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Interaction mechanism of icariin and whey protein based on spectrofluorimetry and molecular docking

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Abstract

Icariin has low bioavailability and poor stability, which limits its wide application. The complexation of icariin and whey protein is expected to solve this problem, but there is no research on their interaction mechanism. In view of this, the related mechanism was studied systematically by spectrofluorimetry and molecular docking method in this study. The fluorescence analysis showed that icariin and whey protein could form a non-covalent complex driven by hydrophobic force, which led to the fluorescence quenching of whey protein. In this process, the microenvironment around the tyrosine residue and tryptophan residue of whey protein changed. The molecular docking analysis confirmed the existence of hydrophobic interaction and hydrogen bonding in the complex, which well confirmed fluorescence results. The obtained results can promote the application of icariin in food.

Keywords: icariin; whey protein; interaction; spectrofluorimetry; molecular docking.

Practical Application: This work systematically studied the interaction mechanism of icariin and whey protein, which provided a theoretical basis for the application of icariin in medicines and functional foods.

1 Introduction

Icariin is one of the main active components of *Herba Epimedii*, which has immunomodulatory, anti-inflammatory, anti-aging, anti-tumor activities and can improve the symptoms of osteoporosis, Alzheimer's disease, Parkinson's disease, cerebral ischemia, atherosclerosis, rheumatism and other diseases (Luo et al., 2022; Zeng et al., 2022). Kim et al. (2018) reported that icariin could inhibit the formation and function of osteoclasts and alleviate osteoporosis (Kim et al., 2018). Zeng et al. (2010) found that icariin could hinder the production of β-amyloid protein, hyperphosphorylation of tau protein and decrease dopamine content to protect nervous system (Zeng et al., 2010). Icariin also can protect cardiovascular system by protecting cardiomyocytes, increasing the number of cardiomyocytes and improving endothelial dysfunction (Qian et al., 2017). Its pharmacological functions of promoting reproductive organs and regulating the immune system are also reported (Amanat et al., 2022). However, the low bioavailability and stability of icariin restrict its wide application.

Whey protein is a kind of protein extracted from milk, which contains β-lactoglobulin and lactalbumin (Graf et al., 2020; Melnikova et al., 2022; Jabeen et al., 2021). It has the characteristics of high nutritional value, easy digestion and absorption, and contains a variety of active components (Barajas-Ramírez et al., 2022; Bolognesi et al., 2022). It is recognized as a high quality protein. Whey proteins are easy to form complexes with polyphenols, so they are widely used in the delivery of polyphenols and the improvement of their bioavailability (Tamargo et al., 2022). However, there is no systematic report on the interaction between icariin and whey protein. In this study, the interaction mechanism of icariin and whey protein was clarified based on spectrofluorimetry and molecular docking, in order to promote the wide application of icariin in food.

2 Materials and methods

2.1 Materials and chemicals

Whey protein (purity, 93.77%) was from Mullins Whey Inc (Mosinee, WI, USA). Icariin was the product of Aladdin (Shanghai, China). All other chemicals are of analytical grade.

2.2 Preparation of sample solution

Whey protein solution (0.5 mg/mL) was prepared by 50 mM phosphate buffer (pH, 6.8), and stored at 4 °C. Icariin was dissolved in 85% ethanol and diluted to 100 mL with ultrapure water to obtain the stock solution of 500 μmol/L.

2.3 Measurement of binding constant

The binding constant of icariin and whey protein at 303 K was measured using an Agilent Cary Eclipse fluorescence spectrophotometer (Santa Clara, CA, USA) based on the previous report (Yue et al., 2019). 1 mL of icariin solution at different concentrations (0-140 μmol/L) and 4 mL of whey protein solution were mixed and kept for 30 min at 303 K until the reaction reached equilibrium. Then, the mixed solution was transferred to the quartz glass test tube to measure its fluorescence spectrum with the excitation wavelength of 280 nm and the scanning range of 300-450 nm. The scanning voltage was set at 650 kV, and the slit width was fixed at 5 nm. The obtained data were corrected by the following Equation 1:

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$$F = FInit \times 10^{(\frac{Aex+Aem}{2})} \quad (1)$$

Where F and F_{Init} are the corrected and measured fluorescence intensity values, respectively. A_{ex} and A_{em} are the absorption values of the mixture at the excitation wavelength and the emission wavelength, respectively.

Then, the quenching constant (K_q) could be calculated to judge the fluorescence quenching type by the Stern-Volmer equation (Equation 2):

$$\frac{F}{F_0} = K_q \cdot \tau_0[Q] + 1 \quad (2)$$

Where F_0 and F are the fluorescence intensity values of the mixture without icariin and with icariin, respectively; K_q is the quenching constant; τ_0 is the lifetime of proteins (10^{-8}s); [Q] is the concentration of icariin.

For static quenching, the binding constant (K_a) and binding-site number (n) of between icariin and whey protein could be calculated based on the double logarithm equation (Equation 3)

$$\lg\frac{(F_0 - F)}{F} = \lg K_a + n\lg[Q] \quad (3)$$

Where [Q] is the icariin concentration.

2.4 Measurement of thermodynamic parameters

In order to understand the binding behavior between icariin and whey protein, the K_a values at 303 K and 310 K were measured according to *2.2*, and the thermodynamic constants for their interaction were calculated by using Van'tHoff equation (Equation 4) and Gibbs free energy equation (Equation 5).

$$\ln K_a = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

2.5 Measurement of synchronous fluorescence spectrum

The synchronous fluorescence spectra can provide changes in the microenvironment of tyrosine and tryptophan residues during the interaction between icariin and whey protein. According to a published method (Geng et al., 2020), the fluorescence spectra with excitation wavelength 265-360nm ($\Delta\lambda$ = 15 nm) and 220-360 nm ($\Delta\lambda$ = 60 nm) were recorded respectively.

2.6 Molecular docking method

Molecular docking between whey protein and icariin was carried out by using the Autodock 4.2 software (He et al., 2019). The crystal structure of bovine β-lactoglobulin (PDB ID: 5io6), the main protein in whey protein, was downloaded from the RCSB Protein Data Bank (https://www.rcsb.org). The 3D structure of icariin was constructed and optimized through the PM3 method of MOPAC 2016 software. For molecular docking, the grid size was fixed at 50×50×50 points, and the grid space was set at 0.375 Å. The Lamarckian GA method (LGA) was used to found the possible docking mode.

3 Results and discussion

3.1 Binding constant

The intrinsic fluorescence of proteins mainly comes from tyrosine, tryptophan and phenylalanine residues (Li et al., 2022). This quenching of intrinsic fluorescence can reflect the interaction between proteins and ligands. Therefore, the interaction between icariin and whey protein was investigated by fluorescence spectroscopy. The fluorescence quenching result is shown in Figure 1. When the excitation wavelength was set at 280 nm, whey protein had the maximum emission peak at 333 nm. There was no fluorescence emission peak of icariin in the range of 300-450 nm, but when whey protein and icariin were mixed together, the fluorescence quenching of whey protein could be observed obviously. With the increase of icariin concentration, the fluorescence intensity of whey protein decreased gradually, and the position of the maximum emission peak was basically unchanged in this process.

In order to determine the type of fluorescence quenching, the fluorescence quenching of whey protein by icariin at 303 and 310 K was studied, and the quenching process was analyzed by fitting Stern-Volmer equation. It could be seen from Figure 2 and Table 1 that there was a good linear relationship between the icariin concentration (C) and F_0/F at different temperatures, and the K_q value was much higher than the maximum diffusion

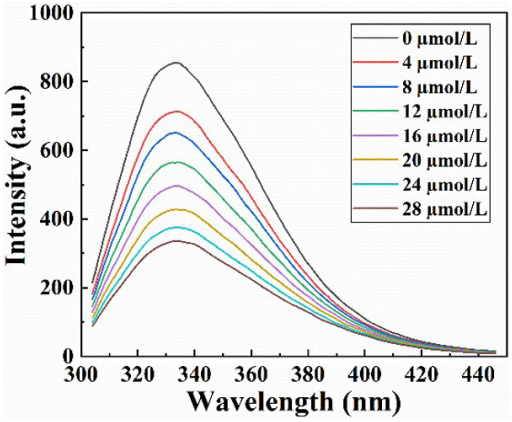

Figure 1. The fluorescence spectra of icariin and whey protein.

Table 1. The quenching constants (K_q), binding constants (K_a), binding-site number (n) and thermodynamic parameters for the interaction of icariin with whey protein.

T (K)	K_q (10^{12} L·mol^{-1}s^{-1})	n	pK_a	ΔG (kJ·mol^{-1})	ΔH (kJ·mol^{-1})	ΔS (J·mol^{-1}·K^{-1})
303	2.55 ± 0.05	0.96 ± 0.04	4.20 ± 0.16	-24.3657	281.8612	1010.65
310	3.26 ± 0.16	1.18 ± 0.05	5.30 ± 0.28	-31.4402		

constant of dynamic quenching, 2×10^{10} L/(mol·s). Therefore, it could be concluded that the quenching type was static quenching (Liu et al., 2022), and the quenching was caused by the formation of icariin/whey protein complex.

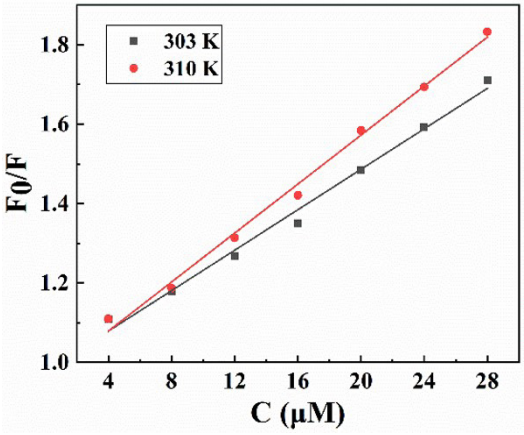

Figure 2. The plot of F_0/F versus C at different temperatures.

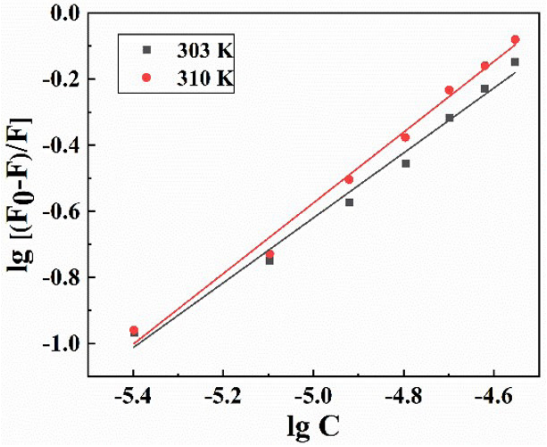

Figure 3. The plots of log [$(F_0$-F)/F] versus log C at different temperatures.

For static quenching, the binding constant (K_a) and binding site (n) between icariin and whey protein could be calculated by double logarithm equation as shown in Table 1, and the corresponding double logarithm diagram is exhibited in Figure 3. The pK_a value increased with the increase of temperature, indicating that the increase of temperature was beneficial to the binding of icariin and whey protein. In addition, the binding site number calculated at different temperatures was close to 1, which meant that there was only one binding site between icariin and whey protein.

3.2 Binding behavior analysis

The non-covalent interactions between proteins and ligands mainly involve electrostatic force, van der Waals force, hydrogen bonding and hydrophobic interaction (Sanver et al., 2016). When $\Delta H > 0$ and $\Delta S > 0$, hydrophobic interaction is dominant; when $\Delta H < 0$ and $\Delta S < 0$, van der Waals force and hydrogen bond are the main driving force; when $\Delta H < 0$ and $\Delta S > 0$, electrostatic force is the main driving force; when $\Delta H > 0$ and $\Delta S < 0$, electrostatic and hydrophobic interaction play an important role (Li et al., 2015). Combined with the results in Table 1, ΔG was negative, indicating that the binding of icariin to whey protein was spontaneous. ΔH and ΔS were greater than zero, suggesting that the hydrophobic force was the main driving force in the binding process of icariin and whey protein.

3.3 Synchronous fluorescence analysis

The synchronous fluorescence spectra at $\Delta\lambda$ = 15 nm and $\Delta\lambda$ = 60 nm can reflect the microenvironmental changes of tyrosine residues and tryptophan residues, respectively (Takahama & Hirota, 2018). The effect of icariin on the synchronous fluorescence spectrum of whey protein is exhibited in Figure 4. With the increase of icariin concentration, the intensity of synchronous fluorescence decreased gradually. In this process, the maximum peak of fluorescence spectrum changed slightly, indicating that the microenvironment around tyrosine residue and tryptophan residue changed. Similar results had previously been reported that whey protein changed its molecular conformation through interaction with puerarin, rutin and phloridin (Li et al., 2021).

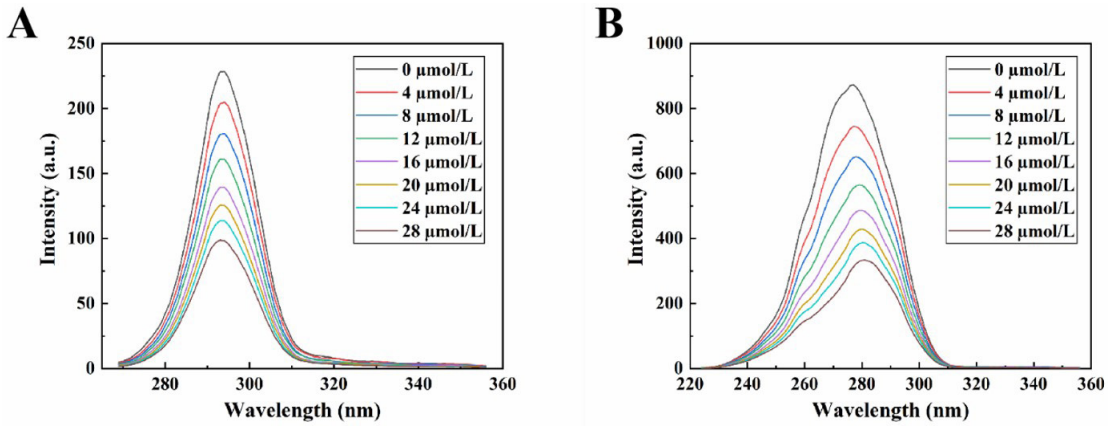

Figure 4. The synchronous fluorescence spectra of icariin and whey protein (A: $\Delta\lambda$ = 15 nm; B: $\Delta\lambda$ = 60 nm).

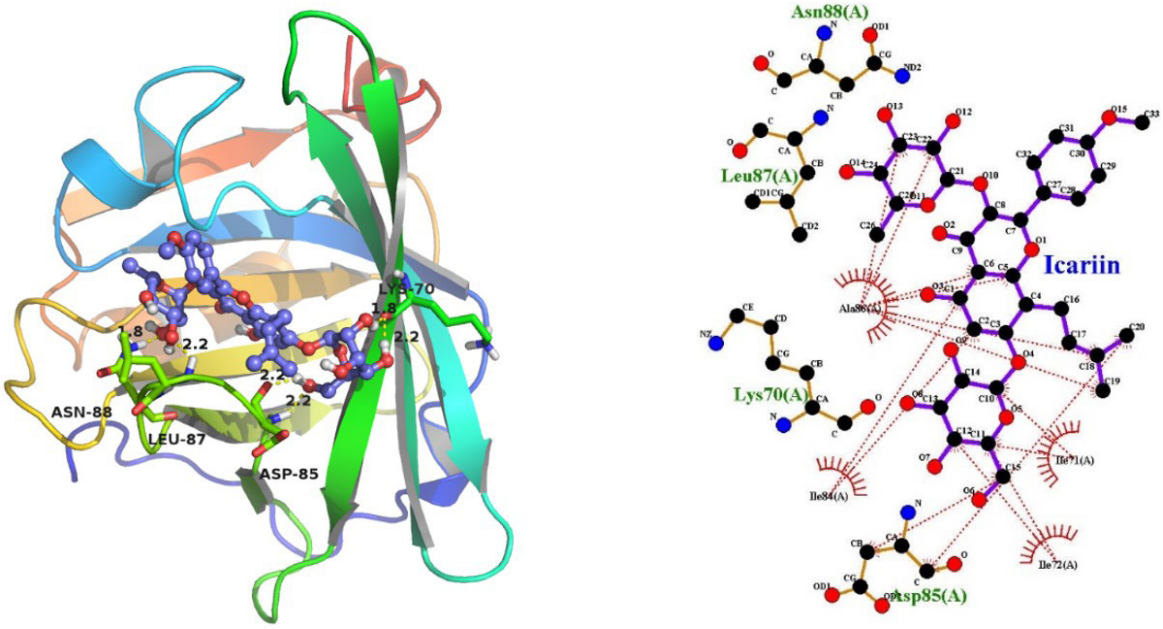

Figure 5. The docking diagrams of icariin/β-lactoglobulin complex.

3.4 Molecular docking analysis

Molecular docking is a method of drug design based on the characteristics of the receptor and the interaction between the receptor and drug molecules, which can investigate the interaction between proteins and drug molecules (such as ligands and receptors) and to predict their binding mode and affinity (Śledź & Caflisch, 2018). In recent years, molecular docking has become an important technology in the field of computer-aided drug research (Liu et al., 2017; Li et al., 2019). In this study, the semi-flexible docking method was adopted, which allowed the conformation of icariin to change to a certain extent, but the conformation of β-lactoglobulin was fixed, and the conformation adjustment of icariin was limited to a certain extent, such as fixing the bond length and bond angle of some non-critical parts, which could take into account the amount of calculation and the prediction ability of the model. The molecular docking of icariin with β-lactoglobulin was shown in Figure 5. During the molecular interaction, residues Asn88, Leu87, Asp85 and Lys70 of β-lactoglobulin participated in forming hydrogen bonding. Ile84, Ala86, Ile71, Ile72, Asn88, Leu87, Asp85 and Lys70 were involved in hydrophobic interaction, which well confirmed fluorescence results.

4 Conclusion

Icariin and whey protein could form a non-covalent complex driven by hydrophobic force, causing the fluorescence quenching of whey protein. The interaction led to the changes of the microenvironment around the tyrosine residue and tryptophan residue of whey protein. The molecular docking analysis confirmed the existence of hydrophobic interaction and hydrogen bonding in the complex, which coincided with the fluorescence results. Our results can provide a theoretical basis for the application of icariin in medicines and foods, and promote the wide application of whey protein in functional foods.

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