Studying the Interactions between Tea Polyphenols and α-Amylase

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Abstract

Dixon, Cornish-Bowden and Lineweaver-Burk plot analyses were applied to study the detailed kinetics of inhibition of porcine pancreatic α-amylase (PPA) by tea polyphenols. Fluorescence quenching (FQ), differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) were combined with the kinetics of inhibition to elucidate the mechanism of binding interactions of tea polyphenols and PPA. Then, the reciprocal of competitive inhibition constant (1/K_{ic}), fluorescence quenching constant (K_{FQ}) and binding constant (K_{itc}) obtained from these measurements were compared and correlated to analyse the relations between PPA inhibition and binding behaviour. The role of the galloyl moiety in binding of catechins and theaflavins with PPA was highlighted as well. In addition, the effects of three soluble polysaccharides (citrus pectin (CP), wheat arabinoxylan (WAX) and oat β-glucan (OOG)) on the inhibition of PPA by tea polyphenols were studied through PPA inhibition, IC_{50} value, inhibition kinetics and fluorescence quenching methods. Furthermore, the effects of tea polyphenols on binding of PPA with normal maize starch granules were studied in terms of binding ratio, dissociation constant (K_d) and binding rate. Then, the association constant (1/K_d), 1/K_{ic} and K_{FQ} of tea polyphenols were correlated to analyse the effects of PPA inhibition on the binding of the enzyme with starch.

The results show that green, oolong and black tea extracts, epigallocatechin gallate, theaflavin-3, 3'-digallate and tannic acid were competitive inhibitors of PPA, whereas epicatechin gallate, theaflavin-3'-gallate and theaflavin were mixed-type inhibitors with both competitive and uncompetitive inhibitory characteristics. Only catechins with a galloyl substituent at the 3-position showed measurable inhibition. K_{ic} values were lower for theaflavins than catechins, with the lowest value for theaflavin-3, 3'-digallate. The lower K_{ic} than the uncompetitive inhibition constant for the mixed-type inhibitors suggests that they bind more tightly with free PPA than with the PPA-starch complex. A 3 and/or 3'-galloyl moiety in catechin and theaflavin structures was consistently found to increase inhibition of PPA through enhanced association with the enzyme active site. 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 supported 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 $1/K_{ic}$, $K_{FQ}$ and $K_{ic}$. Therefore, 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. Interestingly, CP, WAX and OBG could each increase the IC$_{50}$ values and $K_{ic}$, and decrease $K_{FQ}$ of tea polyphenols interacting with PPA. The data show a competitive interaction equilibrium between 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 relationships between IC$_{50}$ and $K_{ic}$ and between $K_{FQ}$ and $1/K_{ic}$ remained stable with and without polysaccharides, indicating that the three constants may be combined to characterize the effects of soluble polysaccharides on the PPA inhibition by polyphenols. Furthermore, polyphenols which have inhibitory activity against PPA increased binding of the enzyme with starch in a polyphenol concentration-dependent manner, while polyphenols without the inhibitory activity did not affect the binding of PPA with starch. The results are consistent with a binding equilibrium between polyphenols, starch granules and PPA. The $K_d$ value for PPA binding with starch was decreased by tea polyphenols, with the effects greater for theaflavins than catechins and for galloylated compared with non-galloylated polyphenols. Tea polyphenols were also shown to increase the binding rate of the enzyme by increasing the observed rate constant and decreasing the half-lives. Tea polyphenols were shown to be adsorbed onto starch granules as well. Additionally, there were positive linear correlations between $1/K_d$ and $1/K_{ic}$ and between $1/K_d$ and $K_{FQ}$. Taken together the data suggest that binding of polyphenols with PPA promotes PPA binding to starch granules. Despite the greater amount of PPA on the granules, starch
hydrolysis is reduced because the polyphenol inhibition of PPA persists after binding to starch.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Contributions by others to the thesis

1. Professor Michael J. Gidley (principal advisor) who assisted in designing research projects and experiments, discussing research problems, providing research advice and instructing research papers and whole thesis.

2. Dr. Fredrick J. Warren (co-advisor) who assisted in guiding research experiments, discussing research problems, solving technical issues, giving research advice and modifying research papers and thesis.

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Statement of parts of the thesis submitted to qualify the award of another degree

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# Table of Contents

Abstract ......................................................................................................................... I

Declaration by author ................................................................................................... IV

Publications during candidature ................................................................................. V

Publications included in this thesis .............................................................................. VI

Contributions by others to the thesis ........................................................................... VII

Acknowledgements ....................................................................................................... VIII

Keywords ...................................................................................................................... IX

List of Figures ............................................................................................................... XV

List of Tables ................................................................................................................. XX

List of Abbreviations .................................................................................................. XXI

Chapter 1: INTRODUCTION ......................................................................................... 1

1.1 Project background ................................................................................................. 1

1.2 Hypotheses ............................................................................................................. 3

1.3 Objectives ............................................................................................................... 3

Chapter 2: LITERATURE REVIEW ............................................................................. 4

Abstract ....................................................................................................................... 4

2.1 The relationship between phenolic structure and inhibitory activity against α-amylase .4

2.1.1 α-Amylase ......................................................................................................... 4

2.1.2 Flavonoids ....................................................................................................... 5

2.1.3 Phenolic acids ................................................................................................. 9

2.1.4 Galloyl moiety ............................................................................................... 13

2.2 Methods used to investigate the inhibitory effect of polyphenols on α-amylase .......14

2.2.1 Determination methods .................................................................................. 14

2.2.2 Characterization methods ............................................................................. 15

2.3 Mechanisms of interactions between polyphenols and α-amylase .......................16

2.3.1 Kinetics of inhibition ..................................................................................... 16

2.3.2 Fluorescence quenching ............................................................................... 17

2.3.3 Differential scanning calorimetry (DSC) ....................................................... 19
2.3.4 Isothermal titration calorimetry (ITC) ......................................................... 20
2.3.5 Molecular docking ......................................................................................... 22
2.4 Effects of polysaccharides on α-amylase inhibition by polyphenols...................... 22
   2.4.1 Potential influence of polysaccharides on the interactions between polyphenols
       and α-amylase .................................................................................................. 22
   2.4.2 Characterization of the effects of polysaccharides on α-amylase inhibition by
       polyphenols ...................................................................................................... 23
2.5 Combined effects of polyphenols with acarbose against α-amylase ...................... 24
   2.5.1 Synergistic effects ...................................................................................... 24
   2.5.2 Antagonistic effects .................................................................................. 25
2.6 Functional foods resources for inhibiting α-amylase ........................................... 25
   2.6.1 Tea ............................................................................................................. 25
   2.6.2 Fruits ......................................................................................................... 27
   2.6.3 Juices ......................................................................................................... 28
2.7 Summary of review .......................................................................................... 29
Chapter 3 .............................................................................................................. 31
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 ..................................................................................................... 31
ABSTRACT .............................................................................................................. 31
3.1. Introduction .................................................................................................... 32
3.2. Materials and methods .................................................................................. 34
   3.2.1 Materials and chemicals ......................................................................... 34
   3.2.2 Preparation of Tea Extracts .................................................................... 34
   3.2.3 Extracts Analysis ..................................................................................... 34
   3.2.4 α-Amylase Assay .................................................................................... 35
   3.2.5 The Kinetics of Inhibition of PPA by Tea Polyphenols ......................... 36
   3.2.6 Statistical Analysis .................................................................................. 37
3.3. Results .......................................................................................................... 38
3.3.1 α-Amylase Inhibition ................................................................. 40
3.3.2 The Kinetics of Inhibition of PPA by TEs and Tea Polyphenols............ 43
3.4. Discussion ............................................................................. 47
3.5. Conclusions .......................................................................... 53
Chapter 4 ..................................................................................... 55
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.................................................. 55
Abstract .................................................................................... 55
4.1. Introduction ............................................................................ 56
4.2. Materials and methods .......................................................... 57
   4.2.1 Materials and chemicals ..................................................... 57
   4.2.2 Kinetics of inhibition.......................................................... 57
   4.2.3 Fluorescence quenching ................................................. 58
   4.2.4 Differential scanning calorimetry...................................... 59
   4.2.5 Isothermal titration calorimetry....................................... 59
   4.2.6 Statistical analysis ............................................................ 60
4.3. Results .................................................................................. 60
   4.3.1 Kinetics of inhibition......................................................... 60
   4.3.2 Fluorescence quenching ................................................. 60
   4.3.3 Differential scanning calorimetry...................................... 63
   4.3.4 Isothermal titration calorimetry....................................... 66
4.4. Discussion ............................................................................. 69
4.5. Conclusion ............................................................................ 75
Chapter 5 ..................................................................................... 76
SOLUBLE POLYSACCHARIDES REDUCE BINDING AND INHIBITORY ACTIVITY
OF TEA POLYPHENOLS AGAINST PORCINE PANCREATIC α-AMYLASE........ 76
Abstract .................................................................................... 76
5.1. Introduction ............................................................................ 77
5.2. Materials and methods ................................................................. 78
  5.2.1 Materials and chemicals ............................................................ 78
  5.2.2 Effects of polysaccharides on PPA inhibition by tea polyphenols .......... 79
  5.2.3 Effects of polysaccharides on IC_{50} values of tea polyphenols .......... 80
  5.2.4 Effects of polysaccharides on kinetics of PPA inhibition by tea polyphenols 80
  5.2.5 Effects of polysaccharides on PPA fluorescence quenching by tea polyphenols 81
  5.2.6 Statistical analysis ................................................................. 81
5.3. Results .................................................................................. 81
  5.3.1 Effects of polysaccharides on PPA inhibition and IC_{50} values of tea polyphenols ................................................................. 81
  5.3.2 Effects of polysaccharides on kinetics of PPA inhibition by tea polyphenols 84
  5.3.3 Effects of polysaccharides on PPA fluorescence quenching by tea polyphenols ................................................................. 86
  5.3.4 Correlations between IC_{50}, K_{ic} and K_{FQ} values ................................ 89
5.4. Discussion ............................................................................. 91
5.5. Conclusion ............................................................................ 95
Chapter 6 ..................................................................................... 96
TEA POLYPHENOLS ENHANCE BINDING OF PORCINE PANCREATIC \( \alpha \)-AMYLASE WITH STARCH GRANULES BUT REDUCE CATALYTIC ACTIVITY .................................................. 96
Abstract ....................................................................................... 96
6.1. Introduction ........................................................................ 97
6.2. Materials and methods ............................................................ 98
  6.2.1 Materials and chemicals ............................................................ 98
  6.2.2 Determination of binding ratio of PPA with starch ......................... 98
  6.2.3 Determination of binding ratio of tea polyphenols with starch .......... 99
  6.2.4 Determination of dissociation constant for PPA binding with starch .... 100
  6.2.5 Determination of binding rate for PPA binding with starch .......... 100
  6.2.6 Statistical analysis ................................................................. 101
6.3. Results .................................................................................. 101
6.3.1 Binding ratios of PPA with starch granules indicated by initial reaction velocity and fluorescence intensity ................................................................. 101
6.3.2 Binding ratios of tea polyphenols with starch granules .......................... 108
6.3.3 Dissociation constants for binding of PPA with starch granules .......... 108
6.3.4 Binding rates of PPA with starch granules ........................................ 109

6.4. Discussion .............................................................................................. 110
6.5. Conclusion ............................................................................................. 114

Chapter 7: GENERAL CONCLUSIONS AND FUTURE WORKS ..................... 116
7.1. General conclusion of the thesis.............................................................. 116
7.2. Future works.......................................................................................... 118

References Cited ......................................................................................... 120
Appendices .................................................................................................... 139
List of Figures

Figure 2.1 Three-dimensional structure, determined by crystallography, of porcine pancreatic α-amylase (Qian, Haser, & Payan, 1993) (A), and the active site of α-amylase (B) (MacGregor, Janecek and Svensson, 2001) ................................................................. 5

Figure 2.2 The molecular structures of flavonoids (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008) ........................................................................................................ 8

Figure 2.3 The molecular structures of phenolic acids (Narita & Inouye, 2011) .................. 12

Figure 2.4 The molecular structures of tea polyphenols .................................................... 14

Figure 3.1. UPLC chromatogram of GTE (A), OTE (B), and BTE (C) ............................... 39

Figure 3.2. Inhibition of PPA by TEs with different concentrations, expressed as mg extracts/mL (A) and mg gallic acid equivalent (GAE)/mL (B). Inhibition of PPA by pure phenolic compounds (C). All the curves are fitted based on the equation (3.2) used for calculating IC_{50} values of inhibitors ................................................................. 42

Figure 3.3. Initial reaction velocity at different concentrations of starch in the absence or presence of GTE (A), TF2 (B) and ECG (C). Various concentrations of the inhibitors are indicated in the respective legend entries. PBS buffer means starch digestion without the inhibitors; Dixon and Cornish-Bowden (insets) plots for PPA inhibition by GTE (D), TF2 (E) and ECG (F). Various concentrations of starch solution are listed in the legend entries; Lineweaver-Burk plots for PPA inhibition by GTE (G), TF2 (H) and ECG (I). The legend entries are the same as A-C ............................................................................................................ 47

Figure 3.4. Correlation between IC_{50} and K_{ic} and K_{iu} (inset) for pure phenolic compounds in tea extracts .......................................................................................................................... 47

Figure 3.5. Mechanisms that produce competitive (A) and mixed-type (B) inhibition. A, E, I and P represent substrate, enzyme, inhibitor and product, respectively ................................................................. 49

Figure 4.1. 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 (4.1), and equations for three TEs, TA, TF2, EGCG and ECG were fitted according to equation (4.2).............63

Figure 4.2. 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.................................................................64

Figure 4.3. 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)).................................................................................................................67

Figure 4.4. 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), EGCG (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.................................................................68

Figure 4.5. The linear correlations between KFQ and $1/K_{ic}$ (A), $K_{itc}$ and $1/K_{ic}$ (B) and KFQ and $K_{itc}$ (C). The respective correlation equations and coefficients ($R^2$) are listed as well ......70

Figure 5.1. 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)..................................................................................................................83

Figure 5.2. Stern-Volmer plots for fluorescence quenching of PPA by GTE (A), TF2 (B) and ECG (C) in the absence and presence of three polysaccharides.................................................88
**Figure 5.3.** The correlation between IC\textsubscript{50} and K\textsubscript{ic} in the absence (A) and presence of WAX (B), CP (C) and OBG (D). ‘NP’ means no polysaccharides ................................................................. 89

**Figure 5.4.** The correlation between 1/K\textsubscript{ic} and IC\textsubscript{50} for GTE (A), TF2 (B) and ECG (C), and that between K\textsubscript{FQ} and IC\textsubscript{50} for GTE (D), TF2 (E) and ECG (F) in the absence and presence of three polysaccharides. ‘NP’ means no polysaccharides ................................................................. 90

**Figure 5.5.** The correlation between K\textsubscript{FQ} and 1/K\textsubscript{ic} in the absence (A) and presence of WAX (B), CP (C) and OBG (D). ‘NP’ means no polysaccharides ................................................................. 91

**Figure 5.6.** Interaction equilibrium among soluble polysaccharides, tea polyphenols and PPA .............................................................................................................................................. 92

**Figure 6.1.** Binding ratios of PPA with starch granules indicated by initial reaction velocity of PPA in the absence and presence of tea polyphenols. The values in the figure are the ratios of the free, unbound PPA in the mixture after binding ................................................................. 103

**Figure 6.2.** Binding ratios of PPA with starch granules indicated by fluorescence intensity of PPA in the absence and presence of tea polyphenols. The values in the figure are the ratios of the free, unbound PPA in the mixture after binding ................................................................. 105

**Figure 6.3.** Effects of different concentrations of tea polyphenols on the binding ratios of PPA with starch granules indicated by initial reaction velocity and fluorescence intensity .. 105

**Figure 6.4.** Effects of mixing order for tea polyphenols, starch and PPA on the binding ratio of PPA with starch in the presence of TA (A), TF2 (B) and ECG (C), respectively. The two mixing methods included mixing polyphenol with PPA followed by the addition of starch suspension to the mixture (labelled as (polyphenol+AA)+S), and mixing polyphenol with starch suspension followed by the addition of PPA to the mixture (labelled as (polyphenol+S)+AA). 0.04 and 0.06 mg/mL polyphenol concentrations were applied, respectively. * means the significantly (P<0.05) different mean values compared to the control .............................................................................................................................................. 107

**Figure 6.5.** Binding ratios of tea polyphenols with starch granules. Different letters represent significant different mean values (P<0.05) .............................................................................................................................................. 108

**Figure 6.6.** Binding ratios of PPA with starch granules with different concentrations for calculating the dissociation constants .............................................................................................................................................. 109
Figure 6.7. Scatchard plots for binding of PPA with starch granules in the absence and presence of tea polyphenols.................................................................................................................113

Figure 6.8. The correlation between 1/K_d and 1/K_ic and that between 1/K_d and K_FQ..............113

Figure 7.1. Interactions in the multi-component system of α-amylase, polyphenols, polysaccharides and starch established in this thesis.................................................................118

Figure S3.1. Initial reaction velocity at different concentrations of starch in the absence or presence of BTE (A), OTE (B), TA (C), EGCG (D), TF1 (E) and TF (F). Various concentrations of these inhibitors are indicated in the respective legend entries. PBS buffer means starch digestion without the inhibitors .................................................................139

Figure S3.2. Dixon and Cornish-Bowden (insets) plots for PPA inhibition by BTE (A), OTE (B), TA (C), EGCG (D), TF1 (E) and TF (F). Various concentrations of starch solution are listed in the respective legend entries .........................................................................................140

Figure S3.3. Lineweaver-Burk plots for PPA inhibition by BTE (A), OTE (B), TA (C), EGCG (D), TF1 (E) and TF (F). Various concentrations of these inhibitors are indicated in the respective legend entries. PBS buffer means starch digestion without the inhibitors ......141

Figure S5.1. Dixon and Cornish-Bowden (insets) plots for PPA inhibition by tea polyphenols in the absence and presence of three polysaccharides.................................................................143

Figure S5.2. Fluorescence quenching of PPA by tea polyphenols in the absence and presence of three polysaccharides ............................................................................................................145

Figure S5.3. The correlation between 1/K_ic and IC_{50} for BTE (A), EGCG (B), TF (C), TF1 (D) and TA (E), and that between K_FQ and IC_{50} for BTE (F), EGCG (G), TF (H), TF1 (I) and TA (J) in the absence and presence of three polysaccharides. ‘NP’ means no polysaccharides ........................................................................................................................................147

Figure S6.1. A flow diagram showing how the binding of PPA with raw starch granules in the absence or presence of tea polyphenols is determined through the initial reaction velocity of cooked starch...........................................................................................................................................................................149

Figure S6.2. Correlation between the initial reaction velocity of PPA and content of PPA (A) and that between the maximum fluorescence intensity and content of the enzyme (B)..............149
**Figure S6.3.** The plots of binding of PPA with starch granules against time in the absence (A) and presence (B-G) of tea polyphenols. The curves in this figure were fitted based on equation (6.5) ................................................................. 151

**Figure S6.4.** The plots of $1/E_{\text{bound}}$ against $1/[S]$ in the absence and presence of tea polyphenols. The equations were fitted with the straight transform of equation (6.3) ........ 151

**Figure S6.5.** The initial reaction velocities of raw starch digestion by PPA in the absence and presence of 0.04 mg/mL TA. For the determination, 1 mL of PBS or 0.04 mg/mL TA was mixed with 2 mL PPA for 15 min at 0 °C, followed by the addition of 2 mL of 10 mg/mL raw starch suspension. The digestion was carried out at 37 °C, and the initial reaction velocity was determined by use of the method described in Chapter 3 ......................................................... 152
List of Tables

Table 3.1. The contents of total and individual polyphenols in GTE, OTE and BTE.............38

Table 3.2. Detailed kinetics of PPA inhibition by TE and pure phenolic compounds..........41

Table 4.1 Fluorescence quenching parameters for the interactions of TE and pure polyphenols with PPA ..................................................................................................................................................61

Table 4.2 Denaturation temperature ($T_d$) and enthalpy ($\Delta H$) of PPA obtained by DSC thermograms in the absence and presence of TE and pure polyphenols.................................65

Table 4.3 Thermodynamic binding parameters for the interactions of tea polyphenols with PPA fitted by single-site binding model .................................................................................................................65

Table 5.1 Detailed kinetics of PPA inhibition by TE and pure phenolic compounds in the absence and presence of three polysaccharides.................................................................................................85

Table 5.2 Constants of fluorescence quenching of PPA by TE and pure phenolic compounds in the absence ($K_{FQ}$ and $k_q$) and presence ($K_{FQ}'$ and $k_q'$) of three polysaccharides................87

Table 6.1. Binding ratios, dissociation constants, observed rate constants and half-lives for binding of PPA with starch granules in the absence and presence of tea polyphenols..........103
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPA</td>
<td>Porcine pancreatic α-amylase</td>
</tr>
<tr>
<td>GTE</td>
<td>Green tea extracts</td>
</tr>
<tr>
<td>BTE</td>
<td>Black tea extracts</td>
</tr>
<tr>
<td>OTE</td>
<td>Oolong tea extracts</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra Performance Liquid Chromatography</td>
</tr>
<tr>
<td>CF</td>
<td>Caffeine</td>
</tr>
<tr>
<td>GA</td>
<td>Gallic acid</td>
</tr>
<tr>
<td>TA</td>
<td>Tannic acid</td>
</tr>
<tr>
<td>C</td>
<td>Catechin</td>
</tr>
<tr>
<td>EC</td>
<td>Epicatechin</td>
</tr>
<tr>
<td>EGC</td>
<td>Epigallocatechin</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin gallate</td>
</tr>
<tr>
<td>ECG</td>
<td>Epicatechin gallate</td>
</tr>
<tr>
<td>TF</td>
<td>Theaflavin</td>
</tr>
<tr>
<td>TF1</td>
<td>Theaflavin-3’-gallate</td>
</tr>
<tr>
<td>TF2</td>
<td>Theaflavin-3, 3’-digallate</td>
</tr>
<tr>
<td>TEs</td>
<td>Tea extracts</td>
</tr>
<tr>
<td>$K_{ic}$</td>
<td>Competitive inhibition constant</td>
</tr>
<tr>
<td>$K_{iu}$</td>
<td>Uncompetitive inhibition constant</td>
</tr>
<tr>
<td>$K_{m}^{app}$</td>
<td>Apparent Michaelis constant</td>
</tr>
<tr>
<td>$V_{max}^{app}$</td>
<td>Apparent maximum initial reaction velocity</td>
</tr>
<tr>
<td>FQ</td>
<td>Fluorescence quenching</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>$K_{FQ}$</td>
<td>Fluorescence quenching constant</td>
</tr>
<tr>
<td>$K_{ic}$</td>
<td>Binding constant</td>
</tr>
<tr>
<td>CP</td>
<td>Citrus pectin</td>
</tr>
<tr>
<td>WAX</td>
<td>Wheat arabinoxylan</td>
</tr>
<tr>
<td>OBG</td>
<td>Oat β-glucan</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>$k_{obs}$</td>
<td>Observed binding rate</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>Half-life</td>
</tr>
</tbody>
</table>
Chapter 1: INTRODUCTION

1.1 Project background

Type II diabetes is defined as a chronic disease due to reduced insulin sensitivity, which may lead to multiple complications. The control of postprandial hyperglycaemia is advisable in the prevention and treatment of this disease. Retarding sugar absorption through delaying the digestion of starchy foods by inhibiting starch-hydrolyzing enzymes, for example α-amylase and α-glucosidase in the digestive tract, has potential as a management and/or therapeutic approach. It has been reported that some plant extracts have bioactivity potentially beneficial to human health by inhibiting the activities of carbohydrate hydrolyzing enzymes, and the main active constituents in these plant extracts are phenolic compounds that have several hydroxyl groups.

Extracts from tea leaves have been shown to strongly inhibit α-amylase activity. Phenolic compounds in tea extracts which are reported to have relatively high α-amylase inhibition include theaflavin-3, 3’-digallate (TF2), theaflavin-3’-gallate (TF1), theaflavin (TF), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG). The inhibition of α-amylase by tea polyphenols has been studied by half inhibitory concentration (IC\textsubscript{50}), Lineweaver-Burk equation, fluorescence quenching and docking analysis. However, there is a relatively large error in the Lineweaver-Burk plot when the substrate concentration is low. Also, the plot cannot distinguish between uncompetitive, non-competitive and mixed-type inhibition. Therefore, additional inhibition analysis methods may be used to assist in analysing the kinetics of inhibition. Besides, the molecular mechanism of the interaction between α-amylase and tea polyphenols requires further investigation using biochemical and biophysical methods.

Usually, aqueous extracts from fruits or plants are a complex mixture, often containing polyphenols and soluble polysaccharides, due to the binding interactions between the two compounds. On the other hand, even though pure phenolics or phenolic extracts containing very high content of polyphenols are consumed, polyphenols, in practice, may interact with digestive juices, tissues and other food components (like proteins, polysaccharides, etc.) in the digestive tract. The presence of soluble polysaccharides in reaction solution may be a
potential factor affecting the interaction between polyphenols and the enzyme because of the potential binding interaction between polysaccharides and polyphenols. It is not yet known whether soluble polysaccharides affect the inhibitory activity of tea polyphenols against α-amylase.

The process of starch digestion by α-amylase mainly includes two key steps; one is binding of the enzyme with starch and the other one is catalytic hydrolysis of starch. The adsorption and binding of α-amylase with starch granules mainly depends on the granule properties, such as the particle size, the surface properties (the presence of pores/crevices), the supramolecular structure (relative percentage of amorphous and ordered α-glucan chains) of the exposed surfaces, etc. As tea polyphenols have been reported to interact with α-amylase, the effects of polyphenols on the binding of the enzyme with starch granules should also be further elucidated.

Therefore, in this thesis, Dixon, Cornish-Bowden and Lineweaver-Burk plots were applied to study the detailed kinetics of inhibition of porcine pancreatic α-amylase (PPA) by tea polyphenols. Fluorescence quenching (FQ), differential scanning calorimetry (DSC), and isothermal titration calorimetry (ITC) were combined with the kinetics of inhibition to elucidate the mechanism of binding interactions of tea polyphenols and PPA. Then, the reciprocal of competitive inhibition constant (1/K_{ic}), fluorescence quenching constant (K_{FQ}) and binding constant (K_{itc}) obtained from these measurements were compared and correlated to analyse the relations between PPA inhibition and binding behaviour. The role of the galloyl moiety in binding of catechins and theaflavins with PPA was highlighted. Besides, the effects of three soluble polysaccharides (citrus pectin (CP), wheat arabinoxylan (WAX) and oat β-glucan (OBG)) on the inhibition of PPA by tea polyphenols were studied through initial enzymic velocity, IC$_{50}$ value, inhibition kinetics and fluorescence quenching methods. In addition, the effects of tea polyphenols on the binding of PPA with starch granules were investigated in terms of binding ratio, dissociation constant (K\textsubscript{d}) and binding rate. Then, the association constant (1/K\textsubscript{d}) and 1/K\textsubscript{ic} and K\textsubscript{FQ} of tea polyphenols were correlated to analyse the effects of PPA inhibition on the binding of the enzyme with starch.
1.2 Hypotheses

- Specific moiety structures play an important role for tea polyphenols in the inhibitory activity against α-amylase.
- There are potential correlations between activity inhibition and parameters obtained from the methods characterizing interactions between tea polyphenols and α-amylase.
- The interactions between soluble polysaccharides and tea polyphenols affect the binding interactions between polyphenols and α-amylase, and thus affect the inhibitory activity against the enzyme.
- The binding interactions between tea polyphenols and α-amylase affect the binding of the enzyme with starch granules.

1.3 Objectives

- To study the detailed kinetics of inhibition of α-amylase by tea polyphenols.
- To investigate the mechanisms of interactions between tea polyphenols and α-amylase through inhibition kinetics, fluorescence quenching, differential scanning calorimetry and isothermal titration calorimetry.
- To characterize the correlations between the constants obtained to derive insights into mechanisms.
- To characterize the key structural groups in tea polyphenols in binding with and inhibiting α-amylase.
- To study the effects of soluble polysaccharides on the inhibitory activity of tea polyphenols against α-amylase.
- To study the effects of tea polyphenols on the binding of α-amylase with starch granules.
Chapter 2: LITERATURE REVIEW

Abstract

α-Amylase plays an important role in the digestion of starch, providing the main source of exogenous glucose in the human diet. Retarding sugar adsorption through delaying the digestion of starchy foods by inhibiting α-amylase in the digestive tract has potential as a management and/or therapeutic approach. Polyphenols have been reported to have inhibitory activity against the enzyme. The aim of this review is to give an overview of research reporting on the structure-activity relationship of polyphenols inhibiting α-amylase and the inhibiting mechanisms. The potential relationships between parameters obtained from the methods applied to investigate the binding interactions between polyphenols and α-amylase will be further discussed. Besides, as polyphenols can interact with both polysaccharides and α-amylase, the potential effects of polysaccharides on the enzyme inhibition by polyphenols may be of interest. The inhibitory activity of the extracts from some food resources are reviewed as well. Most of the work reported is from in vitro studies, so the inhibitory effects on α-amylase by polyphenols in vivo should be further investigated to give a better understanding of the functional components as inhibitors of α-amylase.

2.1 The relationship between phenolic structure and inhibitory activity against α-amylase

2.1.1 α-Amylase

α-Amylase is an enzyme that hydrolyses α-1,4-glucan polysaccharides, such as starch and glycogen, producing glucose oligosaccharides prior to the adsorption of glucose, which triggers an insulin response if glucose adsorption occurs too quickly. It is the major form of amylase found in mammals. It also exists in plant seeds containing starch as a food reserve (Mundy & Rogers, 1986), and is secreted by some fungi (Mohapatra, Banerjee, & Bapuji, 1998). In the three-dimensional structure, determined by X-ray crystallography of α-amylase (Fig. 2.1A), there are four secondary structures, including α-helix, β-sheet, β-turn and random coil (Cai, Yu, Xu, Liu, & Yang, 2015). At the active site of the enzyme (Fig. 2.1B), there are calcium and chloride ions, which are essential for maintenance of the tertiary structure and
catalytic activity of α-amylase (Buisson, Duée, Haser, & Payan, 1987). Porcine pancreatic α-amylase (PPA) is often used to study catalytic digestion of starch in vitro. There are 496 amino acids residuals in the single polypeptide chain of the enzyme (Qian, Haser, & Payan, 1993). Through modelling based on the X-ray crystallographic structure (Fig. 2.1B), Asp$^{300}$, Asp$^{197}$ and Glu$^{233}$ are considered as essential catalytic residues at the active site of PPA (MacGregor, Janecek and Svensson, 2001). In addition, some aromatic residues, like Trp$^{58}$, Trp$^{59}$ and Tyr$^{62}$ are stacking features at the entrance of the active site of the enzyme (Qian, Haser, & Payan, 1993). Notably, amino acid residues at the active site of α-amylase are proposed to be in positions where the interactions between inhibitors and α-amylase are thought to occur (Miao, Jiang, Jiang, Li, Cui, & Jin, 2013).

![Figure 2.1](image)

**Figure 2.1** Three-dimensional structure, determined by crystallography, of porcine pancreatic α-amylase (Qian, Haser, & Payan, 1993) (A), and the active site of α-amylase (B) (MacGregor, Janecek and Svensson, 2001), in which the calcium, chloride ions and essential amino acids (Glu$^{233}$, Asp$^{197}$, Asp$^{300}$) are indicated. The yellow structure is a short chain of five sugar units connected through α-1,4-link (coloured pink).

### 2.1.2 Flavonoids

Hydroxyl groups are essential for the inhibitory activity of flavonoid compounds against α-amylase, as the inhibition is likely to depend on the formation of hydrogen bonds between the -OH groups of phenolics and the side chains of amino acids (such as Asp$^{197}$ and Glu$^{233}$ in human salivary α-amylase) at the active sites of α-amylase (Kawamura-Konishi, Watanabe, Saito, Nakajima, Sakaki, Katayama, et al., 2012; Lo Piparo, Scheib, Frei, Williamson,
Grigorov, & Chou, 2008). Flavonoids without substitution of -CH$_3$ and -OCH$_3$ at -OH in their structures (Fig. 2.2) are more effective in inhibition of α-amylase than those with such substitution patterns (Al-Dabbas, Kitahara, Suganuma, Hashimoto, & Tadera, 2006; Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008). In particular, quercetagetin and scutellarein were demonstrated as potential α-amylase inhibitors, with IC$_{50}$ values of 10.2 and 9.64 µM, respectively (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008). The molecular structures of both flavonoids have four hydroxyl groups at positions 5, 6, 7 in the A ring as well as 4’ in the B ring (Fig. 2.2). The IC$_{50}$ values of some other flavonoids (kaempferol, apigenin, naringenin, daidzein and catechin) with less hydroxyl groups in their molecular structures were found to be much higher (0.5 mM-6.0 mM) than quercetagetin and scutellarein (Barrett, Ndou, Hughey, Straut, Howell, Dai, et al., 2013; Wang, Du, & Song, 2010). Besides, the methylation of hydroxyl groups in flavonoids was also shown to decrease its inhibitory activity as well (Xiao, Ni, Kai, & Chen, 2013). Therefore, these confirm the role of hydroxyl group of flavonoids in inhibiting the enzyme activity.

The 2, 3-double bonds at ring C (Fig. 2.2) also affect the inhibitory activity of flavonoids to some extent. These double bonds can be conjugated to the 4-carbonyl group, which enhances electron delocalization between ring C (pyrone) and ring A (benzene); therefore, it has been proposed that the benzo@pyrone system (ring AC) can form a highly stable conjugated π-π system with the indole ring of Trp at the active sites of α-amylase, promoting its binding to α-amylase and reducing the catalytic activity (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008). Hydrogenation of the 2, 3-double bonds of flavonoids may change the molecular conformation, transforming the near-planar molecular structure (flavonol and flavone) to a more flexible and non-planar stereochemical structure (flavanone and flavanol) (Todorova, Traykov, Tadjer, & Velkov, 2013). As a consequence, flavanone, flavanols and isoflavone compounds showed weaker inhibition against α-amylase than some flavonols and flavones (Kim, Kwon, & Son, 2000; Wang, Du, & Song, 2010).

There are also glycosylation forms of flavonoids in plant extracts (da Silva, Contesini, Sawaya, Cabral, Cunha, Eberlin, et al., 2013). Quercetin is a flavonol (without glycosylation) that was shown to possess competitive inhibitory activity against α-amylase (Li, Gao, Gao, Shan, Bian, & Zhao, 2009). There are some monoglycoside forms of quercetin, such as
quercitrin (rhamnoside), hyperin (galactoside), guaijaverin (arabinopyranoside), avicularin (arabinofuranoside), with rutin (rhamnoside and glycoside) as a disaccharide form. The inhibiting activity of these glycosylated quercetin molecules against α-amylase was determined to be quercetin > guaijaverin > avicularin > hyperin > rutin (Li, Gao, Gao, Shan, Bian, & Zhao, 2009; Ye, Song, Yuan, & Mao, 2010). Komaki et al. (2003) also measured the IC₅₀ of luteolin (0.01 mg/mL) against α-amylase, and found it to be much lower than that of luteolin-7-O-β-glucoside (0.5 mg/mL) and luteolin-4’-O-β-glucoside (0.3 mg/mL). Besides, the inhibiting effect of kaempferol on α-amylase was much stronger than its glycoside form (kaempferol-3-O-β-D-diglucoside) (Ye, Song, Yuan, & Mao, 2010). Therefore, glycosylation on flavonoids was found to decrease the inhibitory activity. The potential reasons for the inhibition change are as follows: (i) spatial structures of flavonoid-glycosides are transferred to bulky non-planar from near-planar, limiting the ability to enter the hydrophobic (active) site of α-amylase due to steric hindrance, and (ii) as the 3-OH is substituted by a glycoside, its affinity for protein is correspondingly affected; therefore, glycosylation of flavonoids decreases their binding affinity to α-amylase (Wang, Huang, Shao, Qian, & Xu, 2012; Xiao, Huo, Jiang, & Yang, 2011).
Figure 2.2 The molecular structures of flavonoids (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008).
2.1.3 Phenolic acids

Phenolic acids, an important polyphenol class, are aromatic phenols of secondary plant metabolites with a carboxylic acid functional group. They are widely distributed throughout the plant kingdom (Shahidi, Janitha, & Wanasundara, 1992). The natural plant phenolic acids mainly contain two molecular groups: the hydroxyl cinnamic and hydroxybenzoic acids (Fig. 2.3). For individual molecular groups, although the essential structure remains the same, the substituents (hydroxyl and methoxyl groups) on the aromatic ring contribute to distinctive molecular properties, such as polarity, stability and binding.

It has been reported that hydroxybenzoic acids, like vanillic acid, salicylic acid, etc., hardly show any inhibitory activity against α-amylase (McDougall, Shpiro, Dobson, Smith, Blake, & Stewart, 2005; Sharma, Sharma, & Rai, 1986), while hydroxyl cinnamic acids were verified to have inhibiting effects to some extent (Narita & Inouye, 2011). The C=C double bonds in the molecular structure is conjugated to the carbonyl group and is responsible for electron transfer between acrylic acid and benzene ring. As a consequence, hydroxyl cinnamic acids could form a highly conjugated system which stabilizes the compounds when binding to the active site of α-amylase. Caffeic acid has been reported to have a relatively strong inhibitory activity ($IC_{50}=0.4$ mM). However, both dehydroxylation and methylation of caffeic acid lowered its inhibiting activity against α-amylase, although these compounds are still structures with a delocalised π-system established through carbonyl, C=C double bonds and benzene (Narita & Inouye, 2011). The $IC_{50}$ of quinic acid was determined as 26.5 mM, much higher than chlorogenic acids and caffeic acid, with 3 hydroxyl groups but has no strong conjugated system in its molecular structure. Therefore, conjugated structural molecules and polyhydroxyl groups are essential for hydroxyl cinnamic acids to show inhibition against α-amylase.

Chlorogenic acids are a set of esters between quinic acid and one or more cinnamic acid derivatives, like caffeic acid, ferulic acid, and $p$-coumaric acid (Fig. 2.3). They are widely distributed in green coffee beans, including three main classes: caffeoylquinic acids, dicaffeoylquinic acids, and feruloylquinic acids (Clifford, Knight, Surucu, & Kuhnert, 2006). Narita et al. (2011) investigated α-amylase inhibitory activity of 16 kinds of chlorogenic acids and cinnamate derivatives from green coffee beans, with $p$-nitrophenyl-α-D-maltoside.
as substrate. The methylation of 3-OH group on caffeoylquinic acids (converting 3-, 4-, and 5-caffeoylquinic acids to 3-, 4-, and 5-feruloylquinic acids, respectively) decreased their inhibition effects against porcine pancreatic α-amylase isozyme I as a result of weakened hydrogen bonding between feruloylquinic acids (3-, 4-, and 5-feruloylquinic acids) and protein residues of the enzyme, showing a similar trend as methylation of flavonoids. Among the chlorogenic acids tested, dicafeoylquinic acids showed strongest inhibitory activity, with IC$_{50}$ of 3,4-, 4,5-, and 3,5-dicafeoylquinic acids on α-amylase being 0.02, 0.02, and 0.03 mM, respectively (Narita & Inouye, 2011). The potential reasons for their comparatively effective inhibition are (i) the dicafeoyl group provides more hydroxyl groups which are essential for inhibition due to the formation of hydrogen bonds between hydroxyl groups and active catalyzing sites. (ii) there exists one more carbonyl, C=C double bond, and benzene ring in dicafeoylquinic acids than caffeoylquinic acids. As a consequence, dicafeoyl molecules are more electron-rich with p-π (between double bonds and benzene) and π-π (carbonyl and double bonds) conjugated systems, leading to possible stronger π-interaction with the indole ring of Trp$^{59}$ (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008; Rohn, Rawel, & Kroll, 2002).

Tannic acid, a type of antioxidative polyphenol, is a widely-spread secondary metabolite in higher plants (Gülçin, Huyut, Elmastaş, & Aboul-Enein, 2010). The chemical structure for standard tannic acid is decagalloyl glucose, but in fact, it is a mixture of polygalloyl glucoses or polygalloyl quinic acid esters with 2-12 galloyl moieties per molecule depending on plant source (Fig. 2.3). There are studies reporting that tannic acid has inhibitory activity against α-amylase (Gin, Rigalleau, Caubet, Masquelier, & Aubertin, 1999; Kandra, Gyemant, Zajacz, & Batta, 2004). It has been suggested that starch digestion was slowed in a dose-dependent manner, and that foods/drinks, like wine, containing comparatively high levels of tannic acid, could moderate postprandial levels of blood glucose and corresponding insulin. The loss or decrease of the ability to inhibit α-amylase in vivo may occur (Kim, Silva, Kim, & Jung, 2010). This may be because: (i) tannic acid might be oxidized by oxygen and oxygen-derived radicals in the stomach; (ii) tannic acid could interact or bind with proteins present in foods and stomach digesta before reaching the small intestine where starch is hydrolyzed by pancreatic α-amylase (Martinez & Moyano, 2003). In addition, orally-taken tannic acid may
cause astringent and unpleasant bitter taste caused by interactions with salivary proteins (Bate-Smith, 1973). According to these unacceptable properties, microencapsulation systems for tannic acid have been developed to control its releasing process and to improve inhibition in the gastrointestinal tract (Xing, Cheng, Yang, & Ma, 2004). The optimum pH value for pancreatic α-amylase is 7.0 (Coronado, Vargas, Hofemeister, Ventosa, & Nieto, 2000), but the pKa of the phenolic carboxylic acid proton is between 4 and 5, making its aqueous solution acid (pH<7.0); therefore, the catalytic activity of α-amylase may be at least partly inhibited due to unfavourable acidity as well (Nielsen, Borchert, & Vriend, 2001).
Figure 2.3 The molecular structures of phenolic acids (Narita & Inouye, 2011).
2.1.4 Galloyl moiety

The galloyl group is a common substitution of polyphenols, especially tea polyphenols (Fig. 2.4). Catechin (C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), theaflavin, theaflavin-3’-gallate (TF1), theaflavin-3, 3’-digallate (TF2) are the prominent polyphenols in aqueous green, oolong or black tea extracts (Serpen, Pelvan, Alasalvar, Mogol, Yavuz, Gokmen, et al., 2012). The inhibitory activities of the tea polyphenols against α-amylase have been studied, and the results showed that polyphenols with a galloyl moiety in the molecular structures showed greater enzyme inhibition than those without galloyl moiety, indicated by IC$_{50}$ values (Hara & Honda, 1990) (Miao, Jiang, Jiang, Li, Cui, & Jin, 2013; Miao, Jiang, Jiang, Zhang, & Li, 2015). Therefore, 3 or 3’-galloyl groups on the C or C’ ring (Fig. 2.4) are shown to be responsible for enhancing the inhibitory activities of catechins and theaflavins against α-amylase. Each galloyl group provides three hydroxyl groups which can potentially interact with the catalytic site amino acid side-chains of amylase (Asp$^{197}$, Glu$^{233}$ and Asp$^{300}$) (Fei, Gao, Zhang, Sun, Hu, Zhou, et al., 2014) upon the formation of hydrogen bonds, while the benzene ring may develop hydrophobic π-π (aromatic-aromatic) interactions in the active site of amylase (Miao, Jiang, Jiang, Li, Cui, & Jin, 2013; Skrt, Benedik, Podlipnik, & Ulrih, 2012). In addition, in the galloyl group, the C=O double bond is conjugated to the benzene ring and is responsible for electron delocalization, which has been proposed to lead to enhanced π-π interactions with the indole ring of Trp$^{59}$ (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008).
2.2 Methods used to investigate the inhibitory effect of polyphenols on α-amylase

2.2.1 Determination methods

In vitro assays to measure enzyme inhibition are relatively convenient to conduct. Generally, a real substrate at physiological concentration is used with human salivary or porcine pancreatic α-amylase, and the assay is carried out at a suitable pH value. The
\(\alpha\)-amylase inhibitory assay could be performed by iodo-starch reaction, reducing sugars method, \(p\)-nitrophenyl-\(\alpha\)-\(\alpha\)-maltoside method, or fluorescently-labelled starch.

The iodo-starch reaction is mainly used for initial screening for the inhibitory activity of phenolic compounds, because it is operationally easy to conduct (Kawamura-Konishi, et al., 2012). For this method, soluble starch is used as substrate. Enzyme solution and polyphenol solution are mixed and incubated for a while, followed by addition of iodine solution [0.1% (w/v) \(I_2\) and 1% (w/v) KI]. The inhibition of polyphenolics is evaluated by the residual content of starch in the solution, which could be monitored by the absorbance of blue starch-iodine complexes at 595 nm.

To study the time course of starch digestion by \(\alpha\)-amylase in the absence or presence of phenolic compounds, substrates such as amylopectin or maize starch, are hydrolysed, and the inhibitory effect of polyphenols is usually estimated by the concentration of released reducing sugar (maltose or glucose) in solution at set intervals (Fei, et al., 2014). An easy alternative method is to use \(p\)-nitrophenyl-\(\alpha\)-\(\alpha\)-maltoside, consisting of maltose linked with a \(p\)-nitrophenyl ligand. After hydrolysis, the yellow nitrophenol anion is released and measured at 405 nm using a spectrophotometer (Karim, Holmes, & Orfila, 2017).

A more relevant and convenient method with greater sensitivity is the Enzcheck \(^{\circledast}\) assay kit (Warren, Royall, Gaisford, Butterworth, & Ellis, 2011). This is based on an artificial maize starch substrate (DQ starch), fluorescently labelled to such a high degree that the fluorescence is quenched. DQ starch is efficiently degraded by \(\alpha\)-amylase, releasing highly fluorescent fragments. The accompanying increase in fluorescence is proportional to \(\alpha\)-amylase activity and is determined using a fluorimeter.

### 2.2.2 Characterization methods

The common methods to characterize the inhibitory activity of polyphenols mainly include the half inhibitory concentration value, \(IC_{50}\) and the Lineweaver-Burk plot. \(IC_{50}\) is the concentration of an inhibitor where the response (or binding) is reduced by half; therefore, lower \(IC_{50}\) value indicates higher inhibitory activity. The \(IC_{50}\) value can be calculated from the following equation (Epand, Shulga, Timmons, Perri, Belani, Perinpanathan, et al., 2007):

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

(2.1)
where, \([I]\) is the inhibitor concentration, \(I\) is the percentage of inhibition determined at a concentration of \([I]\) and \(I_{\text{max}}\) is the maximum percentage inhibition.

The Lineweaver-Burk plot, a double-reciprocal plot of the Michaelis-Menten equation is applied in inhibition analysis to identify the inhibition type, maximum initial reaction velocity \((V_{\text{max}})\) and Michaelis constant \((K_m)\). The parameters can be calculated by the equation as follows (Lineweaver & Burk, 1934):

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m \frac{1}{v_{\text{max}}}}{a}
\]  

(2.2)

where, \(v\) is the initial reaction velocity determined and \(a\) is the concentration of substrate.

Equation (2.2) indicates that a plot of \(1/v\) against \(1/a\) is linear, therefore, \(K_m\) can be calculated from the slope and \(V_{\text{max}}\) can be obtained from the intercept. For the competitive inhibitor, \(V_{\text{max}}\) remains stable and \(K_m\) increases, while for the uncompetitive inhibitor, \(V_{\text{max}}\) decreases and \(K_m\) remains stable. These theoretical deductions were demonstrated by the inhibition of \(\alpha\)-amylase by some polyphenols in competitive or uncompetitive manners, even though the experimental and calculating errors were observed, which affected the full fitting of the Lineweaver-Burk plot (Fei, et al., 2014).

2.3 Mechanisms of interactions between polyphenols and \(\alpha\)-amylase

2.3.1 Kinetics of inhibition

Although the Lineweaver-Burk equation can give the \(V_{\text{max}}\) and \(K_m\), there is a relatively large error in this plot when the substrate concentration is low. Also, the Lineweaver-Burk plot cannot distinguish between uncompetitive, non-competitive and mixed-type inhibition (Cornish-Bowden & Eisenthal, 1974). Therefore, additional inhibition analysis methods may be used to assist in analysing the kinetics of inhibition. The use of a Dixon plot, in which the reciprocal of initial reaction velocity \((1/v)\) is plotted against inhibitor concentrations \((i)\) at various substrate concentrations \((a)\), along with a Cornish-Bowden plot, plotting \(a/v\) against \(i\) at several values of \(a\), are useful in the case that the interaction between inhibitor and enzyme is more complex than competitive or uncompetitive inhibition mechanisms (Cornish-Bowden & Eisenthal, 1974). In addition, the inhibition type, competitive \((K_{ic})\) and uncompetitive inhibition constant \((K_{iu})\) can be obtained by use of these plots. As defined, \(K_{ic}\) suggests the
dissociation of an inhibitor-enzyme complex; therefore $1/K_{ic}$ demonstrates the association of an inhibitor with enzyme. Similarly, $1/K_{iu}$ describes the binding of an inhibitor-enzyme-substrate ternary complex (uncompetitive inhibition). To calculate the competitive inhibition constant, $K_{ic}$ and uncompetitive inhibition constant, $K_{iu}$, Dixon (2.3) and Cornish-Bowden (2.4) equations can be applied as follows (Cornish-Bowden & Eisenthal, 1974):

\[ v = \frac{V_{max}a}{K_m(1+\frac{i}{K_{ic}})+a} \]  
\[ \frac{v}{a} = \frac{V_{max}}{K_m(1+\frac{i}{K_{ic}})+a(1+\frac{i}{K_{iu}})} \]  
\[ (2.3) \]
\[ (2.4) \]

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.

By use of Eq. (2.3) and Eq. (2.4), the kinetics of inhibition of tea polyphenols can be determined. $K_{ic}$, can be given by the following equilibrium equation:

\[ K_{ic} = \frac{[E][I]}{[EI]} \]  
\[ (2.5) \]

Where, $[EI]$, $[E]$ and $[I]$ are the concentrations of enzyme-inhibitor complex, enzyme and inhibitor, respectively. From this formula, a lower $K_{ic}$ value means a shift of equilibrium position in favour of enzyme-inhibitor complex, or in other words, it means the inhibitor binds more tightly with the enzyme. This methodology has not, however been used to characterize polyphenol inhibition of $\alpha$-amylase.

**2.3.2 Fluorescence quenching**

Fluorescence quenching has been used to clarify if and how phenolic compounds interact with proteins at a molecular level (Soares, Mateus, & de Freitas, 2007; Wu, He, Yao, Zhang, Liu, Wang, et al., 2013). As there are fluorophores in amylase, like tryptophan and tyrosine, $\alpha$-amylase can emit fluorescence at certain excitation wavelengths, and the fluorescence intensity is directly related with the amount of active enzyme in solution (Soares, Mateus, & de Freitas, 2007). Polyphenols can interact with the tryptophan or its vicinity at the active sites of $\alpha$-amylase, decreasing the fluorescence properties of the fluorophores; therefore, the
fluorescence of α-amylase would be quenched. From the Stern-Volmer equation \( \frac{F_0}{F} = 1 + k_q \tau_0 [Q] \) Eq. (2.6) or its modified form \( \frac{F_0}{F} = e^{(K_{FQ}[Q])} \) Eq. (2.7) (Soares, Mateus, & Freitas, 2007), the fluorescence quenching constant \( K_{FQ} \) can be calculated. As \( K_{FQ} \) can reflect the interaction (binding) of polyphenols with α-amylase directly, higher \( K_{FQ} \) value corresponds to higher binding properties of polyphenols. 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 (Soares, Mateus, & Freitas, 2007). 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’, \textit{i.e.}, apparent static quenching. 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 \( 1 \times 10^{10} \) M\(^{-1}\)s\(^{-1}\) for the typical dynamic mechanism (collision-controlled quenching) (Soares, Mateus, & de Freitas, 2007). Therefore, in recent studies, EGCG was shown to quench the fluorescence of α-amylase in a dynamic mechanism because its Stern-Volmer plot was linear and its \( k_q \) was determined to be lower than \( 1 \times 10^{10} \) M\(^{-1}\)s\(^{-1}\) (Fei, et al., 2014; Miao, Jiang, Jiang, Zhang, & Li, 2015), while sorghum procyanidins was shown to apparently statically quench the α-amylase fluorescence with an upward Stern-Volmer plot and a \( k_q \) that was much higher than \( 1 \times 10^{10} \) M\(^{-1}\)s\(^{-1}\) (Cai, Yu, Xu, Liu, & Yang, 2015).

It is worth noting that in some fluorescence spectra of enzyme in the presence of polyphenols, a red-shift of maximum emission wavelength (\( \lambda_{em} \)) is observed (Fei, et al., 2014; Sun, Chen, Meng, Yang, Yuan, & Guo, 2016; Wu, et al., 2013). This indicates that partial structural unfolding may occur for α-amylase upon binding with these polyphenols (Soares,
The main mechanism of polyphenol-enzyme interaction is considered to be noncovalent, including hydrogen bonding and hydrophobic forces (He, Shi, & Yao, 2006; Xiao, Kai, Ni, Yang, & Chen, 2011). The interaction between polyphenols and \( \alpha \)-amylase was supposed to make the residues of Trp and Tyr within the aromatic heterocyclic and hydrophobic groups exposed, causing the microenvironmental changes in the aromatic amino acid residues in the spatial structure and conformational changes in the protein (Fei, et al., 2014). In addition, the polyphenol-\( \alpha \)-amylase interaction was found to make the structure of the enzyme more flexible (Cai, Yu, Xu, Liu, & Yang, 2015). The potential structural unfolding of \( \alpha \)-amylase upon binding with polyphenols indicated by the red-shift of \( \lambda_{em} \) may be supported by detecting techniques which can reflect the supramolecular structure of a protein, such as differential scanning calorimetry (DSC), circular dichroism (CD), etc.

As discussed above, the competitive inhibition constant, \( K_{ic} \), represents the dissociation constant of the \( \alpha \)-amylase-polyphenol complex; therefore, the reciprocal of \( K_{ic} \) (1/\( K_{ic} \)) indicates the association constant of polyphenols with the enzyme. Quenching constant, \( K_{FQ} \), also 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/\( K_{ic} \), which may be of interest to be established in future work.

### 2.3.3 Differential scanning calorimetry (DSC)

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 (Tang, Covington, & Hancock, 2003). It offers an objective and comprehensive way of evaluating the thermal stability of proteins (Barrett, et al., 2013). It has been reported that polyphenol interactions may change the thermostability of proteins (Prigent, Gruppen, Visser, van Koningsveld, de Jong, & Voragen, 2003; Raghavendra, Kumar, & Prakash, 2007). Therefore, DSC can be used to study the effect of tea polyphenols on the thermostability of \( \alpha \)-amylase. In DSC analysis, the parameters indicating thermostability of the protein include denaturation temperature, \( T_d \) and denaturation enthalpy, \( \Delta H \ i.e. \) means the energy required to denature a protein. It has been reported that phenolic acids, such as chlorogenic acid and caffeic acid could decrease the thermostability of lipase indicated by a decreased \( T_d \) (Rawel, Rohn, Kruse, & Kroll, 2002). Some earlier studies also
indicated that phenolic compounds decreased thermal stability by binding with proteins (Muralidhara & Prakash, 1995; Prigent, Gruppen, Visser, van Koningsveld, de Jong, & Voragen, 2003; Rawel, Rohn, Kruse, & Kroll, 2002). Decrease in thermal stability of an enzyme is usually associated with protein conformational changes. 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 (Lumry & Eyring, 1954; Violet & Meunier, 1989). The other one is irreversible, leading to the denaturation of the initially-unfolded molecule (Cueto, Dorta, Munguia, & Llabres, 2003). Therefore, the reversible unfolding process of an enzyme under external force is expected to promote the denaturation process of the enzyme during a DSC experiment. As some polyphenols are supposed to partially unfold α-amylase (indicated by the red-shifted λ<sub>em</sub> as described above (Fei, et al., 2014; Sun, Chen, Meng, Yang, Yuan, & Guo, 2016; Wu, et al., 2013)), DSC may be applied to support this. On the other hand, Barrett, et al. (2013) reported that grape tannins could increase the thermal stability of α-amylase, evidenced by a higher <i>T_d</i> and ∆<i>H</i>. They attributed this to the protein aggregation upon interaction between α-amylase and grape tannins that increases the stability of folded protein. If so, a blue-shifted λ<sub>em</sub> in fluorescence quenching spectra may be observed. However, the grape tannins had no effects on the thermostability of α-glucosidase, the enzyme which hydrolyses α-amylase reaction products to glucose. Therefore, the effect of phenolic compounds on thermal properties of enzymes may be dependent on both the enzyme and polyphenol types.

2.3.4 Isothermal titration calorimetry (ITC)

Thermodynamic surveys of complexation may be performed using isothermal titration microcalorimetry, a technique which permits the determination of binding enthalpy and binding constant of the reaction between a macromolecule and a ligand (Jelesarov & Bosshard, 1999). This technique has been successfully applied to characterize the binding interactions between polyphenols and proteins (Frazier, Papadopoulou, & Green, 2006; Jelesarov & Bosshard, 1999; Karonen, Oraviita, Mueller-Harvey, Salminen, & Green, 2015; Wu, et al., 2013). Through ITC analysis, the binding constant (<i>K_{itc}</i>) can be obtained; higher <i>K_{itc}</i> indicates higher binding affinity of polyphenol with protein. Usually, the binding of
polyphenol with protein is an exothermic process, as hydrogen bonding and hydrophobic interactions occur (Poncet-Legrand, Gautier, Cheynier, & Imberty, 2007). During the ITC experiment, a plot of heat flow (µcal/s) against time (min) can be obtained. To calculate the binding energy released by the binding interactions, $\Delta H_{\text{itc}}$, a plot of observed enthalpy change per mole of injectant ($\Delta H_{\text{itc}}$, J/mol) against molar ratio of polyphenol to enzyme is obtained by peak-by-peak integration of a plot of heat flow against time. Here, higher $\Delta H_{\text{itc}}$ corresponds to higher binding interactions (Karonen, Oraviita, Mueller-Harvey, Salminen, & Green, 2015). One feature that should be noted in ITC experiments is that heat flow may be observed for the titration of some polyphenols (especially polymers) into the blank (buffer) solution. This is caused by the heat of dilution, which affects the detected heat resulting from the binding of polyphenol with enzyme. Therefore, the dilution heat should be subtracted from the total observed heat obtained by the titration of polyphenol solution into enzyme solution. Two binding models are usually used to fit ITC data, including one-site binding model by which specific binding is expected and two-site binding model by which both specific and non-specific binding occur (Karonen, Oraviita, Mueller-Harvey, Salminen, & Green, 2015). As the reversible inhibition of an enzyme by polyphenol mainly involves the binding of polyphenol with the active site of the enzyme (Kromann-Hansen, Oldenburg, Yung, Ghassabeh, Muyltermans, Declerck, et al., 2016), the interactions between polyphenol and enzyme more likely involves specific binding. Therefore, to simplify the analysis, a one-site binding model is often used to analyse the interactions between them (Le Bourvellec & Renard, 2012; Wu, et al., 2013). Besides, the stoichiometry ($n$) indicating the molar ratio of polyphenol to protein can be obtained as well. $n$ can be also considered to be the numbers of molecules required to reach binding saturation with a protein. Therefore, $n$ is related with the binding affinity of polyphenol to the protein, and thus higher $K_{\text{itc}}$ corresponding to lower $n$ value have been observed in previous studies (Karonen, Oraviita, Mueller-Harvey, Salminen, & Green, 2015). To our knowledge, the ITC has not been used previously to study the interactions between polyphenols and $\alpha$-amylase.

Interestingly, as $1/K_{\text{itc}}$, $K_{\text{FQ}}$ and $K_{\text{itc}}$ indicate the binding of polyphenols with $\alpha$-amylase, it is possible and preferable to analyse the correlation among the three constants, in order to
evaluate the accuracy of each method and to rationalize the combination of inhibition kinetics, fluorescence quenching, and isothermal titration calorimetry.

2.3.5 Molecular docking

Molecular docking is a useful tool in structural molecular biology and computer-assisted drug design. The aim of ligand-protein docking is to predict the main binding modes of a ligand with a protein of known three-dimensional structure (Morris & Lim-Wilby, 2008). Molecular docking has been applied to investigate the binding interactions between polyphenols and enzymes (Wu, et al., 2013). Through the docking study, hydrogen bonding between the hydroxyl groups of polyphenols and the amino acids residues (Asp\textsuperscript{197}, Glu\textsuperscript{233} and Asp\textsuperscript{300}) at the active sites of α-amylase was suggested, as well as hydrophobic interactions between the aromatic groups of polyphenols (benzene rings) and the enzyme (Trp\textsuperscript{59}) (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008; Miao, Jiang, Jiang, Zhang, & Li, 2015). The total binding energy (kJ/mol) of polyphenol with α-amylase can be obtained from the docking study as well. It was found that the order of the binding energy for four theaflavin compounds was opposite to that of IC\textsubscript{50}, indicating that more binding of theaflavins with α-amylase corresponded to higher inhibitory activity (Miao, Jiang, Jiang, Li, Cui, & Jin, 2013). In addition, as the binding energy can also reflect the binding of polyphenols with the enzyme, it may be combined with 1/K\textsubscript{ic}, K\textsubscript{FQ} and K\textsubscript{itc} to more completely characterize the binding interactions.

2.4 Effects of polysaccharides on α-amylase inhibition by polyphenols

2.4.1 Potential influence of polysaccharides on the interactions between polyphenols and α-amylase

Many plant extracts have been studied for their inhibitory activity against α-amylase, like green coffee extracts (Narita & Inouye, 2011), tea extracts (Fei, et al., 2014), pomegranate extracts (Kam, Li, Razmovski-Naumovski, Nammi, Shi, Chan, et al., 2013), etc. and the main components that had the inhibiting effects were shown to be phenolic compounds. However, aqueous extracts from plants are likely to be a complex mixture containing not only polyphenols but also other components that they may bind to such as soluble polysaccharides (Chamorro, Viveros, Alvarez, Vega, & Brenes, 2012). On the other hand, even though pure
phenolics or phenolic extracts containing very high contents of polyphenols are consumed, polyphenols, in practice, may interact with digestive juices, tissues and other food components (like proteins, polysaccharides, etc.) in the digestive tract (Le Bourvellec, Guyota, & Renard, 2009). However, it is not yet known whether the interaction of polyphenols with other food components would affect the inhibitory activity of polyphenols in vitro.

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, Gonçalves, Fernandes, Mateus, & de Freitas, 2009).

2.4.2 Characterization of the effects of polysaccharides on α-amylase inhibition by polyphenols

As inhibitors, polyphenols are able to develop inhibitory activity against α-amylase through binding with the protein. The presence of polysaccharides in the reaction solution may be a factor affecting the interactions between polyphenols and the protein because of the potential binding interactions between polysaccharides and polyphenols.

The competitive or uncompetitive inhibition of an enzyme is attributed to the reversible (soluble) binding of an inhibitor with the enzyme or enzyme-substrate complex, rather than irreversible (insoluble) aggregation. The initial enzymic reaction velocity, IC$_{50}$ values, kinetics of inhibition and fluorescence quenching are usually applied to characterize the inhibition of enzyme. These methods may also be applied to characterize the influence of soluble polysaccharides on the α-amylase inhibition by polyphenols. Besides, as $1/K_{ic}$ and $K_{FQ}$ reflect the binding of polyphenols with α-amylase, the two values in the presence of
polysaccharides may also demonstrate the influence of polysaccharides on the binding of polyphenols with α-amylase. The binding interactions between polyphenols and soluble polysaccharides have been studied using ITC (Watrelot, Le Bourvellec, Imberty, & Renard, 2013, 2014), from which the binding constant ($K_{\text{itc}}$) between the two compounds can be obtained (Renard, Watrelot, & Le Bourvellec, 2017). The presence of a $K_{\text{itc}}$ for polyphenol binding with proteins (Karonen, Oraviita, Mueller-Harvey, Salminen, & Green, 2015) as well as a $K_{\text{itc}}$’ for polyphenol binding with polysaccharides (Renard, Watrelot, & Le Bourvellec, 2017) indicates that there may be a competitive mechanism between polysaccharides and α-amylase in terms of binding with polyphenols. Furthermore, by comparing the two $K_{\text{itc}}$ values, it should be possible to determine how polysaccharides affect the inhibition of α-amylase by polyphenols.

2.5 Combined effects of polyphenols with acarbose against α-amylase

2.5.1 Synergistic effects

Acarbose is a pseudotetrasaccharide that competitively inhibits α-amylase and glucosidase in the intestinal tract (Kawamura-Konishi, et al., 2012). Taking acarbose is an effective pharmaceutical treatment for controlling postprandial glycaemic level in diabetes patients, but its continued use may cause undesirable gastrointestinal discomfort, such as diarrhea and stomachache (Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012). Therefore, natural phenolic products which can inhibit digestive enzymes with few side effects are attractive alternatives to acarbose or could be co-applied to reduce the dose of acarbose required.

It has been reported that co-application of rowanberry proanthocyanidins and acarbose could reduce the concentration of acarbose required for effective α-amylase inhibition. When rowanberry proanthocyanidins and acarbose were mixed at the concentrations of their individual $IC_{50}$ levels against α-amylase, the co-inhibition effect was much higher than the two individually. Also, it was suggested that the two inhibitors bound to α-amylase at different sites, which prevented binding competition and potentiated overall inhibition (Grussu, Stewart, & McDougall, 2011). Cyanidin-3-glucoside at low concentration was also found to promote the α-amylase inhibition in vitro by acarbose (Akkarachiyasit, Charoenlertkul, Yibchok-anun, & Adisakwattana, 2010) as its binding to α-amylase may alter
the ternary (polyphenol-\(\alpha\)-amylase-acarbose) structure (Wiese, Gärtner, Rawel, Winterhalter, & Kulling, 2009). In addition, cinnamon extracts also produced additive inhibition against pancreatic \(\alpha\)-amylase when combined with acarbose (Akkarachiyasit, Charoenlertkul, Yibchok-anun, & Adisakwattana, 2010). In addition, a synergistic inhibitory effect of polyphenols from berry extracts with acarbose on \(\alpha\)-glucosidase, another important enzyme related to starch digestion and increase in postprandial blood sugar, was found as well (Boath, Stewart, & McDougall, 2012).

2.5.2 Antagonistic effects

In contrast, Gao et al. (2013) found that the combination of green tea extracts, green tea polyphenols or epigallocatechin gallate (EGCG) with acarbose had an antagonistic effect on \(\alpha\)-amylase and \(\alpha\)-glucosidase inhibition at high concentrations. This could arise from competitive binding to certain \(\alpha\)-amylase sites between phenolic compounds and acarbose, or it may be due to polyphenols binding to one of the \(\alpha\)-amylase sites, which affected the affinity of the active sites for acarbose (Boath, Stewart, & McDougall, 2012; Gao, Xu, Wang, Wang, & Hochstetter, 2013). Acarbose is widely prescribed for type II diabetes patients as an inhibitor of \(\alpha\)-amylase and \(\alpha\)-glucosidase. This medicine is usually recommended to be taken with meals. As food is a complicated system, in which one of the components is polyphenols, it is necessary to study the effects of phenolic components on the inhibitory activity of acarbose to promote the effect of acarbose (thus, the dose may be decreased) and to avoid the antagonism effect on the inhibition of the enzyme.

2.6 Functional foods resources for inhibiting \(\alpha\)-amylase

2.6.1 Tea

Tea is one of the most popular drinks in the world, and is usually classified into three main types, \textit{i.e.,} green tea (non-fermented), black tea (fully-fermented) and oolong tea (semi-fermented), of which the consumption ratios across the countries are around 20\%, 78\%, and 2\%, respectively (Grove & Lambert, 2010; Y. Wang & Ho, 2009). Aqueous tea extracts have been shown to have inhibitory activity against \(\alpha\)-amylase, and the active components which most contribute to the inhibition by tea extracts are polyphenols and tannins (Barrett, et al., 2013). The main phenolic components in tea are catechins (in both non-fermented and
fermented teas) and theaflavins (only in fermented teas). It was found that theaflavins had a higher inhibitory activity than catechins, and only catechins with a galloyl substituent at the 3-position exhibited measurable inhibition of α-amylase (Hara & Honda, 1990). A 3 and/or 3'-galloyl moiety in catechin and related structures was consistently found to increase α-amylase inhibition presumably through enhanced association with the active site of the enzyme (Miao, Jiang, Jiang, Li, Cui, & Jin, 2013; Miao, Jiang, Jiang, Zhang, & Li, 2015). Besides, both green and black tea extracts were shown to reduce the hydrolysis kinetics of wheat, potato, corn and rice starches to different extents in vitro, suggesting that the starch hydrolysis was affected by interactions with tea polyphenols and by the impact on specific enzymes based on starch structure (Guzar, Ragaee, & Seetharaman, 2012). In reality, the inhibition of α-amylase by tea extracts is the synergistic result of all the polyphenols in tea that have been reported to be effective individually (McDougall, Shpiro, Dobson, Smith, Blake, & Stewart, 2005). Additionally, tea extracts are complex and may also contain other components besides polyphenols, like polysaccharides, depending on the extraction process (Lv, Yang, Zhao, Ruan, Yang, & Wang, 2009). It has been reported that soluble polysaccharides are able to disturb the binding of polyphenols with protein (de Freitas, Carvalho, & Mateus, 2003). Therefore, the binding interactions of tea extracts with α-amylase may come from not only the synergistic effects of all the tea polyphenols, but also the effects of the polysaccharides in tea extracts.

In addition to the inhibition of starch digestion in vitro, tea extracts or tea polyphenols also showed the inhibitory activity in vivo by use of mice models fed with starch or starchy foods (Abeywickrama, Ratnasooriya, & Amarakoon, 2011; Du, Peng, Liu, Shi, Tan, & Zou, 2012; Miyata, Tamaru, Tanaka, Tamaya, Matsui, Nagata, et al., 2013). The inhibitory activity of tea polyphenols against starch digestion in vivo is supposed to be caused by the inhibition of both starch-hydrolysing enzymes (α-amylase and α-glucosidase) and glucose transporters (GLUT2 and SGLT1) in the small intestine (Forester, Gu, & Lambert, 2012; C. Schulze, A. Bangert, G. Kottra, K. E. Geillinger, B. Schwanck, H. Vollert, et al., 2014). In addition, it is important to estimate the amount of tea polyphenols that can reach the small intestine and that can potentially produce inhibitory activity. Both theaflavins and green tea catechins have been shown to be stable in the acidic environment of the stomach, whereas they are less stable in
the mildly alkaline conditions of the small intestine (Chen, Zhu, Tsang, & Huang, 2001; Su, Leung, Huang, & Chen, 2003). Previous studies using samples from human volunteers who consumed green tea extracts showed that after ingestion of 200 mg GTE, ~40% polyphenols could be recovered in ileal fluid (Auger, Mullen, Hara, & Crozier, 2008; Schantz, Erk, & Richling, 2010). From an estimate based on typical compositions, there is about 0.75 mg/mL GTE in one cup of green tea (one tea bag in 200 mL hot water). Therefore, after ingestion of one cup of green tea, more than 0.3 mg/mL of effective GTE may reach the small intestine, which would approach the IC$_{50}$ values for $\alpha$-amylase inhibition (Miao, Jiang, Jiang, Zhang, & Li, 2015). Even so, in practice, the interactions of tea extracts with complex digestive juices, tissues and food components (like protein, plant fibre, etc.) should be taken into account as well.

2.6.2 Fruits

Grussu, et al. (2011) demonstrated that both raspberry and rowanberry were two polyphenol-rich berries which effectively inhibited $\alpha$-amylase in vitro with their IC$_{50}$ values being 21.0 and 4.5 $\mu$g/mL, respectively. Further investigation demonstrated that it was proanthocyanidins in the two berries that showed strong inhibition of $\alpha$-amylase, rather than anthocyanins or ellagitannins. A comparative study of the $\alpha$-amylase inhibition by five kinds of soft fruit extract indicated that extracts from strawberry and raspberry were more effective than blueberry, blackcurrant and red cabbage due to the relatively higher content of soluble tannins in strawberry and raspberry (McDougall, Shpiro, Dobson, Smith, Blake, & Stewart, 2005). Interestingly, the inhibition of strawberry extract was greatly decreased after removing tannins from the extracts by resin column chromatography. Besides, pomegranate has been found to be beneficial in controlling glycaemic levels as tannins in this fruit had strong inhibition of $\alpha$-amylase, with increasing inhibition of the enzyme with increasing concentration of the tannins (Barrett, et al., 2013). Therefore, tannins play an important role in the $\alpha$-amylase inhibition by fruit extracts. Furthermore, the tannin moiety size, structure distribution and category vary widely with fruit sources (Tessmer, Besada, Hernando, Appezzato-da-Glória, Quiles, & Salvador, 2016) and are expected to affect the possibility and intensity of amylase-tannin interactions (Amoako & Awika, 2016). In addition to tannins, some phenolic acids in fruits, like ellagitannin acid, gallic acid and chlorogenic acid have also
been reported to have inhibitory activities against α-amylase (Das, Dutta, Chaudhury, & De, 2016; Mohammeda, Gbonjubola, Koorbanally, & Islam, 2017), as well as some flavonoids (Liu, Luo, Li, She, & Gao, 2017) and procyanidins (Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012). The different polyphenolic components in fruits may affect different steps in starch digestion in a synergistic manner.

Although tannin-rich extracts from raspberry and strawberry were effective against α-amylase, ellagitannins may not be stable in the neutral to mildly alkaline environment in the small intestine (Daniel, Ratnayake, Kinstle, & Stoner, 1991). In addition, fruit polyphenols could bind with food proteins or digestive enzymes in the small intestine (Lorenz, Alkhafadj, Stringano, Nilsson, Mueller-Harvey, & Uden, 2014), which would reduce the effective concentration available for interacting with α-amylase; therefore, the bioavailability of dietary fruit polyphenols should also be studied in the environment of the gastrointestinal tract. Besides, the inhibition of α-amylase by fruit polyphenols in vivo and the controlling effect of polyphenols on postprandial glucose increase in experimental animals (or human beings) should be further investigated as well.

2.6.3 Juices

It has been reported that intake of cranberry juice was associated with a favourable glycaemic response, which benefited from the abundant trimer and heptamer proanthocyanidins in the juice (Wilson, et al., 2008). The α-amylase inhibition by some fruit and vegetable juices has been studied for systems in which the active components that had the inhibitory activity included flavonoids (Tundis, Bonesi, Sicari, Pellicanò, Tenuta, Leporini, et al., 2016), phenolic acids (Alakolanga, Kumar, Jayasinghe, & Fujimoto, 2015), anthocyanidin (Mojica, Berhow, & Gonzalez de Mejia, 2017), procyanidins (Ho, Kase, Wangensteen, & Barsett, 2017) and tannins (Bordenave, Hamaker, & Ferruzzi, 2014). The inhibitory effect of fresh apple fruit juice (diluted 10 times with distilled water) against α-amylase was also tested (Lanzerstorfer, Wruss, Huemer, Steininger, Muller, Himmelsbach, et al., 2014) and it was shown that the inhibition demonstrated an incubation-time dependency. Interestingly, the observed inhibitory effect was not dependent on the total phenolic content levels of the apple juice varieties, which is not in agreement with a positive correlation between α-amylase inhibitory activity and total phenolic content (Barbosa, Pinto,
This may result from the variances of some single phenolic compounds which have inhibitory effect on α-amylase, such as chlorogenic acid (Narita & Inouye, 2011), caffeic acid (Narita & Inouye, 2011), and flavan-3-ols (Gamberucci, Konta, Colucci, Giunti, Magyar, Mandl, et al., 2006). Hence, identifying the significance of individual phenolic compound accounting for the inhibitory effect of juice on α-amylase is necessary. To achieve this, some data analysis methods could be applied, like principal components analysis, correlation analysis and cluster analysis. It should be noted that although some fruit juices showed the inhibitory activities against α-amylase because of the presence of phenolic components, the high sugar content should be taken into account in order to avoid a high blood sugar response after intake (Imamura, O’Connor, Ye, Mursu, Hayashino, Bhupathiraju, et al., 2015).

2.7 Summary of review

The interaction of polyphenols with α-amylase is a highly investigated field of research in recent years because of their potential application in controlling postprandial blood glucose level and health benefits. Based on the above discussion, the inhibition of α-amylase by polyphenols is likely caused by the binding interactions between them, which include both hydrogen bonding formed by the hydroxyl groups and the amino acid residues at the active sites of the enzyme and hydrophobic forces formed by the aromatic groups of polyphenols and the enzymes (π-π interactions). The mechanisms of interactions between polyphenols and α-amylase can be investigated through inhibition kinetics, fluorescence quenching, differential scanning calorimetry, isothermal titration calorimetry and molecular docking methods, from which inhibition constants ($K_{ic}$ and $K_{iu}$), fluorescence quenching constant ($K_{FQ}$), denaturation temperature and enthalpy ($T_d$ and $\Delta H$), binding constant ($K_{itc}$) and binding energy can be obtained. The establishment of correlations among these parameters to understand how polyphenols interact with the enzyme has not been investigated to a significant extent. Besides, as polyphenols can interact with both polysaccharides and α-amylase, the potential effects of polysaccharides on the inhibition of α-amylase by polyphenols should be further investigated. Some polyphenols-rich foods, such as teas and berries, may be recommended as healthy dietary resources due to their potential inhibitory
activity against α-amylase. The bioavailability and the inhibition of α-amylase by polyphenols in vivo, as well as the control of postprandial blood sugar increase in vivo should be further studied.
Chapter 3

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

ABSTRACT: The inhibitory activities of three tea extracts (TEs) and individual phenolic compounds in TEs against porcine pancreatic α-amylase (PPA) were studied by measuring their half inhibitory (IC\textsubscript{50}) concentrations. The kinetics of inhibition by these extracts and compounds were investigated through Dixon, Cornish-Bowden, and Lineweaver-Burk plots. The results showed that green, oolong and black tea extracts, epigallocatechin gallate, theaflavin-3, 3’-digallate and tannic acid were competitive inhibitors of PPA, whereas epicatechin gallate, theaflavin-3’-gallate and theaflavin were mixed-type inhibitors with both competitive and uncompetitive inhibitory characteristics. Only catechins with a galloyl substituent at the 3-position showed measurable inhibition. The competitive inhibition constants (\(K_{ic}\)) were lower for theaflavins than catechins, with the lowest value for theaflavin-3, 3’-digallate. The lower \(K_{ic}\) than the uncompetitive inhibition constant for the mixed-type inhibitors suggests that they bind more tightly with free PPA than with the PPA-starch complex. A 3 and/or 3’-galloyl moiety in catechin and theaflavin structures was consistently found to increase inhibition of PPA through enhanced association with the enzyme active site.

Keywords: tea polyphenols; α-amylase; inhibition; kinetics; galloyl moiety
3.1. Introduction

Type II diabetes is defined as a chronic disease due to the reduced insulin sensitivity, which may lead to multiple complications (Colhoun, Betteridge, Durrington, Hitman, Neil, Livingstone, et al., 2004). The control of postprandial hyperglycaemia is advisable in the prevention and treatment of this disease (Kawamura-Konishi, et al., 2012). Retarding sugar absorption through delaying the digestion of starchy foods by inhibiting starch-hydrolyzing enzymes, for example α-amylase and α-glucosidase in the digestive tract, has potential as a management and/or therapeutic approach (Bischoff, 1994; Kawamura-Konishi, et al., 2012). It has been reported that some plant extracts can perform bioactivity potentially beneficial to human health by inhibiting the activities of carbohydrate hydrolyzing enzymes, and the main active constituents in these plant extracts are phenolic compounds such as flavonoids that have several hydroxyl groups (Barrett, et al., 2013; Gamberucci, et al., 2006; Kamiyama, Sanae, Ikeda, Higashi, Minami, Asano, et al., 2010). The inhibitory activities of flavonoids have been reported to depend on not only the substitution of hydroxyl groups that can form hydrogen bonds with the catalytic residues of enzyme, but also the unsaturated C-ring with keto-group that is speculated to be responsible for the formation of a conjugated π-system that is proposed to stabilize the interactions with the active site of enzyme (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008). Similarly, the introduction of hydroxyl groups and a conjugated system by combining caffeic acid with quinic acid also alters the inhibitory activities of chlorogenic acids (Narita & Inouye, 2009, 2011). Therefore, the introduction of certain groups to phenolic molecules may be important in determining inhibitory activity.

Commonly, Lineweaver-Burk equation, a double-reciprocal plot of Michaelis-Menten equation is applied in inhibition analysis to identify the inhibition type, maximum initial reaction velocity ($V_{max}$) and Michaelis constant ($K_m$). However, there is a relatively large error in this plot when the substrate concentration is low. Also, the Lineweaver-Burk plot cannot distinguish between uncompetitive, non-competitive and mixed-type inhibition (Cornish-Bowden & Eisenthal, 1974). Therefore, additional inhibition analysis methods may be used to assist in analysing the kinetics of inhibition. The use of a Dixon plot, in which the reciprocal of initial reaction velocity ($1/v$) is plotted against inhibitor concentrations ($i$) at various substrate concentrations ($a$), along with a Cornish-Bowden plot, plotting $ad/v$ against $i$
at several values of $a$, are useful in the case that the interaction between inhibitor and enzyme is more complex than competitive or uncompetitive inhibition mechanisms (Cornish-Bowden & Eisenthal, 1974). In addition, the inhibition type, competitive ($K_{ic}$) and uncompetitive inhibition constant ($K_{iu}$) can be obtained by use of these plots. It has been reported that the mechanism of inhibition of $\alpha$-amylase by polyphenols lies in the binding of polyphenols to the enzyme (Miao, Jiang, Jiang, Li, Cui, & Jin, 2013). As defined, $K_{ic}$ suggests the dissociation of an inhibitor-enzyme complex, therefore $1/K_{ic}$ demonstrates the association of an inhibitor with enzyme. Similarly, $1/K_{iu}$ describes the binding of an inhibitor-enzyme-substrate ternary complex.

Extracts from tea leaves have been shown to strongly inhibit $\alpha$-amylase activity. Phenolic compounds in tea extracts (molecular structures shown in Fig. 2.4) which are reported to have relatively high $\alpha$-amylase inhibition include theaflavin-3, 3’-digallate (TF2), theaflavin-3’-gallate (TF1), theaflavin (TF), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG), and their IC$\text{S}_{50}$ values have also been reported (Fei et al., 2014; Hara & Honda, 1990; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012). However, the detailed kinetics of inhibition which include the inhibition type, $K_{ic}$, $K_{iu}$, apparent maximum reaction velocity ($V_{\text{max, app}}$), and apparent Michaelis constant ($K_{m, app}$) have not been reported. In addition, the role of 3 or 3’-galloyl moieties on the structures of catechins and theaflavins in their interaction with $\alpha$-amylase, which can be obtained from both the inhibitory effects and the kinetics of inhibition, has yet to be elucidated.

In this study, the phenolic components in three TEs (green, oolong, and black) were characterised by UPLC and the inhibition of PPA by TEs and a range of pure catechins, theaflavins and tannic acid were determined. Dixon, Cornish-Bowden and Lineweaver-Burk plots were employed to investigate the kinetics of inhibition. The correlation between the presence and location of galloyl groups on tea polyphenols and the association of the polyphenols (catechins and theaflavins) with PPA assessed by inhibition constants is discussed.
3.2. Materials and methods

3.2.1 Materials and chemicals

Black tea and green tea leaves were obtained from Lipton® regular black and green tea bags. Oolong tea was purchased from a local market in Brisbane, Australia. Normal maize starch (moisture content of 11.2%) was purchased from Penford® Pty Ltd. (Ryde, Australia). Porcine pancreatic α-amylase with the activity of 23 unit/mg (EC 3.2.1.1 A3176) was obtained from Sigma-Aldrich Co. Ltd. (St. Louis, USA). Pure phenolic compounds including tannic acid (TA, C_{76}H_{52}O_{46}), (+)-catechin (C, C_{15}H_{14}O_{6}), (-)-epicatechin (EC, C_{15}H_{14}O_{6}), (-)-epigallocatechin (EGC, C_{15}H_{14}O_{7}), (-)-epigallocatechin gallate (EGCG, C_{22}H_{18}O_{11}), (-)-epicatechin gallate (ECG, C_{22}H_{18}O_{10}), theaflavin (TF, C_{29}H_{24}O_{12}), theaflavin-3’-gallate (TF1, C_{36}H_{28}O_{16}), and theaflavin-3, 3’-digallate (TF2, C_{43}H_{32}O_{20}) were obtained from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). EnzChek® Ultra-α-amylase Assay Kit (E33651) was obtained from Life Technologies Co. Ltd. (Grand Island, NY, USA). Phosphate buffered saline (P4417), p-hydroxybenzoic acid hydrazide (PAHBAH, CAS No.5351-23-5), gallic acid, Folin-Ciocalteu’s phenol reagent (2 M) and chromatographic grade acetonitrile were purchased from Sigma-Aldrich. Other chemicals in this study were of analytical grade.

3.2.2 Preparation of Tea Extracts

Three kinds of dry tea leaves (green, oolong, and black) were extracted with 1:15 (weight/volume) deionized water at 100 °C for 40 min. The extracts were then centrifuged at 3176 g for 15 min, and the resulting insoluble residues were extracted again as described above. The supernatants were combined, concentrated, and lyophilized before use.

3.2.3 Extracts Analysis

The contents of total polyphenols in the three TEs were determined based on a Folin-phenol reagent colorimetric method (Singleton, Orthofer, & Lamuela-Raventos, 1999). The individual contents of phenolic compounds in TEs were measured by use of a Waters® Acquity UPLC equipped with a PDA detector (Massachusetts, USA). A Vision HT C18 column (100×2 mm I.D. 1.5 µm) (Grace Discovery Sciences, Epping, Vic, Australia) was utilized with acetonitrile (solvent A) and deionized water with 0.1% acetic acid (solvent B) as mobile phases. The gradient elution was conducted as follows: solvent B 92-90% from 0 to 5
min: 90-60% from 5 to 8 min; 60-92% from 8-10 min. During the run, the flow rate was 0.4 mL/min, and the column temperature was kept at 30 °C. The injection volume of sample was 2 µL and the detection wavelength was 280 nm. The individual contents of phenolic compounds were calculated through their respective external calibration curves. Both the contents of total polyphenols and individual phenolic compounds were calculated as the mass percentage in lyophilized tea extracts.

3.2.4 α-Amylase Assay

The PPA inhibition assay was performed as follows: TEs and the pure phenolic compounds were dissolved in PBS buffer to a respective stock solution immediately before using in experiments. 25 µL of various dilutions of test compounds were pre-incubated in 96-well plates (Greiner bio-one, Germany) for 30 min at room temperature with 25 µL of 0.19 U/mL PPA. To start the reaction, 50 µL of 200 µg/mL ‘DQ starch’ (dissolved in PBS buffer) from the amylase Assay Kit was added to the respective plates. The initial enzymic reaction velocity, \( v \) in the absence and presence of polyphenols was determined at \( \lambda_{ex} \) of 460 nm and \( \lambda_{em} \) of 520 nm; taking readings at 6 min intervals over 36 mins, and expressed as the slope of the linear region of fluorescence against reaction time (Δfluorescence value/min). To obtain a standard PPA curve, values of \( v \) at various concentrations of PPA (0.765-3.825 U/mL) were obtained. The percentage inhibition \( I (%) \) can be calculated by the following equation:

\[
I = \left( 1 - \frac{C}{C_0} \right) \times 100
\]  

(3.1)

where, \( C \) and \( C_0 \) are the activity of PPA in the presence and absence of tea polyphenols, respectively. Both \( C \) and \( C_0 \) can be calculated from the standard PPA curve. The IC\(_{50}\) values for different tea extracts and polyphenols were calculated by use of the equation as follows (Epand et al., 2007):

\[
I = I_{max}(1 - \frac{IC_{50}}{[I]+IC_{50}})
\]

(3.2)

where, \([I]\) is the inhibitor concentration, and \( I_{max} \) is the maximum percentage inhibition, %.
3.2.5 The Kinetics of Inhibition of PPA by Tea Polyphenols

For the kinetics of PPA inhibition, normal maize starch (20mg/mL) was prepared in PBS buffer, and then cooked at 90 °C for 20 min before dilution to serial concentrations (1.25-15 mg/mL). Then, for each starch concentration, 50 µL of TEs and polyphenols with different concentrations (concentrations presented in Fig. 3.3 A-C and Fig. S3.1 ) were pre-incubated with 50 µL of 7.65 U/mL PPA solution at 4°C for 20 min, followed by the addition of 4 mL of cooked starch, and the process of digestion was carried out at 37 °C. At 0, 4, 8, and 12 min after the addition of starch, the reaction solution was thoroughly mixed, and 300 µL of the mixture was withdrawn into tubes containing 300 µL of 0.3 M Na₂CO₃ solution to stop the reaction (Slaughter, Ellis, & Butterworth, 2001; Tahir, Ellis, & Butterworth, 2010). The mixture in the tubes was then centrifuged at 12470 g for 6 min. After that, 100 µL of the supernatant was withdrawn into 1.5 mL centrifuge tubes. The reducing sugar content in the supernatant was determined using a previously reported method with some modifications (Lever, 1973): ninefold volume of 0.5 M NaOH solution was added to a PAHBAH solution prepared in 0.5 M HCl to give a 0.5% (w/v) final working PAHBAH reagent. 1 mL of PAHBAH reagent was added to duplicate samples (100 µL) taken at interval time points. After heating up for 5 min in boiling water, the absorbance value of each sample was measured at 410 nm by use of a UV-VIS spectrophotometer (Shimadzu®, Japan). 100 µL of maltose solutions (0.01-0.9 mM) were applied to generate a standard curve, and 100 µL of deionized water was used as the blank. The absorbance values obtained were converted to the concentration of reducing sugar (maltose equivalents) through the obtained maltose standard curve. After that, the initial reaction velocity (v) was determined by the slope of a plot of reducing sugar concentration in the reaction solution against time. To calculate \( K_{ic} \) and \( K_{iu} \), a Dixon equation was used to analyse the plot of initial reaction velocity (v) against inhibitor concentration (i). The equation for competitive inhibition is as follows (Dixon, 1953):

\[
v = \frac{V_{\max} \alpha}{K_m (1 + \frac{i}{K_{ic}}) + \alpha}
\]

and the equation for mixed-type inhibition is as follows:

\[
v = \frac{V_{\max} \alpha}{K_m (1 + \frac{i}{K_{ic}}) + \alpha (1 + \frac{i}{K_{iu}})}
\]
where, $V_{\text{max}}$ is the maximum initial reaction velocity, $a$ is the starch concentration, $K_m$ is the Michaelis constant, $i$ is the inhibitor concentration and $v$ is the initial reaction velocity.

Taking the reciprocals of both sides of the equations, the Dixon plot can be demonstrated as the linear plot of $1/v$ against $i$. By including two or more starch concentrations, it can be deduced that $K_{ic} = -i$ for the two equations above, meaning that $K_{ic}$ equals to the absolute value of the intersection abscissa of the Dixon plots with multiple starch concentrations.

Although the value of $K_{iu}$ is not obtained directly by the Dixon plot, a similar derivation shows that it can be achieved by plotting $a/v$ against $i$ at several $a$ values to establish an Eisenthal-Cornish-Bowden plot. The full Cornish-Bowden equation for the mixed-type inhibition is written as follows (Eisenthal & Cornish-Bowden, 1974):

$$\frac{v}{a} = \frac{V_{\text{max}}}{K_m \left(1 + \frac{i}{K_{ic}}\right) + a \left(1 + \frac{i}{K_{iu}}\right)}$$  \hspace{1cm} (3.5)

Taking the reciprocals of both sides of the equation, the Cornish-Bowden plot can be demonstrated as the linear plot of $a/v$ against $i$. By use of the same calculating method above, it can be deduced that $K_{iu} = -i$ for equation (3.5), meaning that $K_{iu}$ equals to the absolute value of the intersection abscissa of the Cornish-Bowden plots with multiple starch concentrations.

For calculating the apparent maximum reaction velocity ($V_{\text{max}}^{\text{app}}$) and the apparent Michaelis constant ($K_m^{\text{app}}$), a double-reciprocal version of the Michaelis-Menten equation can be applied as follows (Lineweaver & Burk, 1934):

$$\frac{1}{v} = \frac{1}{V_{\text{max}}^{\text{app}}} + \frac{K_m^{\text{app}}}{V_{\text{max}}^{\text{app}}} \frac{1}{a}$$  \hspace{1cm} (3.6)

Equation (3.6) indicates that a plot of $1/v$ against $1/a$ at a constant value of $i$ is linear, therefore, $K_m^{\text{app}}$ can be calculated from the slope and $V_{\text{max}}^{\text{app}}$ can be obtained from the intercept.

3.2.6 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 Statistics (Chicago, USA). The mean values were evaluated by Dunnett’s $t$ Test at the 95% significance level ($P<0.05$).
3.3. Results

The contents of total polyphenols in GTE, BTE and OTE investigated here were determined as 30.31%, 26.26% and 22.34% (mass percentage in lyophilized tea extracts), respectively (Table 3.1). Black tea is a fully-fermented product from green tea, while oolong tea is a half-fermented one. During the fermentation process, a large proportion of catechins are polymerized by polyphenol oxidase, forming theaflavins and other high molecular weight components like thearubigins (Del Rio, Stewart, Mullen, Burns, Lean, Brighenti, et al., 2004; Serpen, et al., 2012). This is in agreement with our data in Fig. 3.1 and Table 3.1 that the contents of catechins (C, EC, ECG, EGC and EGCG) decreased from GTE to OTE and BTE, whereas only BTE contained all three theaflavins (TF, TF1 and TF2). There are many kinds of hydrolyzable tannins with hydroxyl groups on a glucose skeleton esterified by galloyl groups (Deaville, Green, Mueller-Harvey, Willoughby, & Frazier, 2007; Hashimoto, Nonaka, & Nishioka, 1992; Nonaka, Sakai, & Nishioka, 1984). Here tannic acid was used as a well-defined hydrolyzable tannin to add to the study of the role of galloyl groups in inhibiting PPA.

Table 3.1. The contents of total and individual polyphenols in GTE, OTE and BTE

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>Contents (%)</th>
<th>GTE</th>
<th>OTE</th>
<th>BTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF</td>
<td>13.27a</td>
<td>13.97a</td>
<td>15.31b</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>2.39c</td>
<td>1.82b</td>
<td>1.17a</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.41a</td>
<td>1.41a</td>
<td>1.22a</td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>1.44c</td>
<td>1.17b</td>
<td>0.69a</td>
<td></td>
</tr>
<tr>
<td>ECG</td>
<td>14.81c</td>
<td>6.21b</td>
<td>2.96a</td>
<td></td>
</tr>
<tr>
<td>EGC</td>
<td>9.93c</td>
<td>5.38b</td>
<td>1.70a</td>
<td></td>
</tr>
<tr>
<td>EGCG</td>
<td>14.30c</td>
<td>7.59b</td>
<td>1.62a</td>
<td></td>
</tr>
<tr>
<td>TF</td>
<td>ND</td>
<td>1.12</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>TF1</td>
<td>ND</td>
<td>ND</td>
<td>1.97</td>
<td></td>
</tr>
<tr>
<td>TF2</td>
<td>ND</td>
<td>ND</td>
<td>7.51</td>
<td></td>
</tr>
<tr>
<td>Total polyphenols</td>
<td>30.31c</td>
<td>22.34a</td>
<td>26.26b</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are expressed as percentage of lyophilized tea extracts weight. 2 Total polyphenols are calculated as the (gallic acid equivalents) content (%). 3 Different letters in the same line represent significantly different mean values (P<0.05). ND, not detected.
Figure 3.1. UPLC chromatogram of GTE (A), OTE (B), and BTE (C).
3.3.1 α-Amylase Inhibition

The inhibitory effects of TEs and individual phenolic compounds against PPA were studied. Fig. 3.2A shows the inhibition curves at various concentrations of TEs. The relative activity of PPA decreased with increasing concentrations of the three TEs in a similar manner. Among the three TEs, GTE showed the highest inhibitory effect, with its IC\textsubscript{50} as 0.197 mg/mL. The inhibition of PPA by TEs, with TE concentrations expressed as gallic acid equivalents (GAE), is shown in Fig. 3.2B. Interestingly, as with calculations based on the inhibitory effect on PPA by phenolic equivalents, GTE still demonstrated the highest inhibition, but there was less difference in inhibitory effect among the three tea extracts.

The inhibition of PPA by individual phenolic compounds in tea is presented in Fig. 3.2C, in which TF2 had the highest inhibitory activity with the lowest IC\textsubscript{50} value (Table 3.2). Both TF1 and TF also exhibited relatively high inhibitory activity, suggesting that the theaflavin family accounted for a high proportion of the PPA inhibition in BTE. Notably, the inhibitory effects of the three theaflavins on PPA were in the following order: TF2>TF1>TF, and for catechins the inhibitory effects were in the order ECG>EGCG>C>EC>EGC.
Table 3.2. Detailed kinetics of PPA inhibition by TE and pure phenolic compounds

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>( K_{m} ) app (mg/mL)</th>
<th>( V_{max} ) app (mM maltose/min)</th>
<th>Inhibition Type</th>
<th>( K_{i} ) (mg/mL)</th>
<th>1/( K_{i} ) (mL/mg)</th>
<th>IC_{50} (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A^1</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTE</td>
<td>1.861</td>
<td>4.286</td>
<td>4.977</td>
<td>8.265</td>
<td>11.776</td>
<td></td>
</tr>
<tr>
<td>BTE</td>
<td>1.861</td>
<td>4.287</td>
<td>4.106</td>
<td>8.313</td>
<td>8.987</td>
<td></td>
</tr>
<tr>
<td>OTE</td>
<td>1.861</td>
<td>2.607</td>
<td>4.422</td>
<td>4.528</td>
<td>5.065</td>
<td></td>
</tr>
<tr>
<td>EGCG</td>
<td>0.608</td>
<td>0.946</td>
<td>1.407</td>
<td>1.637</td>
<td>1.554</td>
<td></td>
</tr>
<tr>
<td>ECG</td>
<td>1.494</td>
<td>1.852</td>
<td>1.872</td>
<td>1.800</td>
<td>1.853</td>
<td></td>
</tr>
<tr>
<td>TF2</td>
<td>1.074</td>
<td>3.167</td>
<td>4.178</td>
<td>4.866</td>
<td>5.689</td>
<td></td>
</tr>
<tr>
<td>TF1</td>
<td>1.026</td>
<td>1.967</td>
<td>2.531</td>
<td>3.754</td>
<td>2.927</td>
<td></td>
</tr>
<tr>
<td>TF</td>
<td>1.294</td>
<td>1.545</td>
<td>1.911</td>
<td>2.521</td>
<td>2.366</td>
<td></td>
</tr>
<tr>
<td>TA</td>
<td>1.145</td>
<td>1.784</td>
<td>0.169</td>
<td>2.566</td>
<td>3.707</td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>9.321</td>
</tr>
<tr>
<td>EGC</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>14.125</td>
</tr>
<tr>
<td>C</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.552</td>
</tr>
<tr>
<td>GA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.170</td>
</tr>
</tbody>
</table>

1 Characters (A to E) represent the concentrations of TEs and pure phenolic compounds, as in Fig. 3.3A-C and Fig. S3.1 (A=0; E=highest concentration; increasing order from A to E). 2 Different letters in the same column represent significantly different mean values (\( P<0.05 \)). NA, not applicable. ND, not detected due to very weak inhibition by EC, EGC and C.
Figure 3.2. Inhibition of PPA by TE s with different concentrations, expressed as mg extracts/mL (A) and mg gallic acid equivalent (GAE)/mL (B). Inhibition of PPA by pure phenolic compounds (C). All the curves are fitted based on the equation (3.2) used for calculating IC$_{50}$ values of inhibitors.
3.3.2 The Kinetics of Inhibition of PPA by TEs and Tea Polyphenols

As enzymic inhibition for each of the TEs and a range of polyphenol components were observed, a more detailed kinetic characterisation of the inhibition was carried out. Example starch digestion cases in the absence and presence of GTE, assessed by the initial reaction velocity ($v$) are presented in Fig. 3.3A. It is shown that at the same starch concentration, $v$ decreased with increasing concentration of GTE, suggesting that the inhibitory effect on PPA by GTE was inhibitor concentration dependent. Similar results were observed for BTE and OTE (Fig. S3.1A and B), as the active ingredients in all three TEs are polyphenols (He, Lv, & Yao, 2007). Notably, a decreasing effect on $v$ by GTE was observed with increasing starch concentrations. For instance (Fig. 3.3A), at 1.25 mg/mL substrate, $v$ was determined as 0.121 mM maltose/min without any PPA inhibition, and after mixing PPA with 24 mg/mL GTE, $v$ was reduced by 68.6 %. However, at 15mg/mL substrate, $v$ was only decreased by 20.2 % from PPA inhibition at 24mg/mL GTE, indicating that the inhibitory effect can be relieved by increasing the substrate amount. Hence, this kind of response meets the characteristics of competitive inhibition as described by Strominger et al (1960). Inhibition caused by OTE and BTE showed a similar effect on $v$ as GTE. This indicates that the three TEs may be competitive inhibitors of PPA, which may be further characterised through kinetic analysis using Dixon and Cornish-Bowden plots (Butterworth, 1972; Cornish-Bowden & Eisenthal, 1974). As for the pure tea polyphenols, all the compounds showed higher inhibition of PPA with increasing phenolic concentrations (Fig. 3.3B and C and Fig. S3.1). Similar inhibition-relieving phenomenon by enhancing substrate concentration was observed for TA, EGCG, and TF2 (Fig. 3.3B and Fig. S3.1C and D).

3.3.2.1 Dixon and Cornish-Bowden Plots

As discussed above, the inhibition type can be analysed and defined by the combined use of Dixon and Cornish-Bowden plots. Both the plots for TEs and the pure phenolics are shown in Fig 3.3D-F and Fig. S3.2. For the three TEs, the Dixon plot showed a clear intersection, while the Cornish-Bowden plot showed lines running parallel with each other (Fig. 3.3D and Fig. S3.2A and B), demonstrating that all three TEs are competitive inhibitors of PPA. For TEs, the order of $K_{ic}$ (GTE<BTE<OTE) was in agreement with that of the IC$_{50}$ values (Table 3.2). Competitive inhibition was also found for TA, EGCG, and TF2 based on their Dixon
and Cornish-Bowden plots (Fig. 3.3E and Fig. S3.2C and D). As the deviation of the intersection points formed by different plots was smaller (more focused on one point), and linear regression correlation for each Dixon plot was better (for instance, $R^2$ ranging from 0.982 to 0.995 for TF2, compared to 0.934-0.970 for OTE) than TEs, the three phenolics behaved as more typical competitive inhibitors. The reason for this better fitting probably lies in the fact that the pure phenolic compounds are simpler in terms of composition, in comparison with TEs which are complex mixtures in which there are many components exhibiting a range of inhibitory effects. The $K_{ic}$ of TF2 was calculated as 1.15 mg/mL (Table 3.2), the lowest among the pure phenolics, which together with the lowest IC$_{50}$ value suggest that TF2 is a strong inhibitor of PPA. As for EGCG, $K_{ic}$ was 47.7 mg/mL, much higher than the other phenolics. Similar to TEs, the order of $K_{ic}$ for the pure compound competitive inhibitors (TF2, EGCG and TA) matched their relative IC$_{50}$ values (Table 3.2). It should be noted that $K_{ic}$ and $K_{iu}$ for EC, EGC and C could not be determined due to their very weak inhibition. Notably, for the pure phenolic compounds, the order of IC$_{50}$ values corresponded to that of the inhibition constants (both for $K_{ic}$ and $K_{iu}$) (Fig. 3.4). Especially, there is a linear positive correlation ($R^2$=0.990) between IC$_{50}$ and $K_{ic}$ values for both the competitive and mixed-type inhibitors, and a positive correlation between IC$_{50}$ and $K_{iu}$ values for the mixed-type inhibitors (Fig. 3.4).

It is found (Fig. 3.3F and Fig. S3.2E and F) that both the Dixon and Cornish-Bowden lines intersect at one point for ECG, TF1, and TF, indicating that these three phenolics are mixed-type inhibitors, including both the competitive and uncompetitive PPA inhibition. This indicates that increasing substrate concentration cannot fully relieve inhibition of PPA by these polyphenolic compounds because of the existence of uncompetitive inhibition.

3.3.2.2 Lineweaver-Burk Plot

Fig. 3.3G-I shows the double-reciprocal Lineweaver-Burk (LB) plots for TEs and the phenolic compounds. For the competitive inhibitors (three TEs, TA, EGCG, and TF2) assigned from Dixon and Cornish-Bowden plots, all had an intersection near the y axis (Fig. 3.3G and H and Fig. S3.3A-D), consistent with competitive inhibition (Kawamura-Konishi, et al., 2012; Yang, He, & Lu, 2014). Due to the existence of uncompetitive inhibition, the LB lines for ECG, TF1 and TF (Fig. 3.3I and Fig. S3.3E and F) were found to intersect farther
from the y axis than the competitive inhibitors (Miao, Jiang, Jiang, Zhang, & Li, 2015). Lines for ECG even intersected on the x axis because its uncompetitive inhibition was comparable to its competitive inhibition, i.e. $K_{iu}$ is close to $K_{ic}$ (Table 3.2).

$K_m^{app}$ and $V_{max}^{app}$ calculated from the modified Michaelis-Menten equation (3.6) are listed in Table 3.2. As for a typical competitive inhibitor, $K_m^{app}$ increases while $V_{max}^{app}$ remains the same, and this is consistent with the values calculated for TEs in this study. $K_m^{app}$ values of EGCG, TF2 and TA also showed an increasing trend with increasing concentrations, while there was a slight decrease in $V_{max}^{app}$ values at high concentrations of inhibitors. This may be caused by the potential for significant errors in the plot when the concentration of substrate is low (Cornish-Bowden, 1974). For the mixed-type inhibitors (ECG, TF2 and TF), decreased $V_{max}^{app}$ was observed, since it takes longer for substrate or product to leave the active site (Kim, Jeong, Wang, Lee, & Rhee, 2005).
Figure 3.3. Initial reaction velocity at different concentrations of starch in the absence or presence of GTE (A), TF2 (B) and ECG (C). Various concentrations of the inhibitors are indicated in the respective legend entries. PBS buffer means starch digestion without the inhibitors; Dixon and Cornish-Bowden (insets) plots for PPA inhibition by GTE (D), TF2 (E) and ECG (F). Various concentrations of starch solution are listed in the legend entries; Lineweaver-Burk plots for PPA inhibition by GTE (G), TF2 (H) and ECG (I). The legend entries are the same as A-C.

Figure 3.4. Correlation between IC_{50} and \( K_{ic} \) and \( K_{iu} \) (inset) for pure phenolic compounds in tea extracts.

3.4. Discussion

The data presented show that three TEs and each of the pure phenolic compounds found in tea had inhibitory effects on α-amylase. The active components which most contribute to the inhibition by TEs have been shown to be polyphenols and tannins (Zhang & Kashket, 1998). The total polyphenols content in tea extracts varies with the cultivar, grade and producing area (Carloni, Tiano, Padella, Bacchetti, Customu, Kay, et al., 2013; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012). The phenolic content in GTE, BTE and OTE investigated here was in the order GTE>BTE>OTE assessed by Folin-Ciocalteu reagent. Therefore, the higher inhibition of PPA by GTE is likely related to its higher phenolic content. When taking into account the concentration of phenolic compounds in the extracts (Fig. 3.2B), the
inhibition behaviours of the three TEs were closer, but subtle differences remained. These differences in PPA inhibition may lie in the various categories of polyphenols with different structures present in different teas. It should be noted that there is an extra hydroxyl group at the 3‴-position on the B ring of EGC and EGCG compared to EC and ECG (Fig. 2.4), respectively, and that the inhibitory activities were determined as EC>EGC and ECG>EGCG.

Therefore, it seems that the substitution of H by OH group at the 3‴-position on the B ring may reduce the α-amylase inhibition by EC and ECG. However, an investigation of 19 kinds of flavonoids has shown that the 3‴-position on the B ring is not essential in determining these compounds inhibitory activities (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008). Interestingly, the IC$_{50}$ value of the galloyl group (gallic acid) itself was determined as 0.170 mg/mL, significantly lower than that of EC, EGC, TF and TF1 (Table 3.2). For the theaflavin family in black tea, the number of galloyl groups at the 3-position on the C ring or the corresponding 3’-position on the C’ ring in the TF, TF1 and TF2 molecular structure is 0, 1, and 2, respectively. The data presented here indicate that the inhibitory potency is enhanced as the number of galloyl moieties is increased. Additionally, ECG and EGCG, the respective 3-gallate forms of EC and EGC (Fig. 2.4), have inhibitory activities against PPA ~5 times greater than EC and EGC respectively. Therefore, 3 or 3’-galloyl groups on the C or C’ ring are shown to be responsible for enhancing the inhibitory activities of catechins and theaflavins against α-amylase, confirming and extending previous reports (Miao, Jiang, Jiang, Li, Cui, & Jin, 2013; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012) by addressing the importance of presence and location of galloyl groups in determining α-amylase inhibition by polyphenols in both fermented and non-fermented teas.

Through the Dixon and Cornish-Bowden plots, TF2, TA and EGCG were shown to be competitive inhibitors, indicating that such polyphenols can bind with the active site of PPA, competing with the starch molecules (Morrison, 1969). The competitive mechanism may be interpreted in the general scheme described in Fig. 3.5A, in which ‘EI’ is a terminal enzyme-inhibitor complex, as the only potential reaction that can take place is reformation of ‘E’ (enzyme) and ‘I’ (inhibitor). Hence, $K_{ic}$, can be given by the following equilibrium equation:
Where, \([EI]\), \([E]\) and \([I]\) are the concentrations of enzyme-inhibitor complex, enzyme and inhibitor, respectively.

\[
K_{ic} = \frac{[EI]}{[E][I]} \quad (3.7)
\]

**Figure 3.5.** Mechanisms that produce competitive (A) and mixed-type (B) inhibition. A, E, I and P represent substrate, enzyme, inhibitor and product, respectively.

From this formula, a lower \(K_{ic}\) value means a shift of equilibrium position in favour of enzyme-inhibitor complex, or in other words, it means the inhibitor binds more tightly with the enzyme. It should be noted that \(K_{ic}\) is an equilibrium constant, which means the amount of inhibitor-enzyme complex depends on the concentrations of the inhibitor and enzyme. Therefore, \(K_{ic}\) should be used as an indicator of the extent of enzyme-inhibitor complex formation under the condition of defined concentrations of enzyme and inhibitors. So, the lowest \(K_{ic}\) for GTE among the three TEs means the greatest amount of PPA-TEs complex at fixed concentrations of the three TEs, and therefore the highest inhibitory effect on PPA. EGCG had the highest \(K_{ic}\) of all the phenolic compounds investigated (the values for EC, EGC and C could not be measured due to the inhibition being too weak), meaning its competitive inhibition was relatively weak. The competitive inhibition of TF2 contributes significantly to the inhibitory activity of BTE due to the relatively low \(K_{ic}\) and considerable content (7.51%). Our data are consistent with the reported result that TF2 had a much higher
inhibitory effect on α-amylase than EGCG (Hara & Honda, 1990). Interestingly, although TF2 was observed to be the strongest inhibitor of PPA in tea polyphenols based on calculation of IC$_{50}$ and $K_{ic}$, BTE had a lower inhibitory effect on a GAE basis than GTE (Fig. 3.2B). The reason may lie in the observation that GTE contained much more ECG and EGCG than BTE, both of which are also effective inhibitors at the high concentrations found in GTE (Fei et al., 2014; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012). In addition, TEs are a complex mixture of compounds, which contain soluble polysaccharides in addition to polyphenols. It has been reported that soluble polysaccharides are able to disrupt the binding of polyphenols to protein (de Freitas, Carvalho, & Mateus, 2003); therefore, although the PPA inhibition by TEs substantially comes from polyphenols and tannins, there may be influences from other components. As GTE was determined to have more content of total polyphenols than BTE, the inhibition by GTE may be less influenced by other non-phenolic components (e.g. polysaccharides).

It is shown that ECG, TF1 and TF were mixed-type inhibitors of PPA. Based on the mixed-type inhibition mechanism in Fig. 3.5B, ECG, TF1 and TF can compete with starch in binding with PPA, as well as can bind with the PPA-starch complex, forming an inhibitor-enzyme-substrate ternary complex. Because $K_{ic}$ was smaller than $K_{iu}$ for the three mixed-inhibition polyphenols (Table 3.2), this suggests that they bind more tightly with free amylase than with the substrate-amylase complex (Narita & Inouye, 2009, 2011). It has been reported that the main mechanisms involved in the binding of polyphenols to enzymes are noncovalent interactions, including hydrophobic association and hydrogen bonding (Deaville, Green, Mueller-Harvey, Willoughby, & Frazier, 2007; Xiao, Kai, Ni, Yang, & Chen, 2011). Each galloyl group provides three hydroxyl groups which can potentially interact with the catalytic site amino acid side-chains of amylase (Asp$^{197}$, Glu$^{233}$ and Asp$^{300}$) (Fei et al., 2014) upon the formation of hydrogen bonds, while the benzene ring may develop hydrophobic π-π (aromatic-aromatic) interactions in the active site of amylase (Miao, Jiang, Jiang, Li, Cui, & Jin, 2013; Skrt, Benedik, Podlipnik, & Ulrih, 2012). In addition, in the galloyl group the C=O double bond is conjugated to the benzene ring and is responsible for electron delocalization, which has been proposed to lead to enhanced π-π interactions with the indole ring of Trp$^{59}$ (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008). Particularly, for EGC and
EGCG, there is a similar molecular structure in the B ring (linked at 2-position) and galloyl group (linked at 3-position): trihydroxy benzene. However, the trihydroxy benzene has been reported not necessarily related to amylase inhibitory activities of flavonoids (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008). Therefore, it is postulated that the carbonyl (C=O) may act as a spacer to promote the access of galloyl group into the active site of enzyme through the potential electron delocalization covering the ester carbonyl group and benzene ring. In our data, the correlation between IC$_{50}$ and inhibition constants ($K_{ic}$ and $K_{iu}$) was shown to be positive, and to be linear for the correlation between IC$_{50}$ and $K_{ic}$ (Fig. 3.4). As $1/K_{ic}$ and $1/K_{iu}$ demonstrate the association of inhibitor with enzyme, and the association of inhibitor with enzyme-substrate complex, respectively, we conclude that the inhibition resulted from the binding (association) of tea polyphenols to PPA. For catechins in TEs, after introduction of a galloyl group at the 3-position (EC to ECG and EGC to EGCG), the association between galloylated catechins and PPA is enhanced, compared with that between non-galloylated catechins and PPA. For theaflavins in TEs, with an increasing number of galloyl groups at the 3 or 3’-position (TF to TF1 and TF2), the association between theaflavins and PPA increased. Furthermore, as discussed, the inhibitory activities of non-galloylated tea polyphenols were enhanced by addition of a galloyl group that itself (as gallic acid) has a relatively high amylase inhibition (IC$_{50}$, 0.170 mg/mL). Therefore, the galloyl moiety on the molecular structures of both catechins and theaflavins plays an important role in association with α-amylase, and thus in inhibiting the enzymic catalytic activity.

Notably, TFs can bind with both the active and non-active sites of PPA. When introducing one galloyl group at the 3’-position of TF (TF to TF1), although TF1 is still a mixed-type inhibitor, the degree of increase in its competitive inhibition character is larger than that in its uncompetitive one (increased by 2.05 and 1.57 times, respectively). When further introducing a galloyl group at the 3-position of TF1, TF2 showed primarily a competitive inhibition character. Similarly, after galloylation, EGCG (galloylated EGC) was demonstrated to be a competitive inhibitor. Although ECG was found to be a mixed inhibitor, it was shown to be more likely to bind with the active site of PPA than with the non-active site. Furthermore, TA, with 10 galloyl groups incorporated in its molecule, was a typical competitive inhibitor of
PPA. Therefore, the data suggest that the introduction of galloyl groups in catechins and theaflavins enhances the association of inhibitors with the active site of PPA.

For the mixed-type inhibitors (ECG, TF and TF1), the uncompetitive inhibition characteristic indicates that these three inhibitors can bind with non-active sites of the enzyme in addition to the active site. Therefore, it is speculated that there might be secondary carbohydrate binding sites (non-active sites) on α-amylase as possible additional binding sites for phenolics distant from the active site. It has been reported that there are two secondary carbohydrate binding sites on the catalytic barrel and noncatalytic C-terminal domains of barley α-amylase, and they have been shown to possess distinct functions and display synergy in hydrolysis of starch, and these secondary carbohydrate binding sites have been hypothesised to exist on other amylases (Nielsen, Bozonnet, Seo, Motyan, Andersen, Dilokpimol, et al., 2009; Nielsen, Seo, Bozonnet, Aghajari, Robert, Haser, et al., 2008). The uncompetitive inhibition observed in our data may suggest the potential for secondary non-carbohydrate binding sites on PPA, which would help to further our understanding of the catalytic hydrolysis of starch by the enzyme.

In order to determine the effectiveness of tea extracts and polyphenols in controlling postprandial blood sugar, it is necessary to carry out studies in human subjects. Therefore, it is important to estimate the amount of tea polyphenols that can reach the small intestine and that can potentially produce inhibitory activity. Both theaflavins and green tea catechins have been shown to be stable in the acidic environment of the stomach, whereas they are less stable in the mildly alkaline conditions of the small intestine (Chen, Zhu, Tsang, & Huang, 2001; Record & Lane, 2001; Su, Leung, Huang, & Chen, 2003). Previous studies using samples from human volunteers who consumed green tea extracts showed that after ingestion of 200 mg GTE, ~40% polyphenols could be recovered in ileal fluid (Auger, Mullen, Hara, & Crozier, 2008; Schantz, Erk, & Richling, 2010). According to our extraction method, there is 0.75 mg/mL GTE in one cup of green tea drink (one tea bag in 200 mL hot water). Therefore, after ingestion of one cup of green tea, more than 0.3 mg/mL of effective GTE may reach at the small intestine, which would approach the IC_{50} values for α-amylase inhibition determined in this study. Even so, in practice, the interaction of TEs with complex digestive
juices, tissues and food components (like protein, cellulose, etc.) should be taken into account as well.

A number of mechanisms have previously been proposed for the anti-diabetic action of tea polyphenols (catechins and theaflavins), for example having inhibitory effects on intestinal sucrase and α-glucosidase, both of which are digestive enzymes related to starch digestion and increases in postprandial blood sugar (Honda & Hara, 1993). It has been reported that tea polyphenols could competitively inhibit the sodium-dependent glucose transporter of intestinal epithelial cells, retarding glucose uptake in the intestine (Kobayashi, Suzuki, Satsu, Arai, Hara, Suzuki, et al., 2000). Additionally, green tea supplementation for 3 months was shown to ameliorate insulin resistance in a fructose-fed rat model resembling the human type 2 diabetes mellitus (Tsuneki, Ishizuka, Terasawa, Wu, Sasaoka, & Kimura, 2004). The results in this study indicate that tea polyphenols can directly inhibit the activity of α-amylase through binding with the active site, as well as secondary binding sites, of the enzyme. This may be an important anti-diabetic mechanism for some of the polyphenolic compounds highlighted in this manuscript.

3.5. Conclusions
Overall, our data suggest green, oolong and black tea extracts can competitively inhibit the activity of α-amylase, thus potentially delaying starch digestion. Non-galloylated catechins, including catechin, EC and EGC showed weak inhibition, whereas galloylated catechins, including ECG and EGCG demonstrated higher inhibition of PPA. Galloylated theaflavins (TF2 and TF1) also showed stronger inhibition than TF in which there is no gallate group. Through kinetic analysis, it is found that the three TEes investigated as well as TA, TF2 and EGCG were competitive inhibitors, while TF, TF1 and ECG were mixed-type inhibitors. By comparing calculated competitive inhibition constants $K_{ic}$ and uncompetitive inhibition constant $K_{iu}$, incorporating a gallate moiety in catechins and theaflavins is proposed to enhance the association of inhibitors with PPA, mainly through the active site of PPA. The galloyl group may be considered as an inhibitory element in pharmaceutical design and synthesis for amylase activity control. Detailed spectroscopic and calorimetric studies of the
interaction of tea polyphenols with PPA are expected to shed further light on the underlying mechanisms by which tea extracts and polyphenols inhibit amylase activity.
Chapter 4

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

Abstract

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: Tea polyphenols; α-Amylase; Interaction mechanism; Fluorescence quenching; Differential scanning calorimetry; Isothermal titration calorimetry
4.1. 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 (Barrett, et al., 2013; Pierson, Dietzgen, Shaw, Roberts-Thomson, Monteith, & Gidley, 2012). Alpha-amylase is present in saliva and secreted by the pancreas into the small intestine; it catalyses the digestion of dietary starch to maltooligosaccharides. These maltooligosaccharides are further degraded to glucose, which is absorbed into the blood stream, triggering glycaemic and insulaemic responses (Prodanov, Seigner, & Marchismouren, 1984). The inhibitory activities of polyphenols are highly dependent on their molecular structures (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008), 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) (Miao, Jiang, Jiang, Li, Cui, & Jin, 2013; Miao, Jiang, Jiang, Zhang, & Li, 2015).

In Chapter 3, the interactions between polyphenols and α-amylase have been characterized by determination of the half inhibition concentration (IC$_{50}$), kinetics of inhibition, fluorescence quenching and docking analysis. 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 (Cornish-Bowden & Eisenthal, 1974). 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 (Miao, Jiang, Jiang, Zhang, & Li, 2015). However, the correlation between $K_{FQ}$ and $1/K_{ic}$ needs further study, as both constants suggest the binding of polyphenols with the enzyme. Tea polyphenols, including (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (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 (Yilmazer-Musa, Griffith, Michels, Schneider, & Frei,
2012). 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 (Tang, Covington, & Hancock, 2003). It offers an objective and comprehensive way of evaluating the thermal stability of proteins (Barrett, et al., 2013). It has been reported that polyphenol interactions may change the thermostability of proteins (Prigent, Gruppen, Visser, van Koningsveld, de Jong, & Voragen, 2003; Raghavendra, Kumar, & Prakash, 2007). 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 (Jelesarov & Bosshard, 1999). This technique has been successfully applied to determine the binding constants of oligomeric ellagitannins with bovine serum albumin (Frazier, Papadopoulou, & Green, 2006; Frazier, Papadopoulou, Mueller-Harvey, Kissoon, & Green, 2003; Karonen, Oraviita, Mueller-Harvey, Salminen, & Green, 2015). To our knowledge, the combination of DSC and ITC has not been previously applied to study the interactions of polyphenols and PPA.

In Chapter 3, the kinetics of inhibition (IC$_{50}$, $K_{ic}$ and $K_{iu}$) of PPA by a range of tea polyphenols were systematically studied. In this chapter, 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 $K_{itc}$ are compared and correlated to analyse the relationships between PPA inhibition and binding behaviour. The role of the galloyl moiety in binding of catechins and theaflavins with PPA is highlighted.

4.2. Materials and methods

4.2.1 Materials and chemicals

All materials and chemicals are as in Chapter 3.

4.2.2 Kinetics of inhibition

Methods used to determine the kinetics of inhibition of PPA by TEs and tea polyphenols are as described in Chapter 3.
4.2.3 Fluorescence quenching

Fluorescence spectra of PPA in the absence and presence of TEs and pure polyphenols were recorded using a Shimadzu® spectrofluorimeter (RF-5301 PC, Tokyo, Japan) according to a previously reported method (Hill, Horowitz, & Robinson, 1986) 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 (\(\lambda_{\text{ex}}\)) set as 282 nm and the emission \(\lambda_{\text{em}}\) recorded from 300 to 500 nm. Both the slit widths were 10 nm.

Fluorescence quenching is described by the Stern-Volmer equation:

\[
\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{FQ} [Q]
\]  

(4.1)

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, \(\tau_0\) is the lifetime of the fluorophore. For \(\alpha\)-amylase, the \(\tau_0\) is 2.97 ns (Prendergast, Lu, & Callahan, 1983); \([Q]\) 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 (Castanho & Prieto, 1998). The modified form of the Stern-Volmer equation
describing this situation is as follows (Ferrer-Gallego, Goncalves, Rivas-Gonzalo, Escribano-Bailon, & de Freitas, 2012):

\[
\frac{F_0}{F} = e^{(K_F q)}
\]

(4.2)

4.2.4 Differential scanning calorimetry

The thermostability of PPA control and that bound with TEs and pure phenolic compounds were 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 method (Barrett, et al., 2013) with some modifications: 50 µL of PPA and 50 µL 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 µL 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 (\(\Delta H\)) were calculated based on the mass of protein in DSC sample pans (J/g protein).

4.2.5 Isothermal titration calorimetry

A GE® ITC instrument (MicroCal iTC200, Stockholm Sweden) was used to determine enthalpy changes associated with polyphenol-PPA interactions at 298K. 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 µL and the duration of each was 4s. The time delay between the injections was 150s. 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 (Karonen, Oraviita, Mueller-Harvey, Salminen, & Green, 2015). 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 (μcal·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 as follows (Poncet-Legrard, Gautier, Cheynier, & Imberty, 2007):

\[
Q_i = \frac{n[M]ΔHV_0}{2} \left\{ 1 + \frac{[P]}{n[M]} + \frac{1}{nK_{itc}[M]} \right\} - \sqrt{(1 + \frac{[P]}{n[M]} + \frac{1}{nK_{itc}[M]})^2 - 4 \frac{[P]}{n[M]}} \right\} (4.3)
\]

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.

### 4.2.6 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 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 analysed to obtain the binding parameters and fit standard deviation using MicroCal Origin software.

### 4.3. Results

#### 4.3.1 Kinetics of inhibition

In Chapter 3, the phenolic constituents of the three TEs were analysed 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. 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 IC\(_{50}\) values, which are shown together with values of \(K_{ic}\) and \(K_{iu}\) in Table 3.2.

#### 4.3.2 Fluorescence quenching

The fluorescence intensity of PPA before and after addition of tea polyphenols was determined to investigate the interactions between them. Fig. 4.1A-K shows the fluorescence...
emission spectra of PPA obtained at $\lambda_{\text{ex}} = 282$ 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 $\lambda_{\text{em}}$ 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 y axis (Fig. 4.1L and M). Hence, the original Stern-Volmer equation (4.1) was applied for EGC, EC, TF1 and TF, and the modified Stern-Volmer equation (4.2) was applied for TEs, EGCG, ECG, TA and TF2 to calculate the quenching parameters. The $K_{\text{FQ}}$ and bimolecular quenching constants ($k_q$) for TEs and pure polyphenols are summarized in Table 4.1, in which the respective orders of $K_{\text{FQ}}$ values for TEs and pure polyphenols are GTE>BTE>OTE and TF2>TA=TF1>TF>EGC>EGCG>EC. Both these orders are similar to those of the inhibitory activities of these compounds against PPA evaluated by IC$_{50}$ values (Table 3.2).

**Table 4.1** Fluorescence quenching parameters for the interactions of TEs and pure polyphenols with PPA

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

*Different letters in the same column represent significantly different mean values ($P<0.05$).
Figure 4.1. 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 $\lambda_{\text{em}}$ 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 (4.1), and equations for three TEs, TA, TF2, EGCG and ECG were fitted according to equation (4.2).

4.3.3 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. 4.2. 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 ($\Delta H$) of 26.91 J/g protein (Table 4.2). 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 $\Delta H$ of PPA bound with TEs was lowered as well. A lower $\Delta H$ demonstrates that a smaller amount of energy is required to unfold the PPA molecule. 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 $\Delta H$ of PPA bound with TA, ECG, ECCG and TF2 decreased to 18.40, 21.32, 20.39 and 23.15 J/g protein, respectively. However, the $\Delta H$ values for TF, TF1, ECG and EC did not change significantly.
Figure 4.2. 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.
Table 4.2 Denaturation temperature ($T_d$) and enthalpy ($\Delta H$) of PPA obtained by DSC thermograms in the absence and presence of TEs and pure polyphenols

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Phenolic compounds</th>
<th>PBS</th>
<th>TA</th>
<th>ECG</th>
<th>EGCG</th>
<th>TF2</th>
<th>EGC</th>
<th>TF</th>
<th>TF1</th>
<th>EC</th>
<th>GTE</th>
<th>BTE</th>
<th>OTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_d$ (°C)</td>
<td></td>
<td>67.54d</td>
<td>61.99a</td>
<td>65.15f</td>
<td>65.33d</td>
<td>65.74d</td>
<td>67.99j</td>
<td>67.42f</td>
<td>67.47d</td>
<td>68.14e</td>
<td>66.42d</td>
<td>65.46d</td>
<td>63.28d</td>
</tr>
</tbody>
</table>

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

Table 4.3 Thermodynamic binding parameters for the interactions of tea polyphenols with PPA fitted by single-site binding model

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

* 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.
4.3.4 Isothermal titration calorimetry

Isothermal titration calorimetry is a powerful technique to analyse the thermodynamics of binding of polyphenols with proteins (Pascal, Poncet-Legrand, Imberty, Gautier, Sarni-Manchado, Cheynier, et al., 2007). The interactions of eight phenolic compounds and three tea extracts with PPA were investigated by ITC. Fig. 4.3A 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. 4.3B) 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 (Poncet-Legrand, Gautier, Cheynier, & Imberty, 2007) was applied to assess the correlation between interaction heat and molar ratio of polyphenol to PPA. The estimated thermodynamic binding parameters are summarised in Table 4.3. Inspection of Fig. 4.4 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 4.3 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. 4.4A-E), while very little heat was generated during the titration of non-galloylated catechins (EC and EGC) into PPA (Fig. 4.4G and H). For the pure polyphenols studied, TF2 showed the highest values of $K_{\text{itc}}$ (11600 M$^{-1}$) and $\Delta H$ (-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 $K_{\text{itc}}$ (482 M$^{-1}$) and $\Delta H$ (-988 J·mol$^{-1}$). In addition, TA showed the lowest, and ECG showed the highest stoichiometry ($n$) i.e., the polyphenol to PPA ratio.
Figure 4.3. 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)).
Figure 4.4. 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.
4.4. 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 (Lakowicz, 1999). 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) (Soares, Mateus, & Freitas, 2007). 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 $1 \times 10^{10}$ M$^{-1}$s$^{-1}$ for the typical dynamic mechanism (collision-controlled quenching) (Lakowicz, 1999). The $k_q$ values of EC, TF1 and TF are 9-150-fold higher than that (Table 4.1), 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 (Cai, Yu, Xu, Liu, & Yang, 2015; Soares, Mateus, & Freitas, 2007). 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 4.1), presumably due to the large number of potential hydrogen bonds and hydrophobic interactions (Miao, Jiang, Jiang, Zhang, & Li, 2015; Okuda, Mori, & Hatano, 1985), consistent with the prevalence of the static mechanism in comparison to the dynamic one for PPA fluorescence quenching by TF2 and TA.
Figure 4.5. The linear correlations between $K_{FQ}$ and $1/K_{ic}$ (A), $Kitc$ and $1/K_{ic}$ (B) and $K_{FQ}$ and $K_{itc}$ (C). The respective correlation equations and coefficients ($R^2$) are listed as well.
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 correlation between $K_{FQ}$ and $1/K_{ic}$ is shown in Fig. 4.5A. There was a positive linear correlation between the two constants ($K_{FQ}=13.97\cdot1/K_{ic}+1517.1, \ R^2=0.9590$), 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 (Soares, Mateus, & Freitas, 2007). In contrast, $1/K_{ic}$ definitely reflects the formation of enzyme-inhibitor complex. 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 (Fei et al., 2015; Miao, Jiang, Jiang, Zhang, & Li, 2015).

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 (Lakowicz, 1999). Therefore, the red-shifted maximum $\lambda_{em}$ of PPA by TEs, EGCG, ECG, TA and TF2 (Fig. 4.1A-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 $\lambda_{em}$ was observed (Fig. 4.1D, 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 (Rathi, Jaeger, & Gohlke, 2015; Vihinen, 1987). 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 4.1, 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 (Lumry & Eyring, 1954; Violet & Meunier, 1989). The other one is irreversible, leading to the denaturation of the initially-unfolded molecule (Cueto, Dorta, Munguia, & Llabres, 2003). 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. 4.1A-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 (Raghavendra, Kumar, & Prakash, 2007). Some earlier studies also indicated that phenolic compounds decreased thermal stability of mono-subunit and multi-subunit proteins by binding with these proteins (Muralidhara & Prakash, 1995; Prigent, Gruppen, Visser, van Koningsveld, de Jong, & Voragen, 2003; Rawel, Rohn, Kruse, & Kroll, 2002). 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 (Fei, et al., 2014; Barrett, et al., 2013). 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. 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 inhibition kinetics, the relationship between the binding constants obtained through the two methods were plotted (Fig. 4.5B). As shown, there was a positive linear correlation between $K_{ic}$ and $1/K_{ic}$ ($K_{ic}=14.634\cdot1/K_{ic}+1746.4$, $R^2=0.8239$), 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 $K_{ic}$). Therefore, the constants obtained through the inhibition kinetics and ITC show a consistent relationship, and the two methods may be combined to analyse the binding of polyphenols with α-amylase.
Very little corrected exothermic heat was observed for the titration of EC and EGC into PPA (Fig. 4.4G 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 3.2 and Fig. 4.1D and G). By introduction of a galloyl group, EC and EGC were transformed to their galloylated forms, ECG and EGCG, respectively (Fig. 2.4). Then, the binding of ECG and EGCG with PPA could be detected by the corrected heat 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 (Fig. 2.4), and both the $\Delta H$ and $K_{ic}$ 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 10 galloyl groups in its molecular structure had a high binding affinity to PPA ($K_{ic}$, 8740 M$^{-1}$; $\Delta H$, -7273 J·mol$^{-1}$). This finding is consistent with the finding in Chapter 3 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. 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 (Frazier, Papadopoulou, & Green, 2006). 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 (Asp$^{197}$, Glu$^{233}$ and Asp$^{300}$), but also through hydrophobic π-π (aromatic-aromatic) interactions with the active site of the enzyme (Trp$^{59}$) (Lo Piparo, Scheib, Frei, Williamson, Grigorov, & Chou, 2008; Miao, Jiang, Jiang, Li, Cui, & Jin, 2013). 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 $K_{\text{FQ}}$ and $K_{\text{itc}}$ demonstrate the binding of polyphenols with PPA, it is of interest to analyse 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. 4.5C, there was a positive linear relationship between $K_{\text{FQ}}$ and $K_{\text{itc}}$ ($K_{\text{FQ}}=0.8335K_{\text{itc}}+431$, $R^2=0.8872$). 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 $K_{\text{FQ}}$ and $K_{\text{itc}}$ 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.

4.5. 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 $\lambda_{\text{em}}$ 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 $1/K_{\text{itc}}$, $K_{\text{FQ}}$ and $K_{\text{itc}}$, indicating that all three constants reflect the binding affinity of polyphenols to $\alpha$-amylase. The galloyl moiety was shown to be an important substituent group in the binding of catechins and theaflavins with $\alpha$-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.
Chapter 5

SOLUBLE POLYSACCHARIDES REDUCE BINDING AND INHIBITORY ACTIVITY OF TEA POLYPHENOLS AGAINST PORCINE PANCREATIC α-AMYLASE

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.

Key words: Pectin; Arabinoxylan; Mixed linkage β-glucan; Kinetics of inhibition; Fluorescence quenching
5.1. 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). 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\textsubscript{50}) value, inhibition kinetics and fluorescence quenching (Fei et al., 2014).

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), 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; Z. F. Zhang, Lv, Jiang, Cheng, & Fan, 2015). The presence of soluble polysaccharides may be a factor affecting the interactions between polyphenols and α-amylase due to the potential of binding interactions between polysaccharides and polyphenols (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, Goncalves, Fernandes, Mateus, & Freitas, 2009; Soares, Mateus, & Freitas, 2012). 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, Gonçalves, Fernandes, Mateus, & de Freitas, 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. 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.

5.2. Materials and methods

5.2.1 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, Gunness, & Gidley, 2016) were purchased from Megazyme Co. Ltd. (Bray, Ireland).

Details of tea extracts and pure polyphenols are given in Chapter 3

5.2.2 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. Specifically, solutions of the polyphenols were prepared in 20% (v/v) dimethyl sulfoxide (DMSO) in PBS buffer to give a final concentration of 12 mg/mL GTE (an example of tea extracts), 5 mg/mL TF2 (an example of competitive inhibitors) and 50 mg/mL ECG (an example of mixed-type inhibitors). Normal maize starch (20 mg/mL) was prepared in PBS buffer and cooked at 90 °C for 20 min, followed by being diluted to 8 mg/mL. 0.4 g of polysaccharide powder was mixed with 20 mL of PBS buffer and incubated at 60 °C water bath for 45 min, followed by dilution with PBS buffer. The concentration series of the three polysaccharides (WAX, CP and OBG) was 2.0%, 1.75%, 1.5%, 1.0%, 0.75%, 0.5% and 0.25%, w/v. 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 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 described in Chapter 3. Then, the percentage of PPA inhibition was calculated by the following equation:

\[
I = \left(1 - \frac{v}{v_0}\right) \times 100\% \quad (5.1)
\]

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 mg/mL cooked solution was added to start the digestion process, and the initial digestion velocity was determined using the PAHBAH method. For the second mixing order, a concentration series of polysaccharide (2.0%, 1.75%, 1.5%, 1.25%, 1.0%, 0.75%, 0.5% and 0.25%) was added to the mixture of PPA and GTE to investigate if the effect was also concentration-dependent.

### 5.2.3 Effects of polysaccharides on IC\textsubscript{50} values of tea polyphenols

For IC\textsubscript{50} value determination, PPA inhibition by tea polyphenols in the absence and presence of polysaccharides was determined as follows: TEs and pure polyphenols were dissolved in PBS buffer to a respective stock solution immediately before using in the experiments. 25 µL of various dilutions of the test polyphenols were pre-incubated with 25 µL of 1% polysaccharides (WAX, CP and OBG) in a 96-well plate (Greiner bio-one, Germany) at 4 °C for 15 min. Then, 25 µL of 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’ (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 λ\textsubscript{ex} of 460 nm and λ\textsubscript{em} of 520 nm at 6 min intervals over 36 mins. 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 the equation (5.1). The IC\textsubscript{50} value was calculated by the IC\textsubscript{50} equation (3.2).

### 5.2.4 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 mg/mL) was prepared in PBS buffer. Each polyphenol solution (50 µL) was pre-incubated with 200 µL of 0.5% polysaccharide solution at 4 °C for 15 min, followed by addition of 50 µL of 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. The competitive inhibition constant, \( K_{ic} \) can be obtained from the Dixon equation (3.3). The uncompetitive constant, \( K_{iu} \) can be obtained from the Cornish-Bowden equation (3.5).

**5.2.5 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 (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 0.5% 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 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 (4.1) or its modified form (4.2)

**5.2.6 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 (\( P<0.05 \)).

**5.3. Results**

**5.3.1 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. 5.1A. 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. 5.1A), 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 0.5% to 1.5% (Fig. 5.1B). 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. 5.1C, both the mixing orders, i.e. 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 0.25% to 1.5% (Fig. 5.1D).

IC50 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 IC50 values of tea polyphenols are shown in Table 5.1. Based on the order of IC50 values, the inhibitory activity of the pure tea polyphenols was in the order of TF2>TF1≈TA>TF>ECG>EGCG, as reported in Chapter 3. It is shown in Table 5.1 that addition of the polysaccharides did not alter the order of the inhibitory activity for the pure polyphenols. All the polysaccharides tested were found to increase the IC50 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.
Figure 5.1. 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).

5.3.2 Effects of polysaccharides on kinetics of PPA inhibition by tea polyphenols

In Chapter 3, the kinetics of inhibition of PPA by tea polyphenols was studied in the absence of polysaccharides, as shown in Fig. S5.1A-C. In this chapter, the effects of the three polysaccharides on the Dixon and Cornish-Bowden plots for inhibition kinetics of GTE, TF2 and ECG (examples of tea extracts, competitive inhibitor and mixed-type inhibitor respectively) are shown in Fig. S5.1D-L. 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 5.1. As shown in Fig. S5.1A-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. It is shown in Fig. S5.1D-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 5.1. 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.
Table 5.1 Detailed kinetics of PPA inhibition by TEs and pure phenolic compounds in the absence and presence of three polysaccharides

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>$K_i$ (mg/mL)</th>
<th>$K_{i'}$ (mg/mL)</th>
<th>$K_{iu}$ (mg/mL)</th>
<th>$K_{iu'}$ (mg/mL)</th>
<th>IC$_{50}$ (mg/mL)</th>
<th>IC$_{50'}$ (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP</td>
<td>WAX</td>
<td>CP</td>
<td>OBG</td>
<td>NP</td>
<td>WAX</td>
</tr>
<tr>
<td>GTE</td>
<td>6.332$^a$</td>
<td>11.594$^c$</td>
<td>8.402$^b$</td>
<td>13.845$^d$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BTE</td>
<td>7.847$^a$</td>
<td>12.169$^c$</td>
<td>10.61$^b$</td>
<td>14.151$^d$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TA</td>
<td>7.124$^a$</td>
<td>12.987$^c$</td>
<td>10.124$^b$</td>
<td>15.997$^d$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ECG</td>
<td>37.490$^a$</td>
<td>67.410$^c$</td>
<td>50.364$^b$</td>
<td>81.643$^d$</td>
<td>44.567$^b$</td>
<td>77.486$^c$</td>
</tr>
<tr>
<td>EGCG</td>
<td>47.690$^a$</td>
<td>85.541$^c$</td>
<td>67.140$^b$</td>
<td>101.254$^d$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TF2</td>
<td>1.148$^a$</td>
<td>1.773$^c$</td>
<td>1.316$^b$</td>
<td>2.261$^d$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TF1</td>
<td>4.255$^a$</td>
<td>5.612$^b$</td>
<td>5.345$^b$</td>
<td>6.751$^d$</td>
<td>19.925$^b$</td>
<td>26.612$^b$</td>
</tr>
<tr>
<td>TF</td>
<td>8.715$^a$</td>
<td>14.66$^c$</td>
<td>11.647$^b$</td>
<td>18.54$^d$</td>
<td>31.355$^b$</td>
<td>53.747$^c$</td>
</tr>
</tbody>
</table>

Different letters in the same line represent significantly different mean values for the corresponding parameters ($P<0.05$). ‘-’ means not available.
5.3.3 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 (as examples of tea extracts, competitive inhibitor and mixed-type inhibitor respectively) in the absence and presence of three polysaccharides are shown in Fig. S5.2. Stern-Volmer plots (Fig. 5.2) 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 5.2. 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. S5.2), 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) mechanisms. Taking into account the high values of \( K_{FQ} \) and \( k_q \), TF2 was suggested to quench the PPA fluorescence through the formation of a complex with the enzyme (the static mechanism). As shown in Fig. 5.2, although the three polysaccharides decreased the coefficients or slope of Stern-Volmer plots, they did not alter the quenching mechanism of tea polyphenols as the types of plots 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 5.2), as also found for the effects on \( K_{ic} \), \( K_{iu} \) and IC\textsubscript{50} values (Table 5.1).
Table 5.2 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

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>$K_{FQ}$</th>
<th>$K_{FQ}'$</th>
<th>$k_q$ ($10^9$)</th>
<th>$k_q'$ ($10^9$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP</td>
<td>WAX</td>
<td>CP</td>
<td>OBG</td>
</tr>
<tr>
<td>GTE</td>
<td>0.704(^a)</td>
<td>0.346(^c)</td>
<td>0.525(^b)</td>
<td>0.278(^d)</td>
</tr>
<tr>
<td></td>
<td>(ml/mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTE</td>
<td>0.648(^a)</td>
<td>0.313(^c)</td>
<td>0.481(^b)</td>
<td>0.271(^cd)</td>
</tr>
<tr>
<td>TA</td>
<td>5285.597(^a)</td>
<td>2483.392(^c)</td>
<td>3714.251(^b)</td>
<td>2133.363(^d)</td>
</tr>
<tr>
<td></td>
<td>(L/mol)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECG</td>
<td>946.849(^a)</td>
<td>458.959(^c)</td>
<td>726.46(^b)</td>
<td>390.215(^d)</td>
</tr>
<tr>
<td>EGCG</td>
<td>630.399(^a)</td>
<td>305.453(^c)</td>
<td>464.311(^b)</td>
<td>265.313(^d)</td>
</tr>
<tr>
<td>TF2</td>
<td>11711.813(^a)</td>
<td>6692.542(^c)</td>
<td>10183.89(^b)</td>
<td>5563.132(^d)</td>
</tr>
<tr>
<td>TF1</td>
<td>4740.243(^a)</td>
<td>2521.464(^c)</td>
<td>3832.458(^b)</td>
<td>2100.233(^d)</td>
</tr>
<tr>
<td>TF</td>
<td>3252.028(^a)</td>
<td>1663.96(^c)</td>
<td>2534.119(^b)</td>
<td>1384.369(^d)</td>
</tr>
</tbody>
</table>

Different letters in the same line represent significantly different mean values for the corresponding parameters ($P<0.05$).
Figure 5.2. Stern-Volmer plots for fluorescence quenching of PPA by GTE (A), TF2 (B) and ECG (C) in the absence and presence of three polysaccharides.
5.3.4 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 (Fig. 5.3A). 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 (Fig. 5.3B-D). 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 (Fig. 5.4 and Fig. S5.3). In addition, there was a linear relationship between K$_FQ$ and 1/K$_{ic}$ in the absence of polysaccharides (Fig. 5.5A), consistent with both constants reflecting the binding of polyphenols with PPA. 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 (Fig. 5.5B-D).

Figure 5.3. The correlation between IC$_{50}$ and K$_{ic}$ in the absence (A) and presence of WAX (B), CP (C) and OBG (D). ‘NP’ means no polysaccharides.
Figure 5.4. The correlation between $1/K_{ic}$ and $IC_{50}$ for GTE (A), TF2 (B) and ECG (C), and that between $K_{FQ}$ and $IC_{50}$ for GTE (D), TF2 (E) and ECG (F) in the absence and presence of three polysaccharides. ‘NP’ means no polysaccharides.
Figure 5.5. The correlation between $K_{FQ}$ and $1/K_{ic}$ in the absence (A) and presence of WAX (B), CP (C) and OBG (D). ‘NP’ means no polysaccharides.

5.4. Discussion

Tea polyphenols have been reported to inhibit the activity of PPA in previous studies (Hara & Honda, 1990). As both polyphenols and polysaccharides are important human dietary constituents (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 in this chapter 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. 5.6), 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 of the polysaccharides with starch (Dhital, Dolan, Stokes, & Gidley, 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.

Soluble polysaccharides $\rightarrow \alpha$-Amylase  

\[
\text{Binding} \quad \text{Binding (Inhibition)}
\]

Tea polyphenols

**Figure 5.6.** Interaction equilibrium among soluble polysaccharides, tea polyphenols 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. 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 5.1). 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 fluorescence quenching results provide further evidence that the three polysaccharides 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 5.2). Notably, the negative linear correlations between $1/K_{ic}$ (or $K_{FQ}$) and $IC_{50}$ for the respective polyphenols (Fig. 5.4) 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 Fig. 5.3 and $K_{FQ}$ and $1/K_{ic}$ in Fig. 5.5) 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 $\alpha$-amylase and polysaccharides (Miao et al., 2013; Renard, Watrelot, & Le Bourvellec, 2017), and there is a competitive relationship between the two binding interactions (Soares, Mateus, & de Freitas, 2012; Soares, Gonçalves, Fernandes, Mateus, & de Freitas, 2009). The interaction equilibrium among polysaccharides, polyphenols and PPA (Fig. 5.6) 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 an oligosaccharide (arabic gum and $\beta$-cyclodextrin) were able to decrease the quenching of $\alpha$-amylase by procyanidins due to potential interactions between polysaccharides and procyanidins which induced a decrease in the size of procyanidin-amylase aggregates (Soares, Gonçalves, Fernandes, Mateus, & de Freitas, 2009).

The three polysaccharides studied showed a consistent pattern with effects in the order OBG$>$WAX$>$CP. WAX has a branched molecular structure, consisting of a linear backbone of $\beta$-(1-4) linked $\alpha$-xylopyranosyl units to which $\alpha$-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, Virkki, Maunu, Lehto, Ekholm, & Varo, 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, Viëtor, Jardine, Apperley, & Jarvis, 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, Gonçalves, Fernandes, Mateus, & de Freitas, 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. S5.2), 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, Flanagan, D'Arcy, & Gidley, 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 are used widely in foods along with (and potentially bound to) other food components like polyphenols, proteins, etc. (Dickinson, 1998; Manach, Scalbert, Morand, Rémésy, & Jiménez, 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 also be taken into account in order to maximise the inhibition ability of phenolic extracts as far as possible.

5.5. Conclusion

The inhibitory activity of polyphenols against α-amylase has been reported previously, but polyphenols are often present alongside other components in foods which may affect the inhibition of digestive enzymes by polyphenols. In this chapter, 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, as 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.
Chapter 6

TEA POLYPHENOLS ENHANCE BINDING OF PORCINE PANCREATIC α-AMYLASE WITH STARCH GRANULES BUT REDUCE CATALYTIC ACTIVITY

Abstract

The effects of tea polyphenols on the binding of porcine pancreatic α-amylase (PPA) with normal maize starch granules were studied through initial rate kinetics, solution depletion assays and fluorescence spectroscopy. Polyphenols which have inhibitory activity against PPA increased binding of the enzyme with starch in a polyphenol concentration-dependent manner, while polyphenols without inhibitory activity did not affect the binding of PPA with starch. The results are consistent with a binding equilibrium between polyphenols, starch granules and PPA. The dissociation constant ($K_d$) for PPA binding with starch was decreased by tea polyphenols, with the effects greater for theaflavins than catechins and for galloylated compared with non-galloylated polyphenols. Tea polyphenols were also shown to increase the binding rate of the enzyme by increasing the observed rate constant and decreasing the half-lives. Tea polyphenols were shown to be adsorbed onto starch granules as well. Additionally, there were positive linear correlations between $1/K_d$ and the reciprocal of the competitive inhibition constant ($1/K_{ic}$) and between $1/K_d$ and PPA fluorescence quenching constant ($K_{FQ}$). Taken together the data suggest that binding of polyphenols with PPA promotes PPA binding to starch granules. Despite the greater amount of PPA on the granules, starch hydrolysis is reduced because the polyphenol inhibition of PPA persists after binding to starch.

Key words: Tea polyphenols; Enzyme binding; Starch granules; Dissociation constant; Binding rate
6.1. Introduction

The rate and extent of starch digestion is an important determination of the increase in postprandial blood sugar and insulin response (Dhital, Warren, Butterworth, Ellis, & Gidley, 2017; Schulze, Bangert, Kottra, Geillinger, Schwanck, Vollert, et al., 2014); therefore, controlling the rise in blood glucose after a meal through delaying the digestion of starchy foods is a potential management and therapeutic approach for type II diabetes (Lu, Demleitner, Song, Rychlik, & Huang, 2016). α-Amylase (EC 3.2.1.1, α-(1, 4)-glucan-4-glucanohydrolase), which catalyses the hydrolysis of α-(1, 4) glycosidic linkages in starches, is one of the main enzymes in the digestive tract (Bompard-Gilles, Rousseau, Rougé, & Payan, 1996; Warren, Zhang, Waltzer, Gidley, & Dhital, 2015). Starch digestion by α-amylase involves two steps: binding of the enzyme with starch and a subsequent hydrolysis process; therefore, the adsorption and binding of α-amylase to starch is necessary before the catalytic step can proceed (Patel, Day, Butterworth, & Ellis, 2014) and affects the digestion of starch to some extent (Reimann, Ziegler, & Appenroth, 2007). For the case of starch granules, as their size (0.5-50 µm, radius) is much greater than that of α-amylase (ca 3nm, hydrodynamic radius) (Dhital, Shrestha, & Gidley, 2010; Larson, Day, & McPherson, 2010), the adsorption of the enzyme onto starch granules depends on the number and accessibility of available binding sites on the granules (Warren, Royall, Gaisford, Butterworth, & Ellis, 2011). The main factors affecting the rate and extent of the binding have been reported to include the exposed surface area of the granule, the pores (crevices) on the granular surface, and the ratio of amorphous to ordered α-glucan chains on the surface (Warren, Royall, Gaisford, Butterworth, & Ellis, 2011).

Tea polyphenols can inhibit the activity of α-amylase through binding with the active sites of the enzyme causing the competitive inhibition or binding with the enzyme-substrate complex resulting in the uncompetitive inhibition (refer to Chapter 3 and 4). The binding of inhibitors with α-amylase results in changes in the structure of the enzyme (Cai, Yu, Xu, Liu, & Yang, 2015), which may affect its binding properties with starch.

Polyphenols have also been reported to interact directly with starch through hydrophobic forces and hydrogen bonding (Zhu, Cai, Sun, & Corke, 2009), and thus influencing the exposure of hydrolysis sites on starch and reducing starch digestibility (Chai, Wang, & Zhang,
In addition, polyphenols that are adsorbed onto granular starch may also have inhibitory activity against α-amylase (Yang, He, & Lu, 2014). Therefore, by both the mechanisms, the binding of polyphenols with starch is expected to decrease starch digestion.

The interaction of polyphenols, starch and α-amylase is a complex tertiary system with multiple possible interaction mechanisms. In Chapter 3 and 4, we used reciprocal of competitive inhibition constant (1/K_{ic}) and fluorescence quenching constant (K_{FQ}) to describe the binding of tea polyphenols with porcine pancreatic α-amylase (PPA). In this chapter the effects of tea polyphenols on the binding of PPA with maize starch granules at 0 °C were investigated by use of solution depletion and fluorescence methods. Then, the binding properties of PPA with starch granules in the presence of tea polyphenols were compared with that of the polyphenols with PPA to determine how the polyphenols affect PPA binding with starch and the residual enzyme activity.

6.2. Materials and methods

6.2.1 Materials and chemicals

All materials and chemicals are as in Chapter 3.

6.2.2 Determination of binding ratio of PPA with starch

6.2.2.1 Initial reaction velocity

The relative binding of PPA with starch granules in the absence or presence of tea polyphenols was measured through the initial catalytic velocity of the enzyme (a flow diagram as shown in Fig. S6.1). Specifically, 1 mL of PBS or 0.04 mg/mL each tea polyphenol (in PBS buffer: all the polyphenols, starch and PPA in this study were dissolved or suspended in PBS buffer) was mixed with 2 mL of 81.1 U/ml PPA at 0 °C on ice for 15 min. Then, 2 mL of 10 mg/mL normal maize starch suspension was added to the mixture at 0 °C on ice for 30 min and vortexed every 5 min. After that, the mixture was centrifuged at 4000 g for 15 min at 4 °C, and the supernatant was taken as the PPA solution after binding. The supernatant of the mixture of 1 mL of PBS or 0.04 mg/mL each tea polyphenol, 2 mL of 81.1 U/ml PPA and 2 mL of PBS buffer was used to represent PPA solution before binding. 1 mL of the PPA solution before or after binding was transferred to a 2 mL tube containing 0.5 mL of PBS. Then, the catalytic activity of the enzyme was determined as the initial reaction
velocity using the method in Chapter 3. The amounts of PPA before and after binding were obtained from the PPA standard curve that was plotted as the relationship between the initial reaction velocity and the concentration of PPA. The binding ratio of PPA with starch was calculated by the following equation:

$$E_{\text{bound}} = \frac{[E]_{\text{before}} - [E]_{\text{after}}}{[E]_{\text{before}}}$$  \hspace{1cm} (6.1)

where, $E_{\text{bound}}$ is the binding ratio of PPA with starch, %, $[E]_{\text{before}}$ is the amount of PPA before binding, U/mL, $[E]_{\text{after}}$ is the amount of PPA after binding, U/mL.

6.2.2.2 Fluorescence intensity

As there are fluorophores in PPA, like tryptophan and tyrosine, PPA can emit fluorescence after excitation at certain wavelengths, and the fluorescence intensity can be used to measure protein concentration in solution (Soares, Mateus, & De Freitas, 2007). Therefore, according to this property, the binding ratios of PPA with starch granules in the absence and presence of tea polyphenols can be measured through the fluorescence intensity of the enzyme. After the respective supernatant of PPA before and after binding was obtained as described above (Fig. S6.1), 3 mL of the supernatant was withdrawn into a quartz cuvette that had been pre-washed with distilled water and sample solution. Then, the cuvette was loaded into the sample cell of a Shimadzu® spectrofluorimeter (RF-5301 PC, Tokyo, Japan), and the spectrum was recorded with fast speed and low sensitivity at $\lambda_{\text{ex}}=282$nm and $\lambda_{\text{em}}$ from 300 to 500 nm. Both the slit widths were 10 nm. The amounts of PPA in the supernatant before and after binding in the absence or presence of tea polyphenols were obtained from the PPA standard curve that was plotted by the correlation between the maximum fluorescence intensity and the concentration of PPA. The binding ratio of PPA with starch was calculated by equation (6.1).

**6.2.3 Determination of binding ratio of tea polyphenols with starch**

To determine the binding ratios of tea polyphenols with starch granules, 1.5 mL of 0.04 mg/mL each polyphenol was added into a tube containing 1.0 mL of 10 mg/mL normal maize starch suspension and mixed thoroughly at 0 °C on ice for 30 min. The polyphenol mixed with 1.0 mL of PBS buffer was used as control. Then, the tube was centrifuged at 14250 g for 5 min to obtain the supernatant. The absorbance of the supernatant was determined using a
UV-VIS spectrophotometer (Shimadzu®, Japan) at 280 nm. The binding ratio of polyphenol with starch was calculated using the following equation:

\[ P_{\text{bound}} = \left(1 - \frac{A_1}{A_0}\right) \times 100\% \] (6.2)

where, \( P_{\text{bound}} \) is the binding ratio of tea polyphenol, \%, \( A_1 \) is the absorbance of polyphenol after binding and \( A_0 \) is the absorbance of control.

**6.2.4 Determination of dissociation constant for PPA binding with starch**

The dissociation constants (\( K_d \)) were determined through the measurement of \( E_{\text{bound}} \) at different concentrations of starch according to the initial reaction velocity method described above. The \( K_d \) value was obtained by non-liner regression analysis with Origin® software, using a one-site binding model:

\[ E_{\text{bound}} = \frac{B_{\text{max}}[S]}{K_d + [S]} \] (6.3)

where, \( B_{\text{max}} \) is the maximum binding capacity, \%, \([S]\) is the starch concentration, mg/mL and \( K_d \) is the dissociation constant, mg/mL. The data can also be transformed into a linear plot using the Scatchard equation (Scatchard, 1949):

\[ \frac{E_{\text{bound}}}{[S]} = \frac{B_{\text{max}}}{K_d} - \frac{E_{\text{bound}}}{K_d} \] (6.4)

If equation (6.4) is fitted, \( 1/K_d \) can be obtained from the slope of the straight line plot of \( E_{\text{bound}}/\[S\] \) against \( E_{\text{bound}}/\[S\] \).

**6.2.5 Determination of binding rate for PPA binding with starch**

The binding rates for PPA binding with starch were determined according to the method established by Warren et al. (2011) with some modification. Specifically, 5 mL of PBS or 0.04 mg/mL each tea polyphenol solution was mixed with 10 mL of 81.1 U/ml PPA at 0 °C on ice for 15 min. Then, 10 mL of 10 mg/mL normal maize starch suspension was added to the mixture and mixed immediately. After 0.5, 1.0, 1.5, 2, 4, 6, 8 and 10 min, 0.5 mL of the mixture of polyphenol-starch-PPA was syringe filtered through a 0.45 µm nylon membrane to rapidly separate the insoluble starch with bound enzyme from the unbound enzyme. The \( E_{\text{bound}} \) values at different time points were determined using the initial reaction velocity method described above. The binding rates were then obtained by non-linear regression analysis with Origin® software, using the following equation:

\[ E_{\text{bound}} = B_{\text{max}} \left(1 - e^{-k_{\text{obs}}}ight) \] (6.5)
where, $k_{obs}$ is the observed binding rate constant, s$^{-1}$ and $t$ is the binding time, s.

Half-lives for the binding interactions were calculated from the $k_{obs}$ values using the following equation:

$$t_{1/2} = \frac{\ln 2}{k_{obs}}$$

(6.6)

where, $k_{1/2}$ is the half-life for the binding interaction, s.

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 Statistics (Chicago, USA). The mean values were evaluated by Dunnett’s $t$ Test at the 95% significance level ($P<0.05$).

6.3. Results

6.3.1 Binding ratios of PPA with starch granules indicated by initial reaction velocity and fluorescence intensity

The binding ratios of PPA with normal maize starch (uncooked) in the absence and presence of tea polyphenols, determined by the decrease in initial reaction velocity, are shown in Fig. 6.1. As the initial reaction velocity of starch (cooked) digestion by PPA was linearly related with the concentration of the enzyme in solution (Fig. S6.2A), the percentage of residual free PPA in the starch suspension can be considered to be equal to the ratio (the percentage values shown in Fig. 6.1) of initial reaction velocity after binding compared to that before binding. In this way, the binding of PPA with starch in the absence of tea polyphenols was determined as 46.9±1.9% under the conditions in this study (Table 6.1). Because of the inhibitory activity of some tea polyphenols (ECG, EGCG, TF, TF1, TF2 and TA) against PPA (Chapter 3), the reaction velocity before binding in the presence of these polyphenols was decreased (Fig. 6.1E-J), while C, EC and EGC did not cause the reaction velocity to change (Fig. 6.1B-D) because these three polyphenols have very weak PPA inhibition (Chapter 3). As shown in Table 6.1, the binding ratios of PPA with starch did not change significantly in the presence of C, EC and EGC, while the ratios in the presence of ECG, EGCG, TF, TF1, TF2 and TA increased to different degrees.
**Figure 6.1.** Binding ratios of PPA with starch granules indicated by initial reaction velocity of PPA in the absence and presence of tea polyphenols. The values in the figure are the ratios of the free, unbound PPA in the mixture after binding.

**Table 6.1.** Binding ratios, dissociation constants, observed rate constants and half-lives for binding of PPA with starch granules in the absence and presence of tea polyphenols.

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>Binding ratio indicated by initial reaction velocity (%)</th>
<th>Binding ratio indicated by fluorescence intensity (%)</th>
<th>$K_d$ (mg/mL)</th>
<th>$k_{obs}$ (s$^{-1}$)</th>
<th>$t_{1/2}$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>46.9$^a$</td>
<td>43.2$^a$</td>
<td>1.031$^e$</td>
<td>0.031$^a$</td>
<td>22.21$^e$</td>
</tr>
<tr>
<td>C</td>
<td>46.0$^a$</td>
<td>42.9$^a$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EC</td>
<td>46.3$^a$</td>
<td>43.0$^a$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EGC</td>
<td>47.2$^{ab}$</td>
<td>43.8$^{ab}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ECG</td>
<td>59.7$^d$</td>
<td>48.9$^c$</td>
<td>0.379$^c$</td>
<td>0.033$^{ab}$</td>
<td>21.00$^e$</td>
</tr>
<tr>
<td>EGCG</td>
<td>51.4$^c$</td>
<td>48.3$^c$</td>
<td>0.485$^d$</td>
<td>0.040$^c$</td>
<td>17.37$^d$</td>
</tr>
<tr>
<td>TF</td>
<td>60.3$^d$</td>
<td>50.7$^{cd}$</td>
<td>0.380$^c$</td>
<td>0.051$^e$</td>
<td>13.61$^c$</td>
</tr>
<tr>
<td>TF1</td>
<td>63.5$^e$</td>
<td>56.9$^e$</td>
<td>0.298$^b$</td>
<td>0.049$^d$</td>
<td>14.06$^c$</td>
</tr>
<tr>
<td>TF2</td>
<td>66.9$^g$</td>
<td>65.2$^g$</td>
<td>0.113$^a$</td>
<td>0.141$^g$</td>
<td>4.90$^a$</td>
</tr>
<tr>
<td>TA</td>
<td>64.2$^f$</td>
<td>61.4$^f$</td>
<td>0.262$^{bc}$</td>
<td>0.107$^f$</td>
<td>6.47$^b$</td>
</tr>
</tbody>
</table>

* Different letters in the same column represent significantly different mean values ($P<0.05$).
‘-’ means not determined because no significant effects of these polyphenols on PPA binding were observed.
A. PBS
- Before binding
- After binding
- 56.8%

B. EC
- Before binding
- After binding
- 57.1%

C. EGC
- Before binding
- After binding
- 57.0%

D. EGCG
- Before binding
- After binding
- 56.2%

E. ECG
- Before binding
- After binding
- 51.1%

F. EGCG
- Before binding
- After binding
- 51.7%

G. TF
- Before binding
- After binding
- 49.3%

H. TF1
- Before binding
- After binding
- 43.1%

I. TF2
- Before binding
- After binding
- 34.8%

J. TA
- Before binding
- After binding
- 38.6%
Figure 6.2. Binding ratios of PPA with starch granules indicated by fluorescence intensity of PPA in the absence and presence of tea polyphenols. The values in the figure are the ratios of the free, unbound PPA in the mixture after binding.

As the maximum fluorescence intensity of PPA is positively related with its concentration (Fig. S6.2B), the binding of PPA with starch can be determined by this method as well (Fig. 6.2). The percentage of residual free PPA in the starch suspension can be calculated from the ratio (the percentage values shown in Fig. 6.2) of fluorescence intensity after binding to that before binding. In this way, the binding ratio of PPA with starch granules without tea...
polyphenols was calculated as 43.2±0.9% (Table 6.1). The fluorescence intensity of PPA was decreased to some extent by each of the polyphenols that have been shown to inhibit the enzyme (ECG, EGCG, TF, TF1, TF2 and TA, Fig. 6.2E-J) due to quenching effects. In contrast, C, EC and EGC hardly quenched the fluorescence of PPA because of weak binding to the enzyme (Fig. 6.2B-D) (Chapter 4). Similar to the reaction velocity method, the fluorescence method also showed that C, EC and EGC did not significantly change the binding ratio of PPA with starch granules, while ECG, EGCG, TF, TF1, TF2 and TA increased the binding ratio to different degrees (Table 6.1). In addition, both the methods showed that the effects of the six polyphenols on the binding of PPA to starch were concentration-dependent (Fig. 6.3). There were positive linear relationships between the binding ratio and the polyphenol concentration from both the initial reaction velocity and fluorescence methods, and for the respective polyphenol, the two slopes of the two correlations were similar (Fig. 6.3).

The effects of mixing order for starch granules, PPA and tea polyphenols on the binding of PPA with starch were also studied. Here, three tea polyphenols, i.e. TA, TF2 and ECG were selected, as TA and TF2 are two examples of typical competitive inhibitors of PPA, and ECG is a typical mixed-type inhibitor of the enzyme (Chapter 3). As shown in Fig. 6.4, both mixing polyphenols with PPA before the addition of starch granules and mixing polyphenols with starch before the addition of PPA, were shown to increase the binding of PPA with starch, although the binding for the former order was slightly higher than that for the latter one. In addition, for both the mixing methods, higher polyphenol concentrations corresponded to higher binding ratio of PPA.
Figure 6.4. Effects of mixing order for tea polyphenols, starch and PPA on the binding ratio of PPA with starch in the presence of TA (A), TF2 (B) and ECG (C), respectively. The two mixing methods included mixing polyphenol with PPA followed by the addition of starch suspension to the mixture (labelled as (polyphenol+AA)+S), and mixing polyphenol with starch suspension followed by the addition of PPA to the mixture (labelled as (polyphenol+S)+AA). 0.04 and 0.06 mg/mL polyphenol concentrations were applied, respectively. * means the significantly (P<0.05) different mean values compared to the control.
6.3.2 Binding ratios of tea polyphenols with starch granules

The binding of nine tea polyphenols with starch granules are shown in Fig. 6.5, in which it can be seen that the theaflavin family (TF2, TF1 and TF) resulted in significantly higher binding than the catechin family (E, EC, EGC, ECG and EGCG). Furthermore, for both the theaflavins and catechins, the introduction of a galloyl group increased the binding of the polyphenols with starch (EGCG>EGC, ECG>EC and TF2>TF1>TF).

Figure 6.5. Binding ratios of tea polyphenols with starch granules. Different letters represent significant different mean values ($P<0.05$).

6.3.3 Dissociation constants for binding of PPA with starch granules

To study the $K_d$ for the binding of PPA with starch granules in the absence and presence of tea polyphenols, the binding of PPA with different concentrations of starch granules were determined as summarized in Fig. 6.6. Tea polyphenols that showed an increasing effect on the binding of PPA with starch, as indicated by initial reaction velocity and fluorescence intensity data (Table 6.1), were selected. It was found that these tea polyphenols could all increase the binding at each starch concentration, with the increasing effect in the order of TF2>TA>TF1>TF>ECG>EGCG (Fig. 6.6). However, when the starch concentration was above 30 mg/mL, there was no significant difference in binding between the different polyphenols, indicating that these polyphenols did not change the maximum binding of PPA.
with starch. Scatchard equations (Fig. 6.7) were used for calculating the $K_d$ values in the absence and presence of the polyphenols at the starch concentrations of 5-20 mg/mL. The maximum binding ($B_{\text{max}}$) of PPA with starch in the absence and presence of the polyphenols remained stable at 100% (Fig. 6.6), and the $K_d$ in this study was taken as the reciprocal of slope ($1/K_d$) of the Scatchard plot. It is shown in Table 6.1 that the tea polyphenols each decreased the $K_d$ value in the order of TF2>TA>TF1>TF≈ECG>EGCG.

Figure 6.6. Binding ratios of PPA with starch granules with different concentrations for calculating the dissociation constants.

### 6.3.4 Binding rates of PPA with starch granules

The observed binding rate constants ($k_{\text{obs}}$) and half-lives ($t_{1/2}$) for binding of PPA with starch granules in the absence and presence of tea polyphenols were determined and fitted based on equation (6.5) (Fig. S6.3). The $k_{\text{obs}}$ value for maize starch without tea polyphenols was determined as 0.031 s$^{-1}$ in this study (Table 6.1), comparable with that (0.012 s$^{-1}$) for maize starch in previous studies (Warren, Royall, Gaisford, Butterworth, & Ellis, 2011); similar values for $t_{1/2}$ were also found (Table 6.1). It was found that all the tea polyphenols increased the $k_{\text{obs}}$ and decreased the $t_{1/2}$ value, and that the theaflavin family (TF, TF1 and TF2) had greater effects on the two values than the catechin family (ECG and EGCG) (Table 6.1).
6.4. Discussion

Tea polyphenols have been reported to inhibit the catalytic activity of α-amylase due to direct binding with the enzyme (Miao, Jiang, Jiang, Zhang, & Li, 2015; Ming Miao, Jiang, Jiang, Li, Cui, & Jin, 2013). As both polyphenols and α-amylase can bind with starch (Tahir, Ellis, & Butterworth, 2010; Yang, He, & Lu, 2014), the effects of tea polyphenols on the binding of PPA with starch granules were explored in this study. The data indicated that only the tea polyphenols that have inhibitory activities against PPA affected PPA binding with starch granules. It should be noted that the method for determining the binding of PPA was through correlations between the catalytic activity (fluorescence intensity) and the concentration of the enzyme (Fig. S6.2). The low PPA protein level used precluded the direct determination of protein content using e.g. bovine serum albumin as a standard because of the limited sensitivity of direct determination (Lees & Paxman, 1972). Therefore, if there is no PPA inhibition by polyphenols, the binding of the enzyme with starch inferred from the reduction in supernatant enzyme activity is the actual one. However, in the presence of PPA-inhibiting polyphenols, such as ECG, EGCG, TF, TF1, TF2 and TA, the resultant PPA supernatant activity reflects both the binding of polyphenols with PPA in solution and the binding of PPA to starch granules. As these polyphenols inhibit PPA, the binding of PPA with starch granules in the presence of tea polyphenols in this study reflects the binding behaviour of the inhibited enzyme system. It should be noted that although the inhibition of PPA activity by polyphenols has been taken into account when calculating the binding of PPA with starch granules by using appropriate controls (Fig. S6.1), tea polyphenols were also found to bind with starch (Fig. 6.5). Therefore, the values for PPA binding to starch in the presence of polyphenols were potentially inaccurate, due to the complexity of modelling the myriad potential binding interactions between starch, polyphenol and enzyme.

As there were positive linear relationships between the binding of PPA and polyphenol concentration through both the reaction velocity and fluorescence spectroscopy methods, and the slopes of the two correlations for the each polyphenol were similar, the two methods can be combined to characterize the effects of polyphenols on PPA binding with starch. It should be noted that the catalytic activity comes from PPA directly, while the fluorescence intensity is attributed to all of the protein solution. Therefore, to indicate the binding of PPA more
directly, the binding parameters \( (K_d, k_{obs} \text{ and } t_{1/2}) \) were measured based on the catalytic activity method (initial reaction velocity) in this study. Furthermore, because of the difference of accuracy for the two determination methods, there is a corresponding difference in the measured percentage of binding of PPA with starch and an offsets for the data sets between the two methods (Fig. 6.3).

The binding ratio of PPA with starch granules was increased by altering the mixing order (Fig. 6.4), consistent with a binding equilibrium among polyphenol, starch and PPA, in which polyphenol can be proposed to act as a bridge promoting the formation of polyphenol-PPA-starch ternary complexes and thus increasing the binding ratio. Binding of PPA with starch and PPA with polyphenols are well established, and the binding of tea polyphenols with maize starch granules is illustrated in Fig. 6.5. Similar to previous studies (Barros, Awika, & Rooney, 2012; Deshpande & Salunkhe, 1982), tea polyphenols investigated in this study could bind with maize starch granules to different degrees. The forces that drive the interactions are proposed to be hydrophobic force and hydrogen bonding (Barros, Awika, & Rooney, 2012; Chai, Wang, & Zhang, 2013). The binding of PPA with starch increased with the starch concentration increasing in both the absence and presence of tea polyphenols, because the starch granules act as an adsorbate in the suspension. The increasing effects of tea polyphenols on PPA binding were mainly focused on the starch concentration rang of 5-20 mg/mL. When the starch concentration was above 30 mg/mL or above, PPA binding to starch was saturated, and no effect of polyphenols was observed (Fig. 6.6). PPA binding in the absence and presence of tea polyphenols apparently conformed to the one-site binding model due to the good positive linear relationships between \( 1/E_{bound} \) and \( 1/S \) (Fig. S6.4) (Munson & Rodbard, 1980).

\( K_d \) is the dissociation constant, therefore \( 1/K_d \) indicates the association of PPA with starch. Tea polyphenols were confirmed to increase the association of PPA with starch granules, as the \( 1/K_d \) values in the presence of tea polyphenols were higher than without the polyphenols. In Chapter 3 and 4, the association of tea polyphenols with PPA was indicated by the reciprocal of competitive inhibition constant \( (1/K_{ic}) \) and fluorescence quenching constant \( (K_{FQ}) \). Positive linear correlations between \( 1/K_d \) and \( 1/K_{ic} \) and between \( 1/K_d \) and \( K_{FQ} \) were observed in this study (Fig. 6.8), indicating that higher binding of tea polyphenols with PPA

111
corresponded to higher binding of PPA with starch granules in mixtures of PPA, tea polyphenols and starch. This may arise from additional PPA that is carried onto the starch by tea polyphenols.
Figure 6.7. Scatchard plots for binding of PPA with starch granules in the absence and presence of tea polyphenols.

![Scatchard plots](image)

\[\frac{1}{K_d} (\text{mL/mg}) \quad \frac{1}{K_{ic}} (\text{mL/mg})\]

\[y = 7.6672x + 2.0918 \quad R^2 = 0.9685\]

Both \(k_{obs}\) and \(t_{1/2}\) can indicate the binding rate of PPA with starch, and higher \(k_{obs}\) or lower \(t_{1/2}\) values correspond to higher binding rate. Therefore, the tea polyphenols that bind to PPA (ECG, EGCG, TF, TF1, TF2 and TA) promoted the PPA binding process with starch granules. Theaflavins (TF, TF1 and TF2) have been reported to have more binding affinity to
α-amylase than catechins (ECG and EGCG) (Chapter 3 and 4), indicating that at the same concentration of these polyphenols, more theaflavins bind (or interact) with PPA than catechins (this can be suggested by the higher $1/K_{ic}$ and $K_{FQ}$). Furthermore, theaflavins were shown to have higher binding affinity to starch than catechins (Fig. 6.5). Therefore, both the higher binding affinity of theaflavins to PPA and to starch than catechins may cause their greater increasing effects on the binding of PPA with starch (Table 6.1).

For different starches, the binding of α-amylase with starch is one of the factors affecting the catalytic hydrolysis rate of starch (Warren, Royall, Gaisford, Butterworth, & Ellis, 2011). It should be noted that starch in the PPA catalytic activity studies was fully-cooked starch, which have relatively plentiful accessible binding structures for α-amylase (Jane, Chen, Lee, McPherson, Wong, Radosavljevic, et al., 1999; Hoover & Zhou, 2003). The binding of gelatinized starch and α-amylase is limited by the enzymic catalytic activity, not availability of binding sites (Buisson, Duee, Haser, & Payan, 1987). Therefore, it is not surprising that although tea polyphenols were found to increase binding of PPA with starch (Fig. 6.6), they could decrease the hydrolysis rate of cooked starch (Chapter 3) due to the inhibition of PPA catalytic activity. In addition, although tea polyphenols could increase the binding of the enzyme at 0 °C, the hydrolysis of the uncooked starch was still delayed (the digestion rate was very low, Fig. S6.5), indicating that the inhibitory effect was greater than the binding increasing effect of the polyphenols.

6.5. Conclusion

Tea polyphenols were shown to increase the binding of PPA with starch granules at 0 °C (i.e. in the effective absence of hydrolysis), with the relative strength of the effect scaling with the inhibitory activity of individual polyphenols against the enzyme. The dissociation constant ($K_d$) and half-lives ($t_{1/2}$) for PPA binding with starch granules were decreased by the polyphenols, while the observed rate constant ($k_{obs}$) was increased accordingly, confirming that the tea polyphenols could promote the binding of PPA with starch. Theaflavins were shown to have higher PPA binding promotion effects than catechins. The more the binding of tea polyphenols with PPA (indicated by $1/K_{ic}$ and $K_{FQ}$), the more PPA binding (indicated by $1/K_d$) was observed. Tea polyphenols could be adsorbed directly by the starch as well. The
greater PPA binding with starch in the presence of tea polyphenols may be attributed to
bound polyphenols enhancing the binding of enzyme to starch. Despite the greater amount of
bound PPA on starch granules, starch digestion was inhibited showing that polyphenol
inhibition of PPA still occurs even when PPA is bound to starch granules.
Chapter 7: GENERAL CONCLUSIONS AND FUTURE WORKS

7.1. General conclusion of the thesis

The data in this thesis show that tea extracts (GTE, BTE and OTE) and pure tea polyphenols inhibit the activity of porcine pancreatic α-amylase, with potential effects on delayed starch digestion. Through inhibition kinetics analysis, the three tea extracts, TA, TF2 and EGCG were found to be competitive inhibitors, while TF, TF1 and ECG were mixed-type inhibitors of the enzyme. The positive linear relationships between IC_{50} and K_{ic}, and between IC_{50} and K_{iu} indicate that the inhibition can be attributed to the binding of polyphenols with the enzyme. Tea polyphenols could quench the fluorescence of α-amylase, and a higher quenching effect corresponded to a stronger inhibitory activity. Through the thermodynamic binding analysis of isothermal titration calorimetry, the equilibrium of competitive inhibition was shown to result from the binding of particularly galloylated polyphenols with specific sites on α-amylase. There were positive linear correlations between 1/K_{ic}, K_{FQ} and K_{ITC}, indicating that the combination of inhibition kinetics, FQ and ITC can reasonably characterize the binding interactions between tea polyphenols and α-amylase. In addition, the galloyl moiety is an important group in catechins and theaflavins in terms of binding with and inhibiting the enzyme; therefore, galloyl substitution should be considered in the extraction and synthesis of pharmaceutical ingredients for type II diabetes. Interestingly, the red-shift of maximum emission wavelength of α-amylase bound with some tea polyphenols in the fluorescence quenching spectra as well as the decreased thermostability of the enzyme with these polyphenols in the DSC thermograms indicate that the interactions between these tea polyphenols and α-amylase could induce a partial structural unfolding of the enzyme.

The effects of three soluble polysaccharides (OG, WAX and OBG) on the inhibitory activity of tea polyphenols against α-amylase were also studied in this thesis. It is shown that the three soluble polysaccharides could increase the IC_{50} and K_{ic}, while decreasing the K_{FQ} values of the tea polyphenols investigated, indicating that they were able to weaken the inhibitory activity of the polyphenols. The reducing effects of the soluble polysaccharides on α-amylase inhibition were proposed to result from the interactions between polysaccharides
and polyphenols that competitively decreased the binding interactions between polyphenols and the enzyme. Through the establishment of correlations between $K_{ic}$ and $IC_{50}$ and between $K_{FQ}$ and $1/K_{ic}$, the combination of inhibitory activity, inhibition kinetics and FQ can reasonably characterize the effects of soluble polysaccharides on $\alpha$-amylase inhibition by tea polyphenols. To extract the phenolic components which have inhibitory activity against $\alpha$-amylase from plants or foods, as well as to evaluate the inhibitory effects of polyphenols in the digestive tract, some food components, such as soluble polysaccharides should be taken into account because of their potential decreasing effects on the enzyme inhibition of polyphenols.

Granular starch digestion includes two steps, i.e., binding of the enzyme with starch granules followed by the catalytic hydrolysis of starch. Tea polyphenols have been shown to delay the hydrolysis process of starch; therefore, the effects of the polyphenols on the binding of $\alpha$-amylase with starch granules were also investigated in this thesis. It is shown that only the polyphenols that had the inhibitory activity against $\alpha$-amylase could increase the binding ratio of the enzyme with starch granules. This led to the proposal of a binding equilibrium between polyphenols, starch granules and $\alpha$-amylase. The $K_d$ and $t_{1/2}$ for $\alpha$-amylase binding with starch granules were decreased, while $k_{obs}$ was increased by tea polyphenols, confirming that the polyphenols could promote the binding of $\alpha$-amylase with starch. Interestingly, the more the binding of tea polyphenols with $\alpha$-amylase was, the more $\alpha$-amylase binding with starch was observed. Furthermore, because tea polyphenols could be adsorbed by the starch as well, the more enzyme binding in the presence of tea polyphenols may result from parts of the adsorbed polyphenols carrying the enzyme. However, although tea polyphenols could increase the binding of $\alpha$-amylase with starch granules, the initial digestion velocity of starch granules in the presence of tea polyphenols was still lower than that without polyphenols. This indicates that the inhibition effect of polyphenols on $\alpha$-amylase was more obvious than the promoting effect on the enzyme binding with starch.

In conclusion, as summarised in Fig. 7.1, interactions within the multi-component system of $\alpha$-amylase, polyphenols, polysaccharides and starch have been established in this thesis. The binding interactions between polyphenols and $\alpha$-amylase inhibit starch catalytic digestion, while increasing the binding of $\alpha$-amylase with starch granules due to the bound
polyphenols carrying the enzyme. In addition, the binding interactions between polyphenols and polysaccharides decrease binding between polyphenols and α-amylase because of the competitive binding of polyphenols with α-amylase and polysaccharides.

Foods are typically highly complex systems, containing many kinds of components, like starch, proteins, polyphenols, lipids, polysaccharides, etc., and it is becoming clear from this thesis and other recent work that there are multiple possibilities for food components to interact or bind with each other. Therefore, to analyse metabolic processes or evaluate the active function of specific food components more completely, the interactions with other food components need to be taken into account.

**Figure 7.1.** Interactions in the multi-component system of α-amylase, polyphenols, polysaccharides and starch established in this thesis.

### 7.2. Future works

In this thesis, the inhibitory activity against α-amylase was shown for tea polyphenols in the *in vitro* buffer environment with pH value of 7.4. To further approach the environment *in vivo*, the inhibition of α-amylase by tea polyphenols in the digestive tract environment
established in vitro may be studied. In addition, there are multiple starch-hydrolysing enzymes in digestive tract, like α-amylase, α-glucosidase, maltase, sucrase, etc.; therefore, the inhibitory activity of tea polyphenols on the multiple enzyme system in vitro is of interest. Furthermore, the application of tea polyphenols has shown the potential to control postprandial hyperglycaemia in this thesis. However, the practical effect of tea polyphenols on postprandial blood sugar level in vivo is still unknown; therefore, future works may be performed on the effect in vivo (mice or human) and its mechanisms.
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Figure S3.1. Initial reaction velocity at different concentrations of starch in the absence or presence of BTE (A), OTE (B), TA (C), EGCG (D), TF1 (E) and TF (F). Various concentrations of these inhibitors are indicated in the respective legend entries. PBS buffer means starch digestion without the inhibitors.
Figure S3.2. Dixon and Cornish-Bowden (insets) plots for PPA inhibition by BTE (A), OTE (B), TA (C), EGCG (D), TF1 (E) and TF (F). Various concentrations of starch solution are listed in the respective legend entries.
Figure S3.3. Lineweaver-Burk plots for PPA inhibition by BTE (A), OTE (B), TA (C), EGCG (D), TF1 (E) and TF (F). Various concentrations of these inhibitors are indicated in the respective legend entries. PBS buffer means starch digestion without the inhibitors.
**Figure S5.1.** Dixon and Cornish-Bowden (insets) plots for PPA inhibition by tea polyphenols in the absence and presence of three polysaccharides.
Figure S5.2. Fluorescence quenching of PPA by tea polyphenols in the absence and presence of three polysaccharides.
Figure S5.3. The correlation between $1/K_{ic}$ and IC$_{50}$ for BTE (A), EGCG (B), TF (C), TF1 (D) and TA (E), and that between $K_{FQ}$ and IC$_{50}$ for BTE (F), EGCG (G), TF (H), TF1 (I) and TA (J) in the absence and presence of three polysaccharides. ‘NP’ means no polysaccharides.
Figure S6.1. A flow diagram showing how the binding of PPA with raw starch granules in the absence or presence of tea polyphenols is determined through the initial reaction velocity of cooked starch. Because there is a positive linear relationship between the initial reaction velocity and the content of PPA, and the linear curve is through the origin, the percentage of PPA binding with starch can be calculated as the equations in the diagram. Here, $v_0$ and $v_0'$ are the initial reaction velocity before binding in the absence and presence of tea polyphenols, respectively. $v_1$ and $v_1'$ are the initial reaction velocity after binding in the absence and presence of tea polyphenols, respectively.
Figure S6.2. Correlation between the initial reaction velocity of PPA and content of PPA (A) and that between the maximum fluorescence intensity and content of the enzyme (B).
Figure S6.3. The plots of binding of PPA with starch granules against time in the absence (A) and presence (B-G) of tea polyphenols. The curves in this figure were fitted based on equation (6.5).
**Figure S6.4.** The plots of 1/E\(_{\text{bound}}\) against 1/[S] in the absence and presence of tea polyphenols. The equations were fitted with the straight transform of equation (6.3).
Figure S6.5. The initial reaction velocities of raw starch digestion by PPA in the absence and presence of 0.04 mg/mL TA. For the determination, 1 mL of PBS or 0.04 mg/mL TA was mixed with 2 mL PPA for 15 min at 0 °C, followed by the addition of 2 mL of 10 mg/mL raw starch suspension. The digestion was carried out at 37 °C, and the initial reaction velocity was determined by use of the method described in Chapter 3.