beads, and 4 mL of 0.1 M acetate buffer. The reaction mixtures were placed in a water bath at 37 °C. Immediately before the digestion reaction, an enzyme solution, containing pancreatin (46.8 mg/mL prior to centrifugation), amyloglucosidase (13 U/mL), and invertase (187.5 U/mL), was prepared by dissolving or diluting the enzymes in deionized water. Enzyme solution (1 mL) was added into each tube at a certain time interval and this time interval was accurately followed for sampling purpose. The test tubes were capped and placed into a shaking water bath at 37 °C and 150 strokes per min. After incubating for 20 and 120 min, aliquots of 200 µL were taken from the reaction mixture and the reaction was stopped by mixing into 900 µL of 66% (v/v) ethanol vigorously. These digesta samples were kept at 4 °C overnight and then centrifuged (2700 g, 3 min). The glucose concentrations in the supernatant were measured using the glucose oxidase-peroxidase (GOPOD) method and used to calculate the amount of digested starch. The starch contents digested during the first 20 min and between 20 and 120 min were regarded as the RDS and SDS proportions, respectively. The amount of RS was determined as the amount of starch remaining after 120 min of digestion. Results were adjusted according to the measured total starch content (81.10 ± 5.72% and 100 ± 2.80% in PS and HAMS, respectively), and expressed as a percentage of total starch.

2.6 Statistical Analysis

All experiments were conducted in duplicates. Data were analyzed by one-way analysis of variance (one-way ANOVA) followed by Tukey multiple comparison test using the GraphPad Prism software (San Diego, CA). The letters a, b, and c indicate statistically significant differences, \( p < 0.05 \) (a > b > c).
3. Results and Discussion

3.1 Effect of teas on starch digestion

In order to evaluate the inhibitory effect of the four different teas on \textit{in vitro} enzymatic starch digestion, two types of starch, i.e., PS and HAMS, which vary in their amylose content, were subject to simulated \textit{in vitro} digestion with and without the presence of teas. Two simulated \textit{in vitro} digestion methods were used to evaluate starch digestion. In the first method, the extent of starch enzymatic digestion after 1 and 2 h was measured by the amount of digestion product, primarily reducing sugars, released from digestion and expressed as maltose equivalent (Figure 1). Samples without tea (NI) served as a control that demonstrated baseline starch digestion. All four teas, when compared to the control, displayed significantly ($p < 0.05$) lower values of reducing sugar from the digestion of both starch types. An exception was noted for OT in PS digestion at 2 h. These results suggested that tea has an inhibitory effect on the digestion of starch by $\alpha$-amylase in the pancreatin.

The digestion of HAMS and PS proceeded to approximately the same extent by 2 h, but the digestion at 1 h was significantly lower for PS. The difference in the digestion behavior of HAMS and PS may be attributed to the regularity and compactness of molecular arrangement in the two types of starch. Zhang et al. suggested that enzymatic resistance of native granular starch was probably due to the densely packed molecular order in the surface region of starch granules (Zhang et al., 2015). This difference in digestion may therefore be explained by the higher amyllopectin content of PS. Compared with amylose, amyllopectin molecules are much bulkier, highly branched and more densely packed in molecular arrangement. These properties make amyllopectin more difficult to hydrate and be accessed by enzymes in the aqueous phase. Hence,
the presence of more amylopectin in PS could have delayed the binding of α-amylase and its substrates, and thus lower the enzymatic digestion rate in PS.

**Figure 1.** Extent of HAMS and PS digested, without inhibitors (NI) and with the presence of green tea (GT), black tea (BT), oolong tea (OT), and white tea (WT), expressed as maltose equivalent, with and without the presence of teas after 1 h and 2 h of digestion. Significant differences among treatments within each figure are denoted by different letters (a > b > c, p < 0.05).

The percent inhibition values of the teas on starch digestion at 1 and 2 h were obtained and compared in **Figure 2.** It shows that the inhibitory ability on α-amylolysis showed no significant difference among the four teas, except for a lesser inhibition on HAMS digestion by OT at 2 h. Furthermore, the inhibitory percentage ranged from 8.87% to 20.45% at 2 h of digestion. The lower limit indicates a particularly mild inhibitory effect.
Figure 2. Percent inhibition of teas on HAMS and PS digestion after 1 h and 2 h of digestion using green tea (GT), black tea (BT), oolong tea (OT), and white tea (WT). Significant differences among treatments within each figure are denoted by different letters (a > b, \( p < 0.05 \)).

Starch digestion is executed primarily by a series of enzymes in the GIT, including \( \alpha \)-amylase and maltase (e.g., \( \alpha \)-glucosidase). The first simulated in vitro digestion method only involved the former enzyme. It is important to note, however, that the latter enzyme has been reported to be potently inhibited by polyphenols. In fact, studies that utilized polyphenols found in oregano (Gutiérrez-Grijalva et al., 2019) and olive oil (Figueiredo-González et al., 2019) demonstrated significantly greater inhibition on the activity of \( \alpha \)-glucosidase than on that of \( \alpha \)-amylase. Another study reported that green tea polyphenol extracts were a stronger inhibitor of \( \alpha \)-glucosidase than acarbose, which is a known \( \alpha \)-glucosidase inhibitor and common medication prescribed to diabetics (Yan et al., 2018). Hence, the second simulated in vitro digestion study was conducted to include the latter enzyme and obtain starch digestibility profiles as affected by different teas.
In the second digestive method, glucose contents of samples at 20 min and 120 min were measured in order to determine starch digestibility profiles, i.e., the amounts of RDS, SDS, and RS (Figure 3). Again, starches with no inhibitor (NI) were used as a control to demonstrate baseline starch digestion. The RS content in HAMS was 63%, which was very close to other reports in high amylose maize starches, e.g., the Hi-Maize 260 starch (Ingredion, 2019) and our recent report (Gutierrez et al., 2020). The RS content in PS was notably low, yet the RDS content was close to our previous reported value (Gutierrez et al., 2020). Both HAMS and PS are regarded as the type 2 RS, or RS2. The resistance mechanism of RS2 was proposed to be due to the densely packed molecular arrangement on the starch granule surface (Zhang et al., 2015). In particular, no other RS except for high amylose starches have been classified as dietary fiber by the United States Food and Drug Administration (Food and Drug Administration, 2018). Similar to the results of the first digestion method, all four teas inhibited the digestion of HAMS, which is expressed as a significantly increased proportion of RS and decreased RDS. The nominal RS content of HAMS was increased to up to 88% by adding BT. Comparable to results of the first digestion method, PS again had exceptions in exhibiting inhibitory effects, where GT and OT had no appreciable effect on RDS and RS contents. When the percent inhibition was calculated from these digestion data (Figure 4), the inhibitory effect of the teas was very different between the two types of starches. In digesting HAMS, the extent of inhibition was much higher than that observed in the first digestion method, but similarly, OT exerted the lowest inhibition upon digestion for 2h. The protocols, including the enzymes and their concentrations, were different between the two methods and could lead to different results. Indeed, upon digesting PS for 2 h, although it agreed with the first method that there was no difference in the percent inhibition values among the teas, the percent inhibition range was slightly lower than that in the first
method, and GT showed the relatively low inhibition. Overall, these results were consistent in that the inhibitory effect of tea is greater in the presence of starch containing higher proportions of amylose. Meanwhile, the inhibitory effect on starches with lower amylose contents depends on the type of tea.

**Figure 3.** Starch digestibility profiles, presented as rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) contents, of raw high amylose maize starch (HAMS) and raw potato starch (PS) without inhibitors (NI) and with the presence of green tea (GT), black tea (BT), oolong tea (OT), and white tea (WT). Error bars show standard deviation; n=2. Significant differences among treatments within each starch digestion fraction are denoted by different letters (a > b > c, p < 0.05).
Figure 4. Percent inhibition of teas on HAMS and PS digestion after 20 min and 2 h of digestion using green tea (GT), black tea (BT), oolong tea (OT), and white tea (WT). Significant differences among treatments within each figure are denoted by different letters (a > b, p < 0.05).

3.2 Phenolic content and inhibitory ability

Measurements for the total phenolic content of the teas demonstrate significant difference \((p < 0.05)\), except for between GT and OT (Figure 5). GT and OT contained the highest total phenolic content, while WT had the lowest. These results are similar to that of Annunziata et al., who measured the total phenolic contents of WT, GT, and BT using the Folin-Ciocalteu method (Annunziata et al., 2018). They found that WT had the lowest and GT had the highest phenolic contents. The classification of tea into GT, BT, OT, and WT is based on the degree of fermentation or oxidation. GT is unfermented or un-oxidized, WT is only slightly fermented, OT tea is a semi-fermented tea, while BT is a fully fermented or oxidized tea (Weerawatanakorn et al., 2015). Fermentation is known to reduce the phenolic content in tea (Jayasekera et al., 2014), yet there are many other factors determining the phenolic content in different tea samples. In the
present study, the total phenolic content was not a predictive variable for the inhibitory ability on α-amylolysis, as there was no significant difference in the activity among the four teas (Figure 2). The inhibitory ability of the teas did not increase with their total phenolic content. One possible reason is the diversity of phenolic compounds and their variance in inhibitory ability. Phenolic compounds are a large group of compounds derived from a phenol, and even within the same sub-group of phenolic compounds, their inhibitory effect may vary. For instance, the inhibitory effect of tannins (Mkandawire et al., 2013) and proanthocyanins (Zhang et al., 2016) both increased with molecular weight and degree of polymerization. Another study that examined two major polyphenols of tea, i.e., (-)-epigallocatechin (EGC) and (-)-epigallocatechin-3-gallate (EGCG), found that the starch digestion rate was unaffected by EGC but was significantly inhibited by EGCG (Xie et al., 2019). Despite the highly related structures of EGC and EGCG, there is an evident disparity in their inhibitory abilities. The total phenolic content determined cannot reveal the structural diversity of all phenolic compounds in the teas. This could be regarded as a limitation of the present study. Quantification of individual components of tea phenolics could help unravel which phenolic components may have a more profound inhibitory effect on starch digestion. Secondly, the digestive processes may have caused the varying results in inhibitory ability. Chen et al. reported that intestinal digestion was able to degrade proanthocyanins from Acacia mearnsii bark (Chen et al., 2018). Subsequently, its α-glucosidase inhibitory activities were reduced by approximately 47% compared to the bark extract after only gastric digestion. The various types of tea polyphenols may be degraded to different extents, and their digestive products likely vary in their bioactivities. Interestingly, the degradation of phenolic compounds can be beneficial. Annunziata et al. found that even though WT had the lowest total phenolic content compared to GT and BT, the intestinal digestion of WT
extract led to the highest duodenal and colonic bioaccessibility (Annunziata et al., 2018). Enhanced bioaccessibility may yield more potent antioxidant effects. Thirdly, the lack of correlation may also be attributed to other components in tea such as caffeine and ions. Caffeine was reported to enhance α-amylase activity (Kashani-Amin et al., 2013). Ions have been shown to generate varying effects on α-amylase activity. For instance, Aghajari et al. (2002) reported chloride ions to enhance α-amylase activity, while nitrate ions had no significant effect (Aghajari et al., 2002). In relation to this theme, the content of chloride and nitrate ions in GT, BT, and WT have been found to differ significantly (Mincă et al., 2013). Accordingly, it is reasonable to speculate that the mild inhibitory effect in many cases was a net result of the inhibitory effect of tea polyphenols and the augmenting effects of other components such as caffeine and ions. Given that the composition of the other components may differ among the teas, their compounding effects could also explain the lack of correlation between total phenolic content and inhibition percentage.

**Figure 5.** Total phenolic content, expressed as mg of GAE per liter of tea, in green tea (GT), black tea (BT), oolong tea (OT), and white tea (WT). Significant differences among samples are denoted by different letters (a > b > c, *p* < 0.05).

**4. Conclusion**

In this study, the inhibitory effect of four types of teas, specifically GT, BT, OT, and WT, on the enzymatic digestion of PS and HAMS was evaluated using two simulated *in vitro* digestion
methods. All teas, regardless of type, were able to significantly decrease the extent of starch digestion, with a few exceptions at some time points. In the first digestion method, the extent of digestion in both starches was the same at 2 h, while the slower digestion of PS at 1 h may be a result of its high amylopectin content. The second method further involved an important starch digestive enzyme, α-glucosidase, on which polyphenolic compounds might exhibit high inhibitory activities. Regarding starch digestibility profiles, RS was increased by all four teas in HAMS but only by BT and WT in PS. Since HAMS displayed consistent and significant decreases in starch digestion by all four teas, but PS did not, this is evidence that the amylose-amylopectin contents and their molecular arrangement might contribute to the extent of inhibition on digestion. Accordingly, the beneficial inhibitory effects may be most applicable in the digestion of high-amylose starches. The total phenolic contents in the teas were significantly different, except for green and oolong teas, but could not predict the inhibitory ability of the teas. It was possibly due to the structural diversity of phenolic compounds and the presence of other compounds, such as caffeine and ions, which could also alter enzymatic activity. Our in vitro method utilized porcine amylase and amyloglucosidase from Aspergillus niger, and therefore the teas may exhibit different results when using human enzymes or in vivo methods. Nevertheless, the net results of including tea in in vitro starch digestion appear to exhibit potential as a means of delaying starch digestion and glucose absorption. In addition to many known health benefits of tea polyphenols, the results of this study would promote tea consumption as a potential way of modulating glycemic response.

Conflict of Interest

The authors confirm that they have no conflict of interest to declare for this publication.


