Inhibitory kinetics and mechanism of kaempferol on α-glucosidase

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Abstract
α-Glucosidase is a therapeutic target for diabetes mellitus, and α-glucosidase inhibitors play a vital role in the treatments for the disease. As a kind of potentially safer α-glucosidase inhibitor, flavonoids have attracted much attention currently. In this study, kaempferol was found to show a notable inhibition activity on α-glucosidase in a mixed-type manner with IC50 value of (1.16 ± 0.04) × 10⁻⁴ mol L⁻¹. Analyses of fluorescence, circular dichroism and Fourier transform infrared spectra indicated that kaempferol bound to α-glucosidase with high affinity which was mainly driven by hydrogen bonds and van der Waals forces, and this binding resulted in conformational alteration of α-glucosidase. Further molecular docking study validated the experimental results. It was proposed that kaempferol may interact with some amino acid residues located within the active site of α-glucosidase, occupying the catalytic center of the enzyme to avoid the entrance of p-nitrophenyl-α-D-glucopyranoside and ultimately inhibiting the enzyme activity.

1. Introduction
Glycemic control is considered as an effective therapy for individuals with diabetes mellitus, in particular, non-insulin-dependent diabetes mellitus. It can reduce the risk of the serious neurological and cardiovascular complications through the inhibition of carbohydrate hydrolyzing enzymes, such as α-glucosidase in the epithelium of small intestine (Schmidt, Nyberg, & Stærk, 2014). This enzyme plays a vital role in maintaining the normal physiological function and participates in carbohydrate metabolism that specifically hydrolyzes the α-glucopyranoside bond to release α-glucose from the non-reducing end of the sugar (Lordan, Smyth, Soler-Vila, Stanton, & Ross, 2013). Effective α-glucosidase inhibitors can significantly retard the dietary complex carbohydrate digestion and decrease the postprandial effect of starch consumption on blood glucose levels (Zhang et al., 2013). Acarbose, miglitol and voglibose widely used in clinic were validated to exert anti-postprandial hyperglycemia effect by inhibiting α-glucosidase activities. Unfortunately, they are often reported to cause diarrhea and flatulence, with corresponding abdominal pain and liver disorders, which is the most common for noncompliance (Feng, Yang, & Wang, 2011). Thus, finding new α-glucosidase inhibitors with minor side effects is of great importance.

Natural products of great structural diversity are considered as a good source for screening α-glucosidase inhibitors (Kumar, Narwal, Kumar, & Prakash, 2011). As a kind of natural α-glucosidase inhibitors widely existed in various foods and plants, flavonoids have attracted much attention. Previous findings have shown that some flavonoids significantly inhibited α-glucosidase. For example, Yan et al. have reported that luteolin reversibly inhibited α-glucosidase with an IC50 value of (1.72 ± 0.05) × 10⁻⁴ mol L⁻¹ through a multi-phase kinetic process (Yan, Zhang, Pan, & Wang, 2014). Naringenin showed a strong α-glucosidase inhibitory activity in a competitive manner with inhibition constant of 3.17 × 10⁻⁴ mol L⁻¹ (Priscilla, Roy, Suresh, Kumar, & Thirumurugan, 2014). Kaempferol (structure shown in Fig. 1A) is a kind of natural flavonoids rich in broccoli, cabbage, beans, tomato, strawberries and apple. Studies found that kaempferol exerted extensive biological activities, including the antioxidant, anti-inflammatory, antibacterial, and antitumor activities (Chen & Chen, 2013). In recent years, kaempferol extracted from guava leaves has been reported to show a potent inhibitory activity on α-glucosidase with IC50 value of 5.2 × 10⁻⁴ mol L⁻¹. It exhibited much stronger α-glucosidase inhibitory activity than acarbose with 95.1% inhibition (Phan, Wang, Tang, Lee, & Ng, 2013). However, to our knowledge, the study was limited to the enzymatic activity assay, and no report is available on the inhibition mechanism of kaempferol on α-glucosidase. In the present study, yeast α-glucosidase was used as a model for evaluating the inhibition of α-glucosidase by kaempferol since it is readily...
available in a pure form. Yeast α-glucosidase is usually used for screening α-glucosidase inhibitors and studying the inhibitory mechanism (Chai, Kwek, Ong, & Wong, 2015; Zhang, Hu, & Pan, 2014). Elucidating the inhibition mechanism of kaempferol on yeast α-glucosidase activity may facilitate the clinical applications of kaempferol as an α-glucosidase inhibitor and a food functional ingredient for the therapy of diabetes mellitus and its complications.

The aim of this work was thus to study in vitro inhibitory effect and mechanism of kaempferol on α-glucosidase using a combination of UV–vis absorption, fluorescence, circular dichroism (CD), Fourier transform infrared (FT-IR), kinetic analysis and molecular modeling approaches. Some binding properties between kaempferol and α-glucosidase were characterized and the changes in the microenvironment and the secondary structure of α-glucosidase induced by kaempferol were determined. The present study is expected to improve the understanding of the inhibitory mechanism of kaempferol on α-glucosidase.
2. Materials and methods

2.1. Materials

α-Glucosidase (EC 3.2.1.20, 15.8 units mg\(^{-1}\)) from Saccharomyces cerevisiae was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and its stock solution (6.76 \(\times\) 10\(^{-6}\) mol L\(^{-1}\)) was prepared with sodium phosphate buffer (pH 6.8). Acarbose (analytical grade), kaempferol (analytical grade) and p-nitrophenyl-α-glucopyranoside (pNPG, purity ≥ 99%) were obtained from Aladdin Chemical Co. (Shanghai, China). Both the stock solutions of pNPG (8.09 \(\times\) 10\(^{-4}\) mol L\(^{-1}\)) and acarbose (8.44 \(\times\) 10\(^{-3}\) mol L\(^{-1}\)) were made in sodium phosphate buffer. The stock solution of kaempferol (5.10 \(\times\) 10\(^{-4}\) mol L\(^{-1}\)) was prepared by dissolving in ethanol and diluted with the sodium phosphate buffer. The amount of ethanol used in this study was less than 0.5% (v/v) with no inhibitory effect on α-glucosidase. All other chemicals were of analytical purity or higher and the freshly ultrapure water was used throughout the whole experiments.

2.2. Procedures

2.2.1. Fluorescence spectra measurements

The fluorescence spectra were performed on a Hitachi spectrofluorometer (model F-7000, Hitachi, Japan) at 298, 304 and 310 K over a wavelength range of 290–500 nm. Both the excitation and emission bandwidths were set at 2.5 nm. A 2.0 mL solution, containing 1.01 \(\times\) 10\(^{-6}\) mol L\(^{-1}\) α-glucosidase, was titrated by successive additions of kaempferol solution (to give a final concentration of 6.82 \(\times\) 10\(^{-4}\) mol L\(^{-1}\); Fig. 2a). The fluorescence spectra of these mixed solutions were measured after standing for 5 min to equilibrate. Due to the re-absorption and inner filter arisen from the UV absorption, the fluorescence data were corrected for the absorption of both excited and emitted lights (Wang, Zhang, & Ji, 2014):

\[
F_c = F_m e^{A_1 + A_2} \quad (1)
\]

where \(F_c\) and \(F_m\) denote the corrected and measured fluorescence. \(A_1\) and \(A_2\) are the absorbance of kaempferol at excitation and emission wavelength, respectively. The synchronous fluorescence spectra were achieved by scanning the excitation and emission wavelength which recorded at \(\Delta \lambda = 15\) and 60 nm, and both excitation and emission slit widths were set at 2.5 nm. Three-dimensional (3-D) fluorescence spectra of α-glucosidase and α-glucosidase-kaempferol complex were obtained by using an excitation wavelength range of 200–600 nm and monitoring the emission spectra between 200 and 600 nm.

2.2.2. CD spectra measurements

CD measurements were performed in the presence and absence of kaempferol in the range of 200–250 nm. The concentration of α-glucosidase was fixed on 1.01 \(\times\) 10\(^{-6}\) mol L\(^{-1}\) and the concentration of kaempferol was increased from 0.1 to 2.1. All CD spectra were recorded on a Bio-Logic MOS 450 CD spectrometer (Bio-Logic, Claix, France) using a 1.0 mm path length quartz cuvette in pH 6.8 sodium phosphate buffer. Then the online SELCON3 program (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) was used to analyze the contents of different secondary structures of α-glucosidase.

2.2.3. FT-IR spectra measurements

FT-IR spectra of free α-glucosidase and its kaempferol complex were recorded with a thermo Nicolet-5700 FT-IR spectrometer (Thermo Nicolet Co., USA) at wavenumbers between 1400 and 1800 cm\(^{-1}\) in pH 6.8 sodium phosphate buffer with the resolution of 4 cm\(^{-1}\) and 60 scans and all spectra were taken via the ATR method. The concentration of free α-glucosidase was 1.01 \(\times\) 10\(^{-6}\) mol L\(^{-1}\), and the molar ratio of kaempferol to α-glucosidase was 2:1. The corresponding absorbance values related to free kaempferol and buffer solutions were recorded and subtracted at the same instrumental parameters. FT-IR analysis was used more readily to obtain resolution on secondary structures of α-glucosidase by curve-fitted results of amide I band.

2.2.4. Enzyme activity assay

α-Glucosidase inhibitory activity was determined as reported earlier (Wan, Min, Wang, Yue, & Chen, 2013; Zhang et al., 2014) with a Shimadzu UV-2450 spectrophotometer (Shimadzu, Japan). A fixed concentration of α-glucosidase (1.01 \(\times\) 10\(^{-7}\) mol L\(^{-1}\)) and various amount of kaempferol were mixed to a series of assay solutions with 0.1 mol L\(^{-1}\) sodium phosphate buffers (pH 6.8) in the 2.0 mL reaction system. After 2.5 h pre-incubation at 37 °C, the assay was initiated by adding the substrate pNPG (the final concentration was 2.02 \(\times\) 10\(^{-4}\) mol L\(^{-1}\)). The absorbance of the mixtures was monitored at 405 nm every 20 s at room temperature. Acarbose, a clinically used α-glucosidase inhibitor was used as a positive control. The enzymatic activity assay without inhibitor was defined as 100%. Relative enzymatic activity (%) = (slope of reaction kinetics equation obtained by reaction with inhibitor)/(slope of reaction kinetics equation obtained by reaction without inhibitor) \(\times\) 100%.

2.2.5. Determination of inhibitory type

The mixed-type inhibition was evaluated by Lineweaver–Burk plots and described by the following equations (Hu et al., 2012):

\[
\frac{1}{v} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i}ight) + \frac{1}{V_{max}} \left(1 + \frac{[I]}{\alpha K_i}\right) \quad (2)
\]

Secondary plots can be constructed as

\[
\text{Slope} = \frac{K_m}{V_{max}} + \frac{K_m [I]}{V_{max} K_i} \quad (3)
\]

and

\[
\text{Y-intercept} = \frac{1}{V_{max}} \left(1 + \frac{[I]}{\alpha K_i} \frac{V_{max}}{V_{max}}\right) \quad (4)
\]

where \(K_i\) and \(K_m\) represent the inhibition constant and the Michaelis–Menten constant, respectively, \(v\) is the enzyme reaction velocity; \([I]\) and \([S]\) denote the concentration of inhibitor and substrate, respectively; \(\alpha\) is the apparent coefficient. The replots of slope and Y-intercept versus \([I]\) were linearly fitted, which suggested that there may be a single inhibition site or a single class of inhibition site (Wang, Zhang, Pan, & Gong, 2015). The kinetic data were analyzed using a computer program for linear regressions (Origin 8.0).

2.2.6. Homology modeling and molecular simulation

Due to the lack of the 3D structure of α-glucosidase from yeast, homology modeling of α-glucosidase was carried out to acquire its proper structural template through searching the Protein Data Bank (http://www.ncbi.nlm.nih.gov/protein/) and using BLAST algorithms with the amino acid sequence of the target as input. The homology model was used as the receptor model in the virtual screening with docking simulation (AutoDock version 4.2) (Escandón-Rivera et al., 2012) of kaempferol binding to α-glucosidase. The 3D structure of kaempferol was constructed in Chem3D Ultra 8.0. In the process of docking, the calculated grid maps were of dimension 100 \(\times\) 100 \(\times\) 100 points with the spacing of 0.403 Å.
2.2.7. Statistical analysis

All data were analyzed using SAS statistical package (version 8.0, SAS Institute, Cary, NC, USA). Results were expressed as means ± standard deviation (n = 3). One-way analysis of variance (ANOVA) was performed by using Origin 8.0 followed by multiple tests, in order to determine the significant difference at p < 0.05.

3. Results and discussion

3.1. Binding characteristics between kaempferol and \(\xi\)-glucosidase

3.1.1. Mechanism of fluorescence quenching

The interaction between kaempferol and \(\xi\)-glucosidase was investigated by the fluorescence quenching experiments. Fluorescence quenching is to measure the decrease of the quantum yield of the fluorescence due to molecular interactions like energy transfer, excited state reactions, molecular rearrangements, ground state complex formation and collisional quenching. As shown in Fig. 1A, \(\xi\)-glucosidase displayed an intrinsic fluorescence emission peak at 342 nm after being excited at a wavelength of 280 nm, while kaempferol did not show fluorescence under the same conditions. The fluorescence intensity of \(\xi\)-glucosidase at 342 nm was gradually quenched with increasing the amounts of kaempferol (298 K), suggesting that kaempferol quenched the intrinsic fluorescence of \(\xi\)-glucosidase which is a direct evidence for the interaction between kaempferol and \(\xi\)-glucosidase. The following Stern–Volmer equation was utilized to describe the fluorescence quenching (Bhofale et al., 2013):

\[
\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + K_0 \cdot \tau_0
\]

(5)

\(F_0\) and \(F\) denote the fluorescence intensities of \(\xi\)-glucosidase before and after the addition of kaempferol, respectively; [Q] represents the concentration of kaempferol; \(\tau_0\) (10\(^{-8}\) s) is the lifetime of the fluorophore in the absence of kaempferol; \(K_{SV}\) means the Stern–Volmer quenching constant (\(K_{SV} = K_0 \cdot \tau_0\)); \(K_0\) displays the quenching rate constant of the biomolecule.

As shown in Fig. 1A and Table 1, the values of \(K_{SV}\) decreased with the increase of temperature (298, 304 and 310 K), and the calculated values of \(K_0\) were 1.34 \(\times\) 10\(^{-12}\), 1.10 \(\times\) 10\(^{-13}\) and 1.01 \(\times\) 10\(^{-13}\) mol\(^{-1}\) s\(^{-1}\) which were much greater than 2 \(\times\) 10\(^{-9}\) mol L\(^{-1}\) s\(^{-1}\) (Wang, Zhang, & Wang, 2014), suggesting that the kaempferol–\(\xi\)-glucosidase complex has been formed, and the fluorescence quenching process was predominated by a static quenching mechanism rather than a dynamic (Shahabadi, Maghsudi, Kiani, & Pourfoulad, 2011).

In the linear range of above Stern–Volmer curve, the quenching data were expressed via the following equation (Feroz, Mohamad, Bakri, Malek, & Tayyab, 2013):

\[
\log \frac{F_0 - F}{F} = n \log K_a - \log \left(\frac{[Q]}{[P]}\right) \frac{1}{\tau_0} \frac{F_0 - F}{F}
\]

(6)

In this equation, \([Q]\) and \([P]\) denote the total concentration of the kaempferol and \(\xi\)-glucosidase, respectively. \(K_a\) represents the binding constant for the accessible fluorophores, \(n\) is the number of the binding sites per \(\xi\)-glucosidase molecule. As shown in Table 1, the value of \(K_a\) was inversely correlated with temperatures, which was in accordance with the variation of \(K_{SV}\). The \(K_a\) values were in the order of 10\(^2\) L mol\(^{-1}\), suggesting that a high affinity existed between kaempferol and \(\xi\)-glucosidase. In addition, the values of \(n\) at the experimental temperatures were approximately equal to 1, inferring that there was just a single class of binding sites on \(\xi\)-glucosidase for kaempferol.

3.1.2. Thermodynamic parameters and nature of the binding forces

The interaction between a small ligand and a biomolecule comes into being a supramolecular complex usually by the four main forces: hydrophobic interaction, electrostatic force, hydrogen bond and van der Waals force. The thermodynamic parameters, enthalpy change (\(\Delta H^0\)) and entropy change (\(\Delta S^0\)) of the reaction are related to the binding force, and their values can be determined according to the van’t Hoff equation:

\[
\log K_a = - \frac{\Delta H^0}{2.303RT} + \frac{\Delta S^0}{2.303R}
\]

(7)

\(R\) is the gas constant with a value of 8.314 J mol\(^{-1}\) K\(^{-1}\); \(T\) is the absolute temperature (298, 304 and 310 K). The values of \(\Delta H^0\) and \(\Delta S^0\) were calculated from the slope and intercept of the linear plot of log\(K_a\) versus 1/\(T\). Thus, the value of free energy change (\(\Delta G^0\)) could be calculated as the following:

\[
\Delta G^0 = \Delta H^0 - T\Delta S^0
\]

(8)

As revealed in Table 1, the binding process was spontaneous since the value of \(\Delta G^0\) is below zero. Furthermore, the values of \(\Delta H^0\) and \(\Delta S^0\) were obtained to be \(-32.46 \pm 0.20\) kJ mol\(^{-1}\) and \(-29.68 \pm 0.30\) J mol\(^{-1}\) K\(^{-1}\), respectively. According to the theory of Ross and Subramanian (Ross & Subramanian, 1981), negative \(\Delta H^0\) and \(\Delta S^0\) values were frequently regarded as evidence that the formation of kaempferol–\(\xi\)-glucosidase complex was an exothermic and enthalpy reaction, and both van der Waals forces and hydrogen bonds were the predominant driving forces in the kaempferol–\(\xi\)-glucosidase binding reaction (Kashanian, Khodaei, & Kheirdoosh, 2013).

3.1.3. Energy transfer between kaempferol and \(\xi\)-glucosidase

The quantum yield of \(\xi\)-glucosidase was evaluated by comparing fluorescence intensity of \(\xi\)-glucosidase with human serum albumin (HSA) (standard solution) under same conditions according to the relationship (Bi, Yan, Wang, Pang, & Wang, 2012):

\[
\phi_a = \phi_{at} \frac{F_x}{A_{at}}
\]

(9)

where \(F_x\) and \(F_a\) mean the fluorescence intensities of HSA and \(\xi\)-glucosidase; \(A_{at}\) and \(A_a\) represent the absorption of HSA and \(\xi\)-glucosidase at the excitation wavelength of HSA; \(\phi_{at}\) and \(\phi_a\) are the fluorescence quantum yields of HSA and \(\xi\)-glucosidase, the value of \(\phi_{at}\) is 0.13 (Bi et al., 2012). According to Eq. (9), the quantum yield of \(\xi\)-glucosidase was calculated to be 0.089. The efficiency of energy transfer \(E\) and the distance between \(\xi\)-glucosidase and kaempferol were determined by Förster’s non-radiative energy transfer theory following Eqs. (10)–(12):

\[
E = \frac{F_0 - F}{F_0} = \frac{R_0^6}{R_0^6 + \frac{R_0^6}{r^6}}
\]

(10)

Table 1: Quenching constants \(K_{SV}\), binding constants \(K_a\) and relative thermodynamic parameters of the kaempferol–\(\xi\)-glucosidase interaction at different temperatures.

<table>
<thead>
<tr>
<th>(T (K))</th>
<th>(K_{SV} \times 10^9) L mol(^{-1})</th>
<th>(R^0)</th>
<th>(K_a \times 10^9) L mol(^{-1})</th>
<th>(n)</th>
<th>(R^0)</th>
<th>(\Delta H^0) (kJ mol(^{-1}))</th>
<th>(\Delta G^0) (kJ mol(^{-1}))</th>
<th>(\Delta S^0) (J mol(^{-1}) K(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>298</td>
<td>1.34 ± 0.01</td>
<td>0.9989</td>
<td>1.46 ± 0.01</td>
<td>0.80 ± 0.02</td>
<td>0.9972</td>
<td>-32.46 ± 0.20</td>
<td>-23.61 ± 0.02</td>
<td>-29.68 ± 0.03</td>
</tr>
<tr>
<td>304</td>
<td>1.10 ± 0.01</td>
<td>0.9968</td>
<td>1.22 ± 0.02</td>
<td>0.80 ± 0.01</td>
<td>0.9987</td>
<td>-32.46 ± 0.20</td>
<td>-23.43 ± 0.02</td>
<td>-29.68 ± 0.03</td>
</tr>
<tr>
<td>310</td>
<td>1.01 ± 0.02</td>
<td>0.9962</td>
<td>1.11 ± 0.02</td>
<td>0.79 ± 0.02</td>
<td>0.9980</td>
<td>-32.46 ± 0.20</td>
<td>-23.26 ± 0.02</td>
<td>-29.68 ± 0.03</td>
</tr>
</tbody>
</table>

\(a\) \(R\) is the correlation coefficient for the \(K_{SV}\) values.

\(b\) \(R\) is the correlation coefficient for the \(K_a\) values.
where $F_0$ and $F$ are the same as in Eq. (5). $R_0$ is the critical distance; $r$ is the distance between acceptor (kaempferol) and donor ($\alpha$-glucosidase); $k^2$ denotes the orientation factor, $N$ is the refractive index of the medium, $\phi$ is the fluorescence quantum yield of $\alpha$-glucosidase, and $J$ represents the overlap integral between the donor fluorescence emission spectrum and the acceptor absorption spectrum. Fig. 1B shows the overlapping of fluorescence emission spectrum of $\alpha$-glucosidase with the UV absorption spectrum of kaempferol. For ligand–$\alpha$-glucosidase, $R^2 = 2/3$, $N = 1.336$, $\phi = 0.089$. According to Eqs. (10)–(12), $J = 1.32 \times 10^{-14}$ cm$^4$ L mol$^{-1}$, $R_0 = 2.45$ nm, $E = 0.21$, and $r = 3.05$ nm. The value of $r$ was smaller than 8 nm and the scale was $0.5R_0 < r < 1.5R_0$, which suggested the non-radiative energy transfer from $\alpha$-glucosidase to kaempferol may occur (Wang, Zhang, Yan, & Gong, 2014). Moreover, the value of $r$ was greater than $R_0$, suggesting that kaempferol may strongly quench the intrinsic fluorescence of $\alpha$-glucosidase and situate at close proximity to $\alpha$-glucosidase fluorophore by a static quenching (Mehranfar, Bordbar, & Parastar, 2013).

3.2. Conformational studies of $\alpha$-glucosidase

3.2.1. Synchronous fluorescence spectra studies

The synchronous fluorescence was introduced to reflect the conformational change of enzyme to be more reliable. As shown in Fig. 1E, Peak 1 represents the Rayleigh scattering peak of $\alpha$-glucosidase ($\lambda_{ex} = \lambda_{em}$), peak 1 ($\lambda_{ex}/\lambda_{em} = 280.0$ nm/341.0 nm) mainly reveals the spectral feature of Trp and Tyr residues, and peak 2 ($\lambda_{ex}/\lambda_{em} = 225.0$ nm/340.0 nm) primarily shows the fluorescence characteristics of polypeptide chain backbone structure of $\alpha$-glucosidase caused by the $\pi \rightarrow \pi^*$ transition (Zhang, Dai, Zhang, Yang, & Liu, 2008). With the addition of kaempferol to $\alpha$-glucosidase, the fluorescence intensities of peak 1 and peak 2 reduced from 445.4 to 338.2 and from 461.5 to 320.0, respectively (Fig. 1F). The decrease in fluorescence intensity and a slight blue shift at about 1.2 nm in position of peak 1 indicated that the binding site for kaempferol was closer to the Try and Trp residues, and the microenvironment was altered slightly during the formation of $\alpha$-glucosidase.

\begin{align*}
R_0^2 &= 8.79 \times 10^{-25} \kappa^2 N^{-4} \phi J \\
J &= \frac{\sum F(\lambda)\sigma(\lambda)\Delta \lambda}{\sum F(\lambda)\Delta \lambda}
\end{align*}

(11) (12)

where $F_0$ and $F$ are the same as in Eq. (5). $R_0$ is the critical distance; $r$ is the distance between acceptor (kaempferol) and donor ($\alpha$-glucosidase); $k^2$ denotes the orientation factor, $N$ is the refractive index of the medium, $\phi$ is the fluorescence quantum yield of $\alpha$-glucosidase, and $J$ represents the overlap integral between the donor fluorescence emission spectrum and the acceptor absorption spectrum. Fig. 1B shows the overlapping of fluorescence emission spectrum of $\alpha$-glucosidase with the UV absorption spectrum of kaempferol. For ligand–$\alpha$-glucosidase, $R^2 = 2/3$, $N = 1.336$, $\phi = 0.089$. According to Eqs. (10)–(12), $J = 1.32 \times 10^{-14}$ cm$^4$ L mol$^{-1}$, $R_0 = 2.45$ nm, $E = 0.21$, and $r = 3.05$ nm. The value of $r$ was smaller than 8 nm and the scale was $0.5R_0 < r < 1.5R_0$, which suggested the non-radiative energy transfer from $\alpha$-glucosidase to kaempferol may occur (Wang, Zhang, Yan, & Gong, 2014). Moreover, the value of $r$ was greater than $R_0$, suggesting that kaempferol may strongly quench the intrinsic fluorescence of $\alpha$-glucosidase and situate at close proximity to $\alpha$-glucosidase fluorophore by a static quenching (Mehranfar, Bordbar, & Parastar, 2013).

3.2.2. Three-dimensional fluorescence spectra analysis

Three-dimensional fluorescence spectroscopy is a powerful fluorescence analysis technique which makes the determination of the conformational change of enzyme to be more reliable. As shown in Fig. 1E, Peak 1 represents the Rayleigh scattering peak of $\alpha$-glucosidase ($\lambda_{ex} = \lambda_{em}$), peak 1 ($\lambda_{ex}/\lambda_{em} = 280.0$ nm/341.0 nm) mainly reveals the spectral feature of Trp and Tyr residues, and peak 2 ($\lambda_{ex}/\lambda_{em} = 225.0$ nm/340.0 nm) primarily shows the fluorescence characteristics of polypeptide chain backbone structure of $\alpha$-glucosidase caused by the $\pi \rightarrow \pi^*$ transition (Zhang, Dai, Zhang, Yang, & Liu, 2008). With the addition of kaempferol to $\alpha$-glucosidase, the fluorescence intensities of peak 1 and peak 2 reduced from 445.4 to 338.2 and from 461.5 to 320.0, respectively (Fig. 1F). The decrease in fluorescence intensity and a slight blue shift at about 1.2 nm in position of peak 1 indicated that the binding site for kaempferol was closer to the Try and Trp residues, and the microenvironment was altered slightly during the formation of $\alpha$-glucosidase.

\begin{align*}
R_0^2 &= 8.79 \times 10^{-25} \kappa^2 N^{-4} \phi J \\
J &= \frac{\sum F(\lambda)\sigma(\lambda)\Delta \lambda}{\sum F(\lambda)\Delta \lambda}
\end{align*}

Fig. 2. (A) CD spectra of $\alpha$-glucosidase in the presence of increasing amounts of kaempferol. c($\alpha$-glucosidase) = 1.01 \times 10^{-6} mol L$^{-1}$, the molar ratios of kaempferol to $\alpha$-glucosidase were 0:1(a), 1:1(b), 2:1(c), respectively. (B) The FT-IR spectra of free $\alpha$-glucosidase (a) and difference spectra [(kaempferol–$\alpha$-glucosidase)–kaempferol solution] (b) at pH 6.8 sodium phosphate buffer in the region of 1800–1400 cm$^{-1}$. c($\alpha$-glucosidase) = 1.01 \times 10^{-6} mol L$^{-1}$, c(kaempferol) = 2.02 \times 10^{-6} mol L$^{-1}$. The curve-fitted amide I region (1700–1600 cm$^{-1}$) of free $\alpha$-glucosidase (C) and its kaempferol complex (D).
kaempferol–α-glucosidase complex. The decreased intensities of peak 2 and its positions changes from 225.0 nm/340.0 nm to 230.0 nm/338.0 nm suggested that the interaction between kaempferol and α-glucosidase may induce slight unfolding of the enzyme polypeptides (Xiao, Gu, Liang, Li, & Luo, 2014).

3.2.3. CD studies

CD spectra were analyzed to determine the change in secondary structure of α-glucosidase induced by kaempferol. As shown in Fig. 2A, α-glucosidase had a high percentage of α-helix structure that exhibited two negative CD bands at around 209 and 222 nm, and these negative bands were both contributed to n → π* transition for the peptide bond (Liu, Yan, Cao, Chong, & Lu, 2014). After addition of kaempferol, the intensities of double minimum were increased that was directly related to the interaction between kaempferol and α-glucosidase. Also, the contents of different secondary structure of α-glucosidase were calculated. With the increase in molar ratios of kaempferol to α-glucosidase (from 0:1 to 2:1), an increasing tendency of α-helix and random coil contents were observed (from 30.8% to 34.2% and from 27.6% to 29.8%, respectively), while the contents of β-sheet and β-turn decreased from 18.3% to 14.9% and from 23.3% to 21.1%, respectively. The results supported the proposition that the binding of kaempferol to the α-glucosidase might destroy the enzyme hydrogen bonding networks and induce some changes in the secondary structure of α-glucosidase, thus hamper active center formation or prevent substrate binding, which may inactivate the enzyme (Wu et al., 2014).

3.2.4. FT-IR spectra analysis

Further evidence for conformational changes in α-glucosidase was obtained by FT-IR spectroscopy. The infrared spectra of proteins exhibit two important amide bands: amide I and amide II. The amide I in the region of 1600–1700 cm⁻¹ is mainly the C=O stretch, while amide II in the 1600–1500 cm⁻¹ is C–N stretch coupled with N–H bending mode. As shown in Fig. 2B, the peak position of amide I band shifted from 1657 to 1655 cm⁻¹ and the amide II bands moved from 1549 to 1543 cm⁻¹ upon the addition of kaempferol to α-glucosidase. This phenomenon suggested that kaempferol interacted with the C=O and C–N groups in the protein structure subunits, resulting in the rearrangement of polypeptide carbonyl hydrogen bonding pattern and finally altering the secondary structure of α-glucosidase (Naik, Chimataadar, & Nandibewoor, 2010). To further characterize the secondary structure change of α-glucosidase, the curve-fitted spectra of α-glucosidase infrared amide I bands in the presence and absence of kaempferol were analyzed (Zhang, Wang, & Pan, 2012). The contents of α-helix (1660–1650 cm⁻¹), random coil (1648–1638 cm⁻¹), β-turn (1680–1660 cm⁻¹), β-sheet (1637–1610 cm⁻¹) and β-antiparallel (1692–1680 cm⁻¹) of free α-glucosidase were 30.6%, 27.5%, 24.1%, 11.6% and 6.2%, respectively (Fig. 2C). Upon kaempferol complexation (Fig. 2D), the α-helix and random coil contents increased to 35.4% and 29.1%, while β-turn, β-sheet and β-antiparallel contents decreased to 21.3%, 9.1% and 5.1%, respectively. These results were consistent with those obtained from the CD measurements, further confirming that the binding of kaempferol to α-glucosidase altered the secondary structure of α-glucosidase, leading to a decrease in the stability of α-glucosidase.
α-glucosidase due to a partial unfolding of the constitutive polypeptides (Zhang et al., 2014).

3.3. Analysis of inhibitory kinetics

3.3.1. Inhibition of kaempferol on α-glucosidase activity

As shown in Fig. 3A, the activity of α-glucosidase was significantly inhibited by kaempferol in a concentration-dependent manner. When the concentrations of kaempferol were increased, the relative enzyme activities were rapidly decreased. The kaempferol and acarbose concentrations leading to a loss of 50% enzyme activity (IC$_{50}$) were calculated to be $(1.16 \pm 0.04) \times 10^{-5}$ and $(2.09 \pm 0.03) \times 10^{-4}$ mol L$^{-1}$ ($n = 3$), respectively, indicating that kaempferol showed a much better inhibitory ability on α-glucosidase than acarbose. The IC$_{50}$ value of kaempferol determined in the present study for yeast α-glucosidase was consistent with earlier report ([IC$_{50}$ = 1.86 × 10$^{-5}$ mol L$^{-1}$]) (Phan et al., 2013). As reported previously, flavonoids quercetin and myricetin with a similar structure as kaempferol exhibited different inhibition effects on α-glucosidase: myricetin (3', 4', 5'-OH) > quercetin (3', 4'-OH) > kaempferol (4'-OH) (Tadera, Minami, Takamatsu, & Matsuoka, 2006). Thus, a number of hydroxyl groups attached to the B-ring may enhance the inhibition of α-glucosidase. As shown in Fig. 3B, the straight lines all passed through origin and the slopes of the lines were decreased with the increasing concentrations of kaempferol, suggesting that the inhibition of the enzyme by kaempferol was reversible (Wang, Curtis-Long, et al., 2014).

3.3.2. Kinetic type of inhibition

As shown in Fig. 3C, all the data lines on the Lineweaver–Burk plots intersected in the second quadrant, indicating that kaempferol induced a mixed-type of inhibition, which was consistent with a previous report (Phan et al., 2013). From the Eqs. (2)–(4), the values of $K_i$ and $\alpha$ were calculated to be $(1.31 \pm 0.03) \times 10^{-5}$ and $5.75 \pm 0.02$ ($n = 3$), respectively, which confirmed that kaempferol tended to be more easily and firmly bound to the free α-glucosidase rather than the α-glucosidase–substrate complex (Phan et al., 2013; Zhang, Chen, Song, & Xie, 2006). Furthermore, the replots of slope and Y-intercept versus the concentration of kaempferol were linearly fitted, suggesting that this inhibitor had a single inhibition site or a single class of inhibition.

### Table 2

<table>
<thead>
<tr>
<th>[Kaempferol] ($\times 10^{-5}$ mol L$^{-1}$)</th>
<th>Inactivation rate constants ($\times 10^{-4}$ s$^{-1}$)</th>
<th>Transition free-energy change (kJ mol$^{-1}$ s$^{-1}$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.51</td>
<td>0.88</td>
<td>24.07</td>
</tr>
<tr>
<td>1.02</td>
<td>0.95</td>
<td>23.87</td>
</tr>
<tr>
<td>2.04</td>
<td>1.25</td>
<td>23.16</td>
</tr>
<tr>
<td>3.06</td>
<td>1.69</td>
<td>22.38</td>
</tr>
</tbody>
</table>

$^a$ $k$ is the first-order rate constant.

$^b$ The transition free-energy change per s is $\Delta G = -RT\ln k$, where $k$ is the time constant for the inactivation reaction.

---

Fig. 4. (A) Cluster analyses of the AutoDock docking runs of kaempferol with α-glucosidase. (B) Predicted binding mode of kaempferol docked into α-glucosidase. The binding location of α-glucosidase (blue domain) interacting with kaempferol was shown as molecular surface structures (on the left); kaempferol interacted with the amino acid residues located within the active site of α-glucosidase (on the right), the white dashed line represents hydrogen bond. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
site on α-glucosidase (Wang et al., 2015). This result was consistent with that of above fluorescence titrations.

3.3.3. Inactivation kinetics and rate constants

Time-interval experiments were performed to determine the kinetic process and rate constants of enzyme inhibition. As shown in Fig. 3D, α-glucosidase activity tended to decrease with the increase of kaempferol concentration, and all of these inactivation processes followed first-order kinetics. Subsequent semi-logarithmic plots analysis indicated that the inactivation induced by kaempferol was a monophasic process (Fig. 3D and Table 2). These results suggested that kaempferol may bind to α-glucosidase and completely inactivate α-glucosidase in a gradual kinetic process. It was also found that the transition free-energy (∆ΔG°) decreased in a kaempferol concentration-dependent manner, which may be due to the inactivation of α-glucosidase (Wang et al., 2013).

3.4. Docking analysis of α-glucosidase

The α-glucosidase structure model was built based on a high sequence homology (PDB ID: 3MAA, gi number 411229) (Lee et al., 2014). After the 100 docking runs successfully, a total of 32 multimember conformational clusters were formed (Fig. 4A). The most energetically cluster contained the highest number of the analyzed conformation (28 out of 100) with the lowest binding energy of −7.12 kcal mol⁻¹ (red histogram) was selected as the final model to be used in the subsequent virtual screening. The predicted lowest binding energy was a little smaller than ∆ΔG° (−5.65 kcal mol⁻¹) which obtained from the thermodynamic determination at 298 K, this maybe due to the lack of desolvation energy as the molecular docking being conducted under simulation of vacuum condition.

As shown in Fig. 4B, kaempferol apparently had easy access to the active site pocket of α-glucosidase and was surrounded by the catalytic amino acid residues Asp69, Phe178, Asp215, Glu277, His351, Arg315, Phe314 and Val410 that aremino acid residues Asp69, Phe178, Asp215, Glu277, His351, Arg315, Phe314 and Val410 that are conserved in human α-glucosidase (Wang et al., 2015b). This result was consistent with that of above fluorescence titrations. Moreover, a hydrogen bond formed (white dashed line) between the oxygen atom of 7-OH on the A ring and the catalytic amino acid residues Asp69, Phe178, Asp215, Glu277, His351, Arg315, Phe314 and Val410. These residues are conserved in human α-glucosidase (Wang et al., 2015b). This result was consistent with that of above fluorescence titrations. Moreover, a hydrogen bond formed (white dashed line) between the oxygen atom of 7-OH on the A ring and the catalytic amino acid residues Asp69, Phe178, Asp215, Glu277, His351, Arg315, Phe314 and Val410.

4. Conclusions

The inhibition mechanism of kaempferol on α-glucosidase activity was investigated by multispectroscopic methods including fluorescence, absorption, CD and FT-IR spectroscopy coupled with kinetic analysis and molecular simulation. The principal results of our study have shown that (i) the fluorescence quenching of α-glucosidase by kaempferol was a static procedure along with the non-radiative energy transfer; (ii) there was a single class of binding site on α-glucosidase for kaempferol, and the binding induced rearrangement and conformational changes of the enzyme; (iii) the values of ∆H° and ∆S° were calculated to be −32.46 ± 0.20 kJ mol⁻¹ and −29.68 ± 0.30 J mol⁻¹ K⁻¹, respectively, indicating that the binding of kaempferol to α-glucosidase was mainly driven by hydrogen bonds and van der Waals forces; (iv) kaempferol had a significant inhibitory activity on α-glucosidase with the IC₅₀ value of (1.16 ± 0.04) × 10⁻⁵ mol L⁻¹ and Kᵢ value of (1.31 ± 0.03) × 10⁻⁵ mol L⁻¹; (v) the molecular docking analysis validated the experimental results and revealed the inhibition mechanism of kaempferol on α-glucosidase which may be due to the insertion of kaempferol into the active site of α-glucosidase occupying the catalytic center of the enzyme to avoid the entrance of pNPG and inducing the conformational changes of α-glucosidase. These results have indicated that kaempferol may be a vital α-glucosidase inhibitor, and the kaempferol-rich foods may be useful for the treatment of diabetes mellitus. The study has provided the basis for development and application of kaempferol as an α-glucosidase inhibitor and a food functional ingredient.

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References


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