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Spectrophotometric studies on the interaction between nevadensin and lysozyme

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Abstract

Interaction between nevadensin and lysozyme (Lys) was studied using spectrophotometric techniques such as steady fluorescence, synchronous fluorescence, circular dichroism (CD) and UV–vis absorption. The fluorescence emission intensity of Lys was strongly quenched by the addition of nevadensin. Spectrophotometric observations are rationalized in terms of a static quenching process at lower concentration of nevadensin (less than 8 μM) and a combined quenching process at higher concentration of nevadensin (8–20 μM). Binding constants and binding sites for the nevadensin–Lys system were evaluated. The distance of 2.28 nm and the energy transfer efficiency of 0.586 between nevadensin and Lys, evaluated from the Förster non-radioactive resonance energy transfer theory, indicated that the energy transfer from Lys to nevadensin occurred with higher possibility. Thermodynamic data showed that nevadensin was included in the hydrophobic cavity of Lys via hydrophobic interactions. UV/vis measurements on the enzymatic activity of Lys in the absence and presence of nevadensin indicated that the interaction between nevadensin and Lys led to a reduction in the activity of Lys. Furthermore, nevadensin binding to Lys had no influence on the molecular conformation of Lys.

Keywords: Nevadensin; Lysozyme; Fluorescence quenching; Combined quenching process

1. Introduction

Nevadensin (Scheme 1), belonging to the flavonoid family, has been widely used in traditional Chinese medicine for the treatment of lymph node tuberculosis, cough with tachypnoea and rheumatic pains [1]. It broadly distributes in plants of *lysiontous pauciflorus* Maxim, which are growing in a vast area of southern China. Otherwise, the therapeutical action of drugs is exerted by the storage in proteins and transportation by proteins in blood plasma. Therefore, studies on it will provide an insight into the chemical nature of the interaction of biomacromolecule with small drug molecules.

Lysozyme (Lys), a small monomeric globular protein, consists of 129 amino acid residues and contains six tryptophan (Trp) and three tyrosine (Tyr) residues [2]. With a molecular weight of about 14,306, its tertiary structure is compact with several helices surrounding a small beta sheet region. Since Lys was recognized by Fleming in 1922 as a bacteriolytic agent having an ability to hydrolyze bacterial cell walls, it has become one of the most intensively studied proteins. The action of Lys on bacteria works cooperatively and synergistically with antibiotics, which has a very important practical value in medicine area. Moreover, Trp or Tyr residues can cure abscess, stomatitis, rheum, etc. *via* binding to antibiotics. Therefore, studies on the interaction between drug and Lys are of importance in view of realizing disposition, transportation and metabolism of drug as well as efficacy process involving drug and Lys. A large amount of results have been published for the interaction of drug with human serum albumin. Yet, few results have been involved in drug binding to Lys.

In this work, we will report our studies on the interaction of nevadensin with Lys using spectrophotometeric spectra. Efforts were made to investigate the quenching mechanism, binding constants, binding sites, binding mode, binding location and the effect of nevadensin on the conformation of Lys.

2. Materials and methods

2.1. Materials and preparation of solutions

Lys was purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Its

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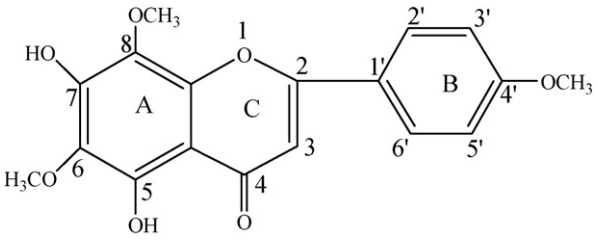

Scheme 1. Chemical structure of nevadensin.

molecular weight was assumed to be 14,306. An assay kit including *Micrococcus lysodeikticus* powder and standard Lys (20000 *U*/mg) with a special solvent for *Micrococcus lysodeikticus* was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Nevadensin was of analytical grade and purchased from the National Institute for Control of Pharmaceutical and Bioproducts, China.

A solution of 1 mol L^{-1} NaCl (analytical grade) was used to maintain the ion strength of samples at 0.1. A buffer solution of 0.05 mol L^{-1} Tris–HCl containing 0.1 mol L^{-1} of NaCl was used to keep the pH of solution at 7.40. A stock solution of Lys was prepared with the Tris–HCl buffer solution and stored in the dark at 4 °C for use. The stock solution of 1×10^{-3} mol L^{-1} nevadensin was prepared by dissolving nevadensin in 50 mL of anhydrous methanol. The suspension of 0.25 mg mL^{-1} *Micrococcus lysodeikticus* and the solution of 0.05 mg mL^{-1} standard Lys were prepared with a special solvent for *Micrococcus lysodeikticus* and doubly distilled water, respectively. All other reagents were of analytical grade. Doubly distilled water was used throughout all experiments.

2.2. Apparatus and methods

Fluorescence measurements were performed on an LS-55 spectrofluorophotometer (Perkin Elmer, America) following an excitation at 282 nm. Fluorescence spectra were recorded over a wavelength range of 290–500 nm. Both excitation and emission bandwidths were adjusted at 10 nm. Synchronous fluorescence spectra were obtained by scanning simultaneously the excitation and emission monochromator. The wavelength interval ($\Delta\lambda$) is fixed individually at 15 and 60 nm, at which the spectrum only shows the spectroscopic behavior of Tyr and Trp residues of Lys, respectively. Circular dichroism (CD) spectra were recorded on an Olis DSM-1000 automatic recording spectropolarimeter (USA) over the range of 200–250 nm at an interval of 0.5 nm in a 1-mm cell equipped with a temperature controlling unit. Each CD spectrum given was an average of three scans at 298 K. CD determinations of pure Lys and nevadensin-Lys mixtures were carried out using the buffer solutions of nevadensin at a corresponding concentration as the reference. Results are expressed as ellipticity (mdeg), which was obtained in mdeg directly from the instrument.

The activity of Lys in the absence and presence of nevadensin was measured on a UV-260 UV–vis spectrophotometer (Japan) by the absorption change at 450 nm of *Micrococcus lysodeikticus*, which was decomposed by Lys along time. The absorptions were recorded at an interval of 15 s within a time span of 5 min at 298 K. The specific activity (*U*/mg) of Lys was calculated to evaluate the effect of drug on the activity of Lys upon addition of nevadensin.

2.3. Fluorometric titration experiments

Into a 3.0 mL of Tris buffer solution (pH 7.40) containing 8×10^{-6} mol L^{-1} Lys was successively titrated using trace syringe the nevadensin stock solution, reaching a final nevadensin concentration of 2×10^{-5} mol L^{-1}. The fluorescence intensities were recorded at 347 nm following an excitation at 282 nm. The measurements were performed at 298, 308, and 318 K.

3. Results and discussion

3.1. Fluorescence quenching studies of Lys

Fig. 1 shows the fluorescence emission spectra of Lys with various amounts of nevadensin following an excitation at 282 nm. Lys exhibits a strong fluorescence emission band at 347 nm. Its intensity decreased gradually with the addition of nevadensin, i.e., the excited Lys was quenched by nevadensin. It was also observed that an increase in the fluorescence intensity at 426 nm assigned to nevadensin, which was well observed at a nevadensin concentration of 20 μM in the absence of Lys. These observations may be referred to a strong binding of nevadensin to Lys and a radiationless energy transfer between nevadensin and Lys [3]. Furthermore, an isoactinic point at 405 nm at less than 8 μM of nevadensin was observed in Fig. 1. It indicated the existence of both bound and free nevadensin at equilibrium [4]. When the concentration of nevadensin rose above 8 μM, no identical isoactinic points were present. This observation implies that the fluorescence quenching mechanism involved may be rationalized in terms of a combined quenching (both static

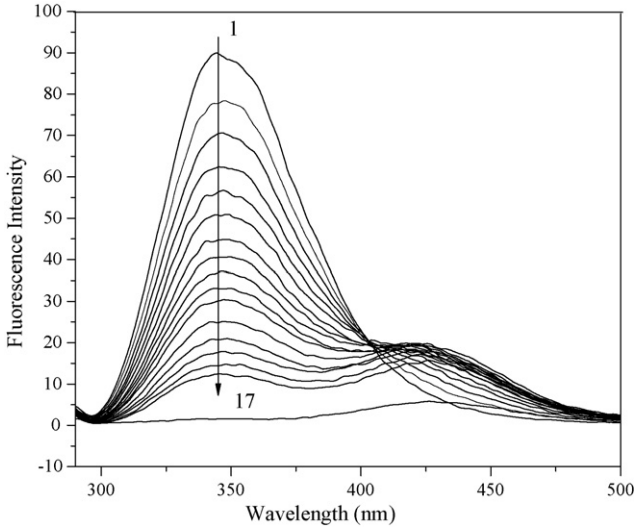

Fig. 1. Fluorescence spectra of Lys (8 μM) following the excitation at 282 nm, at pH 7.40 and 298 K with various amounts of nevadensin: [nevadensin] = 0 (1), 1 (2), 2 (3), 3 (4), 4 (5), 5 (6), 6 (7), 7 (8), 8 (9), 9 (10), 10 (11), 12 (12), 14 (13), 16 (14), 18 (15) and 20 μM (16), respectively. (17) shows the emission spectrum of pure nevadensin at 20 μM.

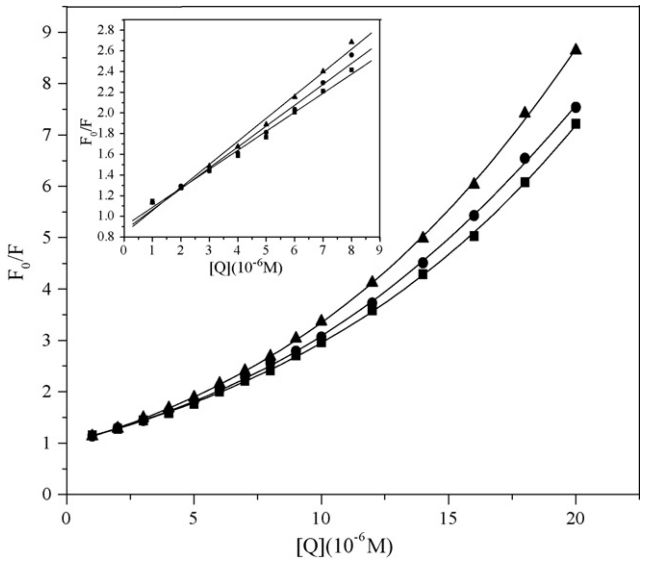

Fig. 2. Plots of F_0/F for Lys against [Q] of nevadensin raning from 1 to 20 μM at: (■) 298 K; (●)308 K; (▲) 318 K. [Lys] = 8 μM, λ_{ex} = 282 nm, λ_{em} = 347 nm, and pH = 7.40. Straight lines in the inset are plots of F_0/F for Lys against [Q] of nevadensin ranging from 1 to 8 μM.

and dynamic) mechanism at higher concentrations (>8 μM) of nevadensin rather than a single static quenching process.

To elucidate further the quenching mechanism induced by nevadensin, fluorescence quenching data are analyzed with the Stern–Volmer equation (Eq. (1)) [5]:

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

where F_0 and F are the relative fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the concentration of quencher, K_{SV} the Stern–Volmer dynamic quenching constant, k_q the bimolecular quenching rate constant, τ_0 the average bimolecular life-time in the absence of quencher evaluated at about 5 ns [6]. Fig. 2 shows the plots of F_0/F for Lys versus [Q] of nevadensin ranging from 1 to 20 μM and 1 to 8 μM (the inset of Fig. 2) of nevadensin, respectively, at 298, 308, and 318 K. Plots in Fig. 2 show upward curvy patterns, being concave towards the y-axis at higher [Q]. This observation may suggest that either the additional binding sites exist or a combined quenching (static and dynamic) process occurs at higher concentrations of nevadensin [7–10] because downward curvy Stern–Volmer plots, being concave towards the x-axis at higher [Q], will be assigned to fluorophores being not equally accessible to quenchers [11,12].

As it is known, a linear Stern–Volmer plot represents a single quenching mechanism, either static or dynamic [7]. In a static quenching process, generally, a linear Stern–Volmer plot indicates either only one drug binding site existing in the proximity of fluorophore, or more than one binding site being all equally accessible to quenchers [8,9]. On the contrary, in a dynamic quenching process, the bimolecular quenching constant K_{SV} is expected to increase with raising temperature because it is closely related to diffusions or diffusion coefficients. In addition, the Stern-Volmer slope is expected to depend on the concentration of donor (Lys in the present case) in a static quenching process, whereas the slope is independent of the concentration of donor in only a dynamic process. Linear fittings (the inset in Fig. 2) of the experimental data obtained at lower concentration of nevadensin (1–8 μM) to Eq. (1) afford K_{SV} and k_q (Table 1). Table 1 shows that K_{SV} increases with raising temperature. It indicates that the fluorescence quenching of Lys by nevadensin at lower concentrations (less than 8 μM) of nevadensin appears to occur *via* a dynamic quenching mechanism. Yet, our values for k_q (Table 1) are 3 orders of magnitude greater than the maximum diffusion collision quenching rate constant (2.0×10^{10} L mol^{-1} s^{-1}) for a variety of quenchers with biopolymer [13]. Therefore, it suggests that the fluorescence quenching process of Lys at low concentrations (less than 8 μM) of nevadensin may be mainly governed by a static quenching mechanism arising from a complex formation rather than a dynamic quenching mechanism. This recognition can be also confirmed by the analysis of Stern–Volmer plot at different concentrations of Lys. Fig. 3 shows plots of F_0/F for Lys versus [Q] of nevadensin at different concentrations of Lys. The value for slopes is presented in Table 1. Table 1 indicates that the value for slopes goes down with increasing concentrations of Lys. It represents an indication that the quenching occurs by a formation of complex rather than a dynamic process.

At higher concentrations (>8 μM) of nevadensin, the large deviations from the linearity of the Stern–Volmer plot might arise from the existence of a combined quenching (both dynamic and static) process rather than additional binding sites present because no obvious turning point was observed from Fig. 2 [9]. In order to obtain a clearer insight into the fluorescence quenching mechanism of Lys caused by nevadensin, the fluorescence data obtained at 308 K for the concentrations of nevadensin ranging from 1 to 20 μM are analyzed with the modified Stern–Volmer equation (Eq. (2)) [11]:

$$\frac{F_0}{F_0 - F} = \frac{1}{[Q]}\frac{1}{fK_{SV}} + \frac{1}{f} \quad (2)$$

where F_0 and F are the steady state fluorescence intensities with various amounts of quencher, respectively; f is the fraction of the initial fluorescence which is accessible to the quencher, K_{SV}

Table 1
Stern–Volmer quenching constants for the nevadensin–Lys system at pH 7.40

T (K)	K_{SV} ($\times 10^5$ L mol^{-1})	k_q ($\times 10^{13}$ L mol^{-1} s^{-1})	R	T (K)	[Lys] ($\times 10^{-6}$ mol L^{-1})	K_{SV} ($\times 10^5$ L mol^{-1})
298	1.84	3.68	0.996		4	1.98
308	2.02	4.04	0.994	298	8	1.84
318	2.23	4.46	0.996		12	1.65

R is the correlation coefficient.

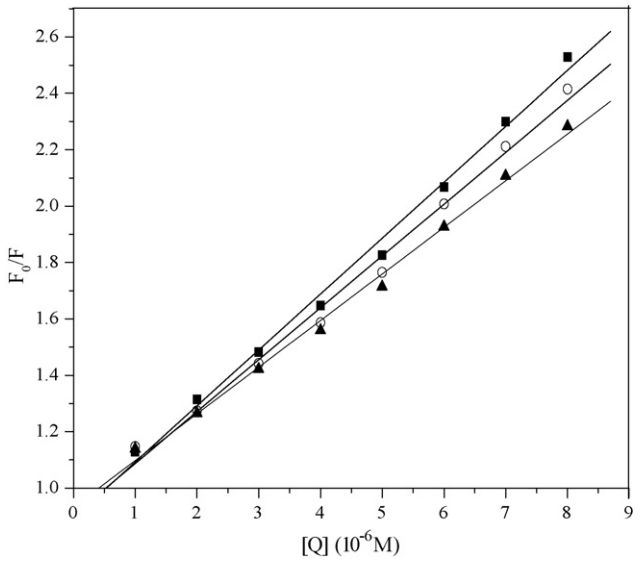

Fig. 3. Stern–Volmer plots for the nevadensin–Lys system at different concentrations of Lys at 298 K and pH = 7.40: (■) [Lys] = 4 μM; (◯) [Lys] = 8 μM; (▲) [Lys] = 12 μM.

the modified Stern–Volmer quenching constant and [Q] the concentration of quencher. It is worth pointing out that the modified Stern–Volmer equation is used for all the cases in which the fractional or total fluorescence of protein is accessible to quencher regardless of quenching mechanism, either a single quenching process (static or dynamic) or a combined quenching process. This implies that modified Stern–Volmer plots should show a good linear relationship for any quenching process in the whole concentration range of drug [14,15] except in the case that drug molecules are bound to additional sites at higher concentrations of drug [16], where the modified Stern–Volmer plot represents a curvy line. If the upward curvy pattern of Stern–Volmer plots, as shown in Fig. 2, arising from the existence of additional binding sites, the modified Stern–Volmer quenching constant K_{SV} at lower drug concentrations would be different from that at higher drug concentrations. Thus, there would appear a sharp turning point on the modified Stern–Volmer plot. Our plots (not shown) of $F_0/F_0 - F$ for Lys versus 1/[Q] in the whole concentration range of nevadensin based on Eq. (2) exhibit a good linearity ($R = 0.99976$) without a sharp turning point, giving a linear regression equation (Eq. (3)) as:

$$\frac{F_0}{F_0 - F} = 7.64097 \times 10^{-6}\,[Q]^{-1} + 0.71874 \tag{3}$$

This indicates that the quenching process is really a combined quenching (both dynamic and static) process rather than additional binding sites present. As such, the values for f and K_{SV} are derived to be 1.39 and 9.41×10^4 L mol^{-1}, respectively. The value of 1.39 for f indicates that 71.9% of the total fluorophores of the excited Lys is accessed by nevadensin [14].

In fact, in many cases, fluorophores can be quenched by both collision and complex formation with the same quencher. Consequently, the Stern–Volmer plot will exhibit an upward curve, being concave towards the y-axis at higher [Q] [17] (Fig. 2). Accordingly, F_0/F is related to [Q] by the following modified form (Eq. (4)) of the Stern–Volmer equation [10,17]:

$$\frac{F_0}{F} = (1 + K_D[Q])(1 + K_S[Q]) = 1 + (K_D + K_S)[Q] + K_D K_S[Q]^2 \tag{4}$$

where K_D and K_S are the dynamic and static quenching constants, respectively. It is second order in [Q] and thus leads to upward curvy plots of F_0/F versus [Q] at higher [Q] arising from a combined quenching (both dynamic and static) process.

3.2. Evaluation of the binding constant and binding site

When small molecules are bound independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by Eq. (5) [18]:

$$\log\frac{(F_0 - F)}{F} = \log K_b + n \log[Q] \tag{5}$$

where K_b is the binding constant and n the number of binding sites. For the nevadensin–Lys system in the lower concentration range of nevadensin (less than 8 μM), the values for K_b and n at different temperatures can be derived from the intercept and slope of plots of log (F_0–F)/F versus log [Q] based on Eq. (5) and presented in Table 2. Linear regression equations (Eqs. (6)–(8) at 298, 308 and 318 K are expressed as follows:

$$\log\frac{(F_0 - F)}{F} = 5.7506 + 1.10 \times \log[Q], \tag{6}$$

$$\log\frac{(F_0 - F)}{F} = 6.1127 + 1.17 \times \log[Q], \tag{7}$$

and

$$\log\frac{(F_0 - F)}{F} = 6.5121 + 1.24 \times \log[Q], \tag{8}$$

respectively. The values for K_b (Table 2) show that there exist a strong interaction between nevadensin and Lys and a complex formation of nevadensin with Lys. Thus, nevadensin can be stored in Lys and removed by Lys. Furthermore, it can be inferred from the values of n that there is an independent class of binding sites on Lys for nevadensin. Otherwise, it appears that the

Table 2
Binding constants K_b, numbers of binding sites n and thermodynamic parameters for the nevadensin–Lys system at different temperatures

T (K)	K_b ($\times 10^5$ L mol^{-1})	n	R	ΔG^0 (kJ mol^{-1})	ΔH^0 (kJ mol^{-1})	ΔS^0 (J mol^{-1} K^{-1})
298	5.63	1.10	0.997	−32.75	–	–
308	12.96	1.17	0.998	−36.17	69.02	341.52
318	32.52	1.24	0.999	−39.58	–	–

R is the correlation coefficient.

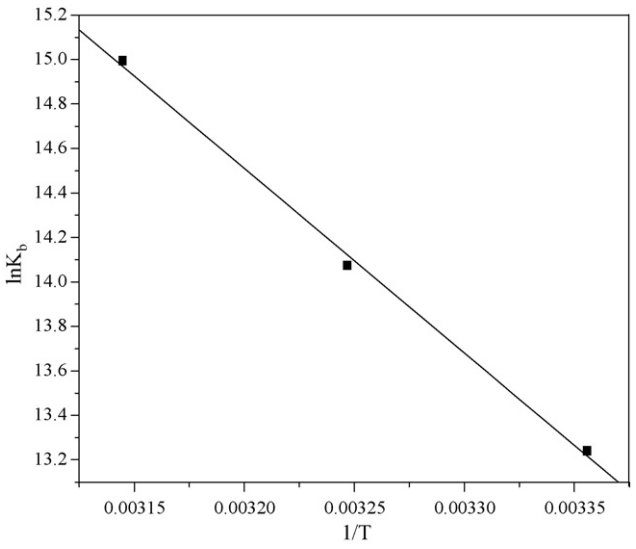

Fig. 4. van't Hoff plot for the interaction of Lys with nevadensin in Tris buffer at pH 7.40.

binding constants and the number of binding sites increase with raising temperature [19,20]. This may be attributed to that the capacity of nevadensin binding to Lys is enhanced with raising temperature.

3.3. Hydrophobic interaction between nevadensin and Lys

Intermolecular interacting forces between a small molecule and a biomacromolecule include hydrogen bond, van der Waals force, electrostatic and hydrophobic interactions, etc. Thermodynamic parameters for a binding interaction can be used as a major evidence to learn the nature of intermolecular forces. Thus, the temperature-dependent thermodynamic parameters for the nevadensin–Lys system are used to characterize the intermolecular forces between nevadensin and Lys. The enthalpy change ΔH^0 and entropy change ΔS^0 for a binding reaction can be derived from the vant't Hoff equations (Eqs. (9) and (10)):

$$\ln K_b = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R} \quad (9)$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (10)$$

where K_b is the binding constant at the corresponding temperature and R the gas constant. The enthalpy change ΔH^0 and entropy change ΔS^0 were obtained from the slope and intercept of the linear vant't Hoff plot (Fig. 4) of ln K_b versus $1/T$ based on Eq. (9). The free energy change ΔG^0 was estimated from Eq. (10). The values of ΔH^0, ΔS^0 and ΔG^0 for nevadensin binding to Lys are listed in Table 2. The negative value of ΔG^0 reveals that the binding process is a spontaneous process. The positive entropy change arises from water molecules arranged more random around Lys and drug, caused by hydrophobic interactions between Lys and drug molecules. Besides, the positive ΔH^0 is considered as another evidence for hydrophobic interactions. Thus, positive values for both ΔH^0 and ΔS^0 indicate hydrophobic interactions playing a major role in nevadensin binding to Lys [21].

3.4. Energy transfer from Lys to nevadensin

According to the Förster non-radioactive resonance energy transfer theory [22], a radiationless energy transfer will occur when two molecules satisfy the following preconditions: (1) The energy donor can produce fluorescence light; (2) the fluorescence emission spectrum of donor overlaps enough with the absorption spectrum of acceptor; and (3) the maximum distance between donor and acceptor, in general, should be less than 8 nm. The nevadensin–Lys system satisfies the above prerequisites. As such, the distance r between nevadensin and Lys could be calculated from the Förster mechanism of non-radiation energy transfer [23], in which the spatial orientation factor of the dipole $k^2 = 2/3$, the refractive index of medium $N = 1.36$ and the fluorescence quantum yield of donor $\Phi = 0.14$ are obtained [24,25]. Consequently, the overlap integral between the emission spectrum of donor and the absorption spectrum of acceptor is calculated to be 8.17×10^{-15} cm^3 L mol^{-1} (Fig. 5) and the critical distance R_0 at the efficiency of transfer for 50% is estimated to be 2.41 nm. Consequently, the distance r between nevadensin and Lys at the efficiency of transfer E for 58.6% is evaluated at 2.28 nm, much shorter than 8 nm, indicating that the energy transfer from Lys to nevadensin occurs with higher possibility.

3.5. Effect of nevadensin on the activity of Lys

In order to further confirm the formation of nevadensin–Lys complex and ascertain the possible influence of nevadensin binding on the activity of Lys, determinations on the activity of Lys were performed in the absence and presence of nevadensin ($C_{drug}/C_{Lys} = 8{:}1$) using UV/vis absorptions. In general, the activity of Lys can be indirectly measured by the change in the UV/vis absorption of *Micrococcus lysodeikticus* which is decomposed by Lys along time. The specific activity (U/mg) of

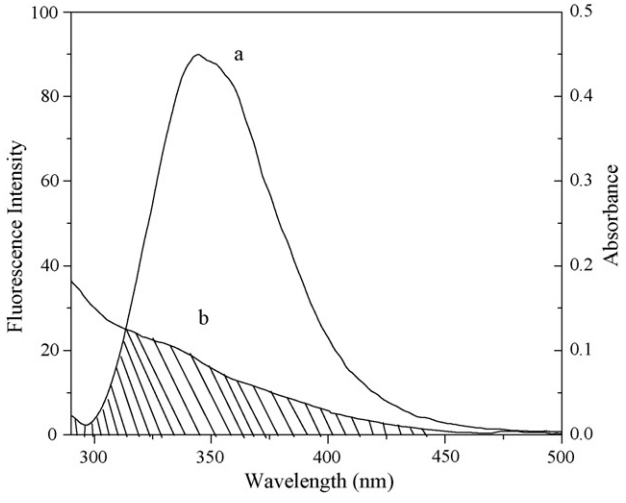

Fig. 5. Overlap of the fluorescence emission spectra of Lys (a) with the absorption spectrum of nevadensin (b). [Lys] = [nevadensin] = 8 μM, T = 298 K.

Lys is estimated from Eq. (11):

$$\frac{\Delta E_{450}}{0.001 \times E_W} = U/\text{mg} \quad (11)$$

where ΔE_{450} are the absorption change per minute at 450 nm and E_W is the mass (mg) of applied Lys. A reduction of 0.001 in absorption is defined as one active unit (U). Eq. (11) is used to evaluate the drug effect on the activity of Lys. ΔE_{450} of *Micrococcus lysodeikticus* decomposed by Lys were obtained from the slope of linear plots (not shown) of the absorption of *Micrococcus lysodeikticus* versus time within an initial time span of 1.5 min. The linear regression equations (Eqs. (12) and (13)) for the absorption A of *Micrococcus lysodeikticus* are given as:

$$A = 1.29761 - 0.10271t \quad (12)$$

and

$$A = 1.29757 - 0.08571t, \quad (13)$$

for the cases in the absence and presence of nevadensin, respectively. The corresponding value of ΔE_{450} was estimated to be 0.10271 and 0.08571, respectively. As such, the specific activity of Lys in the absence and presence of nevadensin were evaluated to be 20542 U/mg and 17142 U/mg, respectively. These results indicate that the addition of nevadensin can exerts influence on the activity of Lys and reduces it, arising from the complex formation of Lys with nevadensin.

3.6. Analysis of the conformation of Lys upon addition of nevadensin

3.6.1. Fluorescence and synchronous fluorescence spectroscopic studies of Lys

It is observed from Fig. 1 that there is no significant wavelength (λ_{em}) shift upon addition of nevadensin. It is well known that λ_{em} at the maximum fluorescence emission intensity of Trp residues is closely related to the polarity of the microenvironment around Trp residues, i.e., the conformation of Lys. Therefore, the changes in λ_{em} of Lys will reflect the conformation changes of Lys. The observations from Fig. 1 suggest that no change in the conformation of Lys took place and nevadensin molecules were only located at the close proximity to Trp residues, by which a quenching process occurred.

In addition, the change in Lys conformation upon addition of nevadensin can be also demonstrated by synchronous fluorescence spectra. As is known, synchronous fluorescence spectra show Tyr residues of Lys only at the wavelength interval ($\Delta\lambda$) of 15 nm and Trp residues of Lys only at $\Delta\lambda$ of 60 nm. As such, Fig. 6A and B show the synchronous fluorescence spectra of Tyr residues in Lys and those of Trp residues in Lys with various amounts of nevadensin, respectively. It can be seen that no apparent shifts occurred in both Fig. 6A and B. It indicates that the interaction of nevadensin with Lys does not significantly affect the conformation of Trp and Tyr residue microregions.

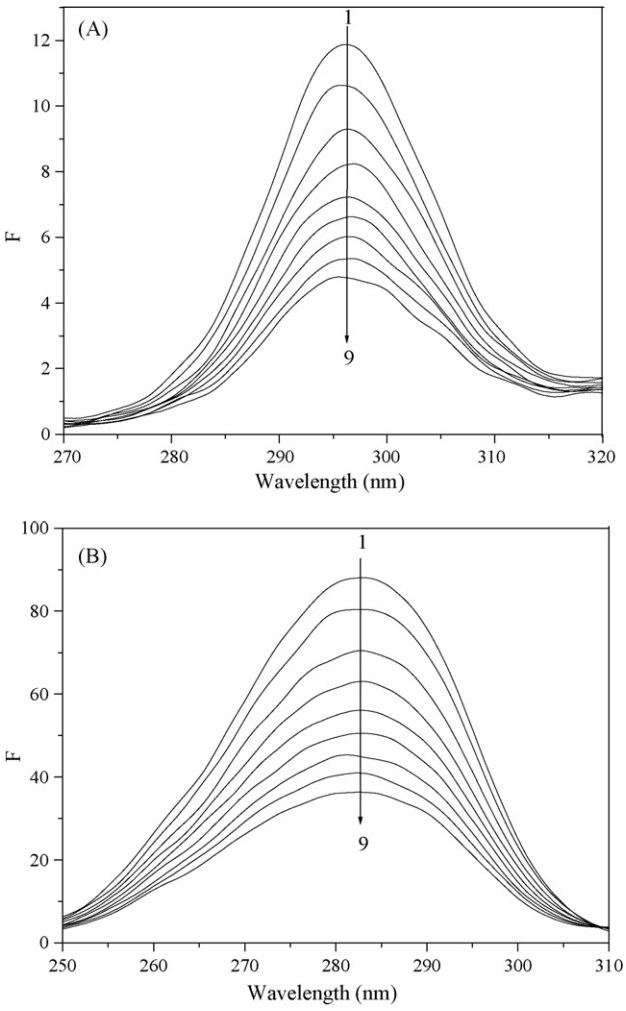

Fig. 6. Synchronous fluorescence spectra of Lys with various amounts of nevadensin. A: $\Delta\lambda = 15$ nm and B: $\Delta\lambda = 60$ nm; [Lys] = 8 μM; [nevadensin] = 0 μM (1), 1 μM (2), 2 μM (3), 3 μM (4), 4 μM (5), 5 μM (6), 6 μM (7), 7 μM (8) and 8 μM (9).

3.6.2. CD spectroscopic studies on nevadensin binding to Lys

Fig. 7 shows the CD spectra of Lys with various amounts of nevadensin at pH 7.40. They display negative CD bands in a wavelength range shorter than 240 nm. The CD spectra of Lys are characterized mainly by two negative bands at 207 and 222 nm. These bands are caused by a negative Cotton effect characteristic of helical structure [26,27]. CD is a relative powerful technique to investigate the secondary structural change of proteins, because they are, in the far ultraviolet region, related to the polypeptide backbone structure of proteins. The negative bands at 207 and 222 nm are rationalized by the $n \rightarrow \pi^*$ transition in the peptide bond of α-helical [28]. It is noticed from Fig. 7 that no detectable effects of nevadensin on the CD spectra of Lys were observed. In other words, the addition of nevadensin exerts essentially no apparent influence on the molecular conformation of Lys. This result is in agreement with those obtained from other spectroscopic techniques mentioned above.

Previous studies on the interaction between BSA and anesthetic chloroform showed that the retention of protein

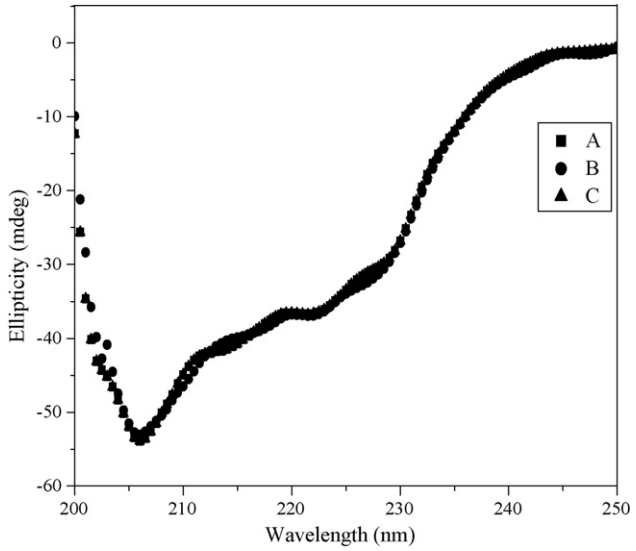

Fig. 7. CD spectra of the nevadensin–Lys system in Tris buffer solution at pH 7.40 and 298 K. [Lys] = 40 μM, [nevadensin] = 0 μM (A), [nevadensin] = 80 μM (B), and [nevadensin] = 320 μM (C).

conformation was crucial to ligands binding [29]. The interaction between Lys and nevadensin is assumed to be similar to it. As such, it can be concluded that the occupancy of Trp sites by binding nevadensin actually leads to stabilize the native conformation of Lys. Frazier and co-workers [17], Dong and co-workers [30] and Simon et al. [31] studied the interaction of flavonoids, NR and wine tannin with protein, respectively, and observed that they could bind tightly to protein without modifying its secondary structure, which may agree well with the results reported here.

4. Conclusions

Several issues on the interaction between nevadensin and Lys could be approached from spectrophotometric observations under simulative physiological conditions and measurements of enzymic activity: (a) The fluorescence of Lys was quenched by nevadensin via a static qhenching mechanism at lower concentrations of nevadensin and a combined quenching mechanism of both dynamic and static process at higher concentrations of nevadensin. Accordingly, the binding constants K_b and the number of binding sites n are obtained; (b) a distance r of 2.28 nm between donor and acceptor was obtained from the Förster non-radioactive resonance energy transfer theory; (c) fluorescence, synchronous fluorescence, and CD spectra show no significant change in the conformation of Lys upon addition of nevadensin under experimental conditions; and (d) the addition of nevadensin led to a reduction of the activity of Lys.

The determinations performed herein may provide an approach to evaluate the toxic effects of chemicals on target proteins and the molecular mechanism of toxicity. Therefore, it is expected to be used for screening and designing appropriate flavonoid-based drugs.

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