Research Article

Liangliang Liu#, Yi Liu#, Aiping Xiao, Shiyong Mei, Yixi Xie*

Influence of Human Serum Albumin Glycation on the Binding Affinities for Natural Flavonoids

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Abstract: Increasing the degree of glycation in diabetes could affect the ability of plasma proteins in binding to small molecules and active compounds. In this study, the influence of glycation of Human serum albumin (HSA) on the binding affinities for six dietary flavonoids was investigated by fluorescence spectra. Glycated HSA was prepared through incubation with glucose and characterized by several methods to confirm the glycation. It was found that the level of glycation increased with the increasing incubation time. The glycation of HSA increased the binding affinities for flavonoids by 1.40 to 48.42 times, which indicates that modifications caused by the glycation may have different influences on the interactions of flavonoids with HSA at separate binding sites on this protein. These results are valuable for understanding the influence of diabetes on the metabolism of flavonoids and other bioactive small molecules in human body.

Keywords: Diabetes; Flavonoids; Fluorescence spectra; Glycation; Human serum albumin.

1 Introduction

Diabetes, which is also known as diabetes mellitus, has become a big challenge in human health all over the world, and the prevalence rate of diabetes is rising quickly in recent years. One of the most typical symptoms of diabetesis the high concentration of glucose in blood. This kind of high level glucose could react with plasma proteins and initiate the Maillard reaction [1]. The Maillard reaction starts with the formation of Schiff base, and then transformed into early glycation product. After a battery of complex reactions, the intermediate finally become the advanced glycation end products (AGEs) [2, 3]. AGEs are harmful to human body and could induce the crosslinking between key proteins in extracellular matrix, alter cellular structure and interact with receptors to initiate a series of signal transactions leading to inflammatory and oxidative stress [4]. The glycations of proteins and lipids play important roles in diabetic complications like nephropathy, diabetic foot, retinopathy and so on [5]. Besides, the system of AGEs and their receptors has been proven to be closely related to the vascular injury in diabetes [6]. Therefore, more research focused on the glycated proteins is helpful for the treatment of diabetes and related diseases.

Serum albumin is an albumin found in blood and is the most common protein in blood. Serum albumin maintains osmotic pressure, buffers the pH and works as a important carrier to transport many bioactive molecules such as vitamins and fatty acids to tissues and organs [7]. The interactions between serum albumin and small active compounds attracts great interests in related research area since it is closely related to the metabolism, availability and kinetic profile of drugs [8]. As reported by the references, about twenty to thrity percent of human serum albumin (HSA) in the body of diabetes patients was glycated. However, the level of glycated HSA is only six to thirteen percent in the body of healthy persons [9], which becomes one of the characteristics for diabetes patients.

As active compounds widely distributed in foods, dietary flavonoids have shown inhibitory effects on formation of AGEs [10-12]. Moreover, they are identified as inhibitors of many human digestive enzymes, which makes them drug candidates for diabetes mellitus [13]. As small molecules, the biological effects of flavonoids depend on their binding behaviors to plasma proteins [14]. The purified HSA, bovine serum albumin, hemoglobin and other commercially acquired proteins are widely used as models to investigate the interaction between proteins and flavonoids [15, 16]. However, the reports on the non-covalent interaction between flavonoids and glycated

***Corresponding author: Yixi Xie,** College of Chemistry, Xiangtan University, Xiangtan 411105, PR China, E-mail: xieyixige@xtu.edu.cn
Liangliang Liu, Yi Liu, Aiping Xiao, Shiyong Mei, Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha 410205, China
#These authors contributed equally to this work

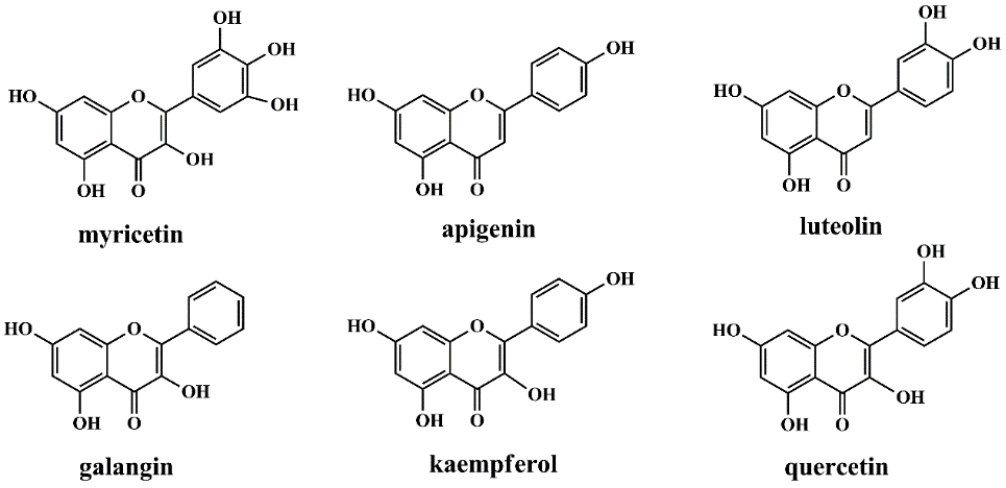

Figure 1: The studied chemical structures of dietary flavonoids.

HSA are few. The mechanism of how glucose affects the binding properties of dietary flavonoids for plasma proteins like HSA is still not clear.

Fluorescence spectroscopy is a typical and appropriate method to investigate the interaction between small molecules like dietary flavonoids and biological macromolecules [17]. HSA molecule contains tryptophan and tyrosine residues which give out intrinsic fluorescence under the excitation light at 280 nm. Through measuring the intensity of emission peak and observing its possible shift, the information about structural fluctuations and micro-environmental alterations of the molecule could be gained. In this study, the glycation of HSA was characterized and the effects of glycated HSA on the affinities for the interaction with dietary flavonoids (**Figure 1**) were studied by fluorescence spectra.

2 Materials and Methods

2.1 Chemicals and apparatus

Human serum albumin (HSA, 96-99%) and 1-deoxy-1-morpholino fructose were bought from Sigma-Aldrich (MO, USA). Nitrotetrazolium blue chloride (NBT) was obtained commercially from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Apigenin and luteolin (99.0%) were purchased from Aladdin Co. Ltd. (Shanghai, China). Galangin, kaempferol, quercetin and myricetin (99.5%) were bought from Shanghai Tauto Biotech Co., Ltd (Shanghai, China). The working solutions of flavonoids (1.0×10^{-3} mol/L) were prepared by dissolving each flavonoid with methanol. All other reagents and solvents were analytical grade and used without further purification.

2.2 Glycation of HSA

Glycation of HSA was carried out by incubating HSA (1.0×10^{-5} mol/L) with glucose (5.0×10^{-2} mol/L) containing 0.02% (w/v) NaN_3 for 20 days at 37°C. As a control group, HSA was incubated with double-distilled water instead of glucose for 20 days at 37°C.

2.3 Analysis of HSA glycation products

The glycation products were quantified by measuring the contents of fructosamine residues as described by Johnson et al. with slight modification [18]. 1-deoxy-1-morpholino fructose at concentrations between 0 and 1.0 mmol/L containing 1.0×10^{-5} mol/L HSA was used for calibration. The ultraviolet (UV) spectra of HSA incubated with glucose were recorded on a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) from 250 nm to 800 nm and the absorbances at 330 nm, 360 nm and 400 nm were monitored. The fluorescence emission spectra of glycated HSA were recorded from 400 to 650 nm using a fluorometer (Hitachi F-7000, Tokyo, Japan) with the excitation wavelength at 375 nm.

2.4 Fluorescence spectra

3.0 mL working solution of HSA or glycated HSA were poured into a 1.0 cm quartz cell. And then, a series amounts of flavonoid solution (1.0×10^{-3} mol/L) was titrated by using trace syringes. When HSA or glycated HSA samples were titrated with different amounts of flavonoids, the fluorescence spectrum of mixture was recorded from 295nm to 420 nm using a fluorometer (Hitachi F-7000, Tokyo, Japan) upon excitation wavelength at 280 nm. The fluorescence quenchings of HSA and glycated HSA with samples were calculated by Stern-Volmer formula shown as follow [19]:

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (1)$$

Where F_0 represents the fluorescence intensities of protein, F represents the fluorescence intensities of protein in the presence of flavonoid. [Q] represents the concentration of flavonoid. K_q is the quenching rate constant, τ_0 is the average lifetime (6.2 ns), and K_{sv} is the Stern-Volmer quenching constant. The binding constants were calculated according to the double-logarithm equation shown as follow [19]:

$$\text{Log}\,(F_0\text{-}F)/F = \log Ka + n\log[Q] \quad (2)$$

Where *Ka* is the binding constant, and *n* is the number of binding sites per HSA molecule. The fluorescence spectra of studied flavonoids were not observed in the same condition and the flavonoids were stable during fluorescence measurements. Each determination was repeated three times and reproducible within experimental errors.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and Discussion

3.1 Characterization of HSA glycation

The generated glycation products during the glycation of proteins are fluorescent and could be used as indicators for the evaluation of glycation degree [20]. As shown in in **Figure 2**, after the incubation with glucose, the fluorescence spectra of HSA showed a characteristic peak at 465 nm. The fluorescence intensities of glycated HSA increased with the increasing incubation time, indicating the modification process of HSA induced by glucose. Schmitt and coworkers investigated the reaction of HSA with glucose and found that glucose modified HSA exhibited the maximum fluorescence intensity at 440 nm [21]. The colorimetric determination of frucotosamine residues was also utilized to further evaluate the glycation level of HSA considering some glycation products of HSA are non-fluorescent [22]. As shown in **Table 1**, no frucotosamine residues was detected in HSA solution at the beginning. However, frucotosamine residues showed up and accumulated as time went on. After 20 days incubation, the content of fructosamine residues was up to 31.3 μmol/L, which was more than three times that in 5 days incubation.

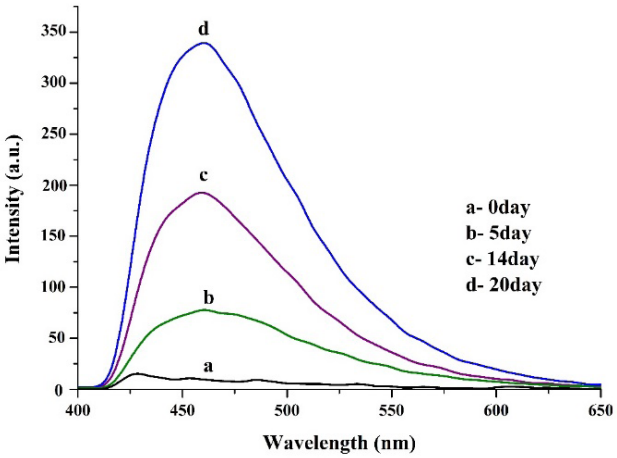

Figure 2: The fluorescence spectra of HSA incubated with glucose for different periods.

Because the chromophores in proteins would be modified during the glycation of HSA, the influence of glycation on the UV spectra of HSA was investigated. The UV adsorptions of HSA at 330 nm, 360 nm and 400 nm spectra are summarized in **Table 1**. Like the increasing of fluorescence intensities, the increased incubation periods led to enhancing absorbance at specified wavelength, which indicated that more structural modifications occurred. Hence, through fluorescence spectra, UV spectra and colorimetric determination, the glycation of HSA was confirmed and increased over time.

3.2 Fluorescence quenching of HSA

The fluorescence of HSA under the excitation at 280 nm was mainly caused by tryptophan residues (Trp214) which were part of subdomain IIA. HSA can bind various ligands in Subdomain IIA in its hydrophobic cavity [23]. When small molecules such as flavonoids are bound to HSA, the microenvironment of Trp214 was affected and its

Table 1: The results of UV measurements and fructosamine contents of HSA incubated with glucose.

Time	Absorbance			Fructosamine residues *
	330 nm	360 nm	400 nm	
0 day	0.002	0.008	0.001	-
5 day	0.052	0.028	0.011	9.36
14 day	0.176	0.082	0.031	20.8
20 day	0.369	0.184	0.064	31.3

* Represented by the equivalent content of 1-deoxy-1-morpholino fructose (1×10^{-6} mol/L)

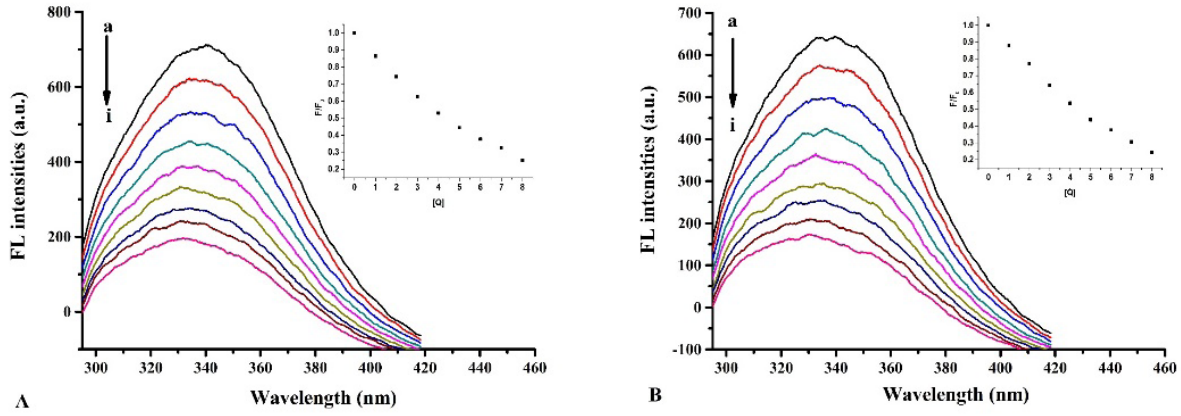

Figure 3: The quenching effect of apigenin on fluorescence spectra of HSA (A) and glycated HSA (B). λex =280 nm; HSA, 1.00×10^{-5} mol/L; a-i: 0.00, 1.00, 2.00 .... 8.00 ($\times10^{-6}$ mol/L) of apigenin. Inset: The Stern-Volmer plots for HSA (A) and glycated HSA (B) fluorescence quenching by apigenin.

fluorescence was consequently quenched. Compared with HSA, the fluorescence spectra of glycated HSA showed a loss of intensity, which might be due to that the local environment of Trp214 on HSA molecule was perturbed during glycation [24].

Glycated HSA was prepared according to the mentioned method and used after 20 days incubation with glucose. The fluorescence quenchings of six flavonoids on HSA and glycated HSA were investigated. As an example shown in **Figure 3**, the fluorescence quenching effect of apigenin was shown. When apigenin was continuously added into HSA and glycated HSA solution, the increasing attenuation in the fluorescence intensities could be observed in **Figure 3A** and **3B**. In addition, similar extents of blue-shifts for the maximum emission of HSA and glycated HSA induced by addition of apigenin were observed as well. The quenching ratios of the HSA fluorescence with addition of apigenin were also shown in insets of **Figure 3**. The fluorescence intensities of both HSA and glycated HSA were found to decline rapidly in the presence of apigenin. It illustrates that after incubated with glucose, the fluorescence quenching properties of HSA were changed to certain degree.

3.3 The binding constants (Ka) and the number of binding sites (n)

The binding processes of six flavonoids with HSA and glycated HSA were tested, and the binding constants (*logKa* and *n*) were summarized in **Table 2**. As shown in the table, all the *logKa* values of flavonoids for HSA were in the range of 1×10^6 to 1×10^8 L/mol, which were in agreement with the reported data [25, 26]. The relationship between *logKa* and *n* for HSA and glycated HSA were calculated and shown in **Figure 4**. The linear regression equations for HSA and glycated HSA were shown as follow:

For HSA, $n = 0.44144 + 0.12455 \log Ka$ (R^2=0.91238).

For glycated HSA, $n = 0.28808 + 0.14912 \log Ka$ (R^2=0.95371).

The values of number of binding sites (*n*) are proportional to log*Ka*. The linear relationship between

Table 2: The affinities (log*Ka*), the number of binding sites (*n*) and corresponding fitting degrees of flavonoids for HSA and glycated HSA.

	0 day						5 days					
Flavonoids	**HSA**			**Glycated HSA**			**HSA**			**Glycated HSA**		
	logKa	***n***	**R^2**	***logKa***	***n***	**R^2**	***logKa***	***n***	**R^2**	***logKa***	***n***	**R^2**
Apigenin	6.760	1.300	0.985	6.762	1.302	0.981	7.035	1.326	0.988	8.066	1.521	0.985
Luteolin	6.680	1.271	0.992	6.800	1.293	0.985	7.183	1.348	0.989	7.450	1.387	0.989
Galangin	6.948	1.318	0.988	7.373	1.389	0.982	7.532	1.415	0.995	7.679	1.436	0.992
Kaempferol	7.289	1.341	0.991	8.313	1.523	0.998	7.536	1.322	0.991	8.172	1.434	0.992
Quercetin	6.668	1.291	0.993	6.986	1.339	0.992	6.709	1.254	0.983	8.057	1.494	0.990
Myricetin	6.513	1.242	0.990	6.846	1.306	0.990	7.166	1.339	0.995	7.390	1.369	0.997
	14 days						20 days					
Flavonoids	HSA			Glycated HSA			HSA			Glycated HSA		
	logKa	***n***	R^2	***logKa***	n	R^2	***logKa***	n	R^2	***logKa***	n	R^2
Apigenin	6.689	1.289	0.998	6.970	1.33	0.996	6.380	1.21	0.989	6.916	1.311	0.996
Luteolin	6.533	1.243	0.982	6.981	1.328	0.995	6.670	1.245	0.988	7.514	1.398	0.988
Galangin	6.566	1.263	0.988	6.933	1.327	0.994	6.434	1.245	0.987	7.273	1.376	0.986
Kaempferol	6.814	1.317	0.985	6.530	1.254	0.994	6.869	1.307	0.997	7.344	1.388	0.995
Quercetin	6.512	1.231	0.997	6.797	1.268	0.989	6.574	1.257	0.994	8.259	1.565	0.997
Myricetin	6.470	1.265	0.995	6.260	1.212	0.988	5.987	1.197	0.991	6.909	1.396	0.990

these two parameters showed the equation used in this calculation is suitable. However, the linear relationships of *n*-log*Ka* for HSA and glycated HSA were different.

3.4 Effect of glycation on the affinities of flavonoids for HSA

It was found that the binding constants of Glycated HSA-flavonoid interaction were clearly higher than that of normal HSA (**Table 2**). The magnitudes of binding constants in HSA-flavonoid interaction after incubation with glucose from 0 to 20 days were mainly in the range of 10^7-10^8 L/mol, which was significantly greater than the corresponding data in the absence of glucose (10^6-10^7 L/mol) and also higher than the *in vivo* tested affinities between drugs and common serum albumins (10^4-10^6 L/mol) [25]. While, the values of log*Ka* for these flavonoids were similar to that in reports [24]. Therefore, the increase of log*Ka* for these interactions is worth attention. These results indicate that the modifications of HSA induced by glycation might affect the interactions between flavonoids and HSA at separate binding sites.

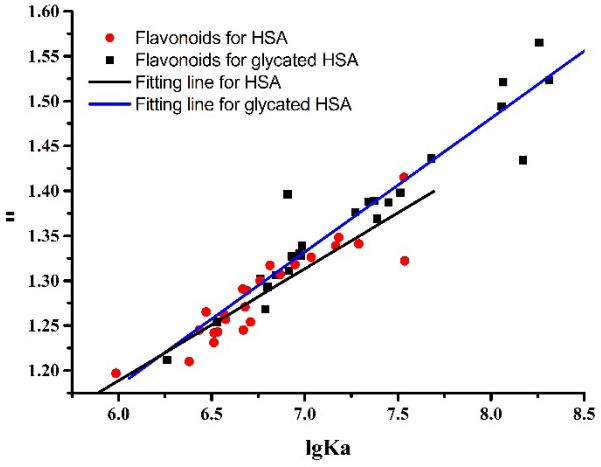

Figure 4: The relationship between the affinities (log*Ka*) and the number of binding sites (*n*) for HSA and glycated HSA.

As shown in **Figure 5**, in most case, the binding affinities for HSA incubated with glucose for 5 or 20 days increased by 1.40 to 48.42 times depending on their structures. The affinities (*Ka* values) of quercetin and apigenin for glycated HSA were apparently increased.

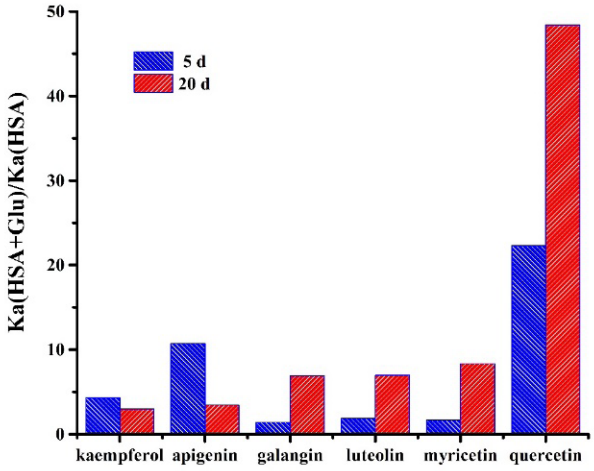

Figure 5: The effect of HSA glycation on the improvement of affinities for flavonoids.

However, the affinities of other flavonoids for glycated HSA were slightly changed. The higher affinities between flavonoids and glycated HSA might be caused by the non-enzymatic process of HSA [27]. With the increasing affinity for glycated HSA, the number of hydroxy groups in structure increased as well (quercetin, 5 -OH; myricetin, 5 -OH; luteolin, 4 -OH; galangin, 3 -OH; apigenin, 3 -OH; kaempferol, 3 -OH). It could be assumed that the affinity for glycated HSA enhanced with the increasing hydroxy groups in flavonoids and the hydrogen bonding might play a crucial part in the interaction between glycated HSA and flavonoids. However, more specific and deeper research needs to be done in future.

The modifications at two binding sites and different levels of glycation of HSA were found to change the glycation pattern of HSA. It also suggested that various kinds of modified forms for HSA may be found at different stages of diabetes [28]. As Joseph et al., reported the binding constants of warfarin for glycated HSA changed little, while the binding affinities for L-tryptophan with glycated HSA increased with a 4.7-5.8 fold [29]. It was also found tolbutamide was bound to both of two Sudlow sites on the glycated HSA and the constants increased by 1.1-1.4 fold [29]. In our case, increasing of binding constants induced by glycation was even more significant (48-fold at maximum) depending on the kinds of flavonoids and the level of glycation of HSA. The results here showed the significant changes of the interactions between flavonoids and HSA induced by glycation of HSA, which might be valuable in further understanding the influence of diabetes on the metabolism of flavonoids and other bioactive small molecules in human body.

4 Conclusions

The effects of HSA and glycated HSA on the binding affinities for dietary flavonoids was investigated by fluorescence spectra. The formation of AGEs was confirmed by observing the increased fluorescence intensity of glycation products during increasing incubation time. And fructosamine residues were accumulated up to 31.3 μmol/L after 20 days' incubation. UV spectra also revealed that the structural modifications of HSA by glucose were going up with time. The glycation was found to increase the binding affinities of HSA for flavonoids by 1.40 to 48.42 times contingent on the flavonoid's structures and the glycation level of HSA. These findings showed the changes in interaction between glycated HSA and flavonoids might be valuable in further understanding the influence of diabetes on the metabolism of flavonoids and other bioactive small molecules in human body.

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Conflicts of Interest: The authors declare no conflict of interest.

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