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Research Article

Investigation of the Interaction Between Ovalbumin and Gallic Acid by Spectroscopic Method

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Abstract

Gallic acid, one of the polyphenolic compounds, has known to have many pharmaceutical and biological properties such as anti-oxidant, anti-inflammatory, anti-virus and anti-tumor activities. The interaction study of gallic acid and protein is very important in drug design, development of drug delivery system as well as immunology research. Among various techniques available for interaction study between polyphenols and small molecule, spectroscopic method is mainly used to assess protein-polyphenol complex due to its simplicity, rapidity and sensitivity. In this study, the interaction between gallic acid and ovalbumin was investigated by fluorescence quenching, synchronous and 3D (three-dimensional) fluorescence spectra. The possibility of quenching of ovalbumin by gallic acid was studied by Stern-Volmer equation. The quenching mechanism, binding constant, the number of binding sites were also investigated. The binding mode was determined using the Van't Hoff and Gibbs-Helmholtz equation. The average distance between gallic acid and ovalbumin was calculated by Förster's theory. The results showed that gallic acid could quench ovalbumin through static quenching mechanism. In the binding process, electrostatic forces were mainly involved. In addition, synchronous and 3D fluorescence spectroscopic analysis revealed that the microenvironments of tryptophan and tyrosine residues in ovalbumin were changed during the complex formation. The result of present study provides the information of binding mechanism of gallic acid with ovalbumin.

Keywords

Ovalbumin, Gallic Acid, Fluorescence Quenching, Förster's Resonance Energy Transfer

1. Introduction

Recently, interaction studies between protein and small molecules have gained a growing interest for scientists. This research plays an important role in the medicine, biology, food industry and chemistry. Among a variety of these studies, the interaction between protein and phenolic compounds seems to be one of the most important issues [1].

Gallic acid (3, 4, 5-trihydroxybenzoic acid) is one of the polyphenolic compounds found in plants. It has been known that gallic acid has many pharmaceutical and biological activities. Gallic acid exhibits antioxidant, anti-allergic, anti-inflammatory, anti-carcinogenic and anti-mutagenic activities. In addition, it can enhance the cerebral antioxidant defense system and be used in the vascular dementia treatment [2, 3].

Ovalbumin, the major protein from egg white, is widely used in interaction study with small molecules. Understanding

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the accurate mechanism between ovalbumin and polyphenols is very important. First, ovalbumin can be used as carrier material for polyphenol. Polyphenols generally have low bioavailability and stability. Therefore, carrier materials are required to encapsulate and deliver polyphenols. Ovalbumin is a good candidate for protein-based carrier material due to its biocompatibility, non-toxicity, low cost, resistant ability for pepsin digestion, high emulsifying ability and water solubility [4]. In previous reports, ovalbumin was used to encapsulate some bioactive agents such as epigallo-catechin 3-gallate (EPCG) and curcumin [5, 6]. Second, it is used in immunological studies as a model protein. Since it is a dominant allergen, studies on binding mechanism of drugs and ovalbumin have been performed to elicit hypersensitivity reactions [7]. Third, it is also important in nutrition study. Polyphenols may act as anti-nutritional factors when they are absorbed in excess because of their binding ability to protein. Both ovalbumin in egg and polyphenols in plants are common ingredients in the food industry, thus it is necessary to study their interaction mechanism [8].

Several methods are utilized to investigate the interaction. The typical methods are spectroscopic, microscopic, thermodynamic, electrophoretic, chromatographic and bioinformatics analyses. Among them, spectroscopic method is mainly used to assess protein-polyphenol complex. It is not only simple, rapid and sensitive but also able to study microenvironment changes of protein in molecular level [1].

Although some results have been reported in terms of gallic acid and serum albumin [2], a few studies were carried out using ovalbumin [9, 10]. To our best knowledge, the present paper is the first attempt to examine the interaction between gallic acid and ovalbumin. In this context, the objective of this paper is to investigate the binding mechanisms by spectroscopic method such as fluorescence quenching, synchronous and 3D fluorescence spectra.

2. Materials and Methods

2.1. Materials

Ovalbumin and gallic acid were purchased from Sigma-Aldrich (USA). Tris was supplied by Solarbio Chemical Co. (China). Ovalbumin stock solution (1 mm) was prepared by dissolving a certain amount of ovalbumin to Tris-HCl buffer (10 mm, pH 7.4). Gallic acid solution was prepared in deionized water. All solution was stored at 4 ℃ before experiment.

2.2. Methods

2.2.1. Fluorescence Spectroscopy

In order to prepare working solution, certain volume of ovalbumin and various concentration of gallic acid stock solution were added into a 10 mL volumetric flask. Then, the flask was filled with Tris-HCl buffer (pH 7.4) to the scale mark. In each working solution, ovalbumin concentration was fixed at 5 μm while gallic acid concentration was changed from 0 to 50 μM by 10 μM. Each flask was stored at 17 ℃ for 5 min before fluorescence measurement.

Fluorescence spectra were measured by Florescence spectrometer (F97Pro, China). The florescence excitation wavelength was fixed at 280 nm whereas emission spectra was measured in the range of 300-450 nm. The silt and the voltage gain were set at 5 nm and 900 V, respectively. Background noises were removed before experiments. The result was analyzed by Stern-Volmer equation [9].

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

F_0: fluorescence intensity in the absence of quencher
F: fluorescence intensity in the presence of quencher
K_{sv}: Stern-Volmer quenching constant
[Q]: concentration of the quencher
k_q: bimolecular quenching rate constant
τ_0: average lifetime of protein without the quencher (10 ns)

The binding constant and the number of binding sites per ovalbumin molecule were calculated by following equation [9].

$$\lg((F_0-F)/F) = \lg K+n\lg[Q] \quad (2)$$

K: binding constant
n: the number of binding sites

2.2.2. Thermodynamic Parameters and Binding Forces

The same concentration of working solutions used for fluorescence spectra measurement were incubated at 31 ℃ and 37 ℃ for 5 min. After calculating binding constant at given temperature using the equation 2, the binding mode was determined using the Van't Hoff (equation 3) and Gibbs-Helmholtz equation (equation 4) [10].

$$\ln K=-\Delta H/RT+\Delta S/R \quad (3)$$

K: binding constant at given temperature
R: gas constant (8.31 J/(mol•K))

$$\Delta G=\Delta H-T\Delta S \quad (4)$$

ΔG: free energy change
ΔH: enthalpy change
ΔS: entropy change

2.2.3. FRET (Förster's Resnonace Energy Transfer)

FRET was obtained from equation (5)-(7) [9].

$$E=1-F/F_0=R_0^6/(R_0^6+r^6) \quad (5)$$

E: the efficiency of energy transfer
r: distance between acceptor and donor
R_0: critical distance when the transfer efficiency is 50%

$$R_0^6=8.79\times10^{-25}K^2n^{-4}\ \varphi J \quad (6)$$

K^2: spatial orientation factor of the dipole
n: refractive index of the medium
φ: fluorescence quantum yield of the donor
J: overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. It can be calculated by following equation.

$$J(\lambda)=(\int_0^\infty F(\lambda)\,\varepsilon(\lambda)\lambda^4 d\lambda)/(\int_0^\infty F(\lambda)\,d\lambda) \quad (7)$$

$\varepsilon(\lambda)$: molar absorption coefficient of the acceptor

UV-vis spectra were recorded using UV-vis spectrometer (UV5200PC) at a wavelength of 300-450 nm.

2.2.4. Synchronous Fluorescence and 3D Fluorescence

Synchronous fluorescence spectra of ovalbumin (10 μm) were recorded with successive additions of gallic acid solution (10 μm). D-value (Δλ) was set at 15 nm and 60 nm, respectively. Excitation wavelength was changed from 225 to 325 nm.

The three-dimensional fluorescence spectra of ovalbumin were carried out in the absense and presence of gallic acid. The range of excitation wavelength was from 240 to 350 nm and emission wavelength was from 300 to 450 nm.

3. Results and Discussion

3.1. Fluorescence Quenching

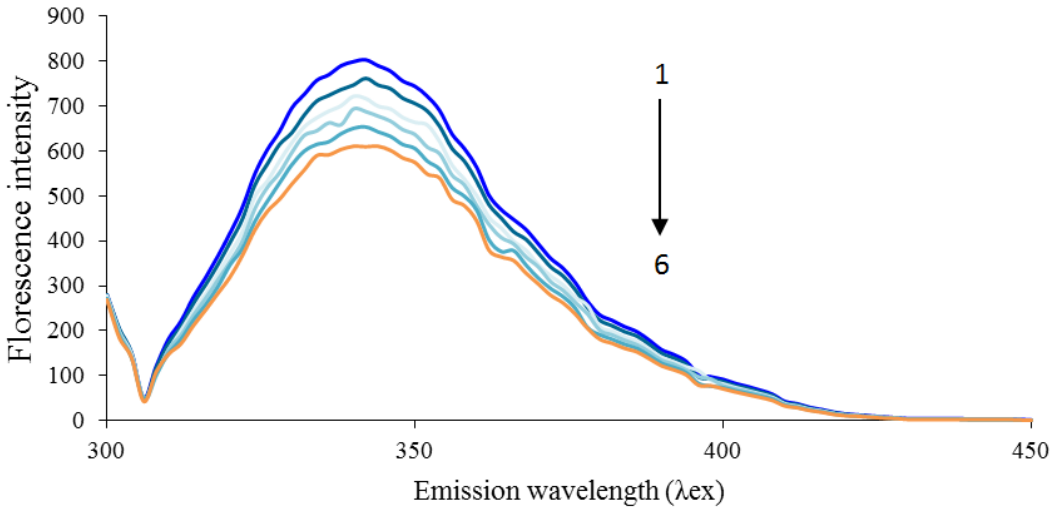

Figure 1. *The fluorescence spectra of ovalbumin in the presence of gallic acid.*

(1: ovalbumin(5 μm), 2:gallic acid(10 μm)+ovalbumin, 3: gallic acid(20 μm)+ovalbumin, 4: gallic acid(30 μm)+ovalbumin, 5:gallic acid(40 μm)+ovalbumin, 6:gallic acid(50 μm)+ovalbumin:λ_{ex}=280 nm)

Fluorescence quenching is the rapid and sensitive technique to study the local microenvironment. To elicit the interaction between gallic acid and ovalbumin, the quenching of the intrinsic emission of ovalbumin was explored and the result was shown in Figure 1.

As can be seen, a peak was observed around 340 nm of emission wavelength. This peak was mainly attributed to tryptophan residues at 148 in helix F, 184 in strand 3A and 267 in helix H [9]. It can be also noted that florescence intensity decreased steadily with the increase of gallic acid concentration. This graph indicated that gallic acid quenched the emission intensity of ovalbumin. In order to verify quenching mechanism, the data were analyzed by the Stern-Volmer equation (Figure 2).

As shown in Figure 2, the Stern-Volmer graph showed there was a positive linear relationship between (Fo-F)/F and quencher concentration (R^2=0.99). This result indicates that the queching mechanism could be static or dymanic. Stern-Vlomer quenching constant(Ksv) was caculated from the intercept and slope of this plot (Table 1). The Kq (bimo-

lecular quenching rate constant) calculated from Kq=Ksv/ τ_0 was much greater than $2.00\times10M^{-1}S^{-1}$ (the maximum scatter collision quenching constant). Therefore, it can be found that the quenching was mainly occured by the formation of compound, i.e. static quenching [7].

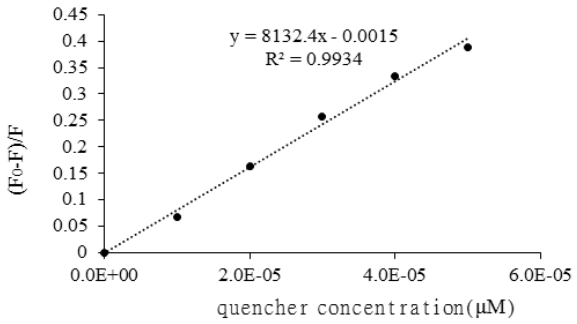

Figure 2. *Stern-Volmer Plot.*

Double logarithmic plot for calculating the binding constant and the number of binding sites was described in Figure 3.

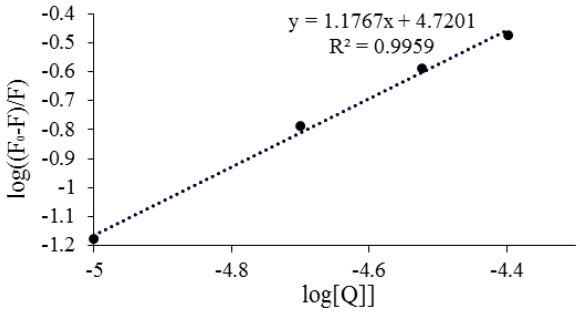

Figure 3. *Double logarithmic plot.*

The double logarimthic plot showed a good liner relationship. The number of binding calculated from this plot was 1.18 for gallic acid.

3.2. Thermodynamic Parameters and Binding Forces

Figure 4 depicts the Van't Hoff plot.

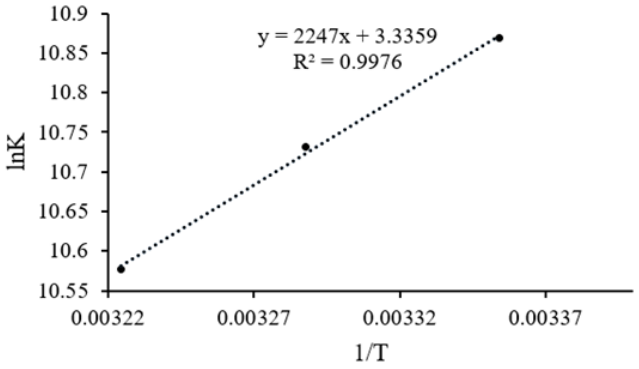

Figure 4. *Van't Hoff plot.*

The binding constant decreased with the increasing temperature. This indicates that the stability of gallic acid-ovalbumin complex gets weaker as the temperature rises. In addition, the higher the temperature, the lower the quenching constant, which reconfirms the result from florescence quenching.

Noncovalent interaction forces between protein and small molecules include hydrogen bonds, van der Waals forces, hydrophobic and electrostatic interactions. The negative value of ΔG(-26.22±0.45 KJ/mol, T=290.15 K) means that the binding process was spontaneous and exothermic. Because ΔH<0 and ΔS>0, electrostatic interactions were mainly involved in the binding process [12].

Table 1. *Binding parameters obtained from fluorescence experiment.*

T (K)	K_{sv} (L/mol)	$k_q\times10^{11}$ (L/mol/s)	$K\times10^5$ (L/mol)	n	ΔH (KJ/mol^{-1})	ΔS (J/mol^{-1}/K)	ΔG (KJ/mol)
290.15	8132.4±98.4	8.13±0.23	5.25±0.32	1.18±0.19			-26.22±0.45
300.15	7128.5±108.9	7.12±0.47	4.58 ±0.25	1.19±0.26	-10.89±0.57	52.79±0.40	-26.74±0.73
310.15	6237.8±76.5	6.23±0.43	3.92±0.18	1.21±0.33			-27.27±0.23

3.3. FRET and Binding Forces

Figure 5 describes that the overlap between UV-vis absorption spectrum and the fluorescence emission spectrum of ovalbumin.

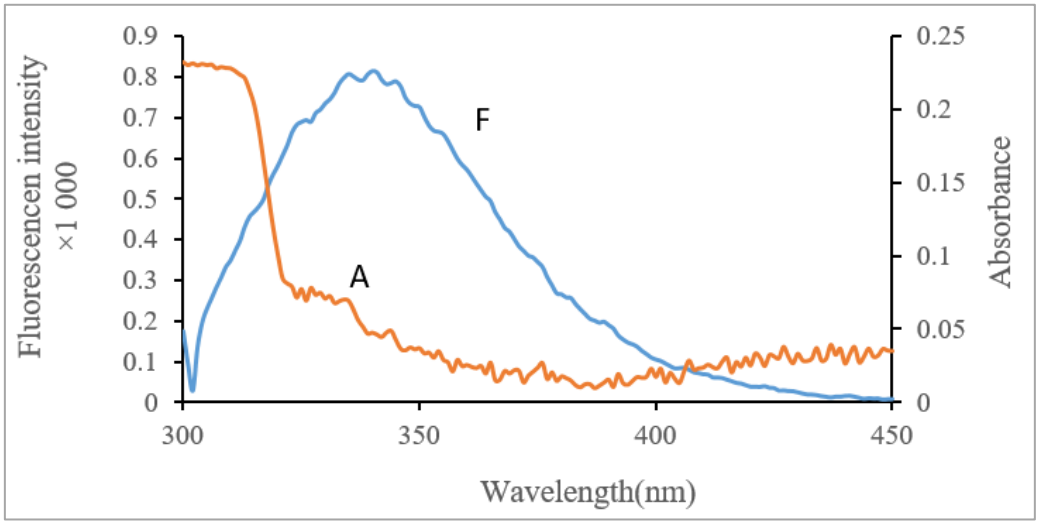

Figure 5. *Overlap of fluorescence emission of ovalbumin(F) and UV-vis absorption spectra of gallic acid(A).*

In the equation (5)-(7), K^2=2/3, n =1.34 and φ=0.118 from previous report [9]. From equations (5)-(7), we calculated that $J=1.59\times10^{-14}$ $cm^3\bullet L\bullet mol^{-1}$, r=3.73 nm and R_0=2.64 nm. Because the average distance is in the range of 2-7 nm and $0.5R_0<r<1.5R_0$, it can be inferred that the energy transfer from ovalbumin to gallic acid with high probability [10, 13].

3.4. Synchronous and 3D Fluorescence

3.4.1. Synchronous Fluorescence Spectra

The information about microenvironment polarity changes around the fluorophore (Tyrosin and Tryptopan) can be provided by synchronous fluorescence. Figure 6 shows the synchronous florescence spectra of ovalbumin with different gallic acid concentration. It can be seen from Figure 6. that there were red shifts in both the maximum emission wavelength of the tyrosine and tryptophan. This result points out that the conformation of tyrosine and trytophan microenvironment in ovalbumin was affected by gallic acid. It also shows that the interaction increased the polarity and the hydrophilicity around tyrosin and tryptophan [14, 15].

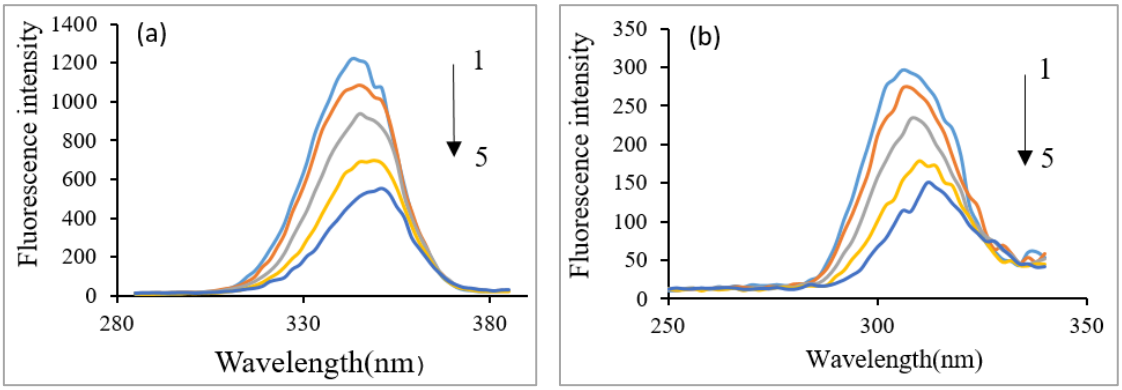

Figure 6. *Synchronous fluorescence spectra of ovalbumin in the absence and presence of gallic acid. a) Δλ=15 nm(tyrosin), b) Δλ=60 nm(tryptophan) 1: Ovalbumin solution(10 μm), 2:gallic acid(10 μm)+ovalbumin, 3: gallic acid(20 μm)+ovalbumin, 4: gallic acid(30 μm)+ovalbumin, 5:gallic aicd(40 μm)+ovalbumin.*

3.4.2. 3D Fluorescence Spectra

The three-dimensional fluorescence spectra of ovalbumin with and without gallic acid were shown in Figure 7. In this figure, two peaks were obtained. Peak 1 was due to Rayleigh scattering because the excitation and emission wavelength were the same. Apart from peak1, peak 2 (λ_{ex}=280, λ_{em}=340) mainly described the spectral characteristics of tyrosin and tryptophan residues [7].

Fluoscence intensity of ovalbumin in the prescence of gallic acid was 44.2% lower than the one in the absence of gallic acid. According to previous report, the change in the

excitation or emission wavelength of fluorescence peak indicates that the protein structure changes due to interacting with the ligand. Therefore, it can be inferred that that the interaction between ovalbumin and gallic acid induces conformational changes in ovalbumin structure [7, 10].

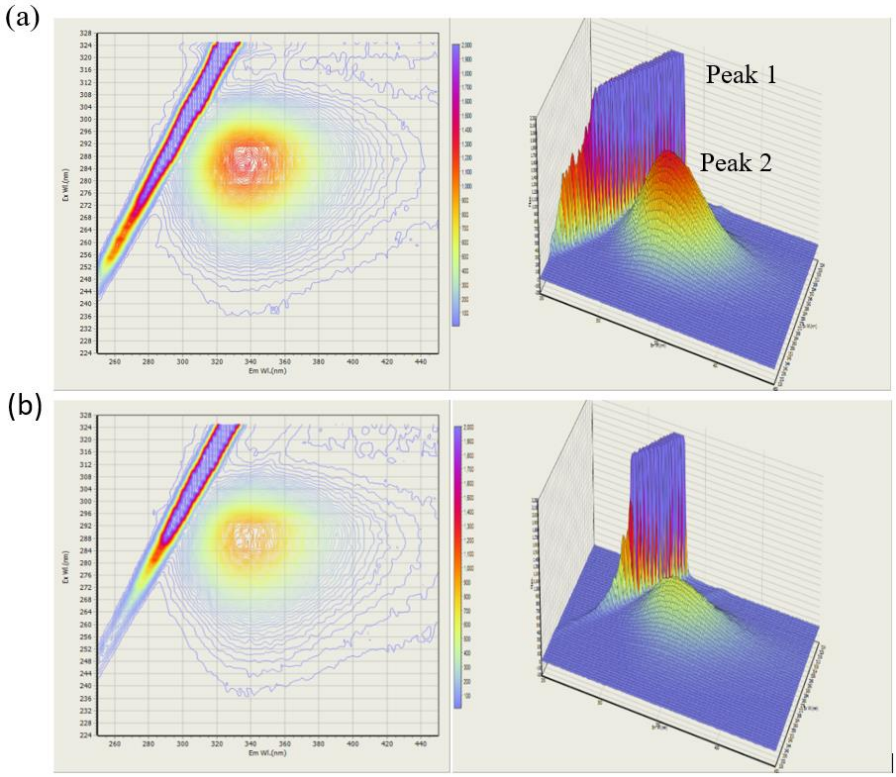

Figure 7. *3D fluorescence spectra of (a) ovalbumin and (b) ovalbumin-gallic acid complex.*

4. Conclusion

A comprehensive study of florescence quenching spectra, synchronous and 3D florescence spectra were applied to investigate the interaction between gallic acid and ovalbumin. It can be drawn that the quenching of ovalbumin by gallic acid was a static mechanism. The negative value of free energy indicated that the binding process was spontaneous. In the binding of gallic acid to ovalbumin, electrostatic interactions were the dominant intermolecular forces. By Förster's resnonace energy transfer theory, the average distance between gallic acid and ovalbumin was found to be 3.73 nm. In addition, the conformational change was mainly caused by the microenvironmental changes of tyrosin and tryptophan residues.

Abbreviations

EPCG	Epigallo-catechin 3-Gallate
FRET	Förster's Resonance Energy Transfer
3D	Three-dimensional

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interests.

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