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Inhibition of α-glucosidases by tea polyphenols in rat intestinal extract and Caco-2 cells grown on Transwell

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ABSTRACT

Inhibition of maltase, sucrase, isomaltase and glucoamylase activity by acarbose, epigallocatechin gallate, epicatechin gallate and four polyphenol-rich tea extract from white, green, oolong, black tea, were investigated by using rat intestinal enzymes and human Caco-2 cells. Regarding rat intestinal enzyme mixture, all four tea extracts were very effective in inhibiting maltase and glucoamylase activity, but only white tea extract inhibited sucrase and isomaltase activity and the inhibition was limited. Mixed-type inhibition on rat maltase activity was observed. Tea extracts in combination with acarbose, produced a synergistic inhibitory effect on rat maltase activity. Caco-2 cells experiments were conducted in Transwells. Green tea extract and epigallocatechin gallate show dose-dependent inhibition on human sucrase activity, but no inhibition on rat sucrase activity. The opposite was observed on maltase activity. The results highlighted the different response in the two investigated model systems and show that tea polyphenols are good inhibitors for α-glucosidase activity.

1. Introduction

Type-2 diabetes is one of the main diet-related diseases in the world (Mann, 2002). The number of type 2 diabetic patients is estimated to increase to more than 600 million by 2040 (Saeedi et al., 2019). A critical strategy for diabetes prevention is the control of postprandial glucose excursions. The hyperglycaemia can be prevented by some antidiabetic drugs, such as acarbose and voglibose via the inhibition of starch digestive enzyme activities (Lee et al., 2016). Recently, some plant-based polyphenols have been reported as alternatives to modulate starch digestibility, which have similar functions of acarbose (Simsek, Quezada-Calvillo, Ferruzzi, Nichols, & Hamaker, 2015).

Many studies have reported the inhibitory effect of tea polyphenols on various disaccharidases, thereby modulating the blood glucose level (Gao, Xu, Wang, Wang, & Hochstetter, 2013; Lim, Kim, Shin, Hamaker, & Lee, 2019). Although human enzymes are the ideal resources for experiments on enzyme inhibition, many *in vitro* studies used the more readily available rat intestinal enzymes (Simsek et al., 2015). However, rat and human α-glucosidases are quite different enzymes. Rat α-glucosidase is composed of two protein complexes, maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI) (Pyner, Nyambe-Silavwe, & Williamson, 2017). Both protein complexes consist of a C-terminal domain (ctMGAM and ctSI) and a *N*-terminal domain (ntMGAM and ntSI). ctMGAM and ntMGAM are glucoamylase and maltase, respectively. ctSI and ntSI are sucrase and isomaltase, respectively (Pyner et al., 2017). Both MGAM and SI have high α-1,4 hydrolytic activity on maltose. Each terminal of them have some unique activity, *eg.*, ctMGAM has high hydrolytic activity on larger starch-based oligomers and polymers, ctSI and ntSI have α-1,2 and α-1,6 hydrolytic activity, respectively (Lee et al., 2016). Besides rat intestinal α-glucosidase, human intestinal epithelial cells (Caco-2 cells) can be a more biological alternative to study the modulating effects of dietary compounds towards maltase and sucrase activity (Pyner et al., 2017). Differently from rat and human intestinal enzymes, Caco-2 cells only provide SI, and while MGAM is absent (Hauri, Sterchi, Bienz, Fransen, & Marxer, 1985). Caco-2 cells are human adenocarcinoma cells that spontaneously differentiate into small intestinal enterocytes expressing brush-border enzymes, *i.e.*, α-glucosidase (Verhoeckx et al., 2015). Enzyme extracts from the Caco-2 cells have been used as sources of sucrase and maltase by some researchers (Pyner et al., 2017). Compared to the use of enzyme extracts from Caco-2, a more physiologically realistic way to simulate disaccharides transport to cells, enzymatic

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hydrolysis by membrane bound brush-border enzymes and absorption of monosaccharides is to use Caco-2 cells grown on Transwell inserts, which have been used to study translocation of nutritional compounds from luminal side to the serosal site which would enter the blood (Pico, Corbin, Ferruzzi, & Martinez, 2019). To the best of our knowledge, few researchers have used intact Caco-2 cells grown on Transwell inserts to provide enzymes. Therefore in this study, rat intestinal enzymes and Caco-2 cells grown on Transwell were chosen as the source of α-glucosidase.

In our previous study, we reported that tea polyphenols can slow down starch digestibility by inhibiting pancreatic α-amylase (Sigma A4268, 1096 unites/mg) and interacting with starch, thus can potentially modulate the glycaemic index of bread (Kan, Capuano, Fogliano, Oliviero, & Verkerk, 2020). In this study, we further investigated the effect of tea polyphenols on α-glucosidase activities. Extracts from white, green, oolong and black tea (WTE, GTE, OTE and BTE, respectively) were chosen as source of tea polyphenols. These teas are popular tea among consumers and they have different polyphenols profile because of different processing method. White and green tea are non-fermented tea and white tea is less processed than green tea. Oolong and black tea are half fermented and fully fermented tea, respectively. Two models were used to provide α-glucosidase, *i.e.,* rat intestinal enzyme and Caco-2 cells in Transwells. Rat intestinal enzyme was used to provide α-glucosidase, and to study the influence of WTE, GTE, OTE, BTE, EGCG, ECG and polymeric fraction from BTE on α-glucosidase inhibition. Four substrates has been used to determine individual hydrolytic properties, *i.e.,* maltose, maltodextrin, sucrose and isomaltose. Kinetics of maltase inhibition and synergy of tea polyphenols and acarbose were carried out to further investigate the inhibition mechanisms. Caco-2 cells grown on Transwells were also used to provide α-glucosidase, and to study the influence of GTE and EGCG on inhibition of compartment Caco-2 derived maltase and sucrase and the effects towards intestinal integrity. The hypothesis of this study is rat and human α-glucosidase exhibit different sensitivity to tea polyphenol inhibition.

2. Materials and methods

2.1. Materials

White tea (LaPlace), green tea (Lipton), and black tea (Pickwick) were purchased from the local supermarket Jumpo in Netherlands. Oolong tea (Pickwick) was provided by Pickwick company in Netherlands. Maltose, sucrose, isomaltose, maltodextrin (dextrose equivalent 4.0–7.0), rat intestinal acetone powders as a source of brush border enzyme, acarbose, EGCG (epigallocatechin gallate), theaflavin, ECG (Epicatechin gallate), theaflavin-3-gallate, theaflavin-3′-gallate, penicillin–streptomycin solution, phosphate buffered saline (PBS), and fetal bovine serum were purchased from Sigma-Aldrich (Netherlands). Acetonitrile, methanol, ethanol, ethyl acetate, chloroform, BCA kit was purchased from Thermo Fisher Scientific. CaCo-2 cell lines were provided by American Type Culture Collection (ATCC).

2.2. Preparation of tea extract and separation of polymeric components

Preparation of tea extracts (TEs), separation of polymeric polyphenols from black tea were done according to our previous method (Kan, Capuano et al., 2020). Briefly, white tea, green tea, oolong tea and black tea were mixed with absolute methanol (1:10, *w/v*). Then the mixture was put in an ultrasound equipment (Sonication, China) for 30 min. The extraction was repeated for three times (Sonication, China). After centrifuging (4000*g*, 15 min), the supernatants were collected as tea extracts and dried on a freeze dryer (Alpha 2–4 LD plus, Christ). Then the polymeric fraction was separated from BTE. Briefly, BTE solution was prepared by dissolving BTE in 5% of aqueous ethanol. The monomeric and oligomeric fractions were removed by adding ethyl acetate into BTE solution. The remaining aqueous fraction was collected as polymeric fraction, and dried via freeze drying (Alpha 2–4 LD plus, Christ).

Quantification of tea polyphenols was performed according to our previously published paper (Kan, Capuano et al., 2020). Monomeric polyphenols composition of tea extract was analysed by HPLC-DAD (diode array detector). The tannins content in the tea extract was measured by BSA precipitation method.

2.3. Preparation of rat intestinal α-glucosidase

The preparation of intestinal α-glucosidase was according to a previous report with some modifications (Shin et al., 2019). Rat intestinal enzyme was mixed with 100 mM sodium phosphate buffer (pH 6.8) to give a final concentration of 25 and 50 mg/mL, respectively. The solution was stored at 4 °C for 24 h to extract crude α-glucosidases. The mixture was centrifuged (13500*g*, 25 min) and the supernatant was stored at −20 °C as the enzyme working solutions. The protein concentration of the enzyme working solution was determined with the BCA protein kit.

2.4. Hydrolytic properties of rat intestinal α-glucosidases

The glucose produced from hydrolysis of maltose (α-1,4), sucrose (α-1,2), isomaltose (α-1,6) and maltodextrin (dextrose equivalent 4.0–7.0) was determined respectively. Briefly, 150 μL of solvent blank (methanol) was mixed with 450 μL of maltose (10 mM), sucrose (30 mM), isomaltose (20 mM) or maltodextrin (2 mg/mL). All these substrates were dissolved in 100 mM sodium phosphate buffer (pH 6.8). Then 150 μL of enzyme working solution was added (25 mg/mL for maltose and maltodextrin, 50 mg/mL for sucrose and isomaltose) to start the reaction. The α-glucosidase activity was stopped by adding 750 μL of 0.5 M TRIS buffer. Several time points were chosen for the reaction (10, 20 30, 40, 50, 60 min) and individual tubes were used for each time point. GOPOD kit was used to measure the amount of produced glucose as previously method (Kan, Capuano et al., 2020). The amount of produced glucose (μmol) from each substrate was plotted against the selected time (10–60 min), which showed a linear curve. The slope (*k*) of the linear curve was used for calculation of enzyme activity. One unit of enzyme activity was defined as the amount of glucose (μmol) hydrolysed from the corresponding substrate per minute in the assay.

2.5. Rat mucosal enzyme inhibition assay

All the inhibitors were dissolved in methanol and diluted to different concentrations: white, green, oolong, black tea extract and polymeric fraction from BTE were diluted to 0.05 ~ 10 mg/mL; EGCG, ECG and theaflavins were diluted to 0.01 ~ 1 mg/mL. Briefly, 150 μL of solvent blank (methanol) or different concentrations of inhibitors was mixed with 450 μL of substrate (maltose (10 mM), sucrose (30 mM), isomaltose (20 mM) or maltodextrin (2 mg/mL)) and 150 μL of enzyme working solution. The reaction time was selected according to the result of section 2.4, to have enough glucose released for GOPOD kit measurement, *i.e.,* 30 min for the hydrolysis of maltose and maltodextrin and 60 min for the hydrolysis of sucrose and isomaltose. Finally, TRIS buffer (0.5 M) was added to stop the reaction. The inhibition of the alpha-glucosidase was calculated according to equation below.

$$\text{Inhibition}(\%) = ((C_{control} - C_{inhibitor})/C_{control}) \times 100$$

where $C_{control}$ was the concentration of glucose produced from individual substrate with solvent blank, and $C_{inhibitor}$ was the concentration of glucose produced from individual substrate with inhibitors. Finally, the inhibition of alpha glucosidase was expressed as IC_{50} value. The IC_{50} value is the concentration of inhibitor required to inhibit 50% of the enzyme activity expressed as milligram sample per millilitre solvent. The IC_{50} values were calculated using the CompuSyn software.

2.6. Kinetics analysis of rat maltase inhibition assay

The inhibition type exerted by phenolic-rich samples on maltase was assessed by a kinetic analysis (Yu, Fan, & Duan, 2019). Michaels-Menten plot, Lineweaver-Burk equation combined with Dixon plot and Cornish-Bowden were applied in this study. The formula models for different inhibition types are as follows:

$$\text{Competitive inhibition } \frac{1}{v}=\frac{K_m}{V_{max}}\left(1+\frac{[i]}{K_i}\right)\frac{1}{[a]}+\frac{1}{V_{max}} \quad (1)$$

$$\text{Non}-\text{competitive inhibition } \frac{1}{v}=\frac{K_m}{V_{max}}(1+\frac{[i]}{K_i})\ \frac{1}{[a]}+\frac{1}{V_{max}}(1+\frac{[i]}{K_i}) \quad (2)$$

$$\text{Un}-\text{competitive inhibition } \frac{1}{v}=\frac{K_m}{V_{max}}\frac{1}{[a]}+\frac{1}{V_{max}}(1+\frac{[i]}{K_i}) \quad (3)$$

$$\text{Mixed inhibition}\frac{1}{v}=\frac{K_m}{V_{max}}\left(1+\frac{[i]}{K_i}\right)\frac{1}{[a]}+\frac{1}{V_{max}}(1+\frac{[i]}{K_{ia}}) \quad (4)$$

where v is the reaction rate. V_{max} and K_m are maximum reaction rate and Michaelis-Menten constant, respectively. K_i and K_{ia} are free enzyme inhibition constant and bound enzyme inhibition constant, respectively. [a] is the concentration of substrate. [i] is the concentrations of the inhibitors.

K_i can be calculated by plotting $1/v$ against i at several a values and it equals the absolute value of the intersection abscissa of the plots. K_{ia} can be achieved by plotting a/v against i and it equals the absolute value of the intersection abscissa of the plots.

To calculate the apparent maximum reaction velocity (V_{max}^{app}) and the apparent Michaelis constant (K_m^{app}), the Eq. (4) can be written as follows:

$$\frac{1}{v}=\frac{1}{V_{max}^{app}}+\frac{K_{max}^{app}}{V_{max}^{app}}\frac{1}{[a]} \quad (5)$$

Eq. (5) indicates that a plot of $1/v$ against $1/[a]$ at a constant value of [i] is linear. Therefore the V_{max}^{app}, K_m^{app}, can be calculated as follows:

$$\text{Slope}=\frac{K_{max}^{app}}{V_{max}^{app}}=\frac{K_m}{V_{max}}(1+\frac{[i]}{K_i}) \quad (6)$$

$$\text{Intercept}=\frac{1}{V_{max}^{app}}=\frac{1}{V_{max}}(1+\frac{[i]}{K_{ia}}) \quad (7)$$

where V_{max}^{app} and K_{max}^{app} are apparent maximum reaction velocity and apparent Michaelis constant, respectively.

2.7. Synergetic effect of phenolic inhibitors and acarbose on rat maltase inhibition

The synergetic effect of the polyphenol-acarbose interaction on maltase inhibition was determined by the combination index (CI) using CompuSyn software (Chou, 2010). It is based on the median-effect principle. The combined inhibition assay of tea polyphenols and acarbose was performed at a constant ratio (1 mg/mL:1 μg/mL). Then a series of concentrations (from 0.25 IC_{50} to 4 IC_{50}) of tea extracts, tea polyphenols and acarbose were prepared. The combined "Dose-effect" relationships of tea extracts or polyphenols with acarbose for α-glucosidase were constructed, and "Dose" represents the total dose of tea extracts or tea polyphenols and acarbose.

The equation for the median-effect principle is as follows:

$$\log(\frac{f_a}{f_u})=mlogD-mlogD_m$$

where D is the dose of the inhibitor, f_a is the fraction affected by dose D (*eg.*, if the enzyme activity is inhibited by 30%, then $f_a=0.3$), f_u is the unaffected fraction ($f_u=1-f_a$), m is the coefficient, and D_m is the median-effect dose (IC_{50} in this paper).

The equation for the CI is expressed as follows:

$$\text{CI}=\frac{(D)_1}{(D_X)_1}+\frac{(D)_2}{(D_X)_2}$$

where $(D)_1$ and $(D)_2$ are the doses of inhibitors that produce a certain level of inhibition in the combination system, and $(Dx)_1$ and $(Dx)_2$ are the doses of inhibitors added alone that lead to the same level of inhibition. The type of polyphenol-acarbose interaction was scored as synergistic (CI < 1), additive (CI = 1) or antagonistic (CI > 1).

2.8. Caco-2 cell culture

Caco-2 cells (American Type Culture Collection) from passage 30 to 40 were cultured and maintained in DMEM containing 10% *v/v* FBS in 75 cm^2 plastic flasks. Cells were seeded in 12-well polyester insert plates (Corning Inc., Oneonta, USA). The cell density was 1.25×10^5 cells per cm^2. The cells were grown and differentiated for 21 days under a humidified atmosphere of air, 5% CO_2 and at 37 °C. Then highly differentiated monolayers with a TEER > 450 O were selected for enzyme studies.

2.9. Maltase and sucrase inhibition using Caco-2

Fresh medium was placed on apical and basolateral sides of Transwells the day before performing the experiments. Before starting the experiment, TEER voltage was measured to ensure membrane integrity prior to enzyme experiments (>450 O). Then phenol medium was replaced with 1500 mL of phenol-free medium with antibiotics at the basolateral side, and 800 μL of prepared inhibitors which dissolved in phenol-free DMEM was added on the apical side. Two wells per plate were employed for the control solution composed of phenol-free DMEM. Another two wells per plate was employed for the blank solution composed of a mixture of maltose (4 mM in phenol-free DMEM) and sucrose (75 mM in phenol-free DMEM). TEER value was measured at 1, 3, 6, 9 and 24 h. After 24 h, samples were collected from the apical and basolateral side.

Maltose and fructose content of the samples collected from apical and basolateral side were detected by HPLC-ELSD as we previously reported (Kan, Oliviero, Verkerk, Fogliano, & Capuano, 2020). The maltase and sucrase inhibition was calculated by the reduction of the amount of maltose and the production of the fructose, respectively.

$$\text{Maltase inhibition}(\%)=\frac{C_0-C_a-C_b}{C_0-C'_a-C'_b}\times100$$

where C_0 is the original amount of maltose added in the apical side, C_a is the amount of maltose detected in the apical side without inhibitors after time 24 h, C_b is the amount of maltose detected in the basolateral side without inhibitors after 24 h, C'_a is the amount of maltose detected in the apical side with inhibitors after 24 h, C_b is the amount of maltose detected in the basolateral side with inhibitors after 24 h.

$$\text{Sucrase inhibition}(\%)=\frac{C'_a+C'_b}{C_a+C_b}\times100$$

where C_a is the amount of fructose detected in the apical side without inhibitors after 24 h, C_b is the amount of fructose detected in the basolateral side without inhibitors after 24 h, C'_a is the amount of fructose detected in the apical side with inhibitors after 24 h, C_b is the amount of fructose detected in the basolateral side with inhibitors after 24 h.

2.10. Statistics analysis

The results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by the Duncan's multiple range test was used to compare the means among different samples by the SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Differences were

considered significant at $P < 0.05$.

3. Results and discussion

3.1. Hydrolytic properties of rat intestinal α-glucosidase on different substrates.

Rat intestinal α-glucosidase shows hydrolytic properties on different α-glycosidic linkages, e.g., α-1,2, α-1,4 and α-1,6 linkages (Lim et al., 2019). First, the specific hydrolytic activity of rat α-glucosidase toward maltose, maltodextrin, sucrose and isomaltose was studied. The rat α-glucosidase is composed of two enzyme complexes, maltase-glucoamylase (MGAM) and sucrase-isomaltose (SI). All four subunits exhibit maltase activities (*i.e.* against α-1,4 bonds) (Shin et al., 2019). This is in line with our results as shown in Table 1, showing the α-glucosidase has the highest activities toward maltose (13.77 U/g protein). The glucoamylase subunit shows higher activity toward maltodextrin. The sucrase subunit shows distinctive α-1,2 glycosidic activity toward sucrose, whereas the isomaltase subunit displays high α-1,6 hydrolytic activity toward isomaltose. Since different enzymatic subunits may exert hydrolytic activity toward the same substrate (e.g., toward maltose), the hydrolytic activity of individual subunits cannot be determined using the rat α-glucosidase that has been used in this study. However, this source of α-glucosidase allows a more realistic assessment of the overall hydrolytic activity. Therefore, the rat intestinal extract is a suitable model to study multiple types of inhibitions on membrane bound disaccharidases. Others also highlighted the advantage of using this model for a more realistic assessment of the hydrolytic activity of disaccharidases (Lim et al., 2019; Shin et al., 2019).

3.2. Inhibition properties of tea extracts on rat α-glucosidase using different substrates

To better understand the hydrolytic inhibition of four TEs on rat α-glucosidase, the polyphenol composition was investigated and the results are shown in Table 2. The total polyphenol content, ECG and EGCG content in TEs was in the order WTE > GTE > OTE > BTE, whereas the tannins content was in the order BTE > OTE > WTE > GTE. This was in line with the previous report, showing that fermented tea (oolong tea and black tea) contains more tannins than non-fermented tea (white and green tea) (Sun, Warren, Netzel, & Gidley, 2016). Then, the individual hydrolytic inhibition of tea polyphenols on individual hydrolytic property toward maltose, maltodextrin, sucrose and isomaltose was studied. As shown in Table 3, all four tea extracts are very effective in the inhibition of maltase and glucoamylase, but only WTE was weakly effective on sucrase and isomaltase. This inhibition was very weak as indicated by the high IC_{50} values, 7.6 and 4.2 mg/mL, respectively. The other three TEs did not show any inhibition on sucrase and isomaltase. WTE was the most efficient inhibitor of α-glucosidase towards maltose and maltodextrin, resulting in IC_{50} value of 0.26 and 0.073 mg/mL, respectively. The higher inhibition of WTE is possibly due to its higher amount of total phenolic content (55 g/100 g) and more specifically to the higher amount of EGCG (31.2 g/100 g) which is the peculiar feature of WTE as shown in Table 2. EGCG had strong inhibition properties towards maltase (IC_{50} = 0.021 mg/mL) and glucoamylase (IC_{50} = 0.018 mg/mL), weak inhibition towards isomaltase (IC_{50} = 0.44 mg/mL) and no effect towards sucrase. However the inhibitory activity of EGCG against maltase is 30 times lower than the positive control acarbose (IC_{50} = 0.0006 mg/mL). Other researchers also reported that EGCG showed strong inhibition on maltase by using *Saccharomyces cerevisiae* with a IC_{50} value of 0.08 mg/mL (Chem & Ii, 2010). Purified rat α-glucosidase was also used by some researcher who found that 0.14 mg/mL of EGCG had similar inhibition properties toward dextrin, maltose, sucrose and isomaltulose with inhibition of 64.2, 79.8, 76.1, and 64.7%, respectively (Lim et al., 2019).

In our previous study, we found that the polymeric fraction from BTE showed strong inhibition on starch digestibility by inhibiting α-amylase and interacting with starch, especially in a model of gluten-free bread (Kan, Capuano et al., 2020). In this study, we further found that polymeric fraction from BTE, *i.e.* the tannins-rich fraction, showed strong inhibition on maltase (0.31 mg/mL) and glucoamylase (0.06 mg/mL) and weak inhibition on sucrase (2.34 mg/mL) and isomaltase (5.14 mg/mL). The tannins fraction was also reported for its α-glucosidase inhibitory capacity. For example, tea tannins from *Ampelopsis grossedentata* leaves inhibited maltase from *Saccharomyces cerevisiae* using p-

Table 1
Specific hydrolytic activities of rat α-glucosidases on different types of substrates.

α-glucosidase	Substrate	Activity (U/g protein)	Activity (U/g solid)
Maltase	maltose	13.77 ± 0.06 a	2.41 ± 0.01 a
Glucoamylase	maltodextrin	7.69 ± 0.09b	1.35 ± 0.01b
Sucrase	sucrose	1.20 ± 0.06 d	0.21 ± 0.01 d
Isomaltase	isomaltose	1.60 ± 0.06c	0.28 ± 0.01c

The protein content of the rat intestinal extract was 17.5 ± 0.14 g/100 g solid. One unit of enzyme activity was defined as the amount of glucose (μmol) produced from corresponding substrate per minute in the assay.
Results were expressed as means ± SD of triplicate analysis. Different letters in the same column indicate a significant difference between means (P < 0.05).

Table 2
Polyphenol composition of WTE, GTE, OTE and BTE (g/100 g).

Polyphenols	WTE	GTE	OTE	BTE
EGCG	31.2 ± 0.2 a	27.2 ± 0.2 a	5.0 ± 0.0 a	7.3 ± 0.1 a
ECG	10.0 ± 0.1b	6.1 ± 0.1b	4.9 ± 0.1 ab	7.1 ± 0.1b
Theaflavin	nd	nd	0.5 ± 0.0 e	2.3 ± 0.1 d
Theaflavin 3-gallate	nd	nd	0.4 ± 0.0f	1.3 ± 0.0f
Theaflavin 3′-gallate	nd	nd	0.8 ± 0.0 d	1.8 ± 0.0 e
tannins	2.2 ± 0.0c	0.8 ± 0.0c	3.3 ± 0.1c	5.0 ± 0.1c
total	55.0 ± 0.1	42.9 ± 0.5	15.0 ± 0.0	24.7 ± 0.1

Values are expressed as mean from triplicate analysis ± standard deviation. Different letters in the same column (except for the total amount) indicate a significant difference between means ($P < 0.05$).
WTE: white tea extract; GTE, green tea extract; BTE, black tea extract; OTE, oolong tea extract; EGCG, epigallocatechin gallate, nd, not detected.

Table 3
IC_{50} value (mg/mL) of the α-glucosidase inhibition using different substrates.

Inhibitors	maltose	maltodextrin	sucrose	Isomaltose
WTE	0.26 ± 0.01f	0.073 ± 0.002f	7.6 ± 0.1 a	4.2 ± 0.3b
GTE	0.67 ± 0.01c	0.10 ± 0.01 e	ND	ND
OTE	1.15 ± 0.01b	0.57 ± 0.02b	ND	ND
BTE	1.59 ± 0.08 a	0.47 ± 0.03c	ND	ND
EGCG	0.021 ± 0.001 / 45.8 μM g	0.018 ± 0.001 / 39.2 μM h	ND	0.44 ± 0.01 / 121.5 μM d
ECG	0.57 ± 0.03 / 1.29 mM d	0.35 ± 0.01 / 0.79 mM d	ND	0.32 ± 0.01 / 0.72 mM e
Theaflavin	ND	ND	ND	ND
Theaflavin 3-gallate	ND	0.48 ± 0.02c	ND	ND
Theaflavin 3′-gallate	ND	0.99 ± 0.02 a	ND	ND
Polymeric fraction	0.31 ± 0.01 e	0.06 ± 0.01 g	2.34 ± 0.03b	5.14 ± 0.06 a
Acarbose	0.00061 ± 0.00001 / 0.94 μM h	0.00025 ± 0.00001 / 0.39 μM i	0.011 ± 0.001 / 15.5 μM c	1.09 ± 0.02 / 1687.3 μM c

WTE: white tea extract; GTE, green tea extract; BTE, black tea extract; OTE, oolong tea extract; EGCG, epigallocatechin gallate. ND, not detected. IC_{50} values of EGCG, ECG and acarbose were expressed as two units, the unit of left value was mg/mL, and the unit of the right value was μM. Values are expressed as mean from triplicate analysis ± standard deviation. Different letters in the same column indicate a significant difference between means ($P < 0.05$).

nitrophenyl-α-D-glucopyranoside as substrate with a IC_{50} value of 1.94 μg/mL (Geng et al., 2016). In our study, ECG showed to have a more selective inhibitory activity towards isomaltose (IC_{50} = 0.32 mg/mL, 0.72 mM) compared to antidiabetic drug acarbose (IC_{50} = 1.09 mg/mL, 1.69 mM). Lim et al. (2019) also found 0.3 mM of ECG and acarbose showed a similar inhibition of 77.7 and 87.1% towards isomaltulose as substrate. Therefore, WTE, EGCG and ECG could reduce postprandial glucose uptake in maltodextrin-rich, maltose-rich and isomaltose-rich food, respectively (Ao et al., 2007).

However, the enzyme working solution used in this study is a crude extract from rat intestine without any purification. Consequently, the enzyme working solution contains not only α-glucosidase but also α-amylase, protease and other impurities (Shin et al., 2019). α-Amylase can also produce glucose from the substrates used in our study. Therefore, the maltase and glucoamylase activities in Table 1 were likely to be overestimated due to the contribution from α-amylolytic activity of the rat extract. In our previous study, we confirmed the inhibitory effect of tea polyphenols on α-amylase (Kan, Capuano et al., 2020). The presence of α-amylase in rat enzyme preparation can thus interfere with the inhibitory effects of polyphenols on maltase and glucoamylase, since α-amylase can generate glucose from maltose and maltodextrins. Therefore, the results of inhibitory effect on maltase and glucoamylase in Table 3 could be a combined inhibition on α-glucosidase and α-amylase. Some researchers have used centrifugal filter unit, with a molecular weight cut-off of 100 kDa to purify the crude α-glucosidase working solution, resulting in a reduction of α-amylase activity from 150.7 U to 101.9 U (Shin et al., 2019). In addition, the presence of other impurities, for instance, non-enzyme proteins may reduce the amount of polyphenols that was able to bind and inhibit brush border enzymes, thus reducing their inhibitory effects.

3.3. Kinetics of α-glucosidase inhibition toward maltose and type of inhibition

As shown before, all four enzymatic subunits of MGAM and SI exhibit maltase activities (*i.e.* against α-1,4 bonds) and maltose is a common product from starch digestion by pancreatic α-amylase. To better understand the inhibitory mechanism on maltase, e.g., the type of inhibition, a more detailed kinetic characterisation of the inhibition using maltose as substrate was carried out (Fig. 1 and Supplementary material). The exact type of inhibition can be analysed and defined by the combined use of Dixon and Cornish-Bowden plots and Lineweaver-Burk plots as summarized in Table 4. Regarding acarbose, the Dixon plots intersect at one point, while the Cornish-Bowden plots run parallel with each other (Fig. 1B & C). This demonstrated that acarbose is a competitive inhibitor for α-glucosidase, which was in line with the previous report (Bischoff, 1995; Calder & Geddes, 1989). This was also confirmed by the values of K_m^{app} and V_{max}^{app} calculated from Lineweaver-Burk plots (Table 5, Fig. 1D). As shown in Table 5, the K_m^{app} increases while V_{max}^{app} remains the same. Besides, the Lineweaver-Burk plots intersected the y axis. Those are the typical characteristics of competitive inhibition (Sun et al., 2016). Regarding all the TEs and tea polyphenols, both the Dixon and Cornish-Bowden lines intersect at one point (Fig. 1B & C), indicating that they are mixed-type inhibitors. This was further confirmed by the decrease of V_{max}^{app} (Table 5). Based on mixed-type inhibition mechanism, TEs and tea polyphenols can compete with maltose in binding with α-glucosidase (competitive), as well as can bind with the α-glucosidase-maltose complex (uncompetitive). Hydrophobic

Table 4
The characteristics of different inhibition types.

Inhibition type	Lineweaver-Burk plots(*1/v* against *1/s*)	Dixon plots (*1/v* against *i*)	Cornish-Bowden plots (*a/v* against *i*)
Competitive	1) Intersection;*2)* K_m^{app} increased, while V_{max}^{app} remains more or less the same.	Intersection	No intersection (parallel lines);
Uncompetitive	1) No intersection (parallel lines);2) Both K_m^{app} and V_{max}^{app} decreased.	No intersection (parallel lines);	Intersection
Non-competitive binding	1) Intersection;2) V_{max}^{app} decreased, K_m^{app} remains the same	Intersection	Intersection
Mixed	1) Intersection;2) V_{max}^{app} decreased, K_m^{app} increase (more closely to competitive binding), or K_m^{app} decrease (more closely to uncompetitive binding).	Intersection	Intersection

The information of this table was summarized according to previous studies (Peng, Zhang, Liao, & Gong, 2016; Sun et al., 2016; Sun, Gidley, & Warren, 2017).

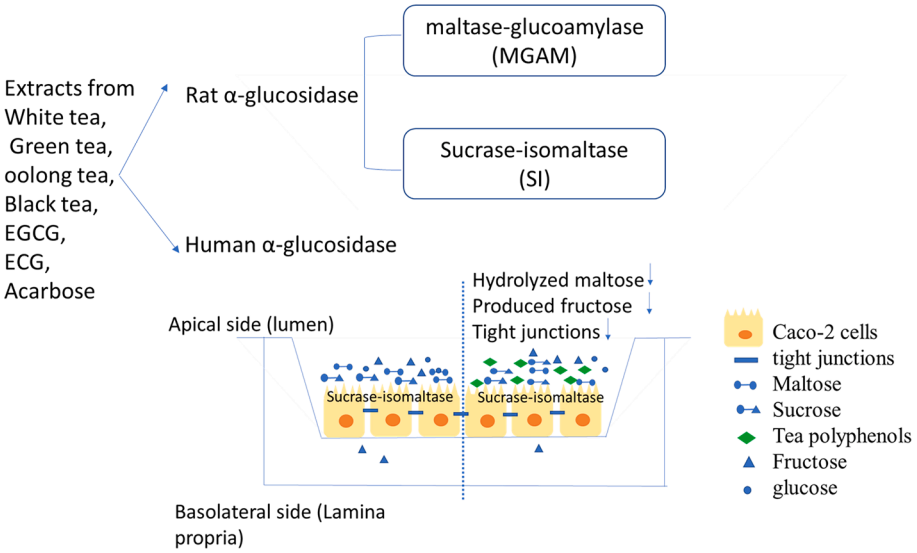

Fig. 1. Kinetics of α-glucosidase inhibition by tea extracts or tea polyphenols using maltose as substrate. A: Michaelis-Menten plots for α-glucosidase inhibition by WTE, EGCG, and acarbose. B: Dixon plots for α-glucosidase inhibition by WTE, EGCG, and acarbose. C: Cornish-Bowden plots for α-glucosidase inhibition by WTE, EGCG, and acarbose. D: Lineweaver-Burk plots for α-glucosidase inhibition by WTE, EGCG, and acarbose; WTE: white tea extract; EGCG, epigallocatechin gallate.

Table 5
Michaelis-Menten parameters for α-glucosidase inhibition by tea polyphenol.

Inhibitor	K_m^{app} (mg/mL)					V_{max}^{app} (mM glucose / min)					K_i	K_{ia}	Inhibition
	A	B	C	D	E	A	B	C	D	E	mg/mL	mg/mL	type
WTE	4.861	5.816	6.098	7.460	8.241	0.062	0.049	0.040	0.029	0.017	0.102 [e]	0.196 [d]	mixed
GTE	4.772	4.639	5.059	5.347	5.933	0.056	0.047	0.040	0.029	0.017	0.735[c]	1.091[c]	mixed
BTE	4.201	4.605	4.938	4.901	4.998	0.056	0.051	0.045	0.035	0.022	1.267[b]	1.509[b]	mixed
OTE	4.525	5.074	5.558	5.534	5.433	0.059	0.055	0.052	0.044	0.029	1.956 [a]	2.366 [a]	mixed
EGCG	4.872	5.426	5.387	5.800	5.823	0.057	0.046	0.034	0.023	0.014	0.026[f]	0.035 [e]	mixed
Polymeric fraction	4.333	4.906	6.121	5.631	6.509	0.084	0.072	0.068	0.041	0.030	0.149 [d]	0.198 [d]	mixed
Acarbose *	3.362	5.532	8.773	20.472	65.700	0.076	0.076	0.074	0.086	0.129	0.121 [g]	NA	competitive

Fig. 2A (A = 0, E = highest concentration, increasing order from A to E). NA, not applicable. WTE, white tea extract; GTE, green tea extract; BTE, black tea extract; OTE, oolong tea extract; EGCG, epigallocatechin gallate.
The characters (A to E) represent the concentrations of inhibitors, as shown in
* The units of Kmapp, Ki and Kia for acarbose is μg/mL. Values are expressed as mean from triplicate analysis. Different letters in the same column indicate a significant difference among means ($P < 0.05$).

association and hydrogen bonding have been reported to be the main mechanisms of polyphenols-enzymes interactions (Martinez-Gonzalez et al., 2017). K_m^{app} of all the TEs and polyphenols increases, indication that the mixed-type inhibition of those inhibitors more closely mimic competitive binding (Tables 4 and 5). This was in line with that K_i was smaller than K_{ia} for all the mixed-type inhibitors (Table 5). This suggests that they bind more tightly with free α-glucosidase than with the α-glucosidase-maltose complex. Interestingly, the order of IC_{50} values of four tea extracts correspond to that of the inhibition constants (both K_i and K_{ia}), this was in line with previous report for α-amylase inhibition (Sun et al., 2016).

As our TEs is a mixture of monomeric and polymeric polyphenols and all the subunits of rat α-glucosidase shows hydrolytic activity towards maltose, the combination of different mechanisms is expected. Our results are consistent with a previous report where black tea extract was shown to exert a mixed-type inhibition for mammalian α-glucosidase, using maltose as substrate (Satoh, Igarashi, Yamada, Takahashi, & Watanabe, 2015). Some researchers reported different results on inhibition of yeast α-glucosidase by TEs or tea polyphenols, using 4-Nitrophenyl β-D-glucopyranoside as substrate. For instance, it was reported that gallocatechin gallate (GCG) and green tea extract inhibited yeast α-glucosidase by non-competitively (Wu et al., 2018; Yang & Kong, 2016). Non-competitive inhibition is sometimes considered as a special case of mixed-type inhibition, *i.e.,* the competitive constant and uncompetitive constant calculated from Dixon and Cornish-Bowden are exactly the same ($K_i = K_{ia}$). Commonly, Lineweaver-Burk plots was used to identify the inhibition type. But the Dixon and Cornish-Bowden can be used to assist in analysing the inhibition to further confirm the inhibition type.

3.4. Synergy determination for maltase inhibition

To investigated the combined effect of polyphenols and acarbose, we used the method reported by Chou and Talalay to distinguish among synergistic, antagonistic, and additive effects (Chou, 2010). In this method, CI value was calculated by Compusy to distinguish the three types of effects. As shown in Fig. 2, except for some lowest concentrations, the combination of all the TEs and EGCG with acarbose showed slight antagonistic inhibition, since the CI values were slightly higher than 1. The combination of polymeric fraction from BTE with acarbose strongly reduced their inhibition, since their CI value was higher than 4. Other researchers also found antagonistic inhibition when combined catechin with acarbose (Zhang et al., 2017). However, some researchers reported a different results using baker's yeast α-glucosidase, *i.e.,* the combination of green tea polyphenols with acarbose had synergistic and antagonistic effects on α-glucosidase at low and high concentrations, respectively (Gao et al., 2013). The different results could be explained by different enzyme resources, *i.e.*, baker's yeast enzymes and rat enzymes have different amino acid sequences at the catalytic site. There are also some in *vivo* reports about the synergistic effect of tea polyphenols and acarbose. One of the examples is black tea extract, in combination with acarbose, produced a synergistic inhibitory effect on sucrase activity, resulting in decrease of plasma glucose levels of Goto Kakizaki rat in *vivo* (Satoh et al., 2015).

3.5. Inhibitory effect of tea polyphenols on human maltase and sucrase

To investigate the effect of tea polyphenols on human maltase and sucrase and their effect on tight junctions, GTE, EGCG and acarbose were tested on Caco-2 grown on Transwell insert plates. First, the hydrolytic properties of maltase and sucrase was measured based on amount of hydrolysed maltose and produced fructose in both apical and basolateral side, respectively. As shown in Fig. 3A, within 24 h, 3.4 mM of maltose was hydrolysed and no maltose was found in basolateral side. Within 24 h, 16.1 and 4.1 mM of fructose were detected in the apical and the basolateral side, respectively. Fructose could be absorbed by Caco-2 cells, which explains its presence in the basolateral side (Andrade, Araújo, Correia-Branco, Carletti, & Martel, 2017). TEER values are strong indicators of the integrity of the cell monolayers before their use to study transport of drugs or chemicals (Srinivasan et al., 2015). As shown in Fig. 3A, cells exposed to 4 mM of maltose and 75 mM of sucrose caused a small (around 5%) reduction of TEER in the first 9 h, but TEER recovered to 100% within 24 h. Therefore, those concentrations of maltose and sucrose were selected for further experiments.

Then, the inhibitory effect of acarbose, EGCG and GTE was investigated. As shown in Fig. 3B, GTE, EGCG and acarbose exhibited an inhibitory effect on sucrase and maltase activity expressed by Caco-2 cells grown on Transwells. The inhibitory activity of all the tested compounds was dose-dependent. Interestingly, the inhibition on sucrase was much higher than on maltase, which was the opposite as found for the experiments with rat enzyme (Table 3). This is possibly because Caco-2 only provide SI, while MGAM is absent (Hauri et al., 1985). Therefore, the use of enzymes produced by Caco-2 can be considered as a better *in vitro* simulation model when evaluating effects for the human intestinal tract, since SI hydrolyses the majority of maltose in human intestine (Pyner et al., 2017). Our data also suggest that the tested inhibitors are more effective on Caco-2 sucrase than rat sucrase. In contrast, the opposite was observed when assessing maltase activity, e.g.: IC_{50} of EGCG was 45.8 μM for rat maltase (Table 3), but 1000 μM of EGCG, the highest concentration used in the study, did not reach 50% of inhibition in human enzyme (Fig. 4B). This is in line with the fact that the homology between human and rat sucrose-isomaltase is only 74%, and so this could possibly explain the difference between human and rat enzyme (Van Beers, Büller, Grand, Einerhand, & Dekker, 1995). Finally the influence of inhibitors on TEER value was measured simultaneously with sugar analysis. As shown in Fig. 4C, cells exposed to 2 ~ 50 μM of acarbose maintained a TEER above 80% during the 24 h measurement and the same happens for the low concentrations of GTE (0.25 and 0.5

mg/mL). Acarbose is commonly used for the type-2 diabetes treatment. The dosage of acarbose that people take as medicine is 25 ~ 50 mg per time, three times per day, *i.e.*, the concentration of acarbose is 1.11 ~ 2.22 μM by considering the volume of intestinal fluids as 105 mL (Rosenstock et al., 1998; Schiller et al., 2005). Therefore, our results about the efficacy of the acarbose in Caco-2 cells were as expected, which indicated the Caco-2 grown on Transwells could be a reliable system for investigating human sucrase and maltase inhibition, as well as the effects on tight junction. The higher concentration of GTE (1 and 2.5 mg/mL) caused a dramatic decrease of TEER during the first 6 h which did not recover after 24 h. Lower concentration of EGCG (0.125 mM) caused an increase of the TEER value during the 24 h. This was in line with the previous reports that EGCG has protective effect towards epithelial integrity in Caco-2 cell monolayers (Carrasco-Pozo, Morales, & Gotteland, 2013). But the high concentrations (0.25, 0.5, 1 and 2 mM) of EGCG caused a significant increase of TEER for the 1st three hours, then decreased dramatically to 75%, 65% and 60% at 6th, 9th, and 24th hour, respectively. Therefore, the enzyme inhibition of high concentrations of EGCG and GTE could be underestimated due to the reduce of TEER. This may happen because the inhibitors may migrate to basolateral compartment due to the reduction of tight junction, and less inhibitors are available in apical compartment to inhibit enzyme activity. This could be one of the reasons that the inhibition curves in Fig. 3B tend to flatten at higher concentrations of inhibitors. The increase of the TEER caused by lower concentration of GTE and EGCG could be used for regulating the intestinal junction and barrier function. Many researchers reported that polyphenols can regulate the intestinal barrier function (Hervert-Hernández & Goñi, 2011; Yang, Bibi, Du, Suzuki, & Zhu, 2017). Although tea polyphenols are promising for sucrase and maltase inhibition, an effective concentration of polyphenol through tea consumption should be considered. As reported previously, one cup of tea could provide 5 ~ 20 mg of EGCG, so the final concentration in the small intestine may less than 10 μM by considering the dilution by gastric and intestinal fluids (Kan, Capuano et al., 2020; Rosenstock et al., 1998). This amount is much lower than the tested concentration as shown in Fig. 3. Although an effective concentration of polyphenol to inhibit human maltase and sucrase can be hardly achieved through daily tea drinking, a feasible strategy may be to combine tea polyphenols or acarbose with sugar-rich or starch-rich food to modulate glucose release in the small intestine.

The main conclusion of this study is that maltase and sucrase activities showed different sensitivity to polyphenols by using rat acetone extract and human Caco-2 cells. That can be mainly attributed to the different intrinsic characteristics of the enzymes and the different experimental system. In human and rat, MGAM and SI are both present on the brush border membrane of small intestinal enterocytes (Semenza, Auricchio, & Rubino, 1965). Both MGAM and SI show maltase activity (Pyner et al., 2017). In humans, the amount of MGAM protein is 40–50 times lower than the amount of SI in the human intestine (Semenza et al., 1965). However, MGAM still contributes 30–40% of the total maltase activity due to its higher Ct subunit hydrolytic activity (Quezada-Calvillo et al., 2008). In rat the MGAM has higher maltase activity and produce higher intestinal glucogenesis, while SI has lower maltase activity and sustains slower glucogenesis (Quezada-Calvillo et al., 2007). Human Caco-2 cells is commonly used to provide human disaccharides, but Caco-2 cells only provide SI (Pyner et al., 2017). Therefore, maltase activity in Caco-2 cells is only from SI. Besides, the different experimental conditions used may also explain the different sensitivity observed. Rat enzyme inhibition was performed in test tubes by mixing enzymes, substrates and inhibitors, whereas human enzyme inhibition was measured by intact Caco-2 cells in Transwells. The rat enzymes are a mixture of enzymes free in solutions, whereas Caco-2 enzymes are located in the membranes of the monolayer of cells. Caco-2 cells have been used by many researchers to provide human α-glucosidase by using either cell homogenates or membrane-enriched preparations (Pyner et al., 2017). In our study, intact cells grown in Transwells were used without damaging the cell monolayer, which is a physiologically realistic way to simulate enzymatic hydrolysis. Moreover, we used different substrate concentrations to determine the enzyme activity of rat and Caco-2 cell enzymes. This is because different detection and calculation method for maltase and sucrase activity are applied to enzymes from rat acetone extract and Caco-2 cells. Regarding enzymes from rat acetone extract, the calculations of maltase and sucrase activity were based on glucose release from corresponding substrate, which were measured by GOPOD kit. Therefore, 10 mM of maltose and 30 mM of sucrose were selected to make sure enough glucose to be produced to have sufficient color reaction with GOPOD reagent. Regarding experiments in Caco-2 cells, the glucose release cannot be measured because large amounts of glucose are present in DMEM cell culture media. Then, the calculations of maltase and sucrase activity in Caco-2 cells were based on the amount of remaining maltose and produced fructose, which are measured by HPLC-ELSD. Therefore, 4 mM of maltose and 75 mM of sucrose were selected to make sure the remaining amount of maltose and produced fructose are within the detection limit of HPLC-ELSD. Clearly, other polyphenols should be tested to draw firm conclusion on the different sensitivity of rat and human enzymes to polyphenols.

4. Conclusion

This study investigated the inhibitory effects of tea polyphenols on α-glucosidase activities, using rat intestinal enzymes and Caco-2 cells. Four tea extracts were selected, *i.e.,* white, green, oolong, and black tea extract. All these tea extracts showed a significant enzyme-inhibiting capacity when using dextrin and maltose as substrate. White tea extract is the most effective inhibitor probably because of the high concentration of EGCG. Mixed-type of inhibition was found for the maltase inhibition, and the tea extracts and EGCG showed slightly antagonism effect when combined with acarbose. Therefore, drinking tea at a different time when taking acarbose is recommended. The inhibition of maltase and sucrase from Caco-2 cells is quite different from rat enzyme. Human sucrase activity was more susceptible than the rat enzyme to inhibition by green tea extract and EGCG, while the opposite result was observed when evaluating maltase activity. Tight junction was also influenced when the cells were exposed to tea polyphenols indicated by the reduction of TEER value. In conclusion, tea polyphenols are potential inhibitors for both rat and human α-glucosidase with different sensitivity. Rat acetone extract is a convenient tool for measuring enzymatic hydrolysis by using various substrates, *i.e.,* maltose, maltodextrin, isomaltose, and sucrose. Caco-2 cells grown on Transwells can be used to simulate enzymatic hydrolysis in a more physiologically realistic way.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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