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# Soluble polysaccharides reduce binding and inhibitory activity of tea polyphenols against porcine pancreatic α-amylase

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ABSTRACT

The effects of three soluble polysaccharides on the inhibitory activity of tea polyphenols against porcine pancreatic α-amylase (PPA) were studied through PPA inhibition, half inhibition concentration ($IC_{50}$), inhibition kinetics and fluorescence quenching. The results show that citrus pectin, wheat arabinoxylan and oat β-glucan could each increase the $IC_{50}$ values and competitive inhibition constants ($K_{ic}$), and decrease the fluorescence quenching constants ($K_{FQ}$) of tea polyphenols interacting with PPA. The data show a competitive interaction equilibrium among polysaccharides, polyphenols and PPA. For individual polyphenols, there were negative linear correlations between both the values of $1/K_{ic}$ and $K_{FQ}$ and that of $IC_{50}$ with and without polysaccharides, indicating that the decreased inhibitory activity of polyphenols induced by the polysaccharides was caused by the reduced binding of polyphenols with PPA. Additionally, the slopes of the linear relationship between $IC_{50}$ and $K_{ic}$ and that between $K_{FQ}$ and $1/K_{ic}$ remained stable with and without polysaccharides, suggesting that these constants may be combined to characterize the effects of soluble polysaccharides on the PPA inhibition by polyphenols.



## 1. Introduction

Dietary polyphenols have been reported to have inhibitory activity against α-amylase, an enzyme that catalyses starch digestion to maltooligosaccharides; therefore, they have been considered as alternatives to pharmaceutical interventions for the treatment of type II diabetes (Barrett et al., 2013; Pierson et al., 2012; Rohn, Rawel, & Kroll, 2002). The inhibition of α-amylase by polyphenols arises as a result of hydrogen bonding between the hydroxyl groups of the phenolic compounds and the catalytic sites of amylase and hydrophobic interactions between the aromatic moieties of polyphenols and the enzyme (Miao et al., 2013). To characterize the mechanism of interactions (binding) between polyphenols and porcine pancreatic α-amylase (PPA), a range of methods have been employed, including half inhibition concentration ($IC_{50}$) value, inhibition kinetics and fluorescence quenching (Fei et al., 2014; Rawel, Frey, Meidtner, Kroll, & Schweigert, 2006; Sun, Warren, Netzel, & Gidley, 2016).

In recent years, many phytochemicals have been studied for their inhibitory activity against α-amylase, like green coffee extracts (Narita & Inouye, 2011), tea extracts (Fei et al., 2014; Sun et al., 2016), pomegranate extracts (Kam et al., 2013), etc., and the main components that demonstrated the inhibitory activity were shown to be phenolic compounds. Aqueous extracts from fruits or plants are generally a mixture of polyphenols and soluble polysaccharides, in part due to binding interactions between the two components (Chamorro, Viveros, Alvarez, Vega, & Brenes, 2012). In addition, even though pure phenolics or phenolic extracts are frequently consumed *e.g.* in tea drinks, polyphenols interact with digestive juices, tissues and other food components (like proteins, polysaccharides, etc.) in the digestive tract (Zhu, 2017). It is not yet known whether the interactions of polyphenols with other food components or polysaccharides in phenolic extracts affect the inhibitory activity of polyphenols either *in vivo* or *in vitro*.

Soluble non-starch polysaccharides (dietary fibres) are indigestible in the upper gastrointestinal tract where amylase is active, and may be recommended as dietary supplements, because they can promote both satiety and microbial fermentation and thereby promote good health and weight management (Yang, Yang, Guo, Jiao, & Zhao, 2013; Zhang, Lv, Jiang, Cheng, & Fan, 2015). The presence of soluble polysaccharides may be a factor affecting the

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interactions between polyphenols and α-amylase due to the potential of binding interactions between polysaccharides and polyphenols (Cai et al., 1990; McManus et al., 1985; Phan, Flanagan, D'Arcy, & Gidley, 2017). Previous studies have suggested that some carbohydrates, like arabinogalactan, dextran, xanthan, etc., were able to interrupt the binding of polyphenols with proteins (de Freitas, Carvalho, & Mateus, 2003). By use of nephelometry, dynamic light scattering and fluorescence spectral methods, some soluble polysaccharides (gum arabic, pectin, and the related polygalacturonic acid) have also been reported to inhibit protein-polyphenol aggregation through two possible mechanisms (Soares, Mateus, & de Freitas, 2012; Soares, Gonçalves, Fernandes, Mateus, & de Freitas, 2009). One is that a ternary protein-polyphenol-polysaccharide complex forms that increases the solubility of protein-polyphenol aggregates. Another is that polysaccharides are able to interact with polyphenols, competing with the binding of polyphenols to protein (Soares et al., 2009).

Pectin, β-glucan and arabinoxylan are three common soluble dietary fibres (non-starch polysaccharides) characteristic of fruits or vegetables (pectin) and cereal grains (β-glucan and arabinoxylan) that have been shown to inhibit lipid and starch digestion *in vitro* (Dhital, Dolan, Stokes, & Gidley, 2014; Zhai, Gunness, & Gidley, 2016). However, limited work has been conducted on the effects of soluble polysaccharides on the inhibitory activity of polyphenols against α-amylase. The competitive or uncompetitive inhibition of an enzyme is attributed to the reversible (solution state) binding of the inhibitor with the enzyme or enzyme-substrate complex, rather than insoluble aggregate formation (Sun, Gidley, & Warren, 2017). Therefore, PPA inhibition, $IC_{50}$ values, kinetics of inhibition and fluorescence quenching can be applied to characterize the inhibition of enzymes and the effects of additional components. The aim of this study is to investigate how three soluble polysaccharides (citrus pectin (CP), wheat arabinoxylan (WAX) and oat β-glucan (OBG)) affect the inhibition of PPA by tea polyphenols by using these methods.

## 2. Materials and methods

### 2.1. Materials and chemicals

Citrus pectin (CP) (galacturonic acid content, 76%; methoxyl content, 8.6%) was obtained from Sigma-Aldrich Co. Ltd. (St. Louis, USA). Wheat arabinoxylan (WAX) and oat β-glucan (OBG) with respective molecular weights of 268 and 245 kDa (Zhai et al., 2016) were purchased from Megazyme Co. Ltd. (Bray, Ireland).

Aqueous green (GTE) and black (BTE) tea extracts were prepared as previously described (Sun et al., 2016). Porcine pancreatic α-amylase (PPA) (EC 3.2.1.1, A6255) was purchased from Sigma-Aldrich Co. Ltd. All the pure polyphenols, including tannic acid (TA), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), theaflavin (TF), theaflavin-3′-gallate (TF1) and theaflavin-3, 3′-digallate (TF2) were products from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Normal maize starch (moisture content of 11.2%) was purchased from Penford Pty. Ltd. (Ryde, Australia). Other chemicals were of analytical grade.

### 2.2. Effects of polysaccharides on PPA inhibition by tea polyphenols

PPA inhibition was obtained through the determination of initial velocity of starch digestion with and without inhibition. All experiments were carried out in phosphate-buffered saline (PBS) at pH 7.4. Specifically, solutions of the polyphenols were prepared in 20% (v/v) dimethyl sulfoxide (DMSO) in PBS buffer to give a final concentration of 12 g/L GTE (an example of tea extracts), 5 g/L TF2 (an example of competitive inhibitors) and 50 g/L ECG (an example of mixed-type inhibitors). Normal maize starch (20 g/L) was prepared in PBS buffer and cooked at 90 °C for 20 min, followed by being diluted to 8 g/L. Polysaccharide powder (0.4 g) was mixed with 20 mL of PBS buffer and incubated at 60 °C water bath for 45 min, followed by being diluted with PBS buffer. The concentration series of the three polysaccharides (WAX, CP and OBG) was 20, 17.5, 15, 10, 7.5, 5 and 2.5 g/L. For each polyphenolic solution, 50 μL was pre-incubated with 200 μL of polysaccharide solution at 4 °C for 15 min, followed by addition of 50 μL of 127.5 nKat/mL (7.65 U/mL) PPA solution and incubation at 4 °C for 15 min. After that, 4 mL of cooked starch at 37 °C was added to the polysaccharide-polyphenol-PPA mixture, and the digestion process was carried out at 37 °C. Starch digestions for the blank control (the mixture of 250 μL of PBS and 50 μL of PPA), polysaccharide control (the mixture of 50 μL of PBS, 200 μL of polysaccharides and 50 μL of PPA) and polyphenol control (the mixture of 50 μL of polyphenols, 200 μL of PBS and 50 μL of PPA) were conducted as well. The initial digestion velocity was obtained from the slope of the plot of reducing sugar content (mM maltose equivalents) in the reaction solution against reaction time (min) using the PAHBAH method established previously (Lever, 1973; Sun et al., 2016). Then, the percentage of PPA inhibition was calculated by the following equation:

$$I = \left(1 - \left(\frac{v}{v_0}\right)\right) \times 100\% \quad (1)$$

where, $I$ is the percentage of PPA inhibition (%), $v$ is the initial reaction velocity with inhibition, and $v_0$ is the velocity without inhibition.

To study the effect of mixing order for polysaccharides, polyphenols and PPA on PPA inhibition by tea polyphenols, the three components were mixed as follows: (i) as described above, *i.e.*, mixing 200 μL of polysaccharide with 50 μL of tea polyphenols (GTE, TF2 and ECG) followed by addition of 50 μL of PPA, and (ii) mixing tea polyphenols with PPA first and then adding polysaccharide to the polyphenol-PPA mixture. After mixing the three components in the two orders, 4 mL of 8 g/L cooked solution was added to start the digestion process, and the initial digestion velocity was determined using the PAHBAH method (Lever, 1973; Sun et al., 2016). For the second mixing order, a concentration series of polysaccharide (20, 17.5, 15, 12.5, 10, 7.5, 5 and 2.5 g/L) was added to the mixture of PPA and GTE to investigate if the effect was also concentration-dependent.

### 2.3. Effects of polysaccharides on $IC_{50}$ values of tea polyphenols

For $IC_{50}$ value determination, PPA inhibition by tea polyphenols in the absence and presence of polysaccharides were determined as follows: TEs and pure polyphenols were dissolved in PBS buffer to a respective stock solution immediately before using in the experiments. Various dilutions (25 μL) of the test polyphenols were pre-incubated with 25 μL of 10 g/L polysaccharides (WAX, CP and OBG) in a 96-well plate (Greiner bio-one, Germany) at 4 °C for 15 min. Then, 25 μL of 3.2 nKat/mL (0.19 U/mL) PPA was added to the wells containing the polyphenol-polysaccharide mixture and incubated at 4 °C for 15 min. The PPA control (the mixture of 50 μL of PBS and 25 μL of PPA) and polyphenol control (mixture of 25 μL of PBS, 25 μL of polyphenols and 25 μL of PPA) were also prepared. To start the reaction, 50 μL of 200 μg/mL 'DQ starch' (an artificial maize starch substrate supplied with the assay kit) dissolved in PBS buffer in an Invitrogen Enzcheck Ultra® α-Amylase Assay Kit was added to the respective wells. The fluorescence intensity of respective wells was determined at $\lambda_{ex}$ of 460 nm and $\lambda_{em}$ of 520 nm at 6 min intervals

over 36 min. The initial enzymic reaction velocity, $v$ was expressed as the slope of a plot of fluorescence intensity against reaction time (Δfluorescence/min). The percentage of PPA inhibition ($I$) was calculated by equation (1). The $IC_{50}$ value was calculated by the following equation (Goodrich & Kugel, 2007):

$$I = I_{max}\left(1 - \frac{IC_{50}}{[I] + IC_{50}}\right) \quad (2)$$

where $I_{max}$ is the maximum percentage of PPA inhibition (%), and [$I$] is the concentration of inhibitor.

### 2.4. Effects of polysaccharides on kinetics of PPA inhibition by tea polyphenols

To study the effects of three polysaccharides (WAX, CP and OBG) on the kinetics of PPA inhibition by tea polyphenols, a concentration series of cooked starch solutions (15, 10, 5, 2.5 and 1.25 g/L) was prepared in PBS buffer. Each polyphenol solution (50 μL) was pre-incubated with 200 μL of 5 g/L polysaccharide solution at 4 °C for 15 min, followed by addition of 50 μL of 127.5 nKat/mL (7.65 U/mL) PPA solution and incubation at 4 °C for 15 min. Then, 4 mL of cooked starch was added to the polysaccharide-polyphenol-PPA mixture, and the digestion process was carried out at 37 °C. The initial digestion velocity at each starch concentration was determined using the PAHBAH method (Lever, 1973; Sun et al., 2016). The competitive inhibition constant, $K_{ic}$ can be obtained from the Dixon equation as follows (Dixon, 1953):

$$v = \frac{V_{max}a}{K_m\left(1 + \frac{i}{K_{ic}}\right) + a} \quad (3)$$

The uncompetitive constant, $K_{iu}$ can be obtained from the Cornish-Bowden equation as follows (Cornish-Bowden & Eisenthal, 1974):

$$\frac{v}{a} = \frac{V_{max}}{K_m\left(1 + \frac{i}{K_{ic}}\right) + a\left(1 + \frac{i}{K_{iu}}\right)} \quad (4)$$

where, $V_{max}$ is the maximum initial digestion velocity, $a$ is the concentration of starch, $K_m$ is the Michaelis constant, $i$ is the concentration of inhibitor, and $v$ is the initial digestion velocity determined.

### 2.5. Effects of polysaccharides on PPA fluorescence quenching by tea polyphenols

The fluorescence spectra of PPA in the presence of polyphenols and polysaccharides were recorded according to a previous study (Susana Soares, Mateus, & de Freitas, 2007) with some modifications. Specifically, 0.1 mL of each polyphenol solution was pre-incubated with 0.1 mL of a 5 g/L solution of polysaccharide at 4 °C for 15 min. The blank control (0.2 mL of PBS), polyphenol control (a mixture of 0.1 mL of polyphenols and 0.1 mL of PBS) and polysaccharide control (a mixture of 0.1 mL of PBS and 0.1 mL of polysaccharide) were prepared as well. Then, 3 mL of 1275.3 nKat/mL (76.5 U/mL) PPA solution was added to the polyphenol-polysaccharide mixture and controls, and incubated at 4 °C for 15 min. The intrinsic fluorescence spectra of PPA were recorded with fast speed and low sensitivity at $\lambda_{ex}$ of 282 nm and $\lambda_{em}$ from 300 to 500 nm. Both the slit width values were set as 10 nm. Fluorescence quenching can be described by the Stern-Volmer equation (Susana Soares et al., 2007):

$$\frac{F_0}{F} = 1 + k_q\tau_0[Q] = 1 + K_{FQ}[Q] \quad (5)$$

where, $F_0$ and $F$ are the fluorescence intensity at $\lambda_{em}$ of 348 nm in the absence and presence of quencher, respectively, $k_q$ is the biomolecular quenching constant, $\tau_0$ is the lifetime of fluorophore, and for α-amylase, the value is 2.97 ns (Prendergast, Lu, & Callahan, 1983), [$Q$] is the concentration of quencher, and $K_{FQ}$ is the fluorescence quenching constant. If there exists a sphere of volume around a fluorophore, within which a quencher can cause the quenching effect, *i.e.* apparent static quenching (Castanho & Prieto, 1998), the Stern-Volmer equation can be modified as follows (Ferrer-Gallego, Gonçalves, Rivas-Gonzalo, Escribano-Bailón, & de Freitas, 2012):

$$\frac{F_0}{F} = e^{(K_{FQ}[Q])} \quad (6)$$

### 2.6. Statistical analysis

The data in this study are expressed as the means of duplicates and analysed through one-way analysis of variance (ANOVA) using SPSS 18.0 Statistic (Chicago, USA). The mean values were evaluated by Dunnett's Test at the 95% significant level ($P < 0.05$).

## 3. Results

### 3.1. Effects of polysaccharides on PPA inhibition and $IC_{50}$ values of tea polyphenols

The effects of the three polysaccharides (WAX, CP and OBG) on PPA inhibition by GTE (an example of tea extracts), TF2 (an example of competitive inhibitors) and ECG (an example of mixed-type inhibitors) are shown in Fig. 1A. In the absence of polysaccharides, the percentages of PPA inhibition by the three polyphenols were 36.2% (GTE), 49.5% (TF2) and 43.5% (ECG) at respective polyphenol concentrations, while the polysaccharides themselves had no inhibitory effects on PPA (data not shown). However, PPA inhibition by the polyphenols was relieved by the polysaccharides to different extents (Fig. 1A), indicating that the PPA activity was protected by mixing the respective polysaccharides with the tea polyphenol solutions before addition of PPA. These effects were shown to be concentration-dependent, and OBG was found to be significantly more effective at protecting PPA activity than WAX and CP at polysaccharide concentrations from 5 to 15 g/L (Fig. 1B). In addition, the effect of mixing order for polysaccharide (WAX), polyphenols and PPA on PPA inhibition by tea polyphenols was studied. As shown in Fig. 1C, both the mixing orders, including mixing WAX with polyphenols before addition of PPA, and mixing polyphenols with PPA before addition of WAX, were shown to reduce PPA inhibition, although the reducing effect by the former mixing order was slightly higher than that by the latter one. In addition, the reducing effect of WAX on the enzyme inhibition by the latter mixing order was shown to be concentration-dependent at polysaccharide concentration from 2.5 to 15 g/L as well (Fig. 1D).

$IC_{50}$ values are usually obtained from inhibition percentages for an inhibitor concentration series, and applied to characterize the inhibitory activity of an inhibitor. The effects of the three polysaccharides on the $IC_{50}$ values of tea polyphenols are shown in Table 1. Based on the order of $IC_{50}$ values, the inhibitory activity of the pure tea polyphenols was in the order of TF2 > TF1 ≈ TA > TF > ECG > EGCG, as previously shown (Sun et al., 2016). It is shown in Table 1 that addition of the polysaccharides did not alter the order of the inhibitory activity for the pure

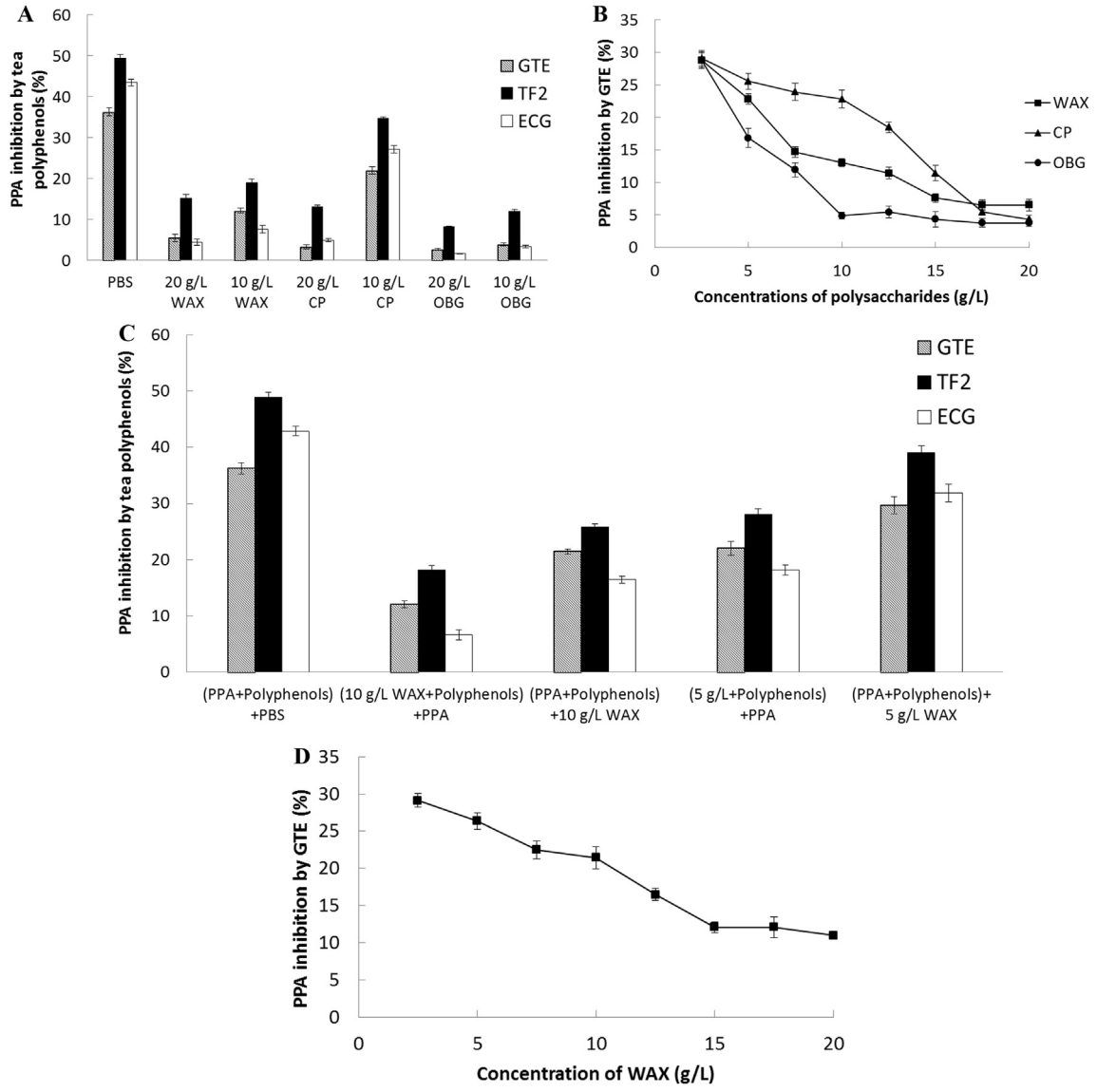


**Fig. 1.** The effects of three soluble polysaccharides (WAX, CP and OBG) on PPA inhibition by tea polyphenols (GTE, TF2 and ECG) (**A**). Polysaccharides were mixed with tea polyphenols before addition of PPA to the mixture; The effects of polysaccharides with different concentrations on PPA inhibition by GTE (**B**). Polysaccharides were mixed with GTE before addition of PPA to the mixture; The effect of mixing order for polysaccharides (WAX), polyphenols (GTE, TF2 and ECG) and PPA on PPA inhibition (**C**). The two mixing methods included mixing polysaccharides with polyphenols before addition of PPA to the mixture (labelled as (WAX + polyphenols)+PPA), and mixing PPA with polyphenols before addition of polysaccharides to the mixture (labelled as (PPA + polyphenols)+WAX); The effects of polysaccharides (WAX) with different concentrations on PPA inhibition by GTE under the condition of mixing PPA with GTE before addition of polysaccharides to the mixture (**D**).

**Table 1**
Detailed kinetics of PPA inhibition by TEs and pure phenolic compounds in the absence and presence of three polysaccharides.

| Phenolics | $K_{ic}$ (g/L) | $K_{ic}'$ (g/L) | | | $K_{iu}$ (g/L) | $K_{iu}'$ (g/L) | | | $IC_{50}$ (g/L) | $IC_{50}'$ (g/L) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NP | WAX | CP | OBG | NP | WAX | CP | OBG | NP | WAX | CP | OBG |
| GTE | $6.332^{a}$ | $11.594^{c}$ | $8.402^{b}$ | $13.845^{d}$ | – | – | – | – | $0.191^{a}$ | $0.343^{c}$ | $0.264^{b}$ | $0.451^{d}$ |
| BTE | $7.847^{a}$ | $12.169^{c}$ | $10.61^{b}$ | $14.151^{d}$ | – | – | – | – | $0.367^{a}$ | $0.694^{c}$ | $0.483^{b}$ | $0.875^{d}$ |
| TA | $7.124^{a}$ | $12.987^{c}$ | $10.124^{b}$ | $15.997^{d}$ | – | – | – | – | $0.294^{a}$ | $0.496^{c}$ | $0.361^{b}$ | $0.601^{d}$ |
| ECG | $37.490^{a}$ | $67.410^{c}$ | $50.364^{b}$ | $81.643^{d}$ | $44.567^{A}$ | $77.486^{C}$ | $60.572^{B}$ | $98.263^{D}$ | $1.834^{a}$ | $3.460^{c}$ | $2.051^{ab}$ | $4.424^{d}$ |
| EGCG | $47.690^{a}$ | $85.541^{c}$ | $67.140^{b}$ | $101.254^{d}$ | – | – | – | – | $2.694^{a}$ | $5.014^{c}$ | $3.996^{b}$ | $7.110^{d}$ |
| TF2 | $1.148^{a}$ | $1.773^{c}$ | $1.316^{ab}$ | $2.261^{d}$ | – | – | – | – | $0.127^{a}$ | $0.201^{c}$ | $0.157^{ab}$ | $0.278^{d}$ |
| TF1 | $4.255^{a}$ | $5.612^{bc}$ | $5.345^{b}$ | $6.751^{d}$ | $19.925^{A}$ | $26.612^{BC}$ | $25.504^{B}$ | $31.880^{D}$ | $0.269^{a}$ | $0.501^{c}$ | $0.394^{b}$ | $0.736^{d}$ |
| TF | $8.715^{a}$ | $14.66^{c}$ | $11.647^{b}$ | $18.54^{d}$ | $31.355^{A}$ | $53.747^{C}$ | $41.986^{B}$ | $67.84^{D}$ | $0.443^{a}$ | $0.884^{c}$ | $0.667^{b}$ | $1.334^{d}$ |

Different letters in the same line represent significantly different mean values for the corresponding parameters ($P < 0.05$). '-' means not available. The values of $K_{ic}$ and $K_{iu}$ were cited from our previous study (Sun et al., 2017).

polyphenols. All the polysaccharides tested were found to increase the $IC_{50}$ values, indicating that they each decreased the inhibitory activity of the polyphenols. In addition, the decreasing effect was in the order of OBG > WAX > CP.

### 3.2. Effects of polysaccharides on kinetics of PPA inhibition by tea polyphenols

In our previous study, the kinetics of inhibition of PPA by tea polyphenols was studied in the absence of polysaccharides, as shown in Figs. S1A–C, through which the inhibition types and constants were obtained from the Dixon and Cornish-Bowden plots (Sun et al., 2016). In the present study, the effects of the three polysaccharides on the Dixon and Cornish-Bowden plots for inhibition kinetics of GTE, TF2 and ECG (the respective example of tea extracts, competitive inhibitor and mixed-type inhibitor) are shown in Fig. S1D–L, respectively. The values of $K_{ic}$ and $K_{iu}$ obtained from the kinetics study in the absence and presence of polysaccharides for all the tea polyphenols investigated (two tea extracts and eight pure polyphenols) are summarised in Table 1. As shown in Figs. S1A–C, GTE and TF2 were suggested to be competitive inhibitors of PPA, while ECG was shown to be a mixed-type inhibitor of PPA, including both competitive and uncompetitive inhibitory characters (Sun et al., 2016). It is shown in Fig. S1D–L that addition of the polysaccharides did not alter the inhibition mechanism of the polyphenols. However, they increased the absolute values of intersection points of both Dixon and Cornish-Bowden equations, *i.e.*, both the values of $K_{ic}$ and $K_{iu}$ of tea polyphenols were enhanced by the three polysaccharides, as shown in Table 1. In addition, for the respective polyphenols, both the values of $K_{ic}'$ and $K_{iu}'$ (the values of $K_{ic}$ and $K_{iu}$ in the presence of three polysaccharides) were increased in the order OBG > WAX > CP, the same as was found for the $IC_{50}$' values.

### 3.3. Effects of polysaccharides on PPA fluorescence quenching by tea polyphenols

Fluorescence quenching is an effective way to describe the binding interactions between enzymes and inhibitors. In this study, the cases of the fluorescence quenching of PPA by GTE, TF2 and ECG (the respective example of tea extracts, competitive inhibitor and mixed-type inhibitor) in the absence and presence of three polysaccharides are shown in Fig. S2. Stern-Volmer equations (Table 2) were used to calculate the fluorescence quenching constants ($K_{FQ}$) and the biomolecular quenching constants ($k_q$) of tea polyphenols. The quenching constants in the absence and presence of three polysaccharides are summarised in Table 3. The tea polyphenols each quenched the fluorescence of PPA in a concentration-dependent manner. However, addition of the polysaccharides decreased the quenching effects of tea polyphenols on PPA intrinsic fluorescence (Fig. S2), while the three polysaccharides themselves had no significant effects on the PPA fluorescence (data not shown). GTE and ECG were able to quench the PPA fluorescence through both the dynamic (collision-controlled quenching) and static (complex-controlled quenching) mechanism. Taking into account the high values of $K_{FQ}$ and $k_q$, TF2 was suggested to quench the PPA fluorescence through the formation of complex with the enzyme (the static mechanism) (Sun et al., 2017). As shown in Table 2, although the three polysaccharides decreased the coefficients or slope of Stern-Volmer equations, they did not alter the quenching mechanism of tea polyphenols as the types of equations remained unchanged. In addition, all the three polysaccharides significantly decreased the values of $K_{FQ}$ and $k_q$, and the decreasing effects for the respective polyphenols were in the order of OBG > WAX > CP (Table 3), as also found for the effects on $K_{ic}$, $K_{iu}$ and $IC_{50}$ values (Table 1).

### 3.4. Correlations between $IC_{50}$, $K_{ic}$ and $K_{FQ}$ values

For our data, the correlation between $IC_{50}$ and $K_{ic}$ was shown to be positive and linear in the absence of polysaccharides (Table 4). This suggests that the inhibition results from the binding (association) of tea polyphenols with the enzyme. It should be noted that although the three polysaccharides could increase both the $IC_{50}$ and $K_{ic}$ values, they did not significantly alter the positive and linear correlation between $IC_{50}$ and $K_{ic}$ values (Table 4). For the respective polyphenols, there were negative linear correlations between both the values of $1/K_{ic}$ and $K_{FQ}$ with that of $IC_{50}$ in the absence and presence of three polysaccharides (Table 5). In addition, there was a linear relationship between $K_{FQ}$ and $1/K_{ic}$ in the absence of polysaccharides (Table 6), consistent with both constants reflecting the binding of polyphenols with PPA (Sun et al., 2017). Interestingly, in the presence of polysaccharides, although both the values of $1/K_{ic}$ and $K_{FQ}$ were decreased, the slopes remained about the same (Table 6).

## 4. Discussion

Tea polyphenols have been reported to inhibit the activity of PPA in previous studies (Hara & Honda, 1990; Sun et al., 2016). As both polyphenols and polysaccharides are important dietary constituents for human beings (Cummings, Bingham, Heaton, & Eastwood, 1992), the effects of polysaccharides (WAX, OBG and CP) on the inhibitory activity of tea polyphenols against PPA have been studied

**Table 2**
Stern-Volmer or its modified equations for fluorescence quenching of PPA by GTE, TF2 and ECG in the absence and presence of three polysaccharides.

| Tea polyphenols | Polysaccharides | equations | $R^2$ | Quenching mechanism |
|---|---|---|---|---|
| GTE | Buffer control | $y = \exp(0.7041x)$ | 0.9941 | Apparent static |
| | WAX | $y = \exp(0.3455x)$ | 0.9978 | |
| | CP | $y = \exp(0.5247x)$ | 0.9872 | |
| | OBG | $y = \exp(0.2777x)$ | 0.9992 | |
| TF2 | Buffer control | $y = 13.482x + 1.235$ | 0.9876 | Static |
| | WAX | $y = 7.704x + 1.109$ | 0.9963 | |
| | CP | $y = 11.723x + 1.188$ | 0.9916 | |
| | OBG | $y = 6.404x + 1.111$ | 0.9946 | |
| ECG | Buffer control | $y = 1.1047\exp(2.1404x)$ | 0.9957 | Apparent static |
| | WAX | $y = 1.0360\exp(1.0375x)$ | 0.9976 | |
| | CP | $y = 1.0457\exp(1.6422x)$ | 0.9983 | |
| | OBG | $y = 1.0136\exp(0.8821x)$ | 0.9977 | |

$y$ means $F/F_0$ (the ratio of the maximum fluorescence intensity of PPA after quenching to that before quenching), and $x$ means the concentration of tea polyphenols (g/L).

**Table 3**
Constants of fluorescence quenching of PPA by TEs and pure phenolic compounds in the absence ($K_{FQ}$ and $k_q$) and presence ($K_{FQ}'$ and $k_q'$) of three polysaccharides.

| Phenolics | $K_{FQ}$ | $K_{FQ}'$ | | | $k_q$ ($10^9$) | $k_q'$ ($10^9$) | | |
|---|---|---|---|---|---|---|---|---|
| | NP | WAX | CP | OBG | NP | WAX | CP | OBG |
| GTE | $0.704^{a}$ (L/g) | $0.346^{c}$ | $0.525^{b}$ | $0.278^{d}$ | $0.237^{a}$ (L/(g·s)) | $0.116^{c}$ | $0.177^{b}$ | $0.094^{d}$ |
| BTE | $0.648^{a}$ | $0.313^{c}$ | $0.481^{b}$ | $0.271^{cd}$ | $0.218^{a}$ | $0.105^{c}$ | $0.162^{b}$ | $0.091^{cd}$ |
| TA | $5285.597^{a}$ (L/mol) | $2483.392^{c}$ | $3714.251^{b}$ | $2133.363^{d}$ | $1779.662^{a}$ (L/(mol·s)) | $921.297^{c}$ | $1401.768^{b}$ | $762.748^{d}$ |
| ECG | $946.849^{a}$ | $458.959^{c}$ | $726.46^{b}$ | $390.215^{d}$ | $318.804^{a}$ | $154.532^{c}$ | $244.599^{b}$ | $131.386^{d}$ |
| EGCG | $630.399^{a}$ | $305.453^{c}$ | $464.311^{b}$ | $265.313^{d}$ | $212.256^{a}$ | $102.846^{c}$ | $156.334^{b}$ | $89.331^{d}$ |
| TF2 | $11711.813^{a}$ | $6692.542^{c}$ | $10183.89^{b}$ | $5563.132^{d}$ | $3943.371^{a}$ | $2253.381^{c}$ | $3428.918^{b}$ | $1873.108^{d}$ |
| TF1 | $4740.243^{a}$ | $2521.464^{c}$ | $3832.458^{b}$ | $2100.233^{d}$ | $1596.041^{a}$ | $848.978^{c}$ | $1290.39^{b}$ | $707.149^{d}$ |
| TF | $3252.028^{a}$ | $1663.96^{c}$ | $2534.119^{b}$ | $1384.369^{d}$ | $1094.959^{a}$ | $560.256^{c}$ | $853.239^{b}$ | $466.118^{d}$ |

Different letters in the same line represent significantly different mean values for the corresponding parameters ($P < 0.05$). The values of $K_{FQ}$ and $k_q$ are from our previous study (Sun et al., 2017).

**Table 4**
Correlations between $IC_{50}$ and $K_{ic}$ of tea polyphenols in the absence and presence of three polysaccharides.

| Polysaccharides | Correlations | $R^2$ |
|---|---|---|
| Buffer control | $y = 18.416x + 0.362$ | 0.9887 |
| WAX | $y = 17.825x - 0.029$ | 0.9874 |
| CP | $y = 17.784x + 1.719$ | 0.9444 |
| OBG | $y = 15.147x + 1.178$ | 0.9645 |

The polyphenols used to calculate each correlation included EGCG, ECG, TA, TF, TF1 and TF2. $y$ means $K_{ic}$ (g/L) and $x$ means $IC_{50}$ (g/L).

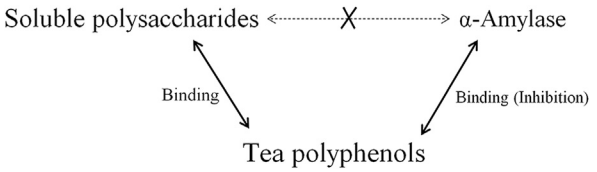


**Fig. 2.** Interaction equilibrium among soluble polysaccharides, tea polyphenols and PPA.

**Table 5**
Correlations between $1/K_{ic}$ and $IC_{50}$ and between $K_{FQ}$ and $IC_{50}$ of tea polyphenols in the absence and presence of three polysaccharides.

| Tea polyphenols | Correlations between $1/K_{ic}$ and $IC_{50}$ | $R^2$ | Correlations between $K_{FQ}$ and $IC_{50}$ | $R^2$ |
|---|---|---|---|---|
| GTE | $y = -0.328x + 0.211$ | 0.9188 | $Y = -1.652X + 0.979$ | 0.9244 |
| BTE | $y = -0.101x + 0.155$ | 0.8574 | $Y = -0.732X + 0.871$ | 0.9152 |
| TA | $y = -0.233x + 0.197$ | 0.8870 | $Y = -5.768X + 4.527$ | 0.8940 |
| EGCG | $y = -0.002x + 0.026$ | 0.8608 | $Y = -0.180X + 1.757$ | 0.8562 |
| ECG | $y = -0.005x + 0.033$ | 0.8450 | $Y = -0.444X + 2.732$ | 0.8806 |
| TF | $y = -0.065x + 0.135$ | 0.8905 | $Y = -3.687X + 6.980$ | 0.8645 |
| TF1 | $y = -0.172x + 0.269$ | 0.8960 | $Y = -7.975X + 8.391$ | 0.8737 |
| TF2 | $y = -2.849x + 1.203$ | 0.9434 | $Y = -47.785X + 18.943$ | 0.8892 |

The polysaccharides used to calculate each correlation included WAX, CP and OBG, as well as a buffer control. $y$ means $1/K_{ic}$ (L/g), $x$ means $IC_{50}$ (g/L), $Y$ means $K_{FQ}$ (L/g) and $X$ means $IC_{50}$ (g/L).

**Table 6**
Correlations between $1/K_{ic}$ and $K_{FQ}$ of tea polyphenols in the absence and presence of three polysaccharides.

| Polysaccharides | Correlations | $R^2$ |
|---|---|---|
| Buffer control | $y = 13.970x + 1517.10$ | 0.9590 |
| WAX | $y = 12.533x + 685.15$ | 0.9765 |
| CP | $y = 13.995x + 1182.10$ | 0.9659 |
| OBG | $y = 13.290x + 561.58$ | 0.9779 |

The polyphenols used to calculate each correlation included EGCG, ECG, TA, TF, TF1 and TF2. $y$ means $K_{FQ}$ (L/mol) and $x$ means $1/K_{ic}$ (L/mol).

in this paper through the analysis of PPA inhibition, $IC_{50}$ value, inhibition kinetics and fluorescence quenching methods.

The three polysaccharides investigated were shown to reduce PPA inhibition by two mixing methods for polyphenols, polysaccharides and enzyme, suggesting that there is an interaction equilibrium among the three components (Fig. 2), in which tea polyphenols could bind both with soluble polysaccharides and with PPA. It should be noted that although some polysaccharides were found to inhibit amylase in some previous studies (Ikeda & Kusano, 1983; Tan & Gan, 2016), under the initial reaction conditions in our study all the three polysaccharides were shown to retain the catalytic activity of the enzyme, suggesting that the interactions between the polysaccharides and PPA were relatively weak compared with interactions of polyphenols with either polysaccharides or PPA. This is consistent with the effect of WAX and OBG on starch digestibility being overcome by efficient mixing the polysaccharides with starch (Dhital et al., 2014), *i.e.* what effect there is of WAX and OBG on restricting starch digestion is likely a rheological one rather than mediated by direct binding interactions with the enzyme. Therefore, the relieving effect of the polysaccharides on PPA inhibition was mainly attributed to the interactions between polysaccharides and polyphenols, rather than that between polysaccharides and PPA. In the analysis of inhibition kinetics, competitive inhibition constant, $K_{ic}$, is the dissociation constant of the inhibitor-enzyme complex, so $1/K_{ic}$ represents the association constant of an inhibitor with an enzyme (Cornish-Bowden, 1974); therefore, a lower value of $K_{ic}$ means a higher binding affinity of an inhibitor with the active sites of the enzyme (Sun et al., 2016). Hence, the higher values of $K_{ic}'$ (in the presence of polysaccharides) than $K_{ic}$ (in the absence of polysaccharides) for the respective polyphenols indicate that the three polysaccharides reduced the binding of tea polyphenols with the catalytic sites of PPA. Further, the reducing effects were in the order of OBG > WAX > CP, because the values of $K_{ic}'$ for the respective polyphenols were in the same order (Table 1). Similarly, in the

analysis of fluorescence quenching of a protein by a quencher, the fluorescence quenching constant, $K_{FQ}$, suggests the binding affinity of the quencher with the fluorophores of the protein (Cai, Yu, Xu, Liu, & Yang, 2015; Skrt, Benedik, Podlipnik, & Ulrih, 2012), and a higher value of $K_{FQ}$ also suggests greater binding interactions between them. Therefore, the three polysaccharides were also shown to weaken the binding of tea polyphenols with PPA, as the values of $K_{FQ}'$ (in the presence of polysaccharides) were lower than that of $K_{FQ}$ (in the absence of polysaccharides) (Table 3). Notably, the negative linear correlations between 1/*K*ic (or $K_{FQ}$) and $IC_{50}$ for the respective polyphenols (Table 5) indicate that the decreased inhibitory activity (indicated by the increased $IC_{50}$) was caused by the reduced binding of polyphenols with PPA (indicated by the decreased $1/K_{ic}$ or $K_{FQ}$) induced by the polysaccharides. Indeed, the consistency in the slopes of correlations between the inhibition constants ($K_{ic}$ and $IC_{50}$ in Table 4 and $K_{FQ}$ and $1/K_{ic}$ in Table 6) in the absence and presence of polysaccharides suggests that these constants were affected to a similar degree by the presence of polysaccharides, and that the inhibitory activity, inhibition kinetics and fluorescence quenching methods can be collectively and reasonably applied to characterize the effects of soluble polysaccharides on PPA inhibition by polyphenols.

In previous studies, polyphenols have been reported to interact with polysaccharides through a combination of hydrogen bonds and hydrophobic interactions (Le Bourvellec & Renard, 2012; Renard, Watrelot, & Le Bourvellec, 2017). Polyphenols can bind with both α-amylase and polysaccharides (two different macromolecules) (Miao et al., 2013; Renard et al., 2017), and there is a competitive relationship between the two binding interactions (Soares et al., 2012; Soares et al., 2009). The interaction equilibrium among polysaccharides, polyphenols and PPA (Fig. 2) along with the decreased $1/K_{ic}$ and $K_{FQ}$ found in our study also suggests this competition. Similarly, one previous study showed that a soluble polysaccharide and oligosaccharide (arabic gum and β-cyclodextrin) were able to decrease the quenching of α-amylase by procyanidins due to potential interactions between polysaccharides and procyanidins which induced a decrease in the size of procyanidin-amylase aggregates (Soares et al., 2009). Notably, as a cereal soluble polysaccharide, WAX has a branched molecular structure, consisting of a linear backbone of β-(1–4) linked $_D$-xylopyranosyl units to which α-$_L$-arabinofuranosyl residues are attached through C (O)-2 and/or C (O)-3 (Dervilly-Pinel, Tran, & Saulnier, 2004), while OBG has a linear glucan backbone joined by β-(1–3) and β-(1–4) carbon linkages (Johansson et al., 2000). CP contains a backbone of partially methyl esterified α-(1–4) linked galacturonosyl residues with occasional rhamnose insertions to which may be attached neutral galactan and/or oligosaccharide side chains. The neutral sugar side chains (especially arabinan) have mobile properties in solution, which may limit the association of the main chain with polyphenols (Ha, Viëtor, Jardine, Apperley, & Jarvis, 2005). As a result, the presence of less sugar branches on the polysaccharide backbone could allow a linear structure to more easily bind/stack with polyphenols (Ha et al., 2005; Watrelot, Le Bourvellec, Imberty, & Renard, 2014). Therefore, OBG is proposed to more likely bind with tea polyphenols than WAX (which contains only monosaccharide branches), which in turn is more likely to bind than CP (which contains longer branches and a charged backbone both of which would limit binding), and thus showed the largest reducing effect on the binding of tea polyphenols with PPA due to the competitive mechanism. In previous work, pectin from citrus peel was shown to inhibit α-amylase-procyanidin aggregation by a mechanism involving formation of a protein-procyanidin-polysaccharide ternary complex. This is consistent with the pectin having no effect on the quenching of α-amylase fluorescence by the polyphenols, but increased the solubility of the α-amylase-procyanidin aggregate, meaning that the number of procyanidins interacting with α-amylase was not changed significantly in the presence of pectin (Soares et al., 2009). However, in our study, although the ternary complex may also exist, it was more likely that PPA was bound with less number of tea polyphenols in the presence of polysaccharides than without them, because the polysaccharides were found to relieve the fluorescence quenching of PPA by the polyphenols (Fig. S2), as also found for OBG and WAX. It is possible that the previously proposed ternary complex involved charge complexation between the positively charged procyanidin and negatively charged pectin (Phan et al., 2017), whereas the polyphenols used in this study were all neutral and less likely to complex with pectin.

Polyphenols have been reported to be a potential alternative to commercial medicines for type II diabetes due to their inhibitory activity against α-amylase (Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012). Soluble polysaccharides widely exist in foods and usually are bound with other food components, like polyphenols, proteins, etc. (Dickinson, 1998; Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). Therefore, both soluble polysaccharides and polyphenols are transported by the digestive system to the small intestine (Duda-Chodak, Tarko, Satora, & Sroka, 2015; Knudsen, Jensen, & Hansen, 1993), where polyphenols may act as inhibitors of pancreatic α-amylase. As shown in our study, soluble polysaccharides could decrease the inhibitory activity of polyphenols, thus soluble polysaccharides should be considered as an important factor that influences the effectiveness of polyphenols as enzyme inhibitors in the digestive tract. In terms of extraction of phyto-polyphenols, eliminating the influence of soluble polysaccharides in phenolic extracts should be also taken into account in order to develop the inhibition ability of phenolic extracts as far as possible.

## 5. Conclusion

The inhibitory activity of polyphenols against α-amylase has been reported, and polyphenols are often found bound to other components in foods. Therefore, there are potential effects of food components on the inhibition of digestive enzymes by polyphenols. In this study, the effects of three soluble polysaccharides on the inhibitory activity of tea polyphenols against PPA were studied. It was found that the polysaccharides were able to reduce the inhibitory activity of polyphenols, which was suggested by the increased $IC_{50}$, increased $K_{ic}$ and decreased $K_{FQ}$ in the presence of soluble polysaccharides. The effectiveness of three polysaccharides on relieving PPA inhibition was in the order of OBG > WAX > CP, and the effects were hypothesised to be caused by the interactions between polysaccharides and polyphenols, which competitively affected the binding of polyphenols with the enzyme. Through establishment of the correlations between $K_{ic}$ and $IC_{50}$ and between $K_{FQ}$ and $1/K_{ic}$, the inhibitory activity, inhibition kinetics and fluorescence quenching methods can be combined to characterize the effects of soluble polysaccharides on the PPA inhibition by polyphenols. Therefore, to develop the inhibitory activity of polyphenols from plant or food extracts, as well as to evaluate the α-amylase inhibition of polyphenols in the digestive tract, some food components, like soluble polysaccharides should be taken into account due to their effects on the enzyme inhibition.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodhyd.2017.12.011.

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