J Fluoresc (2013) 23:333–338
DOI 10.1007/s10895-012-1151-0

ORIGINAL PAPER

Investigation of Binding Properties of Umbelliferone (7Hydroxycoumarin) to Lysozyme

Elmas Gök

Received: 9 March 2012 / Accepted: 20 November 2012 / Published online: 8 December 2012

Abstract The binding interaction of lysozyme and umbelliferone (7hydroxcoumarin, 7HC) was investigated by UV–vis absorption and fluorescence quenching. It was obtained from fluorescence spectra that the fluorescence quenching of lysozyme by 7HC was probably a result of the formation of lysozyme-7HC complex and binding parameters were determined according to the Stern-Volmer equation. The effects of various common metal ions on the binding were also studied. The thermodynamic parameters were calculated at different temperatures which indicated that hydrophobic interaction. The binding distance (r) between the donor (lysozyme) and the acceptor (7HC) was 3.81 nm based on the Förster theory of non-radioactive resonance energy transfer.

Keywords Fluorescence quenching · Lysozyme · Umbelliferone · FRET

Introduction

Coumarins exhibit strong fluorescence in the visible region which makes them suitable for use as colorants, in dye lasers and as nonlinear optical chromophores. They possess distinct biological activity and have been described as agents with potential for anticancer and anticoagulant activity. They can also induce modifications in cell growth, development and intracellular communication mechanisms. The photophysical properties of these compounds depend on the nature and position of a substituent group in the parent molecule and due to a change in the surrounding media [1–4]. Umbelliferone (7hydroxycoumarin, 7HC) is a major biotransformed product of a coumarin derivative (Fig. 1), which is a widely distributed natural product [5–9]. Umbelliferone and its derivatives are suitable for use dyes, fluorescent probes, photoactive agents and used in the synthesis of drugs. It exhibits antioxidant, antidiabetic, antifungal properties.

Lysozyme is a small globular protein belongs to the class of enzymes, consisting of 129 amino acid residues with four disulphide bond. It is capable of destroying of the cell walls many kinds of bacteria and thus it provides some protection aganist infection as an antiseptic. Its intrinsic fluorescence results from especially two residues, Trp 62 and Trp 108, which are located close to the substrate binding sites. Lysozyme is found in tears, saliva, egg white, sweat and many animal fluids. So, it is studied several research fields such as transportation of drugs, and pharmaceutical applications. Fluorescence quenching is a powerful and simple method to study the interaction of small molecule with protein because of its high sensitivity, rapidity and convenience. Measurement of quenching of protein intrinsic fluorescence by a molecule helps to understand the binding properties of small molecule to protein [10–12]. A lot of reports were published on the interactions of small molecules with lysozyme by fluorescence quenching such as cephalosporin analogues [13], bromophenol blue [14], biphenol A [15], cinnamic acid [16], baicalein [17], vitamin B12 [18], silymarin [19], antrhraquinone dyes [20], malachite green [21] etc. In the present study, 7HC was used as small molecule and studied the binding properties of lysozyme-7HC by UV–vis absorption and fluorescence quenching. The binding parameters, thermodynamic functions, intermolecular distance and the effect of common metal ions on the binding constant of lysozyme-7HC were investigated. The studies on the interaction of 7HC with lysozyme will provide an insight into the

E. Gök (✉)
Department of Chemistry, Hacettepe University, 06800, Ankara, Turkey
e-mail: gokoglu@hacettepe.edu.tr

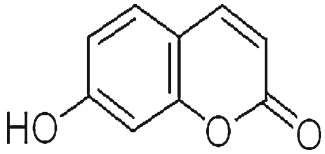

Fig. 1 Molecular structure of 7HC

chemical nature of the interaction of umbelliferone like small molecules and biomacromolecules.

Experimental

Reagents

Chicken egg white lysozyme and umbelliferone (7HC) were obtained from Sigma-Aldrich and Fluka, respectively. Stock solution of lysozyme (0.5 mmolL^{-1}) was prepared in pH7.4 Tris–HCl buffer solution (0.05 molL^{-1} Tris, 0.1 molL^{-1} NaCl). It is kept in a brown flask and stored at 4 °C for further use. Stock solution of umbelliferone (5.0 mmolL^{-1}) was prepared in DMSO. 1.0 mmolL^{-1} common metal ions (Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+}, Mg^{2+}) were prepared from their nitrate salts. The all aqueous solutions used in the experiments were prepared daily from the stock solutions by appropriate dilution in doubly distilled water.

Apparatus

All fluorescence measurements were made on *Hitachi F-4500* spectrofluorometer equipped with a 150 W xenon lamp source and 1.0 cm quartz cell. The excitation and emission slits with a band pass of 2.5 nm were used for all studies. PMT voltage was kept at 700 V and scan speed was 20 nms^{-1}. pH measurements were carried out a *NeoMet* (pH-220 L) pHmeter. The absorption spectra were performed on a *Shimadzu UV1700* (*PharmaSpec*) *UV–vis* spectrophotometer equipped with quartz cells.

Procedure

The binding of 7HC to lysozyme was studied by the fluorescence quenching titration method using the intrinsic fluorescence of lysozyme (Trp 62 and Trp 108) at $\lambda_{ex}/\lambda_{em}$=284/344 nm at different temperatures. The various concentrations of 7HC solution using microliter pipette were added to 2 mL of 2.5 μmolL^{-1} lysozyme in buffer at pH7.4 in fluorescence cell. Then fluorescence intensities and spectra of lysozyme in the presence of absence of 7HC were recorded and titration data analyzed according to *Stern-Volmer* equation.

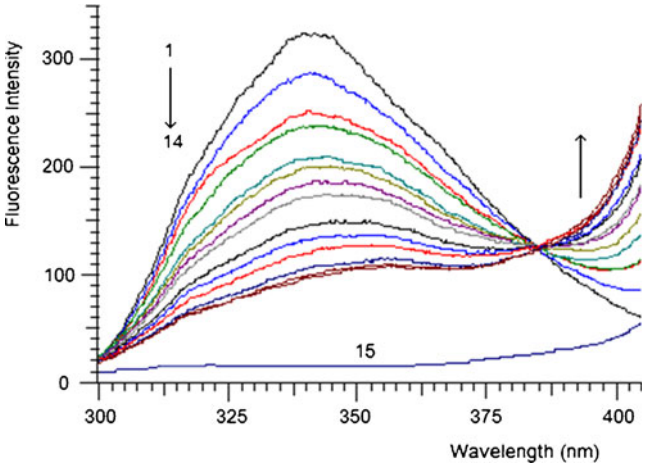

Fig. 2 Fluorescence emission spectra of 2.5 μmolL^{-1} lysozyme in the presence of 7HC. Conditions: λ_{ex}=284 nm. The concentration of 7HC from 1 to14 is 0, 1.24, 2.47, 3.09, 6.10, 9.04, 11.9, 14.7, 20.0, 22.7, 27.8, 32.6, 35.0, 37.2 μmolL^{-1} (Spectrum 15 is 2.5 μmolL^{-1} 7HC)

Results and Discussion

Fluorescence Quenching Mechanism

The fluorescence emission spectra of lysozyme by 7HC were shown in Fig. 2. It is obvious that lysozyme has a strong fluorescence emission peaked at 344 nm after being excited with a wavelength of 284 nm. The intrinsic fluorescence of Trp residues of lysozyme was quenched, when a fixed concentration of lysozyme was titrated with different amounts of 7HC. Furthermore, a new 7HC fluorescence peak developed at about 456 nm with an increase in the concentration of the added 7HC, suggesting that there was a non-radioactive energy transfer and a complex formation between lysozyme and 7HC. An isoactinic point formed at 383 nm indicating equilibrium between the free and bound 7HC.

The fluorescence quenching mechanism is decribed by the *Stern-Volmer* equation [22],

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

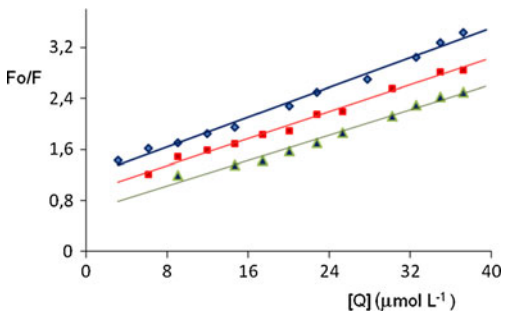

Fig. 3 The Stern-Volmer plots for the binding of 7HC to lysozyme at 20, 30, 37 °C (from high to low). $\lambda_{ex}/\lambda_{em}$=284/344 nm. c(lysozyme)= 2.5 μmolL^{-1}

Table 1 *Stern-Volmer* data for lysozyme-7HC system

T (°C)	Linear regression equation	K_{sv} ($Lmol^{-1}$)x10^4	k_q($Lmol^{-1}s^{-1}$) x10^{13}	R^2
20	$F_o/F=1.183+5.796\times10^4$ [Q]	5.796	3.331	0.9901
30	$F_o/F=0.615+5.249\times10^4$ [Q]	5.249	3.017	0.9911
37	$F_o/F=1.045+5.060\times10^4$ [Q]	5.060	2.908	0.9865

where, F_0 and F are fluorescence intensities of lysozyme before and after the addition of 7HC, respectively, [Q] concentration of 7HC as quencher. K_{sv} is the Stern–Volmer dynamic quenching constant. k_q is the quenching rate constant of the biomolecule, τ_0 is the average lifetime of the biomolecule without quencher. Here, τ_0 of Trp in lysozyme was used as 1.74 ns [16].

In Fig. 3, the linear Stern-Volmer plots were obtained from the fluorescence titrations at different temperatures (20, 30 and 37 °C). K_{sv} values were found from slopes of these graphs. k_q values calculated from $K_{sv}=k_q\tau_0$. The values of K_{sv}, k_q and linear regression equations are shown in Table 1. The results showed that Ksv decreased with increasing temperature, which is consistent with the static quenching mechanism. The maximum scatter collision quenching constant, k_q of various quenchers with the biopolymer is 2×10^{10} $Lmol^{-1}s^{-1}$ [11]. Thus, the rate constants of lysozyme quenching procedure initiated by 7HC are greater than k_q of scatter procedure. This confirms that a static quenching mechanism is operative with the formation of lysozyme-7HC complex.

The activation energy of quenching process could be obtained in accordance with *Arrhenius* equation [9, 23],

$$\ln\ k_q = \ln\ A - E_a/RT \quad (2)$$

where k_q is the quenching rate constant, A is a constant (called frequency or pre-exponential factor), E_a is the activation energy for the quenching process. The plot of ln k_q versus 1/T yields the E_a from the slope of curve and A from intercept. The linear equation was obtained as ln k_q=28.611 +738.66 1/T with R^2=0.9809 from k_q values in Table 1. The calculated E_a for quenchinq is 6.142 $kJmol^{-1}$.

The binding constant K and the binding number n of 7HC with lysozyme can be determined according to the following equation [10],

$$\log\ (F_0-F)/F = \log\ K + n\log[Q] \quad (3)$$

where *K* and n are the binding constant and the number of binding sites for lysozyme-7HC system, respectively. Thus, the plot of log (F_0–F)/F versus log [*Q*] gives K from intercept and n from slope of curve at different temperatures. The values of K and n are shown in Table 2. The values of n approximately equal to 1 suggesting that one molecule of 7HC combines with one molecule of lysozyme.

The Effect of Common Metal Ions on the Binding Constants of Lysozyme-7HC

Metal ions, especially those of *bivalent* type, can affect the interactions of the ligands and the proteins in plasma. Here, the effect of some common metal ions, such as Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+} and Mg^{2+} on the binding constants of lysozyme-7HC complex was investigated at 20 °C by recording the fluorescence intensity in the range 300–450 nm upon excitation at 284 nm. The concentrations of lysozyme and metal ions were fixed 2.5 and 10 $\mu molL^{-1}$ in titrations, respectively. It was found found that the ratio of binding constant in the presence of ions ($K^{\prime}$) to that in the absence of ions(K). The results were shown in Table 3. It can be seen that there is no significant effect of Mg^{2+} on the binding, but the interaction between the other ions and 7HC leads to decrease in binding constants of the lysozyme-7HC complex in the presence of metal ions. Then, metal ions may combine with carboxylic or amino groups of the amino acid residues of lysozyme which resulted in the decrease of binding constants.

Thermodynamic Parameters and Binding Modes

The interaction between a small molecule and biomolecule may involve hydrogen bond, van der Waals force, electrostatic force, hydrofobic interaction force, and so on. The enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) changes are very important for confirming binding modes of species. The ln K (binding constant) values at

Table 2 Binding constant K, binding sites n and thermodynamic parameters for lysozyme-7HC system

*correlation coefficients of double logarithm plots.

T (°C)	K ($Lmol^{-1}$)	n	R^{2*}	ΔH ($kJmol^{-1}$)	ΔG ($kJmol^{-1}$)	ΔS($Jmol^{-1}K^{-1}$)
20	2.130×10^4	0.897	0.9798	233.7	−24.44	846.8
30	5.655×10^5	1.019	0.9930		−32.90	
37	3.146×10^6	1.423	0.9933		−38.82	

Table 3 Effect of common metal ions on the binding constant lysozyme-7HC system at 20 °C

Ion	K′ (×10^{4}Lmol^{-1})	R^2	K′/K
without	2.13	0.9798	-
Cu^{2+}	0.57	0.9847	0.268
Pb^{2+}	1.49	0.9965	0.699
Mg^{2+}	2.12	0.9981	0.995
Co^{2+}	0.46	0.9530	0.216
Ni^{2+}	0.33	0.9995	0.155
Zn^{2+}	0.26	0.9982	0.122

different temperatures are plotted against the reciprocal of temperature according to the *van't Hoff* equation,

$$\ln K = -\Delta H/RT + \Delta S/R \quad (4)$$

The values of ΔH and ΔS were obtained from the slope and interscept of the linear plot in Fig. 4. ΔG was then estimated using ΔG=ΔH–TΔS. Table 2 lists the calculated thermodynamic parameters for the interaction of 7HC and lysozyme. The negative value of ΔG reveal that the interaction process is spontaneous. The positive ΔH and ΔS values indicate that hydrophobic force plays role in the binding of 7HC and lysozyme [24].

UV–vis Absorption Studies

The absorption spectra of lysozyme in the presence and absence of 7HC were recorded and presented in Fig. 5. As can be seen from Fig. 5 with the adition of 7HC, the absorbance intensity increased at 281 nm which indicated that the absorption spectra of lysozyme was changed due to formation of the ground state complex of 7HC-lysozyme.

Quantitative Studies of 7HC

The fluorescence queching of lysozyme had a good relationship with the concenration of 7HC. The calibration graph was used for the determination of 7HC in the presence

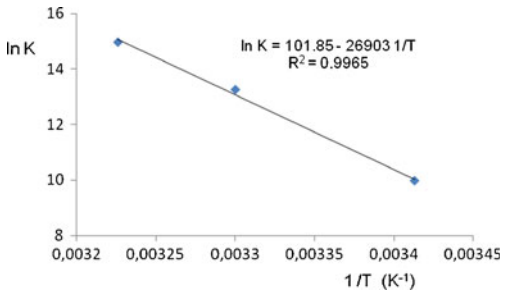

Fig. 4 Van't Hoff plot for the interaction of lysozyme-7HC system

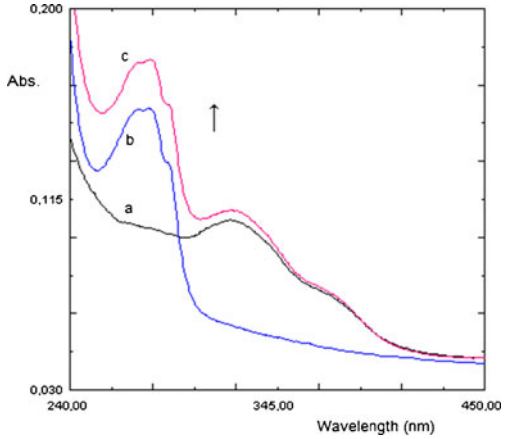

Fig. 5 The absorption spectra of **a**) 2.5 μmolL^{-1} 7HC, **b**) 2.5 μmol L^{-1} lysozyme, **c**) lysozyme -7HC (2.5 μmolL^{-1}–2.5 μmolL^{-1})

of lysozyme under the experimental conditions above described. The linear equation, $F_0/F = 1.183 + 5.796 \times 10^4$ [Q], at 20 °C used for calculations in Table 1. The detection and quantification limits of 7HC were calculated as 3*Sb*/*m* and 10*Sb*/*m*. *Sb* is the standard deviation of the intercept and *m* is the slope of the calibration graph. The analytical results (*n*=11) are given in Table 4.

Energy Transfer Between Lysozyme and 7HC

Fluorescence resonance energy transfer (FRET) relies on the distance dependent of energy transfer from an excited donor to an acceptor [22]. There are several studies about the effect of FRET on the determination of the interaction between proteins and small molecules based on the fluorescence quenching [25, 26]. According to Förster's theory the energy transfer efficiency E is calculated using the equation [22],

$$E = 1 - F/F_0 \quad (\text{or } E = 1 - F_{DA}/F_D) \quad (5)$$

$$E = R_0^6/R_0^6 + r^6 \quad (6)$$

where F (or F_{DA}) and F_0 (or F_D) are the fluorescence intensities of lysozyme in presence and absence of 7HC, r the distance between acceptor and donor and R_0 the

Table 4 The analytical results of 7HC determination in presence lysozyme

Dynamic range of 7HC (μmolL^{-1})	1.24–37.2
Sb standard deviation of the intercept	0.084
Limit of detection (LOD) (μmolL^{-1})	4.35
Limit of quantification (LOQ) (μmolL^{-1})	14.5

critical distance when the transfer efficiency is 50 %. The value of R_0 is calculated using the equation,

$$R_0^6 = 8.79 \times 10^{-25}\left(k^2\, n^{-4} Q_D\, J(\lambda)\right) \quad (7)$$

where κ^2 is the spatial orientation factor of the donor-acceptor dipoles, n the refractive index of the medium, Q_D the fluorescence quantum yield of the donor in the absence of acceptor, $J(\lambda)$ the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor and it is given by the equation,

$$J(\lambda) = \sum F_D(\lambda) e_A(\lambda) \lambda^4 \Delta\lambda / \sum F_D(\lambda) \Delta\lambda \quad (8)$$

where $F_D(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range λ to $\lambda+\Delta\lambda$ with the total intensity (area under curve), $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor at λ. In this study, the overlap of the absorption spectrum of 7HC and the fluorescence emission spectrum of lysozyme was shown in Fig. 6. So $J(\lambda)$ could be calculated by integrating the spectra in Fig. 6 for λ=290–450 nm and was 4.973×10^{-14} cm^3Lmol^{-1} according to Eq. 8. The value of E was obtained from Eq. 5 as 0.296. Using κ^2=2/3, n=1.336 and Q_D=0.14 for lysozyme [21], distance parameters were calculated as R_0=3.30 nm from Eq. 7 and r= 3.81 nm from Eq. 6. The donor to acceptor distance, r is less than 8 nm, and $0.5R_0<r<1.5\ R_0$, which indicates the non-radiative energy transfer occured between lysozyme and 7HC. Also r is bigger than R_0 suggested the presence of a static quenching mechanism.

Conclusion

The binding properties of 7HC to lysozme were studied in physiological buffer solution (pH7.4) by UV–vis absorption and fluorescence quenching. 7HC has a strong ability to quench the lysozyme fluorescence with a static mechanism by forming the lysozyme-7HC complex. The binding constant K and the number binding site n were calculated from the fluorescence data. And the effects of metal ions on the binding constant were studied. The positive values of thermodynamic parameters (ΔH and ΔS) suggested that the binding of 7HC could bind to lysozyme mainly through hydrophobic force. The binding reaction is spontaneous and exothermic process. The binding distance r was 3.81 nm based on Förster theory which indicated that there was a non-radiative energy transfer between 7HC and lysozyme.

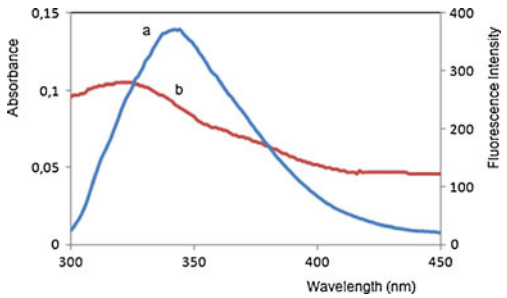

Fig. 6 The overlap of fluorescence spectrum of (**a**) lysozyme and the absorption spectrum of (**b**) 7HC. c(lysozyme)=c(7HC)=2.5 μmolL^{-1}

References

1. Christie RM, Liu C-H (1999) Studies of fuorescent dyes: Part 1. An investigation of the electronic spectral properties of substituted coumarins. Dyes Pigments 42:85–93
2. Christie RM, Liu C-H (2000) Studies of fuorescent dyes: Part 2. An investigation of the synthesis and electronic spectral properties of substituted 3-(2′-benzimidazolyl) coumarins. Dyes Pigments 47:79–89
3. Seixas de Melo J, Fernandes PF (2001) Spectroscopy and photophysics of 4-and 7-hydroxycoumarins and their thione analogs. J Mol Struct 565–566:69–78
4. Preat J, Jacquemin D, Perpete EA (2005) Theoretical investigations of the UV spectra of coumarin derivatives. Chem Phys Lett 415:20–24
5. Gök E, Öztürk C, Akbay N (2008) Interaction of thyroxine with 7 hydroxycoumarin: A fluorescence quenching study. J Fluoresc 18:781–785
6. Toyama DO, Marangoni S, Diz-Filho EBS, Oliveira SCB, Toyama MH (2009) Effect of umbelliferone (7-hydroxycoumarin, 7-HOC) on the enzymatic, edematogenic and necrotic activities of secretory phospholipase A2(sPLA2) isolated from *Crotalus durissus collilineatus* venom. Toxicon 53:417–426
7. Beckmann JD, Burkett RJ, Sharpe M, Giannunzio L, Johnston D, Abbey S, Wyman A, Sung L (2003) Spectrofluorimetric analysis of 7-hydroxycoumarin binding to bovine phenol sulfotransferase. BBA-Proteins Proteom 1648:134–139
8. Li D, Ji B, Sun H (2009) Probing the binding of 8-Acetyl-7-hydroxycoumarin to human serum albumin by spectroscopic methods and molecular modeling. Spectrochim Acta Part A 73:35–40
9. Hussein BHM (2011) Spectroscopic studies of 7, 8-dihydroxy-4-methylcoumarin and its interaction with bovin serum albumin. J Lumin 131:900–908
10. Zhang G, Zhao N, Hu X, Tian J (2010) Interaction of alpinetin with bovine serum albumin: Probing of the mechanism and binding site by spectroscopic methods. Spectrochim Acta Part A 76:410–417
11. Matei I, Hillebrand M (2010) Interaction of kaempferol with human serum albumin: A fluorescence and circular dichroism study. J Pharm Biomed Anal 51:768–773
12. Wang Y-Q, Zhang H-M, Zhang GC (2006) Studies of the interaction between palmatine hydrochloride and human serum albumin by fluorescence quenching method. J Pharm Biomed Anal 41:1041–1046
13. Wang Z, Tan X, Chen D, Yue Q, Song Z (2009) Study on the binding behavior of lysozyme with cephalosporin analogues by fluorescence spectroscopy. J Fluoresc 19:801–808
14. Yue Q, Niu L, Li X, Shao X, Xie X, Song Z (2008) Study on the interaction mechanism of lysozyme and bromophenol blue by fluorescence spectroscopy. J Fluoresc 18:11–15

15. Wang Y-Q, Chen T-T, Zhang H-M (2010) Investigation of the interactions of lysozyme and trypsin with biphenol A using spectroscopic methods. Spectrochim Acta Part A 75:1130–1137
16. Zhang H-M, Chen J, Zhou Q-H, Shi Y-Q, Wang Y-Q (2011) Study on the interaction between cinnamic acid and lysozyme. J Mol Struct 987:7–12
17. Li D, Zhang T, Xu C, Ji B (2011) Effect of pH on the interaction of baicalein with lysozyme by spectroscopic approaches. J Photochem Photobiol B Biol 104:414–424
18. Li D, Yang Y, Cao X, Xu C, Ji B (2012) Investigation on the pH-dependent binding of vitamin B12 and lysozyme by fluorescence and absorbance. J Mol Struct 1007:102–112
19. Pang B, Bi S, Wang Y, Yan L, Wang T (2012) Investigation on the interactions of silymarin to bovine serum albumin and lysozyme by fluorescence and absorbance. J Lumin 132:895–900
20. Paramaguru G, Kathiravan A, Selvaraj S, Venuvanalingam P, Renganathan R (2010) Interaction of anthraquinone dyes with lysozyme: Evidences from spectroscopic and docking studies. J Hazard Mater 175:985–991
21. Ding F, Liu W, Liu F, Li Z-Y, Sun Y (2009) A study of the interaction between malachite green and lysozyme by steady-state fluorescence. J Fluoresc 19:1783–791
22. Lakowicz JR (2006) Principles of Fluorescence Spectroscopy, 3rd edn. Springer, New York
23. Castellan GW (1964) Physical Chemistry, Addison-Wesley Publishing Company.
24. Ross PD, Subramanian S (1981) Thermodynamics of protein association reactions: Forces contributing to stability. Biochemistry 20:3096–3102
25. Aydın BM, Acar M, Arık M, Onganer Y (2009) The fluorescence resonance energy transfer between dye compound in micellar media. Dyes Pigments 81:156–160
26. Xiao J, Wei X, Wang Y, Liu C (2009) Fluorescence resonance energy-transfer affects the determination of the affinity between ligand and proteins obtained by fluorescence quenching method. Spectrochim Acta Part A 74:977–982