Investigation the interaction between procyanidin dimer and α-amylase: Spectroscopic analyses and molecular docking simulation

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1. Introduction

Diabetes is one of the most significant public health issue in the world. According to the statistics from the International Diabetes Federation, diabetes currently affects 425 million adults worldwide and is set to affect close to 700 million people by 2045 [1]. Type 2 diabetes is the most prevalent among diabetes mellitus, and it has been linked to over consumption of foods rich in digestible carbohydrates, such as starch [2]. Generally, dietary carbohydrate digestion is mediated by digestive enzymes such as α-amylase which can rapidly convert digestible starch into maltose. Rapidly digested and absorbed dietary carbohydrates result in a rapid increase in the postprandial blood glucose level. For diabetic patients, the high blood glucose level after a meal face a challenge for managing meal-associated hyperglycemia. However, the dietary carbohydrate digestion and increase of the blood glucose concentration can be inhibited, once the carbohydrate digestive enzymes are inhibited.

Currently, some anti-diabetic drugs available for inhibiting α-amylase, including acarbose, voglibose and miglitol, but these chemical drugs may cause undesirable side effects, such as adverse gastrointestinal symptoms and liver toxicity [3]. Thus, there is a considerable research interest in natural phytochemicals to inhibit the activity of α-amylase [4]. Studies have shown that polyphenolic compounds from plant-based foods or supplements have excellent inhibitory capacity to α-amylase [5]. Proanthocyanidins (PAs) are the most abundant phenolic compounds except lignin [6]. These phenolic compounds are oligomers and polymers of flavan-3-ol monomer units (catechin/epicatechin, afzelechin/epiafzelechin, gallocatechin/epigallocatechin) and the linkage between the different units may be an A-type linkage (containing an ether bond in addition to the C–C bond) or a B-type linkage (C–C bond between monomers) [7–9]. The α-amylase inhibitory capacities of PAs from berry [10], longan pericarp [11], and sorghum [12], red rice [13] have been demonstrated. Numerous studies generally indicated that PAs with higher mean degree of polymerization (mDP) have better α-amylase inhibitory ability than that of lower mDP [14–16]. In addition, B-type oligomeric proanthocyanidins have also exhibited excellent inhibition of α-amylase [13,17,18]. However, these PAs extract are a mixture with different linking type and polymerization degree, the influence of PAs structure on to α-amylase inhibitory ability is not clear. The effect of other active compounds among PAs exact to inhibition of α-amylase is also unclear. Hence, it is necessary to investigate how PAs bind to PPA and the interaction mechanism between PAs and PPA.

In this research, the interaction between PPA (porcine pancreatic α-amylase) and PB2 compound (epicatechin unit and B-linked dimer) were investigated. Spectroscopic methods including fluorescence, circular dichroism, and ultraviolet in combination with molecular docking techniques were employed. This research can offer new insights into the mechanism of PB2 in inhibiting PPA catalysis and provide useful information on dietary recommendation of PB2 for the treatment of type 2 diabetes.
2. Materials and methods

2.1. Materials

The porcine pancreatic α-amylase (PPA, Type VI-B, molecular weight: 51–54 KDa) and 1-anilinonaphthalene-8-sulfonic acid (ANS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). B-type procyanidins dimer (PB2, CAS No: 29106-49-8, HPLC purity: 98.54%) was obtained from Aladdin Industrial Inc. (Shanghai, China). Phosphate buffer solution (PBS) was purchased from Solarbio (Beijing, China). PPA powder was dissolved in PBS (10 mmol/L, pH 6.9) to obtain stock solution (5 mg/mL), and further diluted into 1 mg/mL. PB2 solution were also prepared in PBS (10 mmol/L, pH 6.9), and further diluted into various concentrations ranging from 0.05 to 1.73 mmol/L (0.05, 0.11, 0.21, 0.43, 0.86, and 1.73 mmol/L). The PPA and PB2 stock solutions were filtered through 0.45 μm filters (WondaDisc MCE, Shimadzu-gl) before analysis to remove any large particles. Ultrapure water was used throughout the study.

2.2. Fluorescence spectrum measurements

The fluorescence spectrum at 298, 308 and 318 K were applied to estimate the interaction of PPA with PB2 using a fluorescence spectrophotometer (F-7000, Hitachi, Tokyo, Japan) equipped with 1.0 cm pathlength quartz cell and thermostat bath. The formation of PPA-PB2 complexes was characterized by the fluorescence quenching method previously reported [19] with modifications. The excitation wavelength was set at 295 nm with the width of the excitation and emission slit both adjusted at 2.5 nm and the emission spectra were collected from 300 to 450 nm. Briefly, 0.2 mL of different concentrations (0.05–1.73 mmol/L) of PB2 solution was added into 3 mL 1 mg/mL PPA solution. The maximum fluorescence intensity was used to calculate the binding constant. All of the mixtures were held for 30 min to equilibrate before measurements. Fluorescence intensity was measured, and PB2 solution was used as blank to correct background of fluorescence. To exclude the influence of inner filter effects in UV–vis absorption, the following equation was used to correct fluorescence [20]:

\[ F_c = F_m 10^{(A_1 + A_2)/2} \]

where \( F_c \) and \( F_m \) stand for the corrected and measured fluorescence, respectively; \( A_1 \) and \( A_2 \) represent the absorbance of PB2 at excitation and emission wavelengths, respectively.

2.3. Surface hydrophobicity measurements

The surface hydrophobicity was determined by the hydrophobicity fluorescence probe ANS using a fluorescence spectrophotometer (F-7000, Hitachi, Kyoto, Japan). The excitation slit was 5.0 nm and emission slit was 2.5 nm. The excitation wavelength was 390 nm. 16 μL 8 mmol/L ANS was added into 3.2 mL PPA-PB2 mixture solution (3.0 mL 5 mg/mL of PPA solution with 0.2 mL various concentrations of PB2). PB2 solution was used as blank to correct background of fluorescence. The surface hydrophobicity was remarked as the relative ANS-fluorescence intensity.

2.4. Circular dichroism measurements

Conformational changes during PPA-PB2 interaction were investigated by circular dichroism spectroscopy. The far-UV CD spectra of PPA were measured on CD spectrometer (Bio-Logic MOS 450, Claix, France) according to method previously reported [19]. All spectra of PPA in the presence of PB2 were recorded between 190 and 250 nm under constant nitrogen flush at room temperature after subtracting the background of PB2. The \( \alpha \)-helix, \( \beta \)-sheet, \( \beta \)-turn, and random coil of PPA were analyzed from CD spectroscopic data by the online CONTIN method in DICHROWEB. The Website: (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml).

Fig. 1. (A) Effect of the inner-filter effect on the fluorescence of PPA in a 1 × 1 cm cuvette. Absorbance values of the highest added concentrations are 0.11 mmol/L. Effect of PB2 on the intrinsic fluorescence spectrum of PPA at 298 K (B), 308 K (C), and 318 K (D). (E) The Stern-Volmer plots for the quenching of PPA by PB2 and the \( K_q \) of PPA-PB2 complex in 298 K, 308 K, and 318 K, respectively; (F) The plots for the static quenching of PPA by PB2 (298 K, 308 K, and 318 K), \( C_{PPA} = 1 \text{ mg/mL} \); \( C_{PB2} = 0 \text{ to } 0.108 \text{ mmol/L} \).
2.5. UV spectra determination

UV spectrum were recorded on a spectrophotometer (TU-1901, Persee, Beijing, China) according to method of Cai et al. [12] with some modifications. In brief, 0.2 mL of different concentrations (0.05–1.73 mmol/L) of PB2 solution was added into 3 mL 1 mg/mL PPA solution. The absorption spectrum were recorded after 30 min, and collected from 240 to 330 nm. The appropriate blank responding to the PB2 solution was subtracted to correct background.

2.6. Molecular docking simulation

Molecular docking simulation was performed as [21] with some modification, using Libdock algorithm in Discovery Studio 3.0 (Accelrys Inc., USA). The 3D structure of PPA (PDB: 1DHK) was obtained from the Protein Data Bank (http://www.rcsb.org/pdb). The 3D structure of PB2 was prepared by Chemoffice software, using the MMFF94 force field for energy minimization. After the PPA and PB2 were imported into Discovery Studio, the water molecules in PPA were removed, whereas all hydrogen atoms were added to the PPA file according the “Prepare Protein” function at pH 6.9, temperature 25 °C. Then, the PPA structure was minimized by the algorithm of smart minimizer. The PB2 was optimized according the “Prepare Ligands” function at pH 6.9. Finally, the optimal structure of PPA and PB2 were given to the CHARMm force field. The docking site was defined from the recorded active site. Interaction between identified PPA and PB2 protein structure of 1DHK were observed in the software Discovery Studio 2017 R2.

2.7. Statistical analysis

The statistical analyses were performed using SPSS 16.0 (IBM Inc., USA). The mean values were compared by Tukey’s test at a 5% level of significance using one-way ANOVA.

3. Results and discussion

3.1. Fluorescence emission spectrum

Fluorescence emission spectrum was applied to explore the interaction of PB2 and PPA. PPA consists of 496 amino acid residues, forming a polypeptide chain with 17 tryptophan residues provided intrinsic fluorescence [12]. Thus, the excitation wavelength (295 nm) was selected to measure the fluorescence of PPA. The inner-filter effect refers to the absorption of excitation or emission the fluorophore [22]. The filter effect must be considered before using fluorescence data, firstly. From Fig. 1A, inner filter effect can be excluded because of low absorption for maximum concentration of PB2 (0.11 mmol/L) at the excitation (295 nm) and emission (345 nm) wavelengths. The values were 0.039 (lower 0.1) and 0 at excitation and wavelengths emission, respectively. Thus, fluorescence was not necessary for calibration according to Eq. (1) [22].

The fluorescence spectra of PPA in the presence of PB2 at different concentrations are shown in Fig. 1B (298 K), Fig. 1C (308 K), Fig. 1D (318 K), respectively. It can be observed that PPA exhibited a strong fluorescence emission band at 345 nm. The fluorescence intensity of PPA decreased significantly after addition of PB2, without any distinct shift at maximum band wavelength. This indicated that PB2 had a quenching effect on the intrinsic fluorescence of PPA which may be attributed to the bind of PB2 and PPA molecules to form complex and change the micro-environment of the tryptophan residues [23]. Many studies also found that the fluorescence intensity of enzyme decreased by increasing the phenolic content due to the interaction between digestive enzymes (α-amylase, α-glucosidase) and polyphenols (procyanidin [12], apigenin [24], kaempferol [25]) and forming complexes.

3.2. The fluorescence quenching mechanism and binding constant

Generally, there are several potential factors of quenching, such as the inner-filter effect, dynamic quenching, and static quenching [22]. If the quenching is static, the quenching constant will decrease with the increasing temperature, as the stability of the protein-polypeptide complex will decrease with increasing temperature. On the contrary, for dynamic quenching, the quenching constant increases with the increase of temperature due to the collision effect [26]. The PPA fluorescence quenching data was analyzed using the Stern-Volmer Eq. (2) at three different temperatures (298, 308, and 318 K), which can confirm the type of fluorescence quenching mechanism [27].

$$\frac{F_0}{F} = 1 + K_{SV} [Q] = 1 + K_{SV} [Q]$$

where $F_0$ and $F$ are the fluorescence intensities without and with PB2, respectively; [Q] is the PB2 concentration; $K_{SV}$ is the Stern-Volmer quenching constant; $K_q$ is the biomolecular quenching rate constant and $\tau_0$ is the average lifetime of the biomolecule without a quencher ($\tau_0 = 10^{-8} \text{s}$) [28].

From Fig. 1F, the Stern-Volmer plot showed good linearity at different temperatures, suggesting that the quenching mechanism is either a dynamic or static procedure [29]. The $K_{SV}$ ($K_{SV} = K_q \tau_0$) values [1.60 × 10^{-9} (298 K), 1.48 × 10^{-9} (308 K), and 1.18 × 10^{-9} M^{-1} (318 K)] were found to decrease gradually as the temperature rose, indicating the fluorescence quenching induced by the formation of PPA-PB2 complex (static type). The biomolecular quenching rate constant ($K_q$) was also higher than the maximum diffusion collision quenching constant.

Table 1

<table>
<thead>
<tr>
<th>T/K</th>
<th>Regression equation</th>
<th>$R^2$</th>
<th>$K$ ($×10^1$ L·mol⁻¹)</th>
<th>$n$</th>
<th>$\Delta G$ (kJ·mol⁻¹)</th>
<th>$\Delta H$ (kJ·mol⁻¹)</th>
<th>$\Delta S$ (kJ·mol⁻K⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>298</td>
<td>$\log\left[ \frac{F_0}{F-1} \right] = 1.5678 + 0.5989 \log[\text{PB2}]$</td>
<td>0.947</td>
<td>0.37</td>
<td>0.60</td>
<td>-10.32</td>
<td>99.94</td>
<td>0.37</td>
</tr>
<tr>
<td>308</td>
<td>$\log\left[ \frac{F_0}{F-1} \right] = 2.2014 + 0.7544 \log[\text{PB2}]$</td>
<td>0.990</td>
<td>1.59</td>
<td>0.75</td>
<td>-14.02</td>
<td>99.94</td>
<td>0.37</td>
</tr>
<tr>
<td>318</td>
<td>$\log\left[ \frac{F_0}{F-1} \right] = 2.6682 + 0.8805 \log[\text{PB2}]$</td>
<td>0.966</td>
<td>4.66</td>
<td>0.88</td>
<td>-17.72</td>
<td>99.94</td>
<td>0.37</td>
</tr>
</tbody>
</table>

**Fig. 2.** Changes in ANS-fluorescence intensity of PPA at different concentrations of PB2. $C_{PPA} = 5$ mg/mL; $C_{PB2} = 0$ to 108.03 μmol/L.
value \((2.0 \times 10^{10} \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1})\), which indicated that the quenching type was typical static quenching \[30\].

For the static quenching, the binding constant \((K_a)\) of the interaction between PPA and PB2 and the number of binding sites per protein \((n)\) could be calculated according to a double logarithmic Eq. (3) \[20\]:

\[
\log \frac{F_0 - F}{F} = \log K_a + n \log [Q]
\]

(3)

In Fig. 1F, the high linear correlation coefficient \((R^2 > 0.98)\) at different temperatures indicated the assumptions underlying the derivation of Eq. (3) were valid. The \(n\) values were all approximately equal to 1 suggesting that PPA had a single class of binding sites for PB2. All the obtained experimental results are summarized in Table 1. The \(K_a\) values [0.37 \times 10^2 (298 K), 1.59 \times 10^2 (308 K), and 4.66 \times 10^2 M^{-1} (318 K)] of PPA-PB2 complexes increased with increasing temperature. The calculated \(K_a\) values of PPA-PB2 complexes are lower than of PPA-sorghum procyanidins trimer complexes \((K_a = 4.95 \times 10^2 \text{ M}^{-1})\) \[12\], suggested that there are a moderate affinity between PPA and PB2. Ho et al. \[18\] also reported that B-type procyanidins trimer have the better inhibitory ability to \(\alpha\)-amylase than B-type procyanidins dimer. It can be speculate the B-type procyanidins with higher mDP have better inhibitory and binding ability to \(\alpha\)-amylase. Numerous studies also had indicated that PAs polymers showed a stronger \(\alpha\)-amylase inhibitory activity than that of PAs oligomers \[15,16\].

3.3. Thermodynamic parameters

The thermodynamic parameters were calculated to ascertain the main forces contributing to ligand-protein stability. The main interaction forces between small molecules and proteins may be hydrogen bonding, hydrophobic interactions, and electrostatic forces \[31\]. When \(\Delta S > 0, \Delta H > 0\), hydrophobic forces dominate; when \(\Delta S < 0, \Delta H < 0\), hydrogen bonds dominate; and when, \(\Delta S > 0, \Delta H < 0\) electrostatic forces is

![Fig. 3.](image)
involved in the interaction [32]. The entropy change (ΔS), enthalpy change (ΔH) and free energy change (ΔG) can be obtained by using Van’t Hoff equation [24].

\[
\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}
\]

\[
\Delta G = \Delta H - T \Delta S
\]

Here \(K_a\) is the binding constant obtained from Eq. (3) at the corresponding temperature, \(R\) is the gas constant (8.314 J·mol\(^{-1}\)·K\(^{-1}\)) and \(T\) is the absolute temperature (298, 308, and 318 K). The \(\Delta S\) and \(\Delta H\) values were obtained from the intercept and slope of the linear Van’t Hoff plot based on \(\ln K_a\) versus \(1/T\). The free energy change (\(\Delta G\)) was calculated from Eq. (5).

From Table 1, the negative \(\Delta G\) indicated that the binding process of complex was spontaneous. The \(\Delta S\) and \(\Delta H\) values for the binding interaction between PB2 and PPA were 366 J·mol\(^{-1}\)·K\(^{-1}\) and 99.94 kJ·mol\(^{-1}\), respectively. Both \(\Delta S\) and \(\Delta H\) values were >0, which indicated that the main force acting between PPA and PB2 was hydrophobic interaction and the binding process was entropy driven. This result is consistent with other studies, which have reported that the driving forces for the binding of flavonoids (such as apigenin [24], pelargonidin [33], and anthocyanin [14]) to globular proteins (\(\alpha\)-glucosidase, and \(\beta\)-lactoglobulin) were mainly hydrophobic interactions.

3.4. Protein surface hydrophobicity

The surface hydrophobicity of PPA was characterized by relative ANS-binding fluorescence intensities of PPA with PB2. ANS was utilized to explore conformational changes in PPA and characterized surface...
exposure of hydrophobic sites [34]. In Fig. 2, the surface hydrophobicity for PPA decreased progressively with increasing amount of PB2 from 0 to 108.03 μmol/L. The reduction in surface hydrophobicity of PPA-PB2 complexes speculated that the polarity increased and the surrounding solution polarity changed. The results are as reported by Jia et al. [34], who reported that the surface hydrophobicity of β-lactoglobulin-polyphenols (chlorogenic acid/EGCG) decreased and the forming of complexes might destroy the originally hydrophobic structure of protein and expose hydrophobic regions on PPA surface.

3.5. Circular dichroism spectra

Circular dichroism (CD) was utilized to monitor changes in the secondary structure (α-helix, β-sheet and β-turn) of PPA. The CD spectra of PPA in the absence and presence of PB2 are shown in Fig. 3A. The values of negative ellipticity increased with increasing PB2 concentration, indicating partial changes in the PPA secondary structure. The contents of secondary structures in PPA were calculated in Fig. 3A. The studies showed that free PPA contained around 12.7% α-helix, 7.0% β-sheet, 34.3% β-turn, and 46.0% random coil. After interaction with 108 μmol/L PB2, the contents of α-helix and β-turn increased to 34.7% and 12.7%, respectively, while the contents of β-sheet (27.7%) and random coil (24.9%) significantly decreased. Shu et al. [35] reported that erucic acid inserted into the hydrophobic surface of bovine serum albumin, which resulted in an increase of α-helix content. Xu et al. [36] also reported that lignin molecules inserted into the hydrophobic surface of gelatin which resulted in an increase in the amount of α-helix. Thus, it can be speculate that an increase in the amount of α-helix was caused by PB2 that inserted into the hydrophobic surface of PPA. The results correlated well with the analysis of surface hydrophobicity.

3.6. UV spectrum

UV spectra of protein can be utilized to analyze the PPA structural changes and interaction [37]. Fig. 3B showed the ultraviolet spectra of PPA interacted with PB2 in concentrations ranging 0–108.03 μmol/L. The absorption peak at 275 nm of PPA due to π–π transition of peptide bond C=O group in tryptophan (Trp) and tyrosine (Tyr) aromatic [12]. The absorption of PPA successively increased with the increase of PB2 concentration (3.38 to 108.03 μmol/L), followed by a red shift. Similar results were shown in study of Cai et al. [12], who pointed that the increase of UV spectrum of PPA in the presence of sorghum procyanidins revealed the binding interaction between PPA and procyanidins. The results can also confirm the fluorescence quenching in the interaction of PPA with PB2.

3.7. Computational docking simulation

The computational docking (LibDock) simulation was applied to predict the precise binding sites of PB2 in RG and to confirm the experimental results described above. LibDock is a high throughput docking algorithm that positions catalyst generated ligand conformations in the protein active site, based on polar interaction sites (hotspots) [38]. Based on the LibDock protocol, the pose which have the highest Libdock score (110.327) corresponding to the most stable conformation, was selected for analysis about the interaction between PPA and PB2.

Combined 3D docking mode (Fig. 4A, B) and 2D schematic diagram (Fig. 4C), which can show clearly that PB2 inserted into the cavity of PPA. From Fig. 4C, PB2 interacted with amino acid residues including Lys 200, Ile 235, Val 234, Ser 199, Ala 198, Asp 197, Glu 233, Arg 195, His 201, Asp 300, Leu 162, Tyr 151, Val 163, Ile 148, Gly 147, Glu 161 and Gly 164, which were possible interaction sites between PB2 and PPA. Four hydrophobic interaction forces (one Pi–Pi T-shaped, there Pi-Alkyls) were found between PB2 and amino acid residues (His 201, Ile 235, Val 163, Ile 148). Due to the PB2 have many hydroxyl group, two hydrogen bonds also were found between PB2 and amino acid residue (Tyr 151, Ile 148). The distance of two hydrogen bonds (5.36 Å, 3.94 Å) and hydrophobic forces (6.02 Å, 5.90 Å, 5.41 Å, 4.98 Å) were showed in Fig. 4C. The main interaction force was hydrophobic interaction force which agreed with the result of thermodynamic parameter. From Fig. 4A, the PB2 located in the active site, which included Asp 167, Asn 100, Arg 158, His 201. Specially, His 201 interacted with PB2 through the hydrophobic interaction forces and thus inhibited the activity of PPA.

4. Conclusion

This study demonstrated that bioactive flavonoids (procyanidin dimer) interacted with α-amylase using chemical methods in combination with molecular docking techniques. The fluorescence quenching of PPA was attributed to the formation of a stable PPA-PB2 complex. Hydrophobic interaction dominated the interaction process between PPA and PB2. The CD results showed that PPA-PB2 complex can induce changes in the secondary structure of PPA. The molecular docking simulation provided valuable information about the binding of the PB2 to the PPA surface. This research provides new insights into the inhibitory mechanism of PB2 on α-amylase.

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References
