Spectroscopic Approaches To Define The Interaction Between Gallic Acid And Ovalbumin

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Abstract

Foaming capacity and stability are important characteristics for determining proteins as aerated food items. Surface-active chemicals stabilize the bubbles in foams, in which gas are distributed in aqueous solutions. Protein-polyphenol combinations are common in our routines. Gallic acid (GA) is one of the most abundant polyphenols. An attempt has been made for the stability of the foam in combination with ovalbumin (OVA) and Gallic acid. The results of this study provided some fundamental information on how polyphenols influence the foaming properties of OVA, and proved that polyphenols can be used as stabilizing agent in food industry to improve the functional properties of foam. The interaction between Gallic acid (GA) and ovalbumin (OVA) were studied and characterized by Ultraviolet-visible (UV–Vis). The conformation changes have been noted and quenching the mechanism between Gallic acid (GA) and ovalbumin (OVA) proved to be static quenching by fluorescence. Tryptophan (Trp) residues quenches vibrantly than Tyrosine (Try) which were analyzed by synchronous and the structural and functional group analyzed by FT-IR spectrum. The binding constant and the number of binding sites for GA on OVA were also calculated in this study.

Key words: Ovalbumin (OVA), Fluorescence, Gallic acid (GA), Synchronous fluorescence, FT-IR.

1. Introduction

Ovalbumin from Egg White Protein (EWP) is one of the important protein sources. EWP is highly recognized in the food sector for its exceptional gelling and foaming qualities, which are commonly utilized in food items such as meringues, cakes, cookies, and chocolate mousses. (1)

Ovalbumin (OVA) is the predominant component of EWP (2), with a molecular weight of 45 kDa and 385 single amino acid chains, and it is a typical spherical phosphor - glycoprotein (P-gp). (3)

Foaming is an important functional attribute in a variety of meals, and attractant. It improves the sensational feel, associated foods including meringues, soufflés, whipped toppings, chiffon desserts, and leavened bread goods (4-7) Surface-active chemicals stabilize the bubbles in foams, which are gas distributed in aqueous solutions (8-9). Protein when dissolved in water and agitated rapidly, foam forms and expands quickly, generating a sticky protein film around the gas or air droplet (10). Foaming capacity and stability are important characteristics in determining proteins are suitable for aerated food items. When Bubbles would eventually go through three stages: Ostwald's maturity, drainage, and coalescence, becoming unstable over time, bursting, and minimizing free energy (11). In order to increase foaming capabilities, several research have been undertaken focused on protein modifications, such as high-intensity ultrasound (12), phosphorylation under wet-heating conditions (13), and oxidative modification (14).

Polyphenols, which include flavonoids, stilbenes, phenolic acids, lignans, and other subclasses, are a biologically active class of plant compounds (15). Protein-polyphenol combinations are common in our daily lives. Gallic acid (GA) is one of the most abundant phenolic acids, and it can be found as saccharides in fruits, grains, and nutritional supplements for human use, as well as in traditional Chinese herbs (16). GA is a natural antioxidant for it contains two free phenolic hydroxyl groups in its structure. However, most of the current researches on GA and protein has focused on improving the antioxidant capacity of protein (17), While few studies on how polyphenols affect the functional properties of proteins, such as solubility, foaming and

emulsification properties which are visible to the naked eye and are of greatest concern to consumers and processors .Structural changes of OVA were analyzed by measuring intrinsic tryptophan fluorescence further elucidated the mechanism of interaction between GA and OVA. The study provides some fundamental information on how polyphenols influenced the foaming properties of OVA, and proved that polyphenols could be used to improve the functional properties of food foam.

To understand the binding of gallic acid and ovalbumin through fluorescence techniques of native and polyphenols bound protein in aqueous phase has been studied. In addition, to the experimental studies, UV/ Visible, synchronous fluorescence, and time-resolved analyses assisted the confirmation changes of OVA. The influences of the polyphenols on the secondary structure of protein were studied by FT-IR studies.

2. Materials and Methods

Ovalbumin (from chicken egg white, lyophilized powder, ≥ 98%), Gallic acid were purchased from Sigma-Aldrich Chemical Co. A stock solution of OVA (1×10^{-5}M) was prepared by phosphate buffer. The stock solution of GA (1×10^{-4}M) was prepared by using ethanol. The solutions were prepared just before taking absorption and fluorescence measurements.

2.1. Spectroscopic Instruments Details

Absorption spectra were recorded between 200 and 400 nm with a SHIMADZU model (SHIMADZU 1800 PC UV/Vis Spectrophotometer). Fluorescence spectra of each solution were recorded between 280 and 800 nm using with a RF-5301 PC (Shimadzu Corporation, Kyoto, Japan) fluorescence spectrophotometer. Fluorescence lifetimes were measured by using a time-resolved HARIIBA – JOBIN YVON [SPEX-SF B-III] spectrofluorometer. FT-IR spectra liquid sample of GA, OVA are measured from 4000 to 400 cm^{-1} on an Agilent resolutions pro330 spectrometer.

3. Results and Discussion.

3.1. Absorption spectral characteristics of GA with OVA

UV – Vis spectroscopy was used to study the drug binding interactions (18).The structural changes and an understanding of the formation of a complex between different molecules and proteins were investigated with suitable and effortless spectroscopic technique such as UV – Vis spectroscopy. Therefore, UV – Vis spectral results were shown in Fig.1. The excitation wavelength is observed at 281 nm at pH 7.4. The intensity was recorded for OVA UV – Vis Spectra increased with increasing concentration of GA up to 1.0 [mol L^{-1}] at pH 7.4. The absorption spectral data of OVA with different concentrations of GA were detailed in the Table.1. From Fig.1 UV-visible spectra of OVA with an increasing addition of GA, the strength of UV-absorption system increased gradually, and no obvious change of absorption maximum wavelength was detected. Since the amide group of OVA absorbs UV light, an increase in UV absorption of GA suggests that hydrogen bonding between the phenolic hydroxyl of GA and the amide group of OVA may take place. Hydrogen bonding increased the intensity of the π electron clouds on the aromatic ring of GA and lowered the transition energy, resulting in a hyperchromic effect (19).

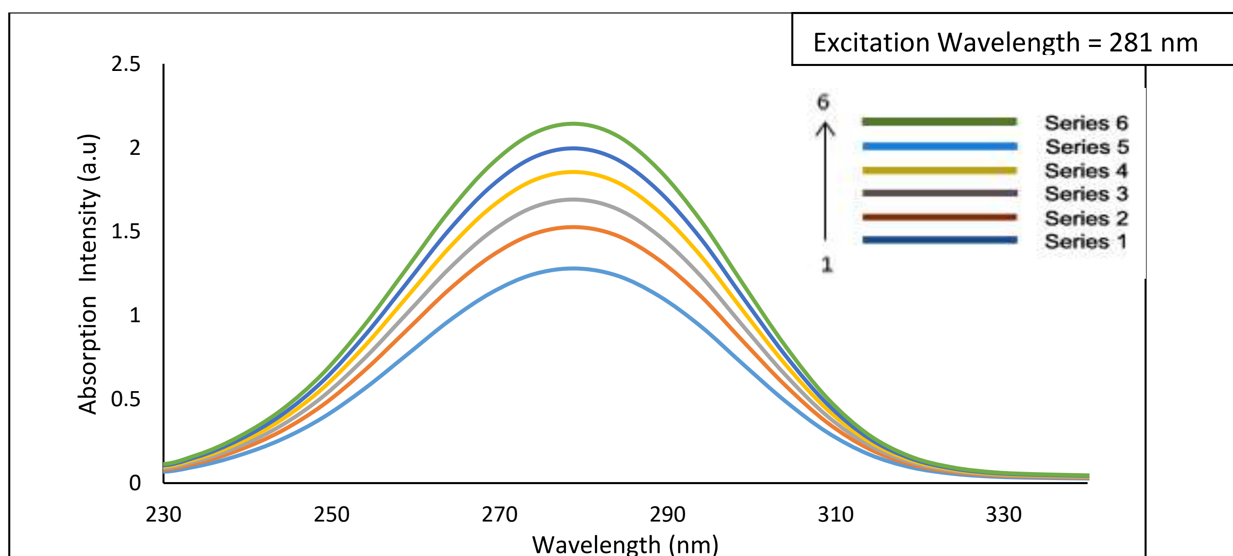


Figure.1. UV-Vis absorption spectra of OVA with GA (1) 0.0, (2) 0.2, (3) 0.4, (4) 0.6, (4) 0.8 and (5) 1.0 (mol L^{-1}) with excitation wavelength = 281 nm in PBS medium (pH 7.4).

Table.1. Absorption spectral data of BLG with different concentrations of GA at pH 7.4.

S.No	Concentration of GA (mol L^{-1})	Absorbance(a.u)
1.	0.0	0.17
2.	0.2	0.25
3.	0.4	0.31
4.	0.6	0.39
5.	0.8	0.47
6.	1.0	0.54

3.2. Fluorescence quenching of OVA by GA

Fluorescence quenching may result from variety of processes such as excited state reactions, molecular rearrangements, energy transfer, ground-state complex formation (static quenching) or collisional interactions (dynamic quenching) (20)

Fluorescence quenching shows the changes in fluorescence emission spectra with different concentration of gallic acid with OVA shown in Fig.2.From the spectrum it was clear that the fluorescence intensity of OVA decreases at around 340 nm with increasing in the concentration of gallic acid which is shown in Table.2.

Fig.2. Shows Fluoresces emission spectra OVA quenched by GA no spectral shift was observed and indicating that interaction with GA have no influence on the environment of fluorophores in protein (21).

Due to the presence of the amino acids Trp, Tyr, Phe, and other individual residues in the molecular structure, most proteins could emit intrinsic fluorescence upon UV light absorption (22). The protein fluorescence intensity could be weakened by various molecular interactions, which is called fluorescence quenching. This phenomenon has been used to study protein-polyphenol binding information. It shows that the OVA fluorescence intensity decreased significantly upon GA addition.

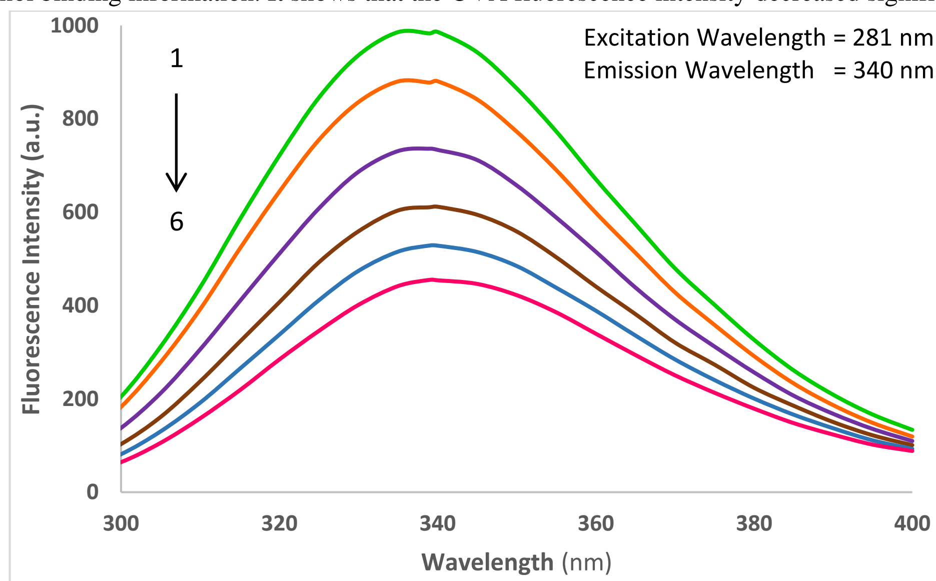


Figure.2. Steady-state fluorescence spectra of OVA with GA (1) 0.0, (2) 0.2, (3) 0.4, (4) 0.6, (5) 0.8 and (6) 1.0 (mol L^{-1}) with emission wavelength = 340 nm in PBS medium (pH 7.4).

Table.2. Fluorescence spectra of OVA with different concentrations of GA at pH 7.4.

S.No	Concentration of GA (mol L^{-1})	Fluorescence intensity (a.u)
1.	0.0	983.0
2.	0.2	877.7
3.	0.4	735.9
4.	0.6	610.5
5.	0.8	528.5
6.	1.0	454.9

The fluorescence quenching for interacted molecule quencher [Q] and fluorescer [F] can be analyzed by the Stern-Volmer equation (23)

$$\frac{F_0}{F} = 1 + K_{sv} [Q] = 1 + K_q \tau_0 [Q]$$

where F_0 and F denotes the steady state fluorescence intensities in the absence and the presence of the quencher [Q] respectively. K_q is the biomolecular quenching rate constant, □□□ is the average lifetime of the protein, [Q] is the concentration of the quencher, K_{sv} is the Stern -Volmer quenching constant (24). The above equation is applied to determine K_{sv} by linear regression of a plot of (F_0/F) against [Q].

The classical Stern-Volmer plots OVA in the presence of gallic acid. The Stern-Volmer quenching constant and biomolecular quenching rate constant (K_q) are shown in Fig.3.

Table.3. Stern - Volmer (K_{sv})and bimolecular quenching rate constant (K_q) of Ovalbumin with gallic acid.

Quenchers	K_{sv} x 10^5(L mol^{-1})	K_q(L $mol^{-1}S^{-1}$)x10^8	R^a	$S.D^b$
Gallic acid	1.19	2.82	0.99	0.64

a→is the correlation coefficient, b→is the standard deviation.

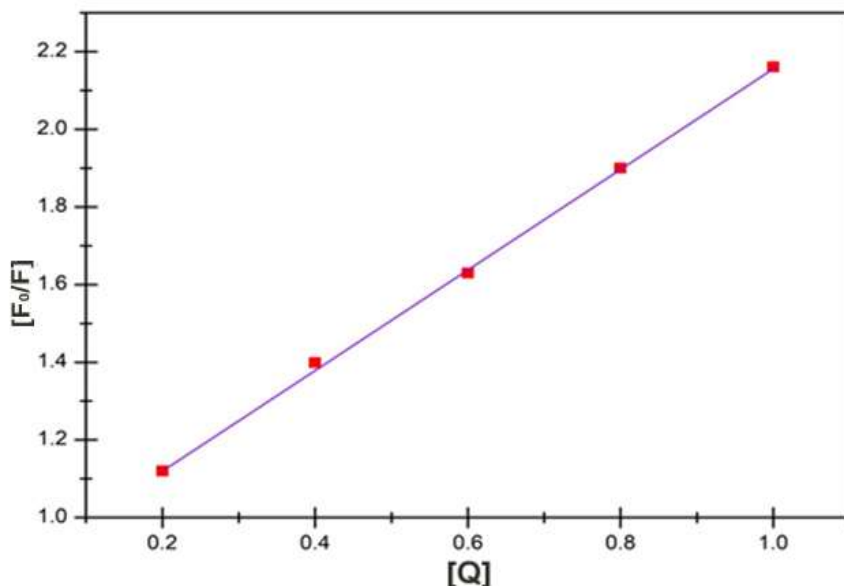
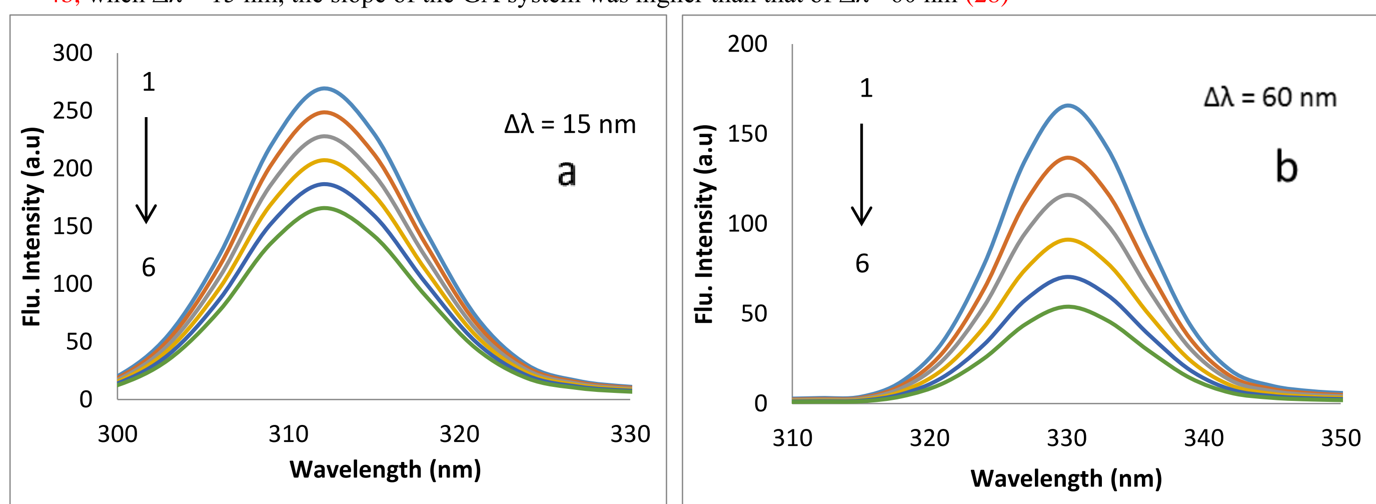


Fig.3.Stern-Volmer plot for the quenching of OVA by Gallic acid concentration

3.3 Synchronous fluorescence Spectroscopy

Synchronous fluorescence spectra (SFS) were used to detect the changes in molecular microenvironment of the fluorophore functional groups. OVA combined with GA were subjected to synchronous fluorescence spectra to get additional information about the conformational changes presented in Fig.4.The effects of GA on the SFS of OVA at the two Δλ are shown in Fig.4 (a) **Δλ = 15 nm** and (b) **Δλ =60 nm**.

The present study results were in agreement with the study by Miller (25-26). The steady state Δλ excitation and emission wavelengths at 15nm and 60nm of synchronous fluorescence spectra represent characteristic information about the Tyr or Trp residues, respectively. As the concentration of GA increased, the fluorescence intensity of OVA decreased. The emission intensities of OVA at Δλ = 15 nm decreased with no shift occurred. The considerable fluorescence quenching showed that the phenolic acids came closer to tyrosine residue during the binding process but no effect on the microenvironment of the Tyr residues (27). When Δλ= 60 nm, the emission intensity of OVA decreased (Fig. 4a and 4b), which indicated that phenolic acids were bound in the vicinity of the Tyr residues. When comparing the binding distance between phenolic acids and chromophore molecules (Tyr and Trp residues), the fluorescence intensity of Tyr and Trp residues in the presence of phenolic acids were obtained when Δλ were stabilized at 15 or 60 nm. As shown in Fig. 4a and 4b, when Δλ = 15 nm, the slope of the GA system was higher than that of Δλ= 60 nm (28)



Fluorescence quenching techniques can be used to determine the binding constant (K_a) and the number of binding sites (n) dependent on the interaction of GA with OVA. Under the presumption that proteins have independent binding sites, the values of Ka and n can be determined by using the formula (29)

$$\log (F_0 - F)/F = \log K_a + n \log [Q]$$

The fluorescence intensities of the protein with and without quencher are F and F_0, respectively, and [Q] is the initial quencher concentration. Fig.5.depicts the results of fluorescence quenching studies, while Table.4. mentions the computed results. In 1:1M concentration ratios, the results suggested that OVA had only one equivalent binding sites with GA, implying

that a complex formed between GA and OVA. In general, if K is greater than 10,000 the binding is strong (30). The K_a value of 1.18 x10^5 from Table.4. demonstrate that GA and OVA had a strong binding interaction. As a result, the GA could quickly interact with OVA biological function. Free energy of ground state (ΔG_g) and excited state (ΔG_e) were calculated and tabulated.

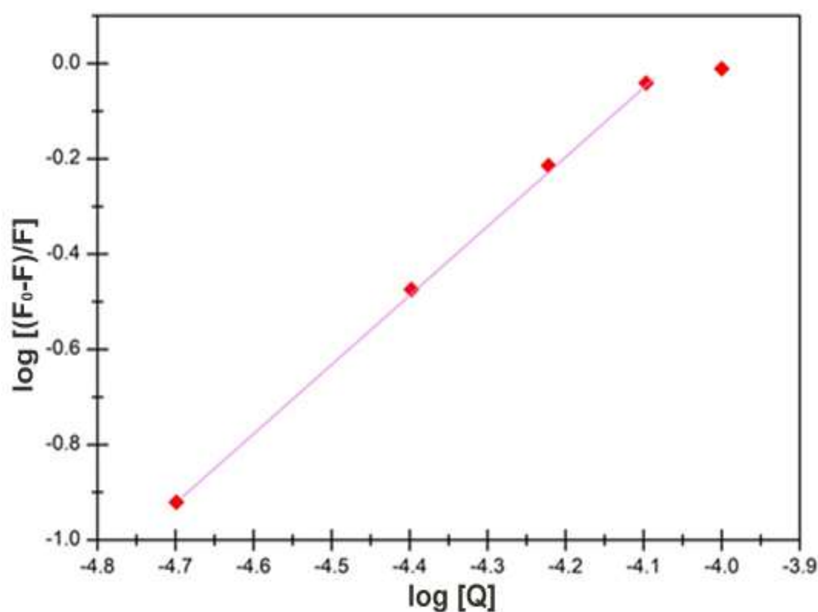


Fig.5.The plot of log (F_0-F)/F versus log [Q]

Table.4. The binding constant (K_a), binding site (n) correlation coefficient(R), change in free energy ΔG_g (for ground state) and ΔG_e (for excited state) of ovalbumin with Gallic acid

Quenchers	K_a x 10^5(L mol^{-1})	n	R	ΔG_g(kJ mol^{-1}) x 10^3	ΔG_e(kJ mol^{-1}) x 10^3
Gallic acid	1.18	1.10	0.98	-1.14	16.95

3.5. Time-resolved fluorescence spectra of OVA with Gallic acid

Time-resolved fluorescence spectral studies were performed to understand how encapsulation within the GA cavity influences the stability and statics in the excited states.GA is carried out to substantiate time-resolved fluorescence decay curves and shown in Table.5. The lifetime and decay curves of OVA and OVA with GA are well displayed in Fig.6. The time-resolved fluorescence decay of OVA combine with GA showed that the tri-exponential decay indicated three lifetimes value (τ_1 =2.8 × 10^{-9}s/1.9 × 10^{-9} s, τ_2 = 6.5× 10^{-9} s/5.1 × 10^{-9} S and τ_3= 7.48× 10^{-9} S/2.2× 10^{-9}s) for the presence and absence of GA respectively.

Table.5. Fluorescence lifetime and relative amplitudes of OVA with different concentrations of Gallic acid

The concentration of Gallic acid (M)	Lifetime (ns)			Average Lifetime τ × 10^{-9} sec	Relative amplitude			χ^2	S.D 10^{-11} sec		
	τ_1	τ_2	τ_3		B_1	B_2	B_3		τ_1	τ_2	τ_3
OVA	2.8	6.5	7.4	4.8	30.7	72.3	21.1	1.2	2.1	5.3	4.1

OVA+GA (0.4)	2.6	5.3	2.7	4.1	23.9	59.9	19.8	1.1	1.2	3.5	1.8
OVA+GA (0.8)	2.5	5.2	2.3	3.3	22.0	54.0	18.8	1.1	1.1	3.1	1.2
OVA+GA (1.2)	1.9	5.1	2.2	3.1	20.5	49.5	16.5	1.0	1.0	2.7	1.0

The consolidated data and the decay curve confirmed the formation of complex based on the hike in lifetime and relative amplitude values by the addition of GA concentration in aqueous solution. The enhanced lifetimes of OVA during the addition of GA leads to the restriction of rotational degrees of freedom with consequent impact on depletion of non - radiative decay channels. The observed enhancement in lifetime indicates that OVA molecule experiences less polar hydrophobic environments within the GA cavity resulting in the reduction of non - radiative decay processes. Further, the increase in fluorescence lifetime is a result of the significant interactions of the OVA with hydrophobic GA. The relative amplitude of free OVA and bound OVA with GA is also continuously enhanced which suggests the formation.

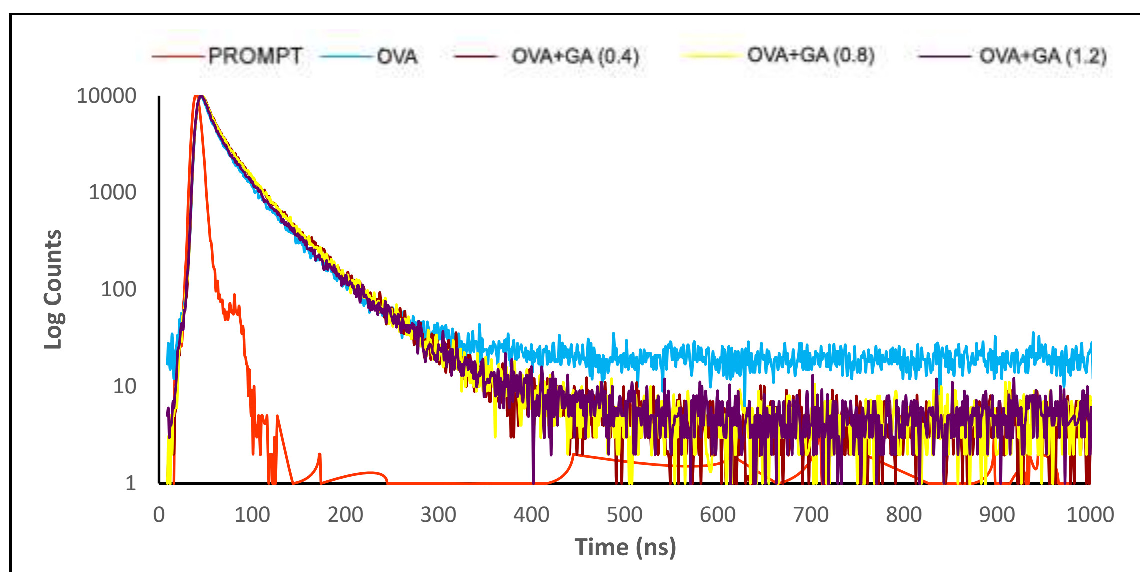


Fig.6. Time-resolved fluorescence spectra of OVA with different concentrations of Gallic acid (mol L^{-1}) 1) 0.0 2) 0.4, 3) 0.8 and 4) 1.2.3.6. FT-IR spectroscopic study of OVA and GA

The conformational changes and its stability of OVA combining at different concentration with gallic acid was determined by its functional and structural compounds which is shown in Table.6 and Fig.7.

Proteins has produced numerous insights into the folding mechanism as well as the identification of the folded protein can be analyzed through FT-IR.

The key elements controlling the conformational sensitivity are the amide bands with hydrogen bonding and coupling between transition dipoles. Amide band was primarily of amide-I detected in the peak range (1600–1700 $cm^{-1)}$ and amide-II in the range (1500-1600 cm^{-1}).

The amide-I band is frequently utilized for visible analysis of protein secondary structure elements and the amide-II band, on the other hand, has a relatively complex association. The C=O stretching vibration of the peptide backbone, which is influenced by the interactions between the amide groups and the strength of the hydrogen bond, creates the amide-I band. Fig.7. (a) show the FT-IR spectra of OVA and Fig.7. (b), 7. (c) OVA with different concentration of GA.

Table 6. shows pure OVA has α - helix rich in confirmation by extreme band in the amide-I province that centers at 1653.284 cm^{-1}. The OVA with GA complex, the typical peak stretched at1653.409 cm^{-1} and 1654.582. In all the three spectra, the fundamental characteristics of the OVA secondary structure were preserved in the presence of GA. According to the FT-IR analysis, it could be conformed that the GA was involved in the complexation and the furan ring is present outside of the OVA cavity.

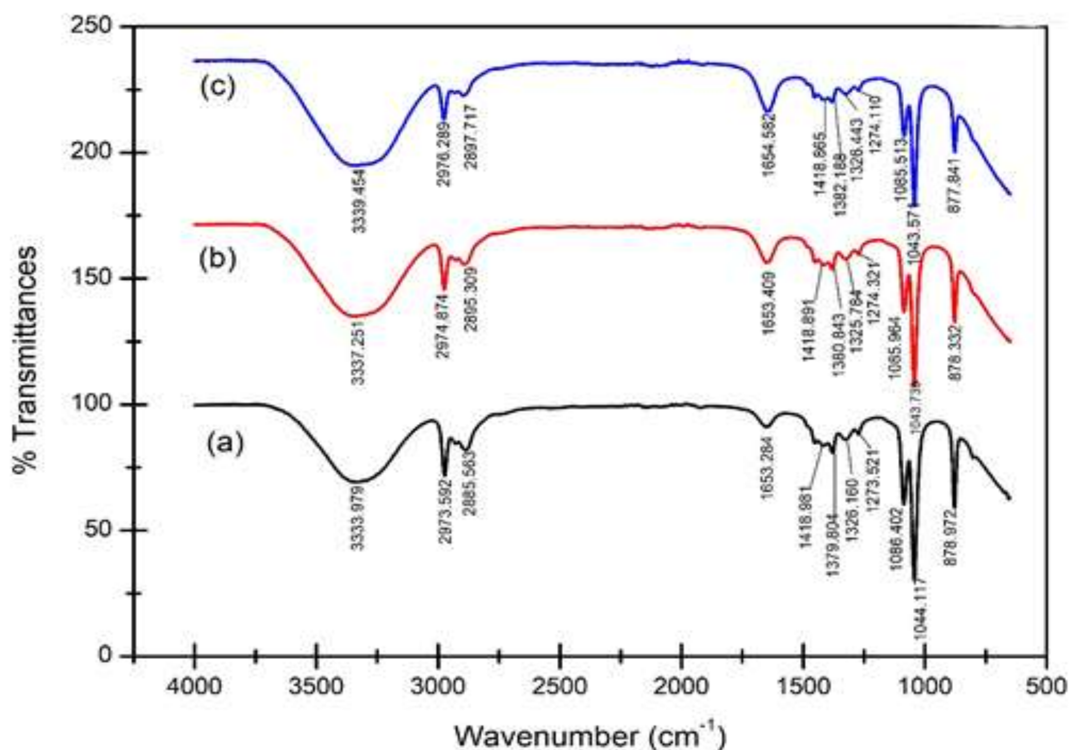


Figure.7. FT-IR spectra of (a) OVA (b) OVA+0.4mol L^{-1}GA (c) OVA+1.0mol L^{-1}GA

Table.6. FT-IR peak assignments of OVA without and with different concentration of Gallic acid

Wavenumber cm^{-1}			**Peak assignments**
OVA	**(OVA+0.4 mol L^{-1}GA)**	**(OVA+1.0 mol L^{-1}GA)**	
3333.979	3337.251	3339.454	O-H Stretching
2973.592	2974.874	2976.289	C-H Stretching
2885.563	2895.309	2897.717	C-H Stretching
1653.284	1653.409	1654.582	C=N Stretching
1418.981	1418.891	1418.865	C-C Stretching
1379.804	1380.843	1382.188	C-N Stretching
1326.160	1325.784	1326.443	CH_3 Implement Bending
1273.521	1274.321	1274.110	CH_2 Implement Bending
1086.402	1085.964	1085.513	C-O Stretching
1044.117	1043.733	1043.571	C-O Stretching
878.972	878.332	877.841	C-H Aromatic

4. Conclusion

The results of multi-spectroscopic techniques demonstrate that polyphenols caused the fluorescence quenching of egg white protein (OVA). The mechanism of fluorescence quenching was mainly static quenching, which was induced by the formation of the protein-polyphenol complexes. The results of UV–Vis absorption spectra, FT-IR spectra, and synchronous fluorescence spectra showed that GA affected the secondary structure of OVA and the fractions of α-helix was increased instead of the fractions of β-sheet. The obtained results might allow a better understanding of the structure-function relationship in terms of obtaining food with desired functionality. The presented study is truly valuable in the dairy processing industry and in daily life.

References

1. 1.Kuan, Y.-H., Bhat, R., & Karim, A. A. (2011). Emulsifying and foaming properties of ultraviolet-irradiated egg white protein and sodium caseinate. Journal of Agricultural and Food Chemistry, 59(8), 4111–4118 https://doi.org/10.1021/jf104050k
2. 2.Yu, Z., Liu, B., Zhao, W., Yin, Y., Liu, J., & Chen, F. (2012). Primary and secondary structure of novel ACE-inhibitory peptides from egg white protein. Food Chemistry, 133(2), 315–322 https://doi.org/10.1016/j.foodchem.2012.01.032
3. Hassan, N., Messina, P. V., Dodero, V. I., &Ruso, J. M. (2011). Rheological properties of ovalbumin hydrogels as affected by surfactants addition. International Journal of Biological Macromolecules, 48(3), 495–500. https://doi.org/10.1016/j.ijbiomac.2011.01.015
4. Beveridge, T., Toma, S., &Nakai, S. (1974). Determination of SH-and SS-groups in some food proteins using Ellman's reagent. Journal of Food Science, 39(1), 49–51. https://doi.org/10.1111/j.1365-2621.1974.tb00984.x
5. WD Annan, W Manson (1969). A fractionation of the os-casein complex of bovine milk. Journal of Dairy Research 36: 259. https://doi.org/10.1017/S0022029900012760
6. Atassi. M.Z., Habeeb, A.F.S.A. and Rystedt. L. (1970). Lack of immunochemical cross-reaction between lysozyme and a-lactalbumin and comparison of their conformations. Biochimica et Biophysica Acta 1970 Vol.200 No.1 pp.184-87
7. H.J.T.Beveridge[2]S.Nakai (1970) . Effects of chemical modification with 2-phenyl-1,4-dibromoacetoin on as,-casein.journal of dairy science. 53: 1532. https://doi.org/10.1021/ja01088a025
8. Brun, M., Delample, M., Harte, E., Lecomte, S., & Leal-Calderon, F. (2015). Stabilization of air bubbles in oil by surfactant crystals: A route to produce air-in-oil foams and air-in-oil-in-water emulsions. Food Research International, 67, 366–375. https://doi.org/10.1016/j.foodres.2014.11.044
9. Dickinson, E. (2020). Advances in food emulsions and foams: Reflctions on research in the neo-pickering era. Current Opinion in Food Science, 33, 52–60. https://doi.org/10.1016/j.cofs.2019.12.009
10. Tang, S., Hettiarachchy, N., Horax, R., &Eswaranandam, S. (2003). Physicochemical properties and functionality of rice bran protein hydrolyzate prepared from heatstabilized defatted rice bran with the aid of enzymes. Journal of Food Science, 68(1),152–157. https://doi.org/10.1111/j.1365-2621.2003.tb14132.x
11. Herd, R. A., & Pinkerton, H. (1997). Bubble coalescence in basaltic lava: Its impact on the evolution of bubble populations. Journal of Volcanology and Geothermal Research, 75 (1–2), 137–157. https://doi.org/10.1016/S0377-0273(96)00039-X
12. Xiong, W., Wang, Y., Zhang, C., Wan, J., Shah, B. R., Pei, Y., … Li, B. (2016). High intensity ultrasound modifid ovalbumin: Structure, interface and gelation properties. Ultrasonics Sonochemistry, 31, 302–309. https://doi.org/10.1016/j.ultsonch.2016.01.014
13. Sheng, L., Wang, Y., Chen, J., Zou, J., Wang, Q., & Ma, M. (2018). Inflence of highintensity ultrasound on foaming and structural properties of egg white. Food Research International, 108, 604–610. https://doi.org/10.1016/j.foodres.2018.04.007
14. Duan, X., Li, M., Shao, J., Chen, H., Xu, X., Jin, Z., et al. (2018). Effect of oxidative modifiation on structural and foaming properties of egg white protein. Food Hydrocolloids, 75. http://dx.doi.org/10.1016/j.foodhyd.2017.08.008
15. Daniel, M. (2006). Medicinal plants: Chemistry and properties. Science publishers.
16. Jiang, R.-W., Lau, K.-M., Hon, P.-M., Mak, T. C., Woo, K.-S., & Fung, K.-P. (2005). Chemistry and biological activities of caffeic acid derivatives from Salvia miltiorrhiza. Current Medicinal Chemistry, 12(2), 237–246 https://doi.org/10.2174/0929867053363397
17. Li, T., Li, X., Dai, T., Hu, P., Niu, X., Liu, C., et al. (2020). Binding mechanism and antioxidant capacity of selected phenolic acid-β-casein complexes. Food Research International, 129, 108802 https://doi.org/10.1016/j.foodres.2019.108802
18. MA Rub, JM Khan, N Azum, AM Asiri (2017) .Influence of antidepressant clomipramine hydrochloride drug on human serum albumin: Spectroscopic study- Journal of Molecular Liquids, https://doi.org/10.1016/j.molliq.2017.05.143
19. Jianhui Ye , Fangyuan Fan , Xinqing Xu , Yuerong Liang (2013) Interactions of black and green tea polyphenols with whole milk - Food Research International http://dx.doi.org/10.1016/j.foodres.2013.05.033
20. BJ Liu, CX Liu, F Liu, F Liu, HH Liu, HM Liu, J Li…,(2007) Production of σ in ψ (2S)→ π+ π− J/ψ…,(- Physics Letters B. http://dx.doi.org/10.1016/j.physletb.2006.11.056
21. Jianhui Ye , Fangyuan Fan , Xinqing Xu , Yuerong Liang (2013) Interactions of black and green tea polyphenols with whole milk - Food Research International http://dx.doi.org/10.1016/j.foodres.2013.05.033
22. changeGA Jones, M Lazarchic (2000) Phosphatidylinositol-specific phospholipase C-γ1 undergoes pH-induced activation and conformational - Biochimica et Biophysica Acta (BBA)-Molecular …, - Elsevier https://doi.org/10.1016/s1388-1981(00)00097-4

23. JBF Lloyd (1971) Synchronized excitation of fluorescence emission spectra - Nature Physical Science, - nature.com
24. .CW Fuller, JN Miller (1979) .Silver medal lectures - Proceedings of the Analytical Division of the …,- pubs.rsc.org
25. DE Lowther, R Dryer-Minnerly, R Wang..(2019) ,Systemic and local immunity following adoptive transfer of NY-ESO-1 SPEAR T cells in synovial sarcoma…,Journal for ImmunoTherapy of Cancer doi: 10.1186/s40425-019-0762-2.
26. J Wang, X Wang (2019) Structural equation modeling: Applications using Mplus books.
27. Fakhrossadat Mohammadi 1, Marzieh Moeeni 2 Study on the interactions of trans-resveratrol and scurcumin with bovine α-lactalbumin by spectroscopic analysis and molecular docking(2010) https://doi.org/10.1016/j.msec.2015.02.007Belatik et.,al.,2012; ulrich,1990 Binding sites of retinol and retinoic acid with serum albumins- http://dx.doi.org/10.1016/j.ejmech.2011.12.002
28. Yada Kanjanapan, Daphne Day, Lisa Wang, Hamad Al-Sawaihey, Engy Abbas, Amirali Namini, Lillian L. Siu(2019).Hyper progressive disease in early-phase immunotherapy trials: clinical predictors and association with immune-related toxicities. Cancer., 1218-20. https://doi.org/10.1002/cncr.31999
29. Belatik et.,al.,2012; ulrich,1990 Binding sites of retinol and retinoic acid with serum albumins- http://dx.doi.org/10.1016/j.ejmech.2011.12.002
30. Guofan Zhang1 *, Xiaodong Fang2 *, Ximing Guo3 *, Li Li1 * 2012 .The oyster genome reveals stress adaptation and complexity of shell formation doi:10.1038/nature11413