

RESEARCH ARTICLE

The mechanism of interactions between tea polyphenols and porcine pancreatic alpha-amylase: Analysis by inhibition kinetics, fluorescence quenching, differential scanning calorimetry and isothermal titration calorimetry

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Scope: This study aims to use a combination of biochemical and biophysical methods to derive greater mechanistic understanding of the interactions between tea polyphenols and porcine pancreatic α -amylase (PPA).

Methods and results: The interaction mechanism was studied through fluorescence quenching (FQ), differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) and compared with inhibition kinetics. The results showed that a higher quenching effect of polyphenols corresponded to a stronger inhibitory activity against PPA. The red-shift of maximum emission wavelength of PPA bound with some polyphenols indicated a potential structural unfolding of PPA. This was also suggested by the decreased thermostability of PPA with these polyphenols in DSC thermograms. Through thermodynamic binding analysis of ITC and inhibition kinetics, the equilibrium of competitive inhibition was shown to result from the binding of particularly galloylated polyphenols with specific sites on PPA. There were positive linear correlations between the reciprocal of competitive inhibition constant ($1/K_{ic}$), quenching constant (K_{FQ}) and binding constant (K_{itc}).

Conclusion: The combination of inhibition kinetics, FQ, DSC and ITC can reasonably characterize the interactions between tea polyphenols and PPA. The galloyl moiety is an important group in catechins and theaflavins in terms of binding with and inhibiting the activity of PPA.

Keywords:

α -Amylase / Differential scanning calorimetry / Fluorescence quenching / Interaction mechanism / Isothermal titration calorimetry / Tea polyphenols



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1 Introduction

Dietary phenolic compounds have been suggested as potential alternatives to medicines for controlling and treating type II diabetes, a chronic disease caused by reduced insulin sensitivity, as they have inhibitory effects on α -amylase [1, 2]. Alpha-amylase is present in saliva and secreted by the pancreas into the small intestine. It catalyses the digestion of

porcine pancreatic α -amylase; **TA**, tannic acid; **TF**, theaflavin; **TF1**, theaflavin-3'-gallate; **TF2**, theaflavin-3, 3'-digallate

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Abbreviations: **BTE**, black tea extracts; **C**, catechin; **DSC**, differential scanning calorimetry; **EC**, epicatechin; **ECG**, epicatechingallate; **EGC**, epigallocatechin; **EGCG**, epigallocatechingallate; **FQ**, fluorescence quenching; **GTE**, green tea extracts; **ITC**, isothermal titration calorimetry; **K_{ic}**, competitive inhibition constant; **K_{iu}**, uncompetitive inhibition constant; **K_{FQ}**, fluorescence quenching constant; **K_{itc}**, binding constant; **OTE**, oolong tea extracts; **PPA**,

dietary starch to maltooligosaccharides. These maltooligosaccharides are further degraded to glucose, which is absorbed into the blood stream, triggering glycaemic and insulaemic responses [3]. The inhibitory activities of polyphenols are highly dependent on their molecular structures [4], and the inhibition of α -amylase results from the interactions and/or binding of polyphenols with the enzyme. Hydrogen bonding between the hydroxyl groups and the active site of the enzyme, as well as hydrophobic interactions between the aromatic moieties of polyphenols and enzyme are considered to be the prime forces that drive the interactions (binding) [5].

Previously, the interactions between polyphenols and α -amylase have been characterized by determination of the half inhibition concentration (IC_{50}), kinetics of inhibition, fluorescence quenching (FQ) and docking analysis [6]. The IC_{50} indicates the strength of inhibition, i.e. lower IC_{50} value means higher inhibitory activity. From the kinetics of inhibition, the inhibition type can be identified, and competitive inhibition constant (K_{ic}) and uncompetitive inhibition constant (K_{iu}) are obtained [7]. Furthermore, the fluorescence quenching constant (K_{FQ}) indicating the binding affinity of polyphenols to the enzyme is obtained from the quenching effects of polyphenols on the intrinsic fluorescence of tryptophan side chains within the structure of α -amylase [8]. However, the correlation between K_{FQ} and $1/K_{ic}$ needs further study, as both constants suggest the binding of polyphenols with the enzyme [6]. Tea polyphenols, including (-)-epicatechingallate (ECG), (-)-epigallocatechingallate (EGCG), theaflavin-3, 3'-digallate (TF2), theaflavin-3'-gallate (TF1) and theaflavin (TF) have all been reported to have α -amylase inhibition activity in vitro [8–11]. However, the precise mechanism of the inhibition of α -amylase by tea polyphenols has not been elucidated in terms of binding interactions between polyphenols and enzyme.

Differential scanning calorimetry (DSC) can be applied to monitor phase and conformational transitions through measurement of specific heat capacity as a function of temperature for a sample [12]. It offers an objective and comprehensive way for evaluating the thermal stability of proteins [13]. It has been reported that polyphenol interactions may change the thermostability of proteins [14, 15]. Therefore, DSC can be used to study the effect of tea polyphenols on the thermostability of α -amylase. Isothermal titration calorimetry (ITC) permits the determination of binding enthalpy and binding constant of the reaction between a macromolecule and a ligand [16]. This technique has been successfully applied to determine the binding constants of oligomeric ellagitannins with bovine serum albumin [17–19]. To our knowledge, the combination of DSC and ITC has not been previously applied to study the interactions of polyphenols and porcine pancreatic α -amylase (PPA).

In our previous study, the kinetics of inhibition (IC_{50} , K_{ic} and K_{iu}) of PPA by a range of tea polyphenols were systematically studied [6]. In this study, FQ, DSC and ITC were combined together with the kinetics of inhibition to elucidate the mechanism of binding interactions of tea polyphenols

and PPA. In this approach, $1/K_{ic}$, K_{FQ} and K_{ic} are compared and correlated to analyze the relationships between PPA inhibition and binding behavior. The role of the galloyl moiety in binding of catechins and theaflavins with PPA is highlighted.

2 Materials and methods

2.1 Materials and chemicals

Aqueous extracts of various teas (TE), green (GTE), black (BTE) and oolong (OTE) were prepared as described [6]. PPA (EC 3.2.1.1 A6255) was purchased from Sigma-Aldrich Co. Ltd. (St. Louis, US). Pure phenolic compounds including tannic acid (TA), (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechingallate (EGCG), (-)-epicatechingallate (ECG), theaflavin (TF), theaflavin-3'-gallate (TF1) and theaflavin-3, 3'-digallate (TF2) were obtained from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Other chemicals were of analytical grade.

2.2 Kinetics of inhibition

The kinetics of inhibition of PPA by TEs and tea polyphenols were as reported in our previous research [6]. In brief, 20 mg/mL normal maize starch was prepared in PBS buffer and cooked at 90°C for 20 min before dilution to serial concentrations (1.25–15 mg/mL). Then for each starch concentration, 50 μ L of TEs and pure polyphenols with different concentrations were pre-incubated with 50 μ L of 7.65 U/mL PPA solution (in PBS buffer) at 4°C for 20 min, followed by the addition of 4 mL of cooked starch, and the digestion process was carried out at 37°C. The initial reaction velocity was determined using the PAHBAH method described in our previous study [6]. To calculate the competitive inhibition constant, K_{ic} and uncompetitive inhibition constant, K_{iu} , Dixon (1) and Cornish–Bowden (2) equations were applied as follows [7]:

$$v = \frac{V_{\max}a}{K_m \left(1 + \frac{i}{K_{ic}}\right) + a} \quad (1)$$

$$\frac{v}{a} = \frac{V_{\max}}{K_m \left(1 + \frac{i}{K_{ic}}\right) + a \left(1 + \frac{i}{K_{ic}}\right)} \quad (2)$$

where v is the initial reaction velocity determined in the experiment, V_{\max} is the maximum initial reaction velocity, a is the concentration of starch, K_m is the Michaelis constant, i is the concentration of inhibitor, K_{ic} is the competitive inhibition constant and K_{iu} is the uncompetitive inhibition constant.

2.3 Fluorescence quenching

Fluorescence spectra of PPA in the absence and presence of TEs and pure polyphenols were recorded by using a Shimadzu[®] spectrofluorimeter (RF-5301 PC, Tokyo, Japan) according to a previously reported method [20] with some modifications. Briefly, three TEs were dissolved in PBS buffer and diluted to 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0 mg/mL. Concentration series of pure polyphenols were prepared at 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 mg/mL, respectively. Polyphenol solution (0.2 mL) (or TE solution) was added to a tube containing 3 mL of 0.07 mg/mL PPA solution and mixed thoroughly, followed by incubation at 4°C for 30 min. The control comprised 3 mL of the enzyme solution plus 0.2 mL of PBS buffer. After incubation, each sample solution was transferred into a quartz cuvette pre-washed using distilled water and sample solution in sequence. Then, the cuvette was loaded into a small cell and the fluorescence spectra were recorded immediately at fast speed and low sensitivity with the excitation wavelength (λ_{ex}) set as 282 nm and the emission λ_{em} recorded from 300 to 500 nm. Both the slit widths were 10 nm.

Fluorescence quenching is described by the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{FQ} [Q] \quad (3)$$

where F_0 and F are the fluorescence intensity in the absence and presence of the quencher (TE or tea polyphenol), respectively; k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore. For α -amylase, the τ_0 is 2.97 ns [21]; $[Q]$ is the concentration of the quencher; K_{FQ} is the fluorescence quenching constant.

Usually, a linear Stern–Volmer plot indicates that there is a single class of fluorophore in the protein interacting with the quencher in the same way and that only one quenching mechanism (dynamic or static) takes place. However, positive deviations for the equation are frequently observed when the quenching extent is large. In this case, the plot of F_0/F against $[Q]$ describes an upward curve, concave towards the y axis. Commonly, the upward curvature indicates that there are several mechanisms responsible for the quenching effects on fluorophores in protein, or it suggests the existence of a ‘sphere of action’, i.e. apparent static quenching [22]. The modified form of the Stern–Volmer equation describing this situation is as follows [23]:

$$\frac{F_0}{F} = e^{(K_{FQ}[Q])} \quad (4)$$

2.4 Differential scanning calorimetry

The thermostability of the PPA control and that bound with TEs and pure phenolic compounds was studied by use of a differential scanning calorimeter (DSC Q2000, TA[®] Instrument, New Castle, DE). Solutions of eight individual phenolic

compounds (60 mg/mL), concentrations of the three TEs (40, 80 and 120 mg/mL) and 47 mg/mL PPA were prepared in PBS (20% DMSO) buffer. The DSC procedure was performed according to a previous method¹³ with some modifications: 50 μ L of PPA and 50 μ L of each polyphenol (or TE) solution were mixed thoroughly in a 1.5 mL microcentrifuge tube. After incubation for 30 min at 4°C, 15 μ L of the mixture was pipetted into a TA[®] Tzero pan and tightly sealed with a Tzero Hermetic lid. 15 mg of each sample solution was loaded. The control was PPA with PBS (20% DMSO) buffer. Thermograms were recorded from 10 to 100°C with 5°C/min heating rate, using an empty pan as the reference. The enthalpy values (ΔH) were calculated based on the mass of protein in DSC sample pans (J/g protein).

2.5 Isothermal titration calorimetry

A GE[®] ITC instrument (MicroCal iTC₂₀₀, Stockholm Sweden) was used to determine enthalpy changes associated with polyphenol–PPA interactions at 298 K. In a typical ITC experiment, a polyphenol solution in the injection syringe was titrated into 1.175 mg/mL PPA solution in the sample cell of the calorimeter with stirring at 1000 rpm. All the solutions were prepared in PBS buffer. Each polyphenol solution (10 mg/mL for TEs, ECG, EGCG, TF1 and TF; 5 mg/mL for TA and TF2) was titrated as a sequence of 20 injections. The volume of each injection was 2 μ L and the duration of each was 4 s. The time delay between the injections was 150 s. Control experiments included the titration of polyphenol solutions into PBS buffer, the titration of PBS buffer into PPA solution and the titration of PBS buffer into PBS buffer. The latter two control experiments resulted in no measurable enthalpy changes; therefore, they were not considered in the data analysis [19]. The control data of polyphenols titrated into PBS buffer was always subtracted from the sample data. The raw data were obtained as a plot of heat flow (μ cal s⁻¹) against time (min). Then, the data was integrated peak-by-peak and normalized using MicroCal Origin (MicroCal Inc.) to obtain a plot of corrected enthalpy change per mole of injection (ΔH kJ mol⁻¹) against molar ratio of polyphenol to PPA (or weight ratio of TEs to PPA). The data obtained were fitted using a single-site binding model. The equation for this binding model is [24]

$$Q_i = \frac{n[M] \Delta H V_0}{2} \left\{ 1 + \frac{[P]}{n[M]} + \frac{1}{nK_{\text{itc}}[M]} - \sqrt{\left(1 + \frac{[P]}{n[M]} + \frac{1}{nK_{\text{itc}}[M]} \right)^2 - 4 \frac{[P]}{n[M]}} \right\} \quad (5)$$

where Q_i is the total heat released after injection i , V_0 is the volume of the calorimeter cell, $[M]$ is the total concentration of PPA, $[P]$ is the total concentration of polyphenols, n is the molar ratios of interacting species, ΔH is the enthalpies, K_{itc} is the equilibrium binding constants.

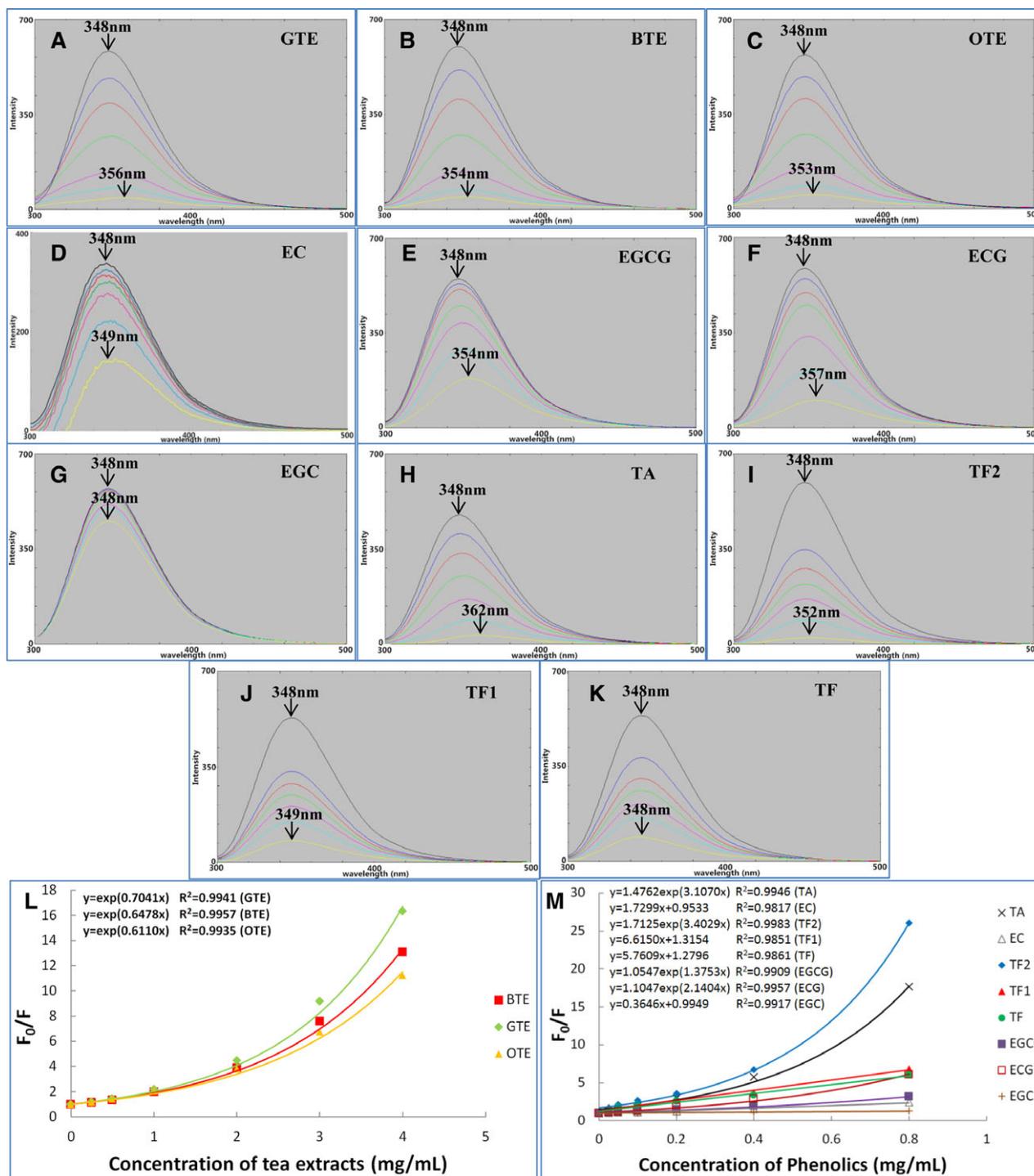


Figure 1. Fluorescence spectra of PPA in the absence (black line) and presence (coloured lines) of GTE (A), BTE (B), OTE (C), EC (D), EGCG (E), ECG (F), EGC (G), TA (H), TF2 (I), TF1 (J) and TF (K). From top down, the concentrations of three TEs are 0, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0 mg/mL, and the concentrations of eight pure polyphenols are 0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 mg/mL. The values labelled in plot (A–K) are the maximum λ_{em} at 0 and highest concentrations of phenolic compounds, respectively; Stern-Volmer plots for fluorescence quenching of PPA by three TEs (L) and eight pure polyphenols (M). The equations for EC, TF1, TF and EGC were fitted according to equation (3), and equations for three TEs, TA, TF2, EGCG and ECG were fitted according to equation (4).

Table 1. Fluorescence quenching parameters for the interactions of TEs and pure polyphenols with PPA

Phenolic compounds	FQ parameters		
	K_{FQ} (M^{-1})	k_q ($10^{11} M^{-1} s^{-1}$)	Red-shift of maximum λ_{em} (nm)
GTE	0.70 ^C (mL/mg)	2.37 ^C (10^8 mL mg ⁻¹ s ⁻¹)	8 ^B
BTE	0.65 ^B (mL/mg)	2.18 ^B (10^8 mL mg ⁻¹ s ⁻¹)	6 ^A
OTE	0.61 ^A (mL/mg)	2.06 ^A (10^8 mL mg ⁻¹ s ⁻¹)	5 ^A
EC	273.07 ^b	0.92 ^b	1 ^{ab}
EGCG	630.4 ^c	2.12 ^c	6 ^d
ECG	946.85 ^d	3.19 ^d	9 ^e
EGC	111.67 ^a	0.38 ^a	0 ^a
TA	5285.60 ^g	17.80 ^g	14 ^f
TF2	11711.81 ^h	39.43 ^h	4 ^c
TF1	4740.24 ^f	15.96 ^f	1 ^{ab}
TF	3252.03 ^e	10.95 ^e	0 ^a

*Different letters in the same column represent significantly different mean values ($P < 0.05$).

2.6 Statistical analysis

The data in this study are expressed as the means of duplicates and analyzed through one-way analysis of variance (ANOVA) using SPSS 18.0 Statistics (Chicago, USA). The mean values were evaluated by Dunnett's *t*-test at the 95% significance level ($P < 0.05$). The ITC data were analyzed to obtain the binding parameters and fit standard deviation using MicroCal Origin software.

3 Results

3.1 Kinetics of inhibition

In our previous study [6], the phenolic constituents of the three TEs were analyzed by UPLC. All TEs contained different contents of catechins, including C, EC, ECG, EGC and EGCG. Both black tea extract (BTE) and oolong tea extract (OTE) contained TF, and only BTE contained TF1 and TF2 [6]. The inhibitory activities against PPA of pure phenolic compounds were in the order of TF2>TF1≈TA>TF>ECG>EGCG>EC>EGC as indicated by IC₅₀ values, which are shown together with values of K_{ic} and K_{iu} in Supporting Information Table S1 as reported previously [6].

3.2 Fluorescence quenching

The fluorescence intensity of PPA before and after addition of tea polyphenols was determined to investigate the interactions between them. Figure 1A–K shows the fluorescence emission spectra of PPA obtained at $\lambda_{ex} = 282$ nm with addition of three TEs; (GTE, BTE and OTE) and eight pure phenolic compounds (EGCG, ECG, EGC, EC, TA, TF2, TF1, and TF). In all cases a decrease in the fluorescence intensity, albeit to different extents, was observed from the quenching.

Notably, there was a red-shift of the maximum λ_{em} for PPA with three TEs, EGCG, ECG, TA and TF2, while no significant shift was observed for EC, TF1, TF or EGC. The Stern–Volmer plots presented for EC, TF1, TF and EGC showed a linear character, and the plots for TEs, EGCG, ECG, TA and TF2 had an upward curvature, concave toward the γ axis (Fig. 1L and M). Hence, the original Stern–Volmer Eq. (3) was applied for EGC, EC, TF1 and TF, and the modified Stern–Volmer Eq. (4) was applied for TEs, EGCG, ECG, TA and TF2 to calculate the quenching parameters. The K_{FQ} and bimolecular quenching constants (k_q) for TEs and pure polyphenols are summarized in Table 1, in which the respective orders of K_{FQ} values for TEs and pure polyphenols are GTE>BTE>OTE and TF2>TA≈TF1>TF>ECG>EGCG>EC. Both these orders are similar to those of the inhibitory activities of these compounds against PPA evaluated by IC₅₀ values (Supporting Information Table S1).

3.3 Differential scanning calorimetry

DSC was applied to assess interactions with PPA in terms of thermal stability. The effects of TEs and pure polyphenols on DSC characteristics of PPA are presented in Fig. 2. All the PPA–TE (or PPA–phenolic) complexes showed endotherms, meaning no complete denaturation due to polyphenol binding was observed. The DSC thermogram of the PPA control showed a single transition with a peak denaturation temperature (T_d) at 67.54°C and denaturation enthalpy (ΔH) of 26.91 J/g protein (Table 2). The thermal stability of bound PPA, as reflected by T_d , decreased as a function of the concentration of GTE, reaching 63.28°C at the highest GTE concentration applied (120 mg/mL). The lowest T_d of PPA bound with BTE and OTE were also observed at their respective highest concentrations and determined as 64.21 and 64.40°C, respectively. Generally, the ΔH of PPA bound with TEs was lowered as well. A lower ΔH demonstrates that a smaller amount of energy is required to unfold the PPA molecule.

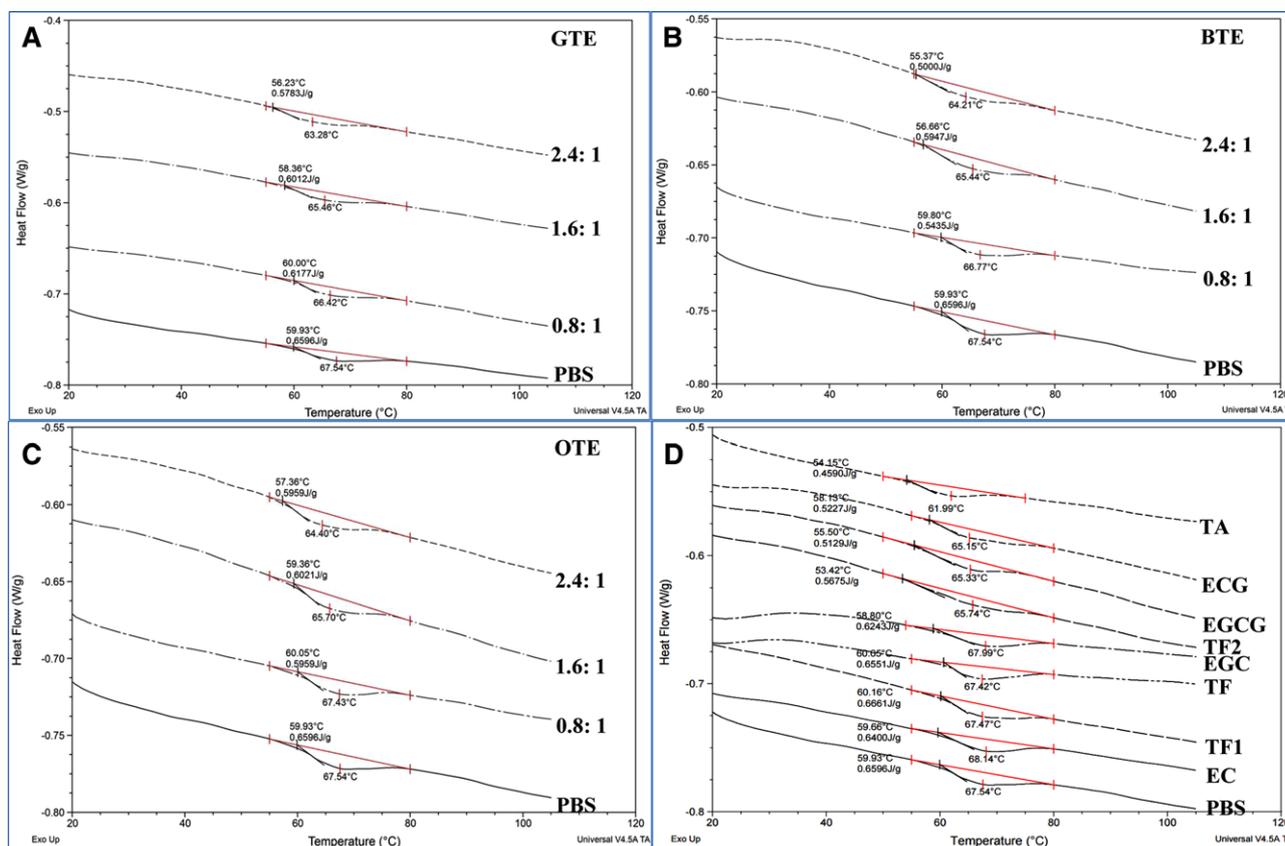


Figure 2. DSC thermograms of PPA treated with GTE (A), BTE (B), OTE (C) and eight pure polyphenols (D). The mass ratios of TEs to PPA are 0.8:1, 1.6:1 and 2.4:1, and the concentration of each pure polyphenol used is 60 mg/mL.

Together with the decreased T_d by TEs, this indicates that PPA molecules bound with TEs were less thermally stable than non-bound PPA. For pure phenolic compounds, the T_d of PPA bound with TA, ECG, EGCG and TF2 decreased by 5.55, 2.39, 2.21 and 1.80°C, respectively, while TF, TF1, EGC and EC had no significant effects on the T_d of PPA. Consistent with the changing tendency of T_d , the ΔH of PPA bound with TA, ECG, EGCG and TF2 decreased to 18.40, 21.32, 20.39 and 23.15 J/g protein, respectively. However, the ΔH values for TF, TF1, EGC and EC did not change significantly.

3.4 Isothermal titration calorimetry

ITC is a powerful technique to analyze the thermodynamics of binding of polyphenols with proteins [18, 25]. The interactions of eight phenolic compounds and three tea extracts with PPA were investigated by ITC. Figure 3A shows a typical plot of heat flow against time for titration of tannic acid into PBS buffer (green line) and PPA solution (black line). The positive energy flow for titration of tannic acid into buffer was caused by the heat that was released by the dilution of tannic acid. The corrected heat flow for binding of tannic acid with PPA (Fig. 3B) was obtained by subtracting the dilution heat from

the apparent titration heat. The heat flow plots for titration of other polyphenols into PPA solution showed similar dilution responses. For each polyphenol–PPA system studied, an exothermic binding was observed as the corrected heat flow was negative.

The single-site binding model that assumes a single set of multiple binding sites [24] was applied to assess the correlation between interaction heat and molar ratio of polyphenol to PPA. The estimated thermodynamic binding parameters are summarized in Table 3. Inspection of Fig. 4 indicates that the single-site binding model could fit the binding of pure polyphenols better than the three TEs, and the fit standard deviations (the indicator of the goodness of fit) in Table 3 also suggest this. It should be noted that heat was generated during the process of titration of galloylated polyphenols (TA, EGCG, TF2, ECG and TF1) into PPA (Fig. 4A–E), while very little heat was generated during the titration of non-galloylated catechins (EC and EGC) into PPA (Fig. 4G and H). For the pure polyphenols studied, TF2 showed the highest values of K_{itc} (11600 M⁻¹) and ΔH (−8790 J mol⁻¹), indicating the strongest binding with PPA and the greatest amount of heat released during the binding process. Similarly, EGCG was shown to have the weakest interaction with PPA with the lowest values of K_{itc} (482 M⁻¹) and ΔH (−988 J mol⁻¹).

Table 2. Denaturation temperature (T_d) and enthalpy (ΔH) of PPA obtained by DSC thermograms in the absence and presence of TEs and pure polyphenols

Parameters	Phenolic compounds																	
	PBS	TA	ECG	EGCG	TF2	EGC	TF	TF1	EC	GTE	BTE	OTE						
T_d (°C)	67.54 ^f	61.99 ^a	65.15 ^d	65.33 ^d	65.74 ^d	67.99 ^f	67.42 ^f	67.47 ^f	68.14 ^{fg}	66.42 ^e	65.46 ^d	63.28 ^b	66.77 ^e	65.44 ^d	64.21 ^c	67.43 ^f	65.70 ^d	64.40 ^c
ΔH (J/g protein)	26.91 ^g	18.40 ^a	21.32 ^c	20.39 ^b	23.15 ^d	25.47 ^f	26.72 ^g	27.17 ^g	26.11 ^{fg}	25.20 ^{ef}	24.52 ^e	23.59 ^d	26.90 ^g	24.26 ^e	20.40 ^b	24.30 ^e	24.56 ^e	24.30 ^e

* Different letters in the same line represent significantly different mean values ($P < 0.05$). ΔH was calculated based on the mass of proteins in DSC sample pans.

In addition, TA showed the lowest, and ECG showed the highest stoichiometry (n), i.e. the polyphenol to PPA ratio.

4 Discussion

The difference in fluorescence quenching activities of three TEs indicates that the changes in the micro-environment of Trp residues caused by interactions of TEs and PPA may depend on the phenolic constituents in TEs. In comparison with the data of PPA inhibition, it was found that a higher quenching effect of polyphenols corresponded to a stronger inhibitory activity.

Fluorescence quenching can be classified into dynamic and static patterns, in which the former results from collisional encounters between fluorophore and quencher, and the latter is caused by formation of a ground state (complex) between the two compounds [26]. As mentioned, the upward curve characteristics of Stern–Volmer plots for TEs, TF2, TA, EGCG and ECG suggest that PPA fluorescence was able to be quenched through both dynamic and static mechanisms by these compounds or it might describe the existence of a ‘sphere of action’ (apparent static quenching) [27]. On the other hand, the linear characteristics of Stern–Volmer plots for EC, EGC, TF1 and TF mean that only one mechanism (dynamic or static) of quenching occurred for these polyphenols. The bimolecular quenching constant, k_q , which reflects the efficiency of quenching or the availability of quenchers to fluorophores, can be used to determine if the quenching results from complex formation between proteins and quenchers. The k_q is close to $1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ for the typical dynamic mechanism (collision-controlled quenching) [26]. The k_q values of EC, TF1 and TF are 9–150-fold higher than that given in Table 1, suggesting fluorescence quenching of PPA by the three polyphenols involves a static mechanism (complex-controlled quenching). As fluorescence quenching describes how a quencher affects Trp or its micro-environment dynamically or statically upon the interactions of the quencher and protein, a higher fluorescence quenching constant suggests stronger affinity of a quencher to protein [27, 28]. TF2 and TA showed stronger binding affinity to PPA than the other phenolic compounds in this study due to their higher K_{FQ} values (Table 1), presumably due to the large number of potential hydrogen bonds and hydrophobic interactions [29, 30], consistent with the prevalence of the static mechanism in comparison to the dynamic one for PPA fluorescence quenching by TF2 and TA.

The competitive inhibition constant, K_{ic} , represents the dissociation constant of the PPA–polyphenol complex; therefore, the reciprocal of K_{ic} ($1/K_{ic}$) indicates the association constant of polyphenols with PPA. As discussed above, quenching constant, K_{FQ} , indicates the binding affinity of a quencher to protein; therefore, if the quencher binding is related to enzyme inhibition, there should be a relationship between K_{FQ} and $1/K_{ic}$. Based on this concept, the

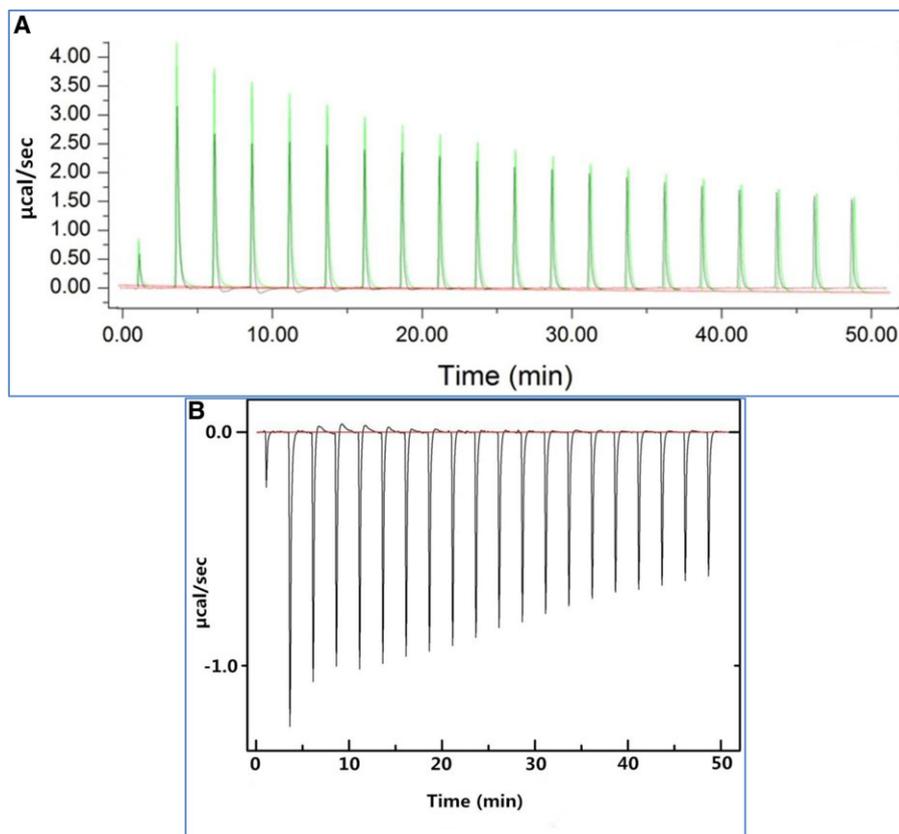


Figure 3. Typical raw (A) and corrected (B) plots of heat flow against time for titration of TA into PPA. Plot (B) was obtained by subtracting the heat flow of titration of TA into PBS buffer (green line in plot (A)) from the heat flow of titration of TA into PPA solution (black line in plot (A)).

correlation between K_{FQ} and $1/K_{ic}$ is shown in Fig. 5A. There was a positive linear correlation between the two constants ($K_{FQ} = 13.97 \cdot 1/K_{ic} + 1517.1$, $R^2 = 0.9590$), suggesting that lower K_{ic} corresponds to higher K_{FQ} . Hence, K_{FQ} and $1/K_{ic}$ obtained through fluorescence quenching and inhibition kinetics methods, respectively, may be combined to characterize the binding of polyphenols with PPA. FQ reflects the change of micro-environment in the vicinity of Trp residues caused by both collisional encounters and complex formation between Trp and quenchers. In addition, other amino acid residues near Trp may also affect the fluorescence of a fluorophore [27]. In contrast, $1/K_{ic}$ definitely reflects the formation of enzyme–inhibitor complex [6]. Therefore, FQ can be more sensitive than inhibition kinetics in analysis of interactions between enzyme and polyphenol, which is reflected by the fact that a lower polyphenol concentration was needed to quench PPA fluorescence than to inhibit PPA activity. This may explain why the coefficient (the slope of the plot of K_{FQ} against $1/K_{ic}$) is much higher than 1. Previous studies also suggest that K_{FQ} values can be much higher than the inhibition constants [8, 31].

The dominant fluorophore of proteins is the indole group of the Trp residue. The indole aromatic heterocyclic residue shows a UV adsorption peak near 270 nm and a fluorescent emission peak near 340 nm. The emission wavelength of indole may be blue-shifted (shorter wavelength) if the group

is buried within a native protein, while it may be red-shifted (longer wavelength) when the protein is unfolded [32]. Therefore, the red-shifted maximum λ_{em} of PPA by TEs, EGCG, ECG, TA and TF2 (Fig. 1A–C, E, F, H and I) indicates that partial structural unfolding occurs for PPA upon binding with these polyphenols. There was no evidence for structural unfolding of PPA bound with EC, EGC, TF1 or TF, as no significant red-shifted maximum λ_{em} was observed (Fig. 1D, G, J and K).

The thermal stability of a protein is strongly dependent on its spatial structure, meaning that partial structural disruption for a protein may decrease its heat stability [33, 34]. In this study, DSC was applied to provide supportive data for the potential structural unfolding of PPA indicated by red-shifted maximum λ_{em} . As shown in Table 1, TA induced 14 nm of red-shift in maximum λ_{em} at its highest concentration. This was the largest shift for all the pure polyphenols investigated and was followed by ECG (9 nm), EGCG (6 nm) and TF2 (4 nm). This indicates that the order of the extent of potential structural unfolding for PPA upon binding with the four polyphenols was TA > ECG > EGCG > TF2. The denaturation process for a protein usually takes place in two steps. One is reversible, arising from the protein unfolding process. In this step, there is a partial loss of activity for the protein due to the disruption of intramolecular non-covalent interactions [35, 36]. The other one is irreversible, leading to the

Table 3. Thermodynamic binding parameters for the interactions of tea polyphenols with PPA fitted by single-site binding model

Parameters	TA	EGCG	TF2	EGC	TF1	TF	GTE	BTE	OTE
$K_{itc} (M^{-1})$	8740 ± 292^d	482 ± 106^a	11600 ± 1750^e	486 ± 105^a	5345 ± 1014^c	1965 ± 553^b	0.70 ± 0.35 ($L \cdot g^{-1}$) ^b	0.65 ± 0.42 ($L \cdot g^{-1}$) ^a	0.61 ± 0.10 ($L \cdot g^{-1}$) ^a
$\Delta H (J \cdot mol^{-1})$	-7273 ± 2022^e	-988 ± 189^a	-8790 ± 1250^{ef}	-1562 ± 569^b	-5214 ± 1586^{cd}	-4256 ± 996^c	-0.54 ± 0.24 ($J \cdot g^{-1}$) ^a	-0.56 ± 0.32 ($J \cdot g^{-1}$) ^a	-0.56 ± 0.14 ($J \cdot g^{-1}$) ^a
N	4 ± 1^a	52 ± 4^f	15 ± 1^b	38 ± 3^e	17 ± 4^c	24 ± 6^d	NA	NA	NA
$\Delta S (J \cdot mol^{-1} \cdot K^{-1})$	6 ± 2^a	14 ± 3^{cd}	5 ± 1^a	13 ± 1^{cd}	10 ± 1^b	12 ± 5^{bc}	0.07 ± 0.01 ($J \cdot g^{-1} \cdot K^{-1}$) ^a	0.07 ± 0.01 ($J \cdot g^{-1} \cdot K^{-1}$) ^a	0.08 ± 0.01 ($J \cdot g^{-1} \cdot K^{-1}$) ^a
SD	30.1	43.6	10.6	4.6	8.9	36.5	359.6	396.4	284.6

*Different letters in the same line represent significantly different mean values ($P < 0.05$). NA, not available. SD is the standard deviation around fit obtained by MicroCal Origin software.

denaturation of the initially unfolded molecule [37]. Therefore, the reversible unfolding process of an enzyme under external force (here, the interactions between polyphenols and PPA) is expected to promote the denaturation process of the enzyme during a DSC experiment. This was confirmed as TA which caused the most extensive PPA structural unfolding (reflected by the most red-shift of maximum λ_{em}), also made PPA most vulnerable to heat during DSC (reflected by the lowest denaturation temperature, T_d , and energy required, ΔH). Also, the T_d of PPA bound with ECG, EGCG, and TF2 corresponded to the extent of structural unfolding caused by binding of the three polyphenols, i.e. lower T_d corresponded to a higher red-shift of maximum λ_{em} . In addition, the T_d and ΔH of PPA incubated with EGC, TF1, TF and EC remained similar to the PPA control, indicating that these four polyphenols did not change the thermostability of PPA. This is because they induced hardly any structural unfolding for PPA, since there was no significant red-shift of maximum λ_{em} for PPA interacting with these polyphenols. Therefore, as no structural unfolding occurred, the thermostability of PPA did not change. A similar relationship of red-shift of maximum λ_{em} and T_d (ΔH) was also observed for PPA bound with three TEs, in which the higher mass ratio of TEs to protein caused lower thermostability of PPA. This is because higher concentration of TEs induced more extensive structural folding of the PPA molecule (Fig. 1A–C).

A similar observation has been reported for rice bran lipase, in which a decrease in apparent thermal denaturation temperature of the enzyme in the presence of chlorogenic acid and caffeic acid was observed [14]. Some earlier studies also indicated that phenolic compounds decreased thermal stability of mono-subunit and multi-subunit proteins by binding with these proteins [15, 38, 39]. Decrease in thermal stability of enzyme is usually associated with protein conformational changes. Tea polyphenols investigated in this study, as compounds with polyhydroxyl groups and aromatic rings in molecular structures, were able to interact with α -amylase through both hydrogen bonding and hydrophobic forces [13, 31]. This might induce conformational changes of PPA, leading to a shift in both intrinsic fluorescence and thermal stability of PPA. It should be noted that the potential unfolding of an enzyme by a phenolic compound is not necessarily related to its fluorescence quenching nor to enzyme inhibition. For example, both TF1 and TF could cause strong fluorescence quenching and inhibition of PPA, but no potential unfolding was observed by DSC thermograms for PPA bound with the two polyphenols.

The kinetics of inhibition of PPA indicates that TA, EGCG and TF2 were competitive inhibitors of PPA, specifically binding with the active site of the enzyme [6]. In ITC binding analysis, the fitted single-site binding model for the three polyphenols suggests that they were most likely to bind at a single set of binding sites. Therefore, the ITC results were consistent with the inhibition kinetics, in terms of specific binding sites on PPA for the competitive inhibitors. To further elucidate the correlation between the binding analyses by ITC and

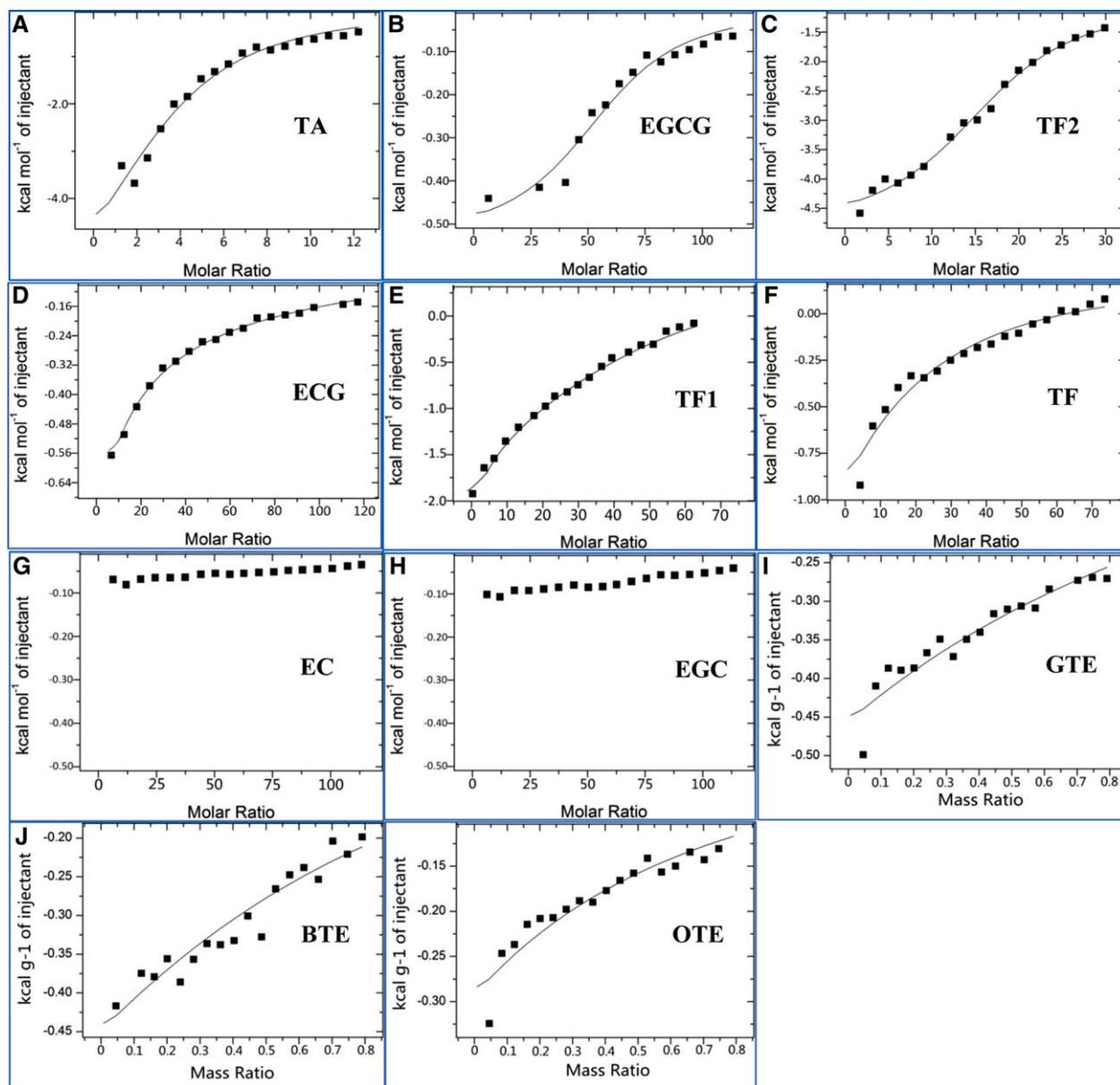


Figure 4. Single-site binding model fitted to the experimental ITC data for the interaction of TA (A), EGCG (B), TF2 (C), ECG (D), TF1 (E), TF (F), EC (G), EGC (H), GTE (I), BTE (J) and OTE (K) with PPA. The model could not fit the data for EC and EGC due to the very small level of heat generated during the titration process.

inhibition kinetics, the relationship between the binding constants obtained through the two methods were plotted (Fig. 5B). As shown, there was a positive linear correlation between K_{itc} and $1/K_{ic}$ ($K_{itc} = 14.634 \cdot 1/K_{ic} + 1746.4$, $R^2 = 0.8239$), indicating that the equilibrium of competitive inhibition (suggested by $1/K_{ic}$) actually resulted from the binding of polyphenols with specific sites on the enzyme (suggested by K_{itc}). Therefore, the constants obtained through the inhibition kinetics and ITC show a consistent relationship, and the two methods may be combined to analyze the binding of polyphenols with α -amylase.

Very little corrected exothermic heat was observed for the titration of EC and EGC into PPA (Fig. 4G and H), indicating that non-galloylated catechins hardly interacted with PPA. This is in agreement with the very weak binding of non-galloylated catechins with PPA that was demonstrated by the low inhibition and fluorescence quenching of PPA by EC and EGC (Table S1 and Fig. 1D and G). By introduction of a galloyl group, EC and EGC were transformed to their galloylated forms, ECG and EGCG, respectively (Supporting Information Fig. S1). Then, the binding of ECG and EGCG with PPA could be detected by the corrected heat

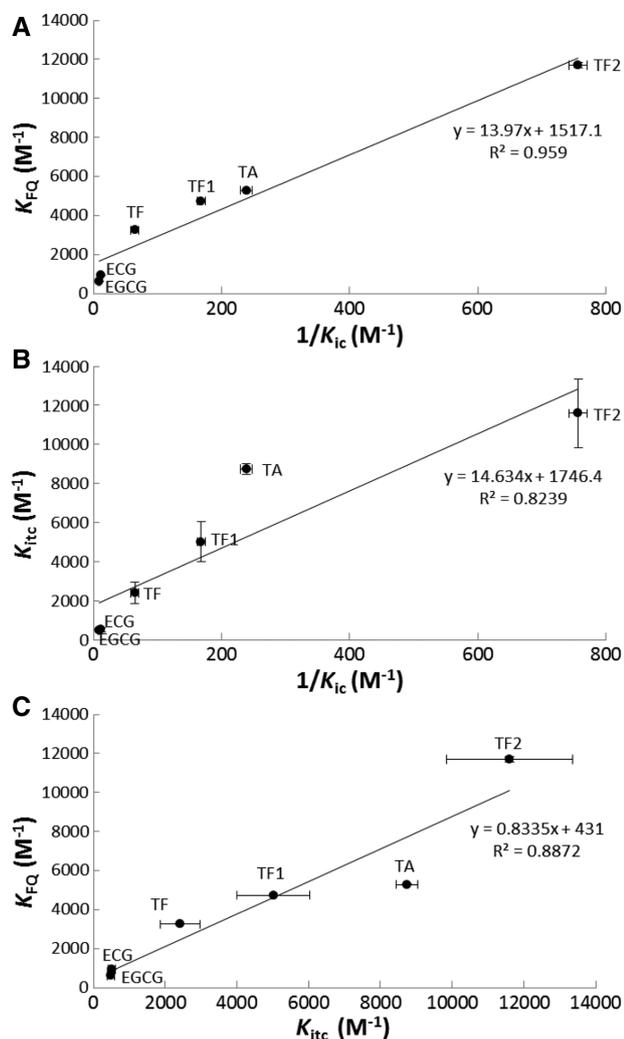


Figure 5. The linear correlations between K_{FQ} and $1/K_{ic}$ (A), K_{itc} and $1/K_{ic}$ (B) and K_{FQ} and K_{itc} (C). The respective correlation equations and coefficients (R^2) are listed as well.

released for the respective titration of the two polyphenols into PPA. Similarly, for the theaflavin family, TF1 and TF2 were the mono- and di-galloylated forms of TF (without a galloyl group in its molecule), respectively (Supporting Information Fig. S1), and both the ΔH and K_{itc} values of the three compounds were in the order of TF2>TF1>TF. Therefore, for catechin and theaflavin families, the introduction of galloyl groups in the molecular structures could enhance the binding of these polyphenols with PPA. Additionally, TA, with ten galloyl groups in its molecular structure had a high binding affinity to PPA (K_{itc} , 8740 M⁻¹; ΔH , -7273 J mol⁻¹). This finding is consistent with our previous results that galloyl substitution plays an important role in association of catechins and theaflavins with PPA, as deduced from the kinetics of inhibition of PPA by tea polyphenols [6]. Interestingly, the role of galloyl moiety in binding with PPA is also reflected by the apparent stoichiometry (n), i.e. the polyphenol to PPA

ratio [17]. Tannic acid which has the most galloyl groups in all the polyphenols investigated, had the lowest stoichiometry (n , 4), indicating that the fewest TA molecules are needed to saturate the binding with PPA. In addition, as the number of galloyl moieties decreased in the theaflavin molecules, n increased (n for TF2, TF1 and TF were 15, 17 and 24, respectively). The changing tendencies of n for both ECG to EC and EGCG to EGC were the same as that for theaflavins. Through molecular docking analysis it appeared that the galloyl moiety interacts with PPA, not only through hydrogen bonds between its three hydroxyl groups and the catalytic amino acid side-chains (Asp197, Glu233 and Asp300), but also through hydrophobic π - π (aromatic-aromatic) interactions with the active site of the enzyme (Trp59) [4,5]. Therefore, based on our results, it is likely that the galloyl moiety in tea polyphenols binds with PPA and further promotes polyphenols entering and associating with the active site of the enzyme.

As both K_{FQ} and K_{itc} demonstrate the binding of polyphenols with PPA, it is of interest to analyze the correlation between the two constants in order to evaluate the accuracy of each method and the rationality of combining fluorescence quenching and ITC. As shown in Fig. 5C, there was a positive linear relationship between K_{FQ} and K_{itc} ($K_{FQ} = 0.8335K_{itc} + 431$, $R^2 = 0.8872$). Because both constants directly reflected the interactions between polyphenols and PPA, the correlation coefficient was close to 1. This also indicates that the binding constants obtained through fluorescence quenching and ITC are comparable and that analysis of the interactions between polyphenols and enzyme by combination of the two methods is feasible. In addition, the fact that K_{FQ} and K_{itc} are quantitatively similar suggests that tea polyphenols binding to PPA mainly include the interactions with the tryptophan and with the vicinity of tryptophan at the active sites of the enzyme.

5 Conclusion

Overall, our data show that tea polyphenols which had higher inhibitory effects on PPA showed higher fluorescence quenching effects on the enzyme. Our study shows, for the first time, the potential structural unfolding of PPA upon binding of polyphenols with the enzyme as indicated by the red-shift of maximum λ_{em} in FQ and the decreased thermostability of PPA in DSC. Through ITC analysis and inhibition kinetics, it was shown that the equilibrium of competitive inhibition resulted from the binding of polyphenols with specific sites on the enzyme. There were positive and linear correlations among $1/K_{ic}$, K_{FQ} and K_{itc} , indicating that all three constants reflect the binding affinity of polyphenols to α -amylase. The galloyl moiety was shown to be an important substituent group in the binding of catechins and theaflavins with α -amylase and thus in inhibiting the catalytic activity of the enzyme; therefore, galloyl substitution should be considered in the extraction and synthesis of pharmaceutical ingredients for type II diabetes.

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6 References

- [1] Pierson, J. T., Dietzgen, R. G., Shaw, P. N., Roberts-Thomson, S. J. et al., Major Australian tropical fruits biodiversity: bioactive compounds and their bioactivities. *Mol. Nutr. Food Res.* 2012, *56*, 357–387.
- [2] Barrett, A., Ndou, T., Hughey, C. A., Straut, C. et al., Inhibition of α -amylase and glucoamylase by tannins extracted from cocoa, pomegranates, cranberries, and grapes. *J. Agr. Food Chem.* 2013, *61*, 1477–1486.
- [3] Prodanov, E., Seigner, C., Marchis-Mouren, G., Subsite profile of the active center of porcine pancreatic α -amylase. Kinetic studies using maltooligosaccharides as substrates. *Biochem. Biophys. Res. Commun.* 1984, *122*, 75–81.
- [4] Lo Piparo, E., Scheib, H., Frei, N., Williamson, G. et al., Flavonoids for controlling starch digestion: structural requirements for inhibiting human α -amylase. *J. Med. Chem.* 2008, *51*, 3555–3561.
- [5] Miao, M., Jiang, H., Jiang, B., Li, Y. G. et al., Elucidation of structural difference in theaflavins for modulation of starch digestion. *J. Funct. Foods* 2013, *5*, 2024–2029.
- [6] Sun, L. J., Warren, F. J., Netzel, G., Gidley, M. J., 3 or 3'-Galloyl substitution plays an important role in association of catechins and theaflavins with porcine pancreatic α -amylase: the kinetics of inhibition of α -amylase by tea polyphenols. *J. Funct. Foods* 2016, *26*, 144–156.
- [7] Cornish-Bowden, A., A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors (Short Communication). *Biochem. J.* 1974, *137*, 143–144.
- [8] Miao, M., Jiang, B., Jiang, H., Zhang, T. et al., Interaction mechanism between green tea extract and human alpha-amylase for reducing starch digestion. *Food Chem.* 2015, *186*, 20–25.
- [9] Williamson, G., Possible effects of dietary polyphenols on sugar absorption and digestion. *Mol. Nutr. Food Res.* 2013, *57*, 48–57.
- [10] Hara, Y., Honda, M., The inhibition of α -amylase by tea polyphenols. *Agr. Biol. Chem.* 1990, *54*, 1939–1945.
- [11] Yilmazer-Musa, M., Griffith, A. M., Michels, A. J., Schneider, E. et al., Grape seed and tea extracts and catechin 3-gallates are potent inhibitors of α -amylase and α -glucosidase activity. *J. Agr. Food Chem.* 2012, *60*, 8924–8929.
- [12] Tang, H. R., Covington, A. D., Hancock, R. A., Use of DSC to detect the heterogeneity of hydrothermal stability in the polyphenol-treated collagen matrix. *J. Agr. Food Chem.* 2003, *51*, 6652–6656.
- [13] Barrett, A., Ndou, T., Hughey, C. A., Straut, C. et al., Inhibition of alpha-amylase and glucoamylase by tannins extracted from cocoa, pomegranates, cranberries, and grapes. *J. Agr. Food Chem.* 2013, *61*, 1477–1486.
- [14] Raghavendra, M. P., Kumar, P. R., Prakash, V., Mechanism of inhibition of rice bran lipase by polyphenols—A case study with chlorogenic acid and caffeic acid. *J. Food Sci.* 2007, *72*, E412–E419.
- [15] Prigent, S. V. E., Gruppen, H., Visser, A. J. W. G., van Koningsveld, G. A. et al., Effects of non-covalent interactions with 5-O-caffeoylquinic acid (chlorogenic acid) on the heat denaturation and solubility of globular proteins. *J. Agr. Food Chem.* 2003, *51*, 5088–5095.
- [16] Jelesarov, I., Bosshard, H. R., Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. *J. Mol. Recognit.* 1999, *12*, 3–18.
- [17] Frazier, R. A., Papadopoulou, A., Green, R. J., Isothermal titration calorimetry study of epicatechin binding to serum albumin. *J. Pharmaceut. Biomed.* 2006, *41*, 1602–1605.
- [18] Frazier, R. A., Papadopoulou, A., Mueller-Harvey, I., Kissoon, D. et al., Probing protein-tannin interactions by isothermal titration microcalorimetry. *J. Agr. Food Chem.* 2003, *51*, 5189–5195.
- [19] Karonen, M., Oraviita, M., Mueller-Harvey, I., Salminen, J. P. et al., Binding of an oligomeric ellagitannin series to bovine serum albumin (BSA): analysis by isothermal titration calorimetry (ITC). *J. Agr. Food Chem.* 2015, *63*, 10647–10654.
- [20] Hill, B. C., Horowitz, P. M., Robinson, N. C., Detection, characterization, and quenching of the intrinsic fluorescence of bovine heart cytochrome-C-oxidase. *Biochem.-Us* 1986, *25*, 2287–2292.
- [21] Prendergast, F. G., Lu, J., Callahan, P. J., Oxygen quenching of sensitized terbium luminescence in complexes of terbium with small organic-ligands and proteins. *J. Biol. Chem.* 1983, *258*, 4075–4078.
- [22] Castanho, M. A. R. B., Prieto, M. J. E., Fluorescence quenching data interpretation in biological systems—The use of microscopic models for data analysis and interpretation of complex systems. *BBA-Biomembranes* 1998, *1373*, 1–16.
- [23] Ferrer-Gallego, R., Goncalves, R., Rivas-Gonzalo, J. C., Escribano-Bailon, M. T. et al., Interaction of phenolic compounds with bovine serum albumin (BSA) and alpha-amylase and their relationship to astringency perception. *Food Chem.* 2012, *135*, 651–658.
- [24] Poncet-Legrand, C., Gautier, C., Cheynier, V., Imbert, A., Interactions between flavan-3-ols and poly(L-proline) studied by isothermal titration calorimetry: effect of the tannin structure. *J. Agr. Food Chem.* 2007, *55*, 9235–9240.
- [25] Pascal, C., Poncet-Legrand, C., Imbert, A., Gautier, C. et al., Interactions between a non glycosylated human proline-rich protein and flavan-3-ols are affected by protein concentration and polyphenol/protein ratio. *J. Agr. Food Chem.* 2007, *55*, 4895–4901.
- [26] Lakowicz, J. R., *Principles of Fluorescence Spectroscopy* (2nd Ed.), Kluwer Academic/Plenum Publishers, New York 1999, pp. 237–255.

- [27] Soares, S., Mateus, N., De Freitas, V., Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary alpha-amylase (HSA) by fluorescence quenching. *J. Agr. Food Chem.* 2007, 55, 6726–6735.
- [28] Cai, X., Yu, J., Xu, L., Liu, R. et al., The mechanism study in the interactions of sorghum procyanidin trimer with porcine pancreatic α -amylase. *Food Chem.* 2015, 174, 291–298.
- [29] Miao, M., Jiang, H., Jiang, B., Li, Y. et al., Elucidation of structural difference in theaflavins for modulation of starch digestion. *J. Funct. Foods* 2013, 5, 2024–2029.
- [30] Okuda, T., Mori, K., Hatano, T., Effects of the interaction of tannins with coexisting Substances .4. Relationship of the structures of tannins to the binding activities with hemoglobin and methylene-Blue. *Chem. Pharm. Bull.* 1985, 33, 1424–1433.
- [31] Fei, Q. Q., Gao, Y., Zhang, X., Sun, Y. et al., Effects of oolong tea polyphenols, EGCG, and EGCG3 " Me on pancreatic alpha-amylase activity *in vitro*. *J. Agr. Food Chem.* 2014, 62, 9507–9514.
- [32] Lakowicz, J. R., *Principles of Fluorescence Spectroscopy* (3rd Ed.), Springer, New York 2006.
- [33] Rathi, P. C., Jaeger, K. E., Gohlke, H., Structural rigidity and protein thermostability in variants of lipase A from bacillus subtilis. *PLoS ONE* 2015, 10, e0130289.
- [34] Vihinen, M., Relationship of protein flexibility to thermostability. *Protein Eng.* 1987, 1, 477–480.
- [35] Lumry, R., Eyring, H., Conformation changes of proteins. *J. Phys. Chem.-Us* 1954, 58, 110–120.
- [36] Violet, M., Meunier, J. C., Kinetic-study of the irreversible thermal-denaturation of bacillus-licheniformis alpha-amylase. *Biochem. J.* 1989, 263, 665–670.
- [37] Cueto, M., Dorta, M. J., Munguia, O., Llabres, M., New approach to stability assessment of protein solution formulations by differential scanning calorimetry. *Int. J. Pharm.* 2003, 252, 159–166.
- [38] Rawel, H. M., Rohn, S., Kruse, H. P., Kroll, J., Structural changes induced in bovine serum albumin by covalent attachment of chlorogenic acid. *Food Chem.* 2002, 78, 443–455.
- [39] Muralidhara, B. K., Prakash, V., Interaction of 3'-O-caffeoyl-D-quinic acid with human serum-albumin. *Int. J. Pept. Prot. Res.* 1995, 46, 1–8.