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Interaction mechanism between green tea extract and human α-amylase for reducing starch digestion

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ABSTRACT

This study evaluated the inhibitory effects of the green tea extract on human pancreatic α-amylase activity and its molecular mechanism. The green tea extract was composed of epicatechin (59.2%), epigallocatechin gallate (14.6%) and epicatechin gallate (26.2%) as determined by HPLC analysis. Enzyme activity measurement showed that % inhibition and IC_{50} of the green tea extract (10%, based on starch) were 63.5% and 2.07 mg/ml, respectively. The Michaelis–Menten constant remained unchanged but the maximal velocity decreased from 0.43 (control) to 0.07 mg/(ml × min) (4 mg/ml of the green tea extract), indicating that the green tea extract was an effective inhibitor against α-amylase with a non-competitive mode. The fluorescence data revealed that the green tea extract bound with α-amylase to form a new complex with static quenching mechanism. Docking study showed the epicatechin gallate in the green tea extract presented stronger affinity than epigallocatechin gallate, with more number of amino acid residues involved in amylase binding with hydrogen bonds and Van der Waals forces. Thus, the green tea extract could be used to manipulate starch digestion for potential health benefits.

1. Introduction

The incidences of diabetes mellitus and of obesity are increasing dramatically worldwide. In the latest DIABETES ATLAS, International Diabetes Federation estimated that there were approximately 382 million diabetics worldwide in 2013; and the data was expected up to 592 million by 2035 (http://www.diabetesatlas.org). The basic characteristic of diabetes is hyperglycemia: uncontrolled high glucose levels in the blood (Aston, 2006; Blonde, 2012; Manuel-y-Keenoy & Perez-Gallardo, 2012; O'Keefe, Abuannadi, Lavie, & Bell, 2011). Poorly controlled blood glucose level can lead to complications, including impaired fasting glycemia and impaired glucose tolerance, resulting from resistance to insulin action or inadequate insulin secretion (Blonde, 2012; O'Keefe et al., 2011). Glucose from starch digestion is not only the biological fuel preferred by the central nervous system but also a signal molecule regulating gene transcription related to glucose homeostasis and energy metabolism (Englyst & Englyst, 2005; Jiang, Miao, Ye, Jiang, & Zhang, 2014; Marshall, 2006; Miao, Jiang, Cui, Zhang, & Jin, 2013). Thus, delaying glucose release from dietary carbohydrate is an effective approach for glycemic control. In the past decades, considerable research efforts have been devoted to several novel methods for effective controlling of blood glucose and prevention of related diseases (Aston, 2006; Englyst & Englyst, 2005; Hanhineva et al., 2010; Li et al., 2014; Miao, Zhang, Mu, & Jiang, 2011; Miao et al., 2013). Compared with the synthetic drugs, the natural molecules have become a more acceptable source of anti-diabetic agents (Boggs, Rosenberg, Ruiz-Narvaez, & Palmer, 2010; Hanhineva et al., 2010; Miao et al., 2013; Miao et al., 2014; O'Keefe et al., 2011).

Green tea is one of the most popular and widely consumed dietary supplements in the world (Senanayake, 2013). Regular intake of green tea is associated with improved antioxidant status *in vivo*, which may contribute to lower the risk of coronary heart disease, stroke, inflammation, and cancers (Ho, Lin, & Shahidi, 2008; Thielecke & Boschmann, 2009; Zdunczyk et al., 2002). Considerable interest has developed in unraveling the benefits of the green tea extract, particularly in epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) and anti-diabetic activities (Boggs et al., 2010; Fukino et al., 2008; Josic, Olsson, Wickeberg, Lindstedt, & Hlebowicz, 2010; MacKenzie, Leary, & Brooks, 2007; Tang, Li, Liu, Huang, & Ho, 2013; Tsuneki et al., 2004). However, the anti-diabetic claims are supported by weak scientific evidence due to the lack of understanding/information on the multiple pathways of tea catechin metabolism (Thielecke & Boschmann, 2009). Also, there are some reports focusing on the tea catechins as α-amylase inhibitors

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(Kawakami, Aketa, Nakanami, Iizuka, & Hirayama, 2010; Piparo et al., 2008), and the interactions between the active components in the green tea extract and human α-amylase for reduction of starch digestibility have not been clearly demonstrated. Herein, we investigated the inhibitory effects of the green tea extract on human pancreatic α-amylase activity and its molecular mechanism.

2. Materials and methods

2.1. Materials and chemicals

The green tea extract sample was obtained from Taiyo Green Power Co., Ltd. (Wuxi, China). Human pancreatic α-amylase (Cat. No. A9972), EC (purity ⩾ 90%), ECG (purity ⩾ 98%), EGC (purity ⩾ 95%) and EGCG (purity ⩾ 97.0%) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Commercial maize starch was from Changchun Dacheng Industrial Group Co. (Jilin, China). The hexokinase kit for glucose assay was obtained from Megazyme International Ltd. (Wicklow, Ireland). Methanol (HPLC grade) and orthophosphoric acid (analytical grade) were purchased from Fisher Scientific (Essex, UK). All other chemicals were reagent grade from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. High-performance liquid chromatography (HPLC) analysis

The green tea extract was dissolved with water to form a 0.4 mg/ml solution and centrifuged prior to HPLC analysis according to the method of Wang, Provan, and Helliwell (2003). The prepared solution was analyzed using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). The isolation was carried out on a Waters RP-C18 (250 × 4.6 mm, 5 μm) column (Waters Corporation, Milford MA, USA). The mobile phase was composed of solvent A (0.1% orthophosphoric acid in water, v/v) and solvent B (0.1% orthophosphoric acid in methanol, v/v). The injection volume was 5 μl and the flow rate was 1.0 ml/min. The determination wavelength was 280 nm. The gradient was as follows: 0–5 min, 20% B; 5–7 min, linear gradient from 20% to 24% B; 7–10 min, 24% B; 10–20 min, linear gradient from 24% to 40% B; 20–25 min, linear gradient from 40% to 50% B. Working standard solution, approximately 2 mg of each catechin reference standard were accurately weighed into a 25-ml volumetric flask, dissolved in water by sonication for 1 min, and made to volume with water prior to HPLC test.

2.3. α-Amylase assays

The digestive enzyme solution was prepared by suspending human pancreatic α-amylase (12.0 g) in phosphate buffer (100 ml, 0.2 M, pH 5.2) with magnetic stirring for 10 min, centrifuging the mixture for 10 min at 1 500×*g*, then transferring a portion (50 ml) of the supernatant into a beaker. The maize starch sample (200 mg) was gelatinised in 15 ml of phosphate buffer (0.2 M, pH 5.2) by heating at 95 °C for 30 min. After the starch solution was cooled and equilibrated at 37 °C for 5 min, the enzyme solution (5.0 ml) and the green tea extract (10%, based on starch) were added. Then, the samples were shaken in a 37 °C water bath at 150 rpm. After 10 min, aliquot of hydrolyzed solution (0.5 ml) was taken and mixed with 4 ml of absolute ethanol to deactivate the enzymes. The reducing sugar content was determined with the Nelson-Somogyi procedure by measuring the absorbance at 540 nm (Somogyi, 1945). A control vial was prepared by replacing the inhibitor (tea extract) solution with phosphate buffer. Percentage of pancreatic α-amylase inhibition was calculated according to the equation below:

$$\%\ \text{Inhibition} = \frac{(A_{\text{control}} - A_{\text{controlblank}}) - (A_{\text{sample}} - A_{\text{sampleblank}})}{A_{\text{control}} - A_{\text{controlblank}}} \times 100,$$

where A_{control}, $A_{\text{controlblank}}$, A_{sample} and $A_{\text{sampleblank}}$ refer to the absorbance value of reaction vial containing native enzyme and buffer, denatured enzyme and buffer, native enzyme and inhibitor and denatured enzyme and inhibitor, respectively. The denatured enzyme solution was obtained from native enzyme treated in a boiling water bath for 15 min. Substrate was present in all these vials. IC_{50} value (half maximal inhibitory concentration) was obtained graphically by an inhibition curve.

The Michaelis–Menten kinetic model: $v = \frac{V_{\max}[S]}{K_m + [S]}$ was employed to evaluate the effect of the green tea extract on starch digestion, where v is the reaction rate, $[S]$ was the concentration of a substrate S, $V_{\max}$ was maximum enzyme reaction rate and K_m was Michaelis–Menten constant. The amount of glucose liberated under different starch concentrations (5, 10, 15, 20, 25 mg/ml) of cooked maize starch in the presence of the green tea extract (0, 1, 2, 4 mg/ml) was analyzed using a hexokinase kit for measuring the type of inhibition. A Lineweaver–Burk plot was used to examine the inhibitory type of the green tea extract on amylase.

2.4. Fluorescence quenching studies

A HITACHI fluorescence spectrometer (Model 650–60, Hitachi, Tokyo, Japan) was used for fluorescence quenching assays. The sample (1 ml) was excited at 280 nm, with 1 nm excitation and emission slits, and spectra were recorded between 300 and 400 nm at 0.1 nm resolution. A stock solution of α-amylase (0.2 mg/ml) and the quenchers of the green tea extract (1 mg/ml) were prepared by dissolving in phosphate buffer (pH 5.2). The fluorescence intensities were obtained at different green tea extract concentrations and plotted according to the Stern–Volmer equation (Lakowicz, 2006).

2.5. Docking studies

The three-dimensional structure of human pancreatic α-amylase was imported from the Protein Data Bank (1HNY). The structure of EGCG and ECG was generated with the Cambridge Soft ChemBioDraw Ultra (Version 12.0) and energy minimized with the MM2 calculations using a conjugate gradient. Before the docking procedure, water molecules were removed from the crystal structure of digestive enzyme using Accelrys Discovery Studio 3.0 software. Automated molecular docking studies of the inhibitory ligand at the amylase-binding site were performed with the AutoDock 4.2 package, in the presence of cofactors (calcium and chloride ions). The Binding Site tool was used to determine the active site. The docking runs were performed with a radius of 9 Å with coordinates x: 11.563, y: 46.792, and z: 44.400. The best pose for the green tea extract was obtained.

2.6. Statistical analysis

All analyses were performed in triplicate, and the results were expressed as the mean value ± standard deviation. Analysis of variance (ANOVA) was performed, and the mean separation was analyzed by Duncan's test ($p < 0.05$). All statistical analyses were conducted using Version 18.0 SPSS Statistics (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. The catechins contents in the green tea extract

The HPLC chromatography of the green tea extract is illustrated in Fig. 1. Three major catechins forms including EC (59.2%), EGCG (14.6%) and ECG (26.2%) were detected in tea extract. According to Ho et al. (2008), there were six major catechins found in green tea, including catechin (C), EC, ECG, EGC, EGCG and gallocatechin gallate (GCG). In general, EGCG is the most powerful and abundant of the six major catechins found in green tea. However, only three catechins with a lower EGCG content were present in tea extract, which may be attributed to biological origin, physiology of tea plant, soil characteristics and isolation method as previously reported by Yang et al. (2009) and Senanayake (2013).

3.2. α-Amylase inhibition studies

Inhibition activity of the green tea extract against human α-amylase is listed in Table 1. The % inhibition and IC_{50} of the green tea extract (10%, based on starch) were 63.5% and 2.07 mg/ml, respectively. According to He, Lv, and Yao (2006), the inhibition ratio of α-amylase was approximately 61% when the concentration of tea polyphenols (including 5.1% C and EGC, 40.9% EGCG, 30.4% ECG, 10.9% GCG and 6.3% EC) was 0.05 mg/ml. The reported inhibition ratio was comparable with our data of % inhibition, although the concentration of green tea extract we used was 20-fold higher than the concentration of tea polyphenol used by He et al. (2006). Koh, Wong, Loo, Kasapis, & Huang (2010) reported that there was no solid relationship between total phenolic content and enzyme inhibition activity, and green tea showed relatively low inhibitory activity with IC_{50} range from 2.8 to 4.4 mg/ml. IC_{50} of an inhibitor was dependent on enzyme concentration and origin, substrate type and concentration along with other experimental conditions (reaction duration, temperature and pH) (Cer, Mudunuri, Stephens, & Lebeda, 2009). In this study, the reaction condition of α-amylase assay at a pH of 5.2 was used to examine the inhibitory effect and different with the optimum condition for enzyme activity in previous literatures (Hanhineva et al., 2010; Hara & Honda, 1990; Koh et al., 2010).

From the double-reciprocal (Lineweaver–Burk) plots between 1/[*S*] (starch concentration) and 1/[*V*] (reaction rate) (Fig. 2), the type of inhibitive mode of the green tea extract towards α-amylase belonged to the non-competitive type. According to the Michaelis–Menten equation, K_m of α-amylase was calculated as 5.90 mg/ml. A decreased reaction velocity without affecting the enzyme's affinity for substrate was also observed in Table 1 ($p < 0.05$). Similar behavior is reported in the literature for enzyme kinetic studies of millet phenolics (Shobana, Sreerama, & Malleshi, 2009), which indicated that the Michaelis–Menten constant remained unchanged but the maximal velocity decreased, revealing a non-competitive type of inhibition on α-amylase. The inhibitory activity of the green tea extract against amylase was associated with hydrogen bonds between the hydroxyl groups of the ligands and the catalytic residues of the binding site and formation of a conjugated π-system that stabilizes the interaction with the active site (Miao et al., 2013; Xiao, Ni, Kai, & Chen, 2013). Hara and Honda (1990) also reported that the inhibitory pattern of α-amylase by four kinds of the green tea catechins and their isomers belonged to non-competitive inhibition. EGCG might modulate amylase-mediated starch digestion and non-competitively inhibited pancreatic amylase activity by 34% at 20 μM as suggested by Forester, Gu, and Lambert (2012).

3.3. Human pancreatic α-amylase binding studies

The fluorescence emission spectra of human α-amylase at various concentrations of the green tea extract following the excitation at 280 nm is shown in Fig. 3. The tryptophan (Trp) residue was responsible for the fluorescence intensity of proteins (Lakowicz, 2006). As shown in Fig. 3, there was a fluorescence emission peak at near 348 nm, which belongs to Trp residues located at protein interior. With the increased concentration of the green tea extract, the fluorescence intensity of α-amylase was significantly reduced, which was caused by protein–polyphenol interaction (Xiao et al., 2013). Meanwhile, a faint blue shift of the maximum emission wavelength from 348 to 342 nm was observed, which suggested that the interaction between α-amylase and the catechins in the green tea extract resulted in a polarity variation for Trp and quenching the intrinsic fluorescence intensity.

Fluorescence quenching process can be divided up into 2 types, namely a collision process, dynamic quenching mechanism; and a formation of a ground-state complex between quencher and fluorophore, static quenching mechanism. Both mechanisms can be distinguished by their different dependence on the temperature and excited life-time (Lakowicz, 2006). For the dynamic quenching, higher temperatures will result in faster diffusion and larger amounts of collisional quenching, hence the quenching constant values will increase with increasing temperature, but the reverse effect would be observed for static quenching. Dynamic quenching follows the Stern–Volmer equation: $F_0/F = 1 + K_{sv}[Q] = 1 + K_q \tau_0 [Q]$, where F_0 and F are fluorescence intensity of amylase in the absence and in the presence of tea extract, $[Q]$ is the concentration of the green tea extract, τ_0 is the average life of the emissive excited state of amylase (about 10^{-8} s), K_q is the quenching rate constant, K_{sv} is the dynamic quenching constant. The values of K_q and K_{sv} by the plots of linear equation (F_0/F vs. $[Q]$) were present in Table 2. The value of K_{sv} at 293 K and 313 K was 1.19×10^3 and 0.67×10^3 l/mol, respectively, which showed that the values of K_{sv} decreased with increasing temperature. The K_q value at 293 K or 313 K was higher

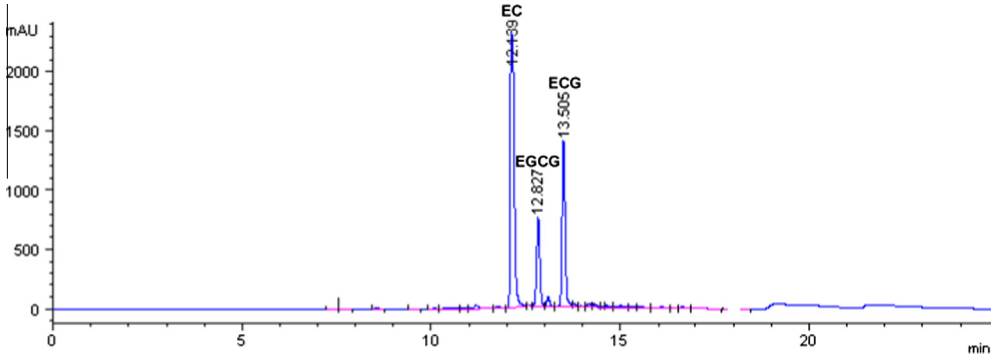

Fig. 1. HPLC chromatogram of the green tea extract. EC: (−)-epicatechin; EGCG, (−)-epigallocatechin gallate; ECG: (−)-epicatechin gallate.

Table 1
The inhibiting parameters of the green tea extract against α-amylase.

		Green tea extract
% Inhibition		63.5 ± 3.8
IC_{50} (mg/ml)		2.07 ± 0.19
Type		Non-competitive
V_{max} (mg/(ml × min))[1]	Control	0.43 ± 0.02[a]
	1 mg/ml of tea extract	0.29 ± 0.05[a]
	2 mg/ml of tea extract	0.18 ± 0.12[c]
	4 mg/ml of tea extract	0.07 ± 0.01[b]
K_m (mg/ml)		5.90 ± 0.12

[1] V_{max} was determine at control, 1, 2, and 4 mg/ml of tea extract, respectively. The values having different superscripts in the same column are significantly different ($p < 0.05$) by Duncan's test.

than 2.0×10^{10} l/(mol × s), which is the maximal value for macromolecule-participating quenching rate constant in dynamic quenching (Zhang & Ma, 2013). Therefore, the process of quenching is not the dynamic quenching induced by the collision of molecules, but the static quenching by forming a complex.

Assuming the catechins in the green tea extract binds independently to a set of equivalent sites on a starch-active enzyme, the binding constant (K_a) and number of binding sites per amylase (n) were calculated according to the double-logarithm equation: $\lg\frac{F_0-F}{F} = \lg K_a + n \lg[Q]$ (Lakowicz, 2006). The calculated values of K_a and n at 293 K or 313 K were presented in Table 2. The increasing trend of K_a with rising temperature [K_a(313 K) > K_a(293 K)] indicated that the capacity of the catechins in the green tea extract binding to human α-amylase was enhanced and the binding was an endothermic reaction. The value of n approximately equals to 1, which suggested that there was only one binding site in human pancreatic α-amylase for the catechins in the green tea extract.

3.4. Docking studies

Based on a large amount of literature (Forester et al., 2012; Ho et al., 2008; Thielecke & Boschmann, 2009), the tea catechins, as bioactive phytochemicals, have been suggested to have a variety of health benefits such as anti-cancer, anti-tumor, anti-cardiovascular disease, anti-obesity, and anti-diabetic properties. It is known that the catechin possesses two benzene rings (A ring and B ring) and a dihydropyran heterocycle (C ring) with a hydroxyl group on carbon 3 (Ho et al., 2008). Among them, EGCG, known as epigallocatechin-3-gallate, is the ester of epigallocatechin and gallic acid,

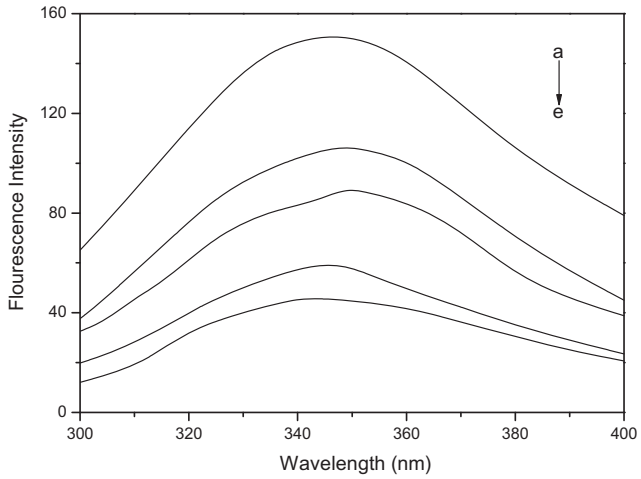

Fig. 3. The fluorescence spectra of α-amylase at different concentrations of the green tea extract: (a) 0, (b) 0.25, (c) 0.5, (d) 1.25, (e) 2 mM measured in phosphate buffer, pH 5.2, λ_{ex} = 280 nm.

Table 2
The quenching parameters of the green tea extract against α-amylase.

	Temperature (K)	K_{sv} (10^3 l/mol)	K_q (10^{11} l/(mol × s))	n	K_a (mol/l)
Green tea extract	293	1.19	1.19	0.89	277.59
	313	0.67	0.67	1.06	970.51

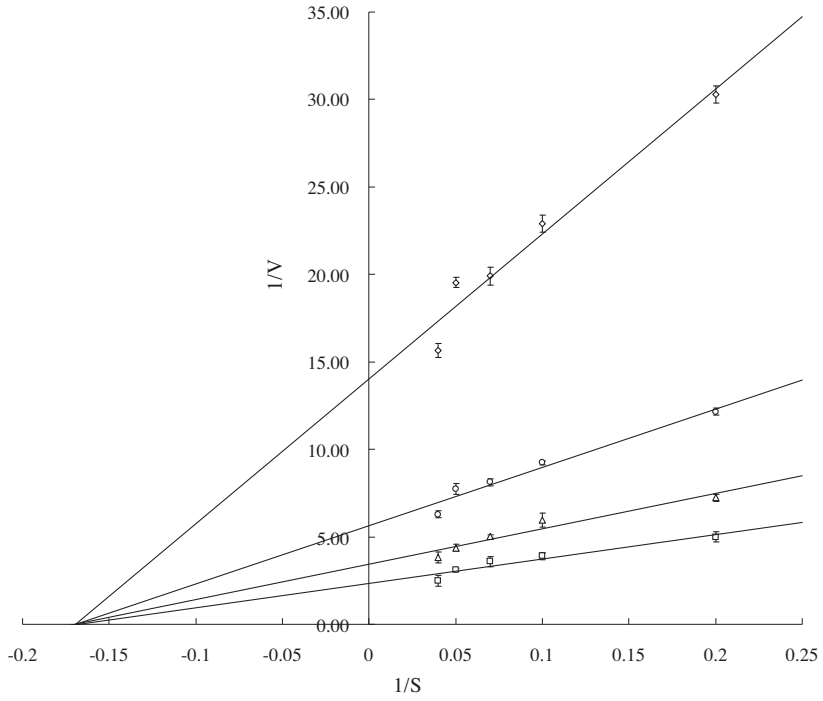

Fig. 2. Lineweaver–Burk plots of the green tea extract for the α-amylase inhibitory activity. □: control, ▽: 1 mg/ml, ○: 2 mg/ml, ◇: 4 mg/ml.

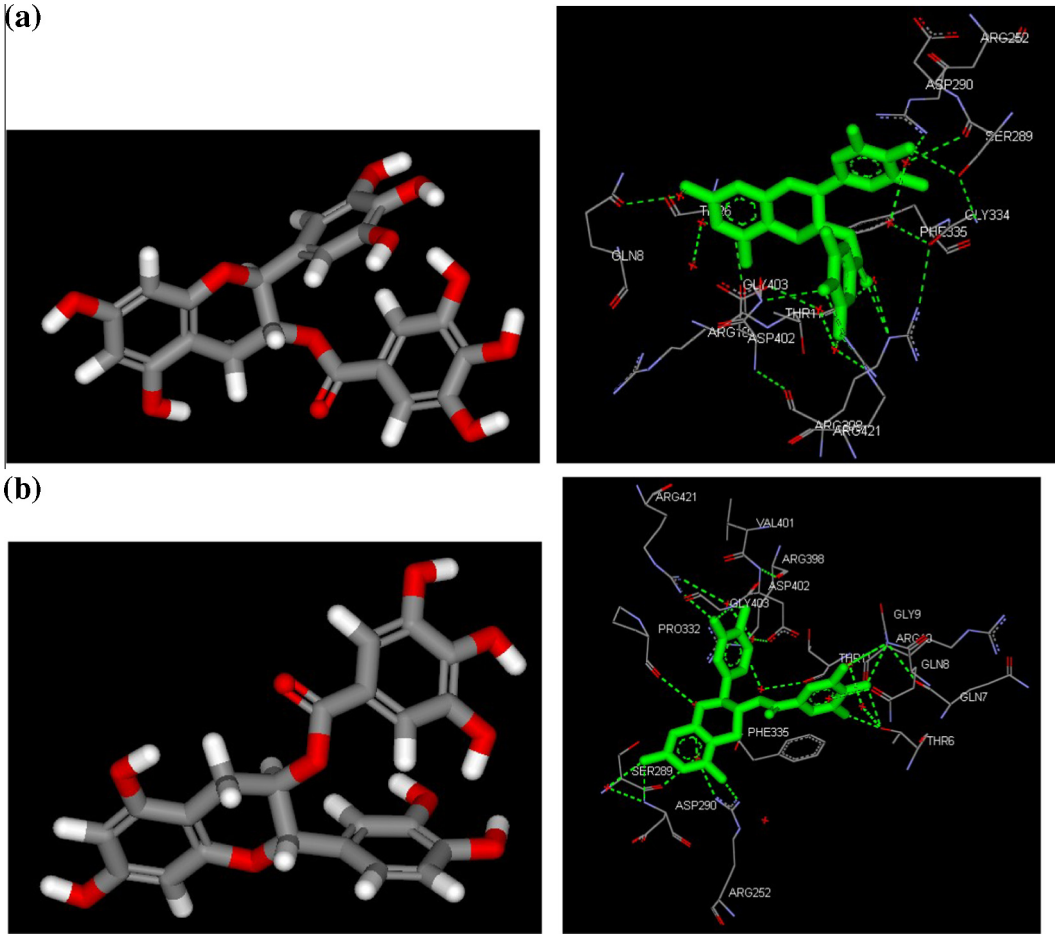

Fig. 4. Details of the interaction between human α-amylase and the catechins in the green tea extract: (a) EGCG and (b) ECG.

and ECG, known flavan-3-ol, is a derivative of flavans that use 2-phenyl-3,4-dihydro-2 H-chromen-3-ol skeleton (Fig. 4). The structure of the A, B and C rings were related with the inhibitory activity against amylase. However, not all tea catechin monomers showed positive results for glycemic control as suggested by Hara and Honda (1990) and Xiao et al. (2013). Xiao et al. (2011) reported that EC hardly quenched the fluorescence of amylase and galloylated catechins have higher binding affinities with α-amylase than non-galloylated catechins. The amino acid residues of amylase participated in hydrogen bonding and π–π interaction with the phenolic backbone, and the glycosylation at position R3 of C ring increasing the inhibition on enzyme.

In docking study, only EGCG and ECG were applied for the following experiments. The docking studies of the EGCG and ECG at the human pancreatic α-amylase catalytic site are present in Fig. 4. As shown in Fig. 4, the EGCG was surrounded by 12 amino acid residues, including Gln-8, Thr-11, Arg-252, Ser-289, Asp-290, Pro-332, Gly-334, Phe-335, Arg-398, Asp-402, Gly-403 and Agr-421, while ECG interacted with 13 amino acid residues, such as Gln-7, Gly-9, Arg-10, Thr-11, Arg-252, Ser-289, Asp-290, Pro-332, Gly-334, Arg-398, Asp-402, Gly-403 and Arg-421. A best docked conformation was stabilized both by hydrogen bonds and Van der Waals forces. It is known that human pancreatic α-amylase contain 3 conservative domains (named A, B, C) (Brayer et al., 2000). Domain A (residues 1–99 and 169–404) in a tubby shape is the center of catalysis and composed of $(\beta/\alpha)_8$ with three putative active site residues Asp197, Glu233, and Asp300. Domain B (residues 100–168) and domain C (residues 405–496) are relevant to specificity and stability of substrate, while the carboxyl terminal of α-amylase can bind with starch. It could be concluded that the green tea extract occupied domain A of α-amylase in a non-competitive inhibitory mode, and interacted with the side chain of Asp300, which was in agreement with the only one binding site in fluorescence quenching studies and the inhibition kinetic studies. Hara and Honda (1990) found that the inhibitory effect was in the order of ECG > EGCG, indicating catechol type catechins have higher inhibition than pyrogallol-type catechins, which was consistent with our results of ECG containing stronger affinity with more number of amino acid residues involved in amylase binding. Moreover, Xiao et al. (2013) gave an overview of the structure–activity relationship of polyphenols inhibiting α-amylase. The hydroxylation and galloylation of catechins increased the inhibitory effects against α-amylase, whereas the glycosylation of hydroxyl group, methylation, methoxylation, and hydrogenation of the C2=C3 double bond on flavonoids decreased the inhibition. Those observations were fairly compatible with our results, revealing that the catechol-type catechins (ECG) have higher inhibition than pyrogallol-type catechins (EGCG).

4. Conclusions

The data demonstrate specific interactions between the catechins in the green tea extract and human α-amylase. Compared to EGCG, ECG contained stronger affinity with amylase and appeared to be mainly responsible for the inhibitory effect on human α-amylase activity through binding active site residues, which suggest the use of the green tea extract as viable alternatives

to natural inhibitor for glycemic control. Further work is underway to design functional foods with the proper utilization of tea extract for reducing glucose release of cereal products while providing added health benefits.

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