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 attached much attention currently. In this study, kaempferol was found to show a notable inhibition activity on α-glucosidase in a mixed-type manner with IC 50 value of \((1.16 \pm 0.04) \times 10^{-4} \text{ mol L}^{-1}\). 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-D-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-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 IC 50 value of \((1.72 \pm 0.05) \times 10^{-4} \text{ mol L}^{-1}\) 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 \(3.17 \times 10^{-4} \text{ mol L}^{-1}\) (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 antioxidation, 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 IC 50 value of \(5.2 \times 10^{-4} \text{ mol L}^{-1}\). 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

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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.

Fig. 1. (A) Fluorescence spectra of α-glucosidase in the presence of kaempferol with different concentrations [pH 6.8, T = 298 K, λex = 280 nm]. c(α-glucosidase) = 1.01 × 10^{-6} mol L^{-1}, and c(kaempferol) = 0, 0.64, 1.27, 1.89, 2.52, 3.15, 3.77, 4.38, 5.00, 5.61, 6.22 and 6.82 × 10^{-6} mol L^{-1} for curves a → l, respectively. Curve m shows the emission spectrum of kaempferol only. The inset depicts Stern–Volmer plots for the fluorescence quenching of α-glucosidase by kaempferol at 298 ( ), 304 ( ) and 310 K ( ). (B) The spectral overlaps of the fluorescence spectrum of α-glucosidase (a) with the absorption spectrum of kaempferol (b); c(α-glucosidase) = 1.01 × 10^{-6} mol L^{-1}, and c(kaempferol) = 0, 0.64, 1.27, 1.89, 2.52, 3.15, 3.77, 4.38, 5.00, 5.61, 6.22 and 6.82 × 10^{-6} mol L^{-1} for curves a → l, respectively. Three-dimensional fluorescence spectra of α-glucosidase (E) and kaempferol–α-glucosidase system (F): c(α-glucosidase) = 1.01 × 10^{-6} mol L^{-1}, c(kaempferol) = 5.00 × 10^{-6} mol L^{-1}. 

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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-D-glucopyranoside (pNPG, purity ≥ 99%) were obtained from Aladdin Chemical Co. (Shanghai, China). Both the stock solutions of pNPG ($8.09 \times 10^{-3}$ 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 $6.82 \times 10^{-6}$ mol L$^{-1}$). The fluorescence spectra of these mixed solutions were measured after standing for 5 min to equilibrate. Because of the re-absorption and inner filter arisen from the substrate, respectively; [I] and [S] denote the concentration of inhibitor and substrate, respectively; V is the enzyme reaction velocity; [I] and [S] denote the concentration of inhibitor and substrate, respectively; x 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).

2.2.4. Enzyme activity assay

α-Glucosidase inhibitory activity was determined as reported earlier (Wang, 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) × 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):

$$\frac{1}{V} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \left( \frac{1}{[I]} + \frac{1}{K_I} \right)$$

(2)

Secondary plots can be constructed as

$$\text{Slope} = \frac{K_m}{V_{max}} + \frac{K_m[I]}{V_{max}K_I}$$

(3)

and

$$\text{Y-intercept} = \frac{1}{V_{max}} + \frac{1}{I}$$

(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; x 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 × 100 × 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 mean ± 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 α-glucosidase

3.1.1. Mechanism of fluorescence quenching
The interaction between kaempferol and α-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, α-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 α-glucosidase at 342 nm was gradually quenched with increasing the amounts of kaempferol (298 K), suggesting that kaempferol quenched the intrinsic fluorescence of α-glucosidase which is a direct evidence for the interaction between kaempferol and α-glucosidase. The following Stern–Volmer equation was utilized to describe the fluorescence quenching (Bhogale et al., 2013):

\[
\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + K_{q}^*\tau_0 [Q]
\]

\( F_0 \) and \( F \) denote the fluorescence intensities of α-glucosidase before and after the addition of kaempferol, respectively; [Q] represents the concentration of kaempferol; \( \tau_0 \) (10\(^{-5}\) s) is the lifetime of the fluorophore in the absence of kaempferol; \( K_{SV} \) means the Stern–Volmer quenching constant (\( K_{SV} = K_q^*\tau_0 \)); \( K_q^* \) displays the quenching rate constant of the biomolecule.

As shown in Fig. 1A and Table 1, the values of \( K_q^* \) decreased with the increase of temperature (298, 304 and 310 K), and the calculated values of \( K_q \) were 1.34 \( \times \) 10\(^{13}\), 1.10 \( \times \) 10\(^{13}\) and 1.01 \( \times \) 10\(^{13}\) L mol\(^{-1}\) s\(^{-1}\) which were much greater than 2 \( \times \) 10\(^{-9}\) L mol\(^{-1}\) s\(^{-1}\) (Wang, Zhang, & Wang, 2014), suggesting that the kaempferol–α-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):

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

In this equation, [Q] and [P]\(_t\) denote the total concentration of the kaempferol and α-glucosidase, respectively. \( K_q \) represents the binding constant for the accessible fluorophores, \( n \) is the number of the binding sites per α-glucosidase molecule. As shown in Table 1, the value of \( K_q \) was inversely correlated with temperatures, which was in accordance with the variation of \( K_{SV} \). The \( K_q \) values were in the order of \( 10^3 \) L mol\(^{-1}\), suggesting that a high affinity existed between kaempferol and α-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 α-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 (\( \Delta H^\circ \)) and entropy change (\( \Delta S^\circ \)) of the reaction are related to the binding force, and their values can be determined according to the van't Hoff equation:

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

\( R \) 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 \( \Delta H^\circ \) and \( \Delta S^\circ \) 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 (\( \Delta G^\circ \)) could be calculated as the following:

\[
\Delta G^\circ = \Delta H^\circ - \Delta S^\circ
\]

As revealed in Table 1, the binding process was spontaneous since the value of \( \Delta G^\circ \) is below zero. Furthermore, the values of \( \Delta H^\circ \) and \( \Delta S^\circ \) 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 \( \Delta H^\circ \) and \( \Delta S^\circ \) values were frequently regarded as evidence that the formation of kaempferol–α-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–α-glucosidase binding reaction (Kashanian, Khodaei, & Kheirdoosh, 2013).

3.1.3. Energy transfer between kaempferol and α-glucosidase
The quantum yield of α-glucosidase was evaluated by comparing fluorescence intensity of α-glucosidase with human serum albumin (HSA) (standard solution) under same conditions according to the relationship (Bi, Yan, Wang, Pang, & Wang, 2012):

\[
\phi_x = \phi_{st} \times \frac{F_x}{A_{st}} \times \frac{A_{st}}{A_x}
\]

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

\[
E = \frac{F_0 - F}{F_0} = \frac{R_0^6}{R_0^6 + R^6}
\]
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, \( \varphi \) 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, \( K^2 = 2/3, N = 1.336, \varphi = 0.089 \). According to Eqs. (10)–(12), \( J = 1.32 \times 10^{-14} \text{cm}^2 \text{mol}^{-1} \), \( R_0 = 2.45 \text{nm}, \) \( E = 0.21 \), and \( r = 3.05 \text{nm} \). The value of \( r \) was smaller than 8 nm and the scale was 0.5\( R_0 < r < 1.5 R_0 \), 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 change of the polarity microenvironment around the tyrosine (Tyr) and tryptophan (Trp) residues when the D-value (\( \Delta \lambda \)) between excitation and emission wavelength was stabilized at 15 or 60 nm, respectively. The fluorescence intensities of Trp decreased more obviously than that of Tyr after addition of kaempferol (Fig. 1C and D), implying that kaempferol may quench \( \alpha \)-glucosidase fluorescence mainly by quenching the fluorescence of Trp residue. A slight blue shift (from 296.0 to 294.8 nm) of maximum emission wavelength of Tyr residue was observed (Fig. 1C), suggesting that kaempferol bound to \( \alpha \)-glucosidase and induced the increase in hydrophobicity around Tyr residue, resulting in the amino acid residue less exposed to the solvent. In contrast, the maximum emission wavelength of Trp residue did not show any shift (Fig. 1D), indicating that the microenvironment around the Trp residue displayed no discernable change during the binding process (He et al., 2011).

#### 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 a represents the Rayleigh scattering peak of \( \alpha \)-glucosidase (\( \lambda_{ex} = \lambda_{em} \)) peak 1 (\( \lambda_{ex}/\lambda_{em} = 280.0 \text{nm}/341.0 \text{nm} \)) mainly reveals the spectral feature of Trp and Tyr residues, and peak 2 (\( \lambda_{ex}/\lambda_{em} = 225.0 \text{nm}/340.0 \text{nm} \)) 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 kaempferol–\( \alpha \)-glucosidase complex.

Fig. 2. (A) CD spectra of \( \alpha \)-glucosidase in the presence of increasing amounts of kaempferol, c(\( \alpha \)-glucosidase) = 1.01 \times 10^{-6} \text{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 \text{cm}^{-1}, c(\( \alpha \)-glucosidase) = 1.01 \times 10^{-6} \text{mol L}^{-1}, c(kaempferol) = 2.02 \times 10^{-6} \text{mol L}^{-1}. The curve-fitted amide I region (1700–1600 \text{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 in the ultraviolet region at 209 and 222 nm, and these negative bands were both contributed to n → π* transition for the peptide bond (Liu, Yan, Cao, Chong, & Lü, 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, Chimatadar, & 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 in the presence of kaempferol at 0.51, 1.02, 1.53 and 2.04 × 10⁻⁵ mol L⁻¹ for curves a → d, respectively. Semi-logarithmic plots analysis for kaempferol at 0.51 and 2.04 × 10⁻⁵ mol L⁻¹; to the right, the slopes of the curves indicate the inactivation rate constants; c/(α-glucosidase) = 1.01 × 10⁻⁷ mol L⁻¹; c(pNPG) = 2.02 × 10⁻⁸ mol L⁻¹.

![Fig. 3](image-url)

Fig. 3. (A) Effect of kaempferol and acarbose on the activity of α-glucosidase (pH 6.8, T = 310 K). (B) Plots of v vs. [α-glucosidase]. The kaempferol concentrations were 0, 0.25, 0.51, 1.02, 1.53 and 2.04 × 10⁻⁵ mol L⁻¹ for curves a → f, respectively. c(pNPG) = 2.02 × 10⁻⁴ mol L⁻¹; (C) Lineweaver–Burk plots. The kaempferol concentrations were 0, 0.25, 1.27, 1.53, 1.78 and 2.04 × 10⁻⁵ mol L⁻¹ for curves a → f, respectively. c(α-glucosidase) = 1.01 × 10⁻⁷ mol L⁻¹; the secondary plots of slope and Y-intercept vs. [kaempferol] were in the inset. (D) Kinetic time-courses for α-glucosidase in the presence of kaempferol at 0.51, 1.02, 1.53 and 2.04 × 10⁻⁵ mol L⁻¹ for curves a → d, respectively. Semi-logarithmic plots analysis for kaempferol at 0.51 and 2.04 × 10⁻⁵ mol L⁻¹; to the right, the slopes of the curves indicate the inactivation rate constants; c/(α-glucosidase) = 1.01 × 10⁻⁷ mol L⁻¹; c(pNPG) = 2.02 × 10⁻⁸ mol L⁻¹.
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 ± 0.04) × 10$^{-5}$ and (2.09 ± 0.03) × 10$^{-5}$ 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 ± 0.03) × 10$^{-5}$ and 5.75 ± 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.
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 ran 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, Asp 215, Glu277, His351, Asp352, Arg315, Phe314 and Val410 that are believed to play critical roles in the catalytic mechanism. Moreover, a hydrogen bond formed (white dashed line) between the hydrogen atom of 7-OH on the A ring of kaempferol and the active-site residues of α-glucosidase (Xu, 2010). It is likely that adjacent hydroxyl groups on the B ring of flavonoids directly interacted with the active-site residues of α-glucosidase (Vaya et al., 2003). However, kaempferol has only one hydroxyl group on the B ring (lacking adjacent hydroxyl groups), this structure should decrease the electron cloud density of B ring, which caused that the hydroxyl group on the B ring did not interact with α-glucosidase and the hydrogen bond was formed between 7-OH on the A ring and α-glucosidase. This may also be the main reason that the inhibitory ability of kaempferol on α-glucosidase is lower than that of myricetin and quercetin with adjacent hydroxyl groups on the B ring (Tadera et al., 2006). These results indicated that kaempferol had the ability to penetrate into the activity site of the enzyme, stay in the binding residues, and inhibit the catalytic action of α-glucosidase through the hydrogen bond. The docking simulation provided supportive data for kaempferol-induced inhibition by allowing us to predict the binding site in the active site pocket, and the results were in accordance with the experimental results shown above.

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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