

Spectroscopic Study of Dipicolinic Acid Interaction with Bovine Serum Albumin

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Abstract

Spectral changes of bovine serum albumin (BSA) in the presence of dipicolinic acid (DPA) were studied with UV/Vis absorption and fluorescence spectroscopy. The change in UV/vis spectra of BSA in the presence of DPA indicated the protein weakly interacting with DPA. The DPA quenching to the fluorescence of BSA leads to the calculation of the BAS-DPA association constant K of $8.9 \times 10^4 \text{ Lmol}^{-1}$ at 295 K and $4.0 \times 10^4 \text{ Lmol}^{-1}$ at 308 K. From the synchronous spectra, the results show that the tryptophan site has a greater impact on the spectral properties of BSA than the tyrosine; DPA interacts with BSA through Van der Waals force, hydrogen bonding, and electrostatic interactions. Only 40% of the tryptophan is accessible for the ionic DPA quenching.

Keywords: Bovine serum albumin (BSA), dipicolinic Acid (DPA), fluorescence spectroscopy, static quenching, binding constant, conformation change.

1. Introduction

Dipicolinic Acid (DPA), chemical name 2,6 pyridinedicarboxylic acid; is a universal and specific chemical component of bacterial spore (endospore).

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DPA is synthesized and incorporated into the spore core as 1:1 calcium complex. Endospores typically contain 5 to 14 percent DPA by weight in the core (Powell 1953; Gould et al., 1969; Setlow et al., 2006). Endospores are capable of going into dormant state, exhibited by gram positive bacteria such as the toxic bacillus anthrax (anthrax), and clostridium perfringen when they are under stress or deprived of nutrient. They are resistant to heat and chemical changes and can survive for decades or centuries. Endospores can germinate into active cells when the appropriate nutrients and chemical environmental conditions for germination are present. DPA is released from the endospore body upon germination, chemical or physical rupture, thus chemical detection of the presence of spores is usually done through the detection of DPA (Fichtel et al., 2007; Setlow et al., 2008; Yang et al., 2009). Beside the toxicity of the bacterial spores, DPA alone if inhaled can cause systemic and central nervous system effects, abnormal fatigue, memory difficulties and dizziness.

This work aims to investigate whether DPA will bind to and alter the conformation of bovine serum albumin (BSA). BSA is the most abundant protein in the plasma in cows. As a soluble protein constituent of the circulatory system, it contributes to colloid osmotic blood pressure and is chiefly responsible for the maintenance of blood pH (Peter, 1996; Carter et al., 1994). The most outstanding property of albumin is its ability to reversibly bind to a large variety of endogenous and exogenous ligand such as fatty acids, aromatic neutral molecules and carboxylate and sulfonate anionic species as well as metal ions through its many different specific binding sites to these species (Klotz et al., 1946; Teresi et al., 1948; Reynolds et al., 1968; U-Krah- Hansen 1981; Ghuman et al., 2005; Yang et al., 2014). The binding greatly influences the absorptions, distributions, and metabolism and excretion properties of these types of substances. Thus determining the binding parameters is essential in understanding the toxicological profile and other pharmacodynamics of the ligand molecules.

BSA has high homology and similarity to the human serum albumin in sequence and confirmation (He et al., 1992), it is cheap and readily available, and thus it is widely used as a model protein to study the ligand-protein interactions. BSA is a single chain amino acids globular nonglycoprotein, it is divided into three linearly arranged domains, and has two tryptophans (W) embedded into two different domains: W134 is located on the surface of domain I within the hydrophobic pocket and W213 located of domain II that has a similar chemical micro environment to the W214 in human serum albumin (HSA) (see Fig. 1). These two tryptophan residues and together with one tyrosine residue in BSA makes the protein an intrinsic fluorophore (Chen, 1967; Lakowicz et al., 1973). However, the fluorescence emission of these residues is highly susceptible to its local chemical environment and temperature. Solvents and ligands in the solution could induce the protein conformational change and reduce florescence emission through dynamic collision and binding (Eftinik et al., 1981; Willis et al., 1991). In recent years, fluorescence quenching measurements have been widely used to study the interactions of organic compounds with this protein, a sample of the studies is listed here (Sulkowska, 2002; Tian et al., 2004; Barbero et al., 2009; Rownicka-Zubik et al., 2013; Rub et al., 2014; Yu et al., 2015; Islam et al., 2016). Depending on the chemical properties of the compounds, e.g. size, polarity, charge and UV/vis absorption ability; the fluorescence quenching ability, binding strength (with binding constant), accessibility (the number of binding sites) and binding forces to the two Tryptophan sites are significantly different.

Here we report our recent studies using UV-Vis and fluorescence spectroscopy on the binding parameters between BSA and anionic DPA. Synchronous and regular fluorescence spectra at two temperatures and two wavelengths show that the aromatic anion DPA binds moderately strong at the tyrosine and one of the tryptophan site with the correction of the absorption of DPA at 280 nm. The interaction forces between DPA and BSA fluorescence sites are through Van der Waals force and hydrogen

bonding, while electrostatic interaction cannot be excluded.

2. Experimental

2.1 Reagents

BSA (bovine serum albumin, lyophilized powder) and 2, 6 pyridine dicarboxylic acid (DPA) were purchased from Sigma Aldrich. The structures of BSA and DPA are shown in Fig. 1. All other chemicals used were of analytical grade and were used as received at temperatures of 295 K and 308 K. Aqueous solutions were prepared with ultrapure deionized water.

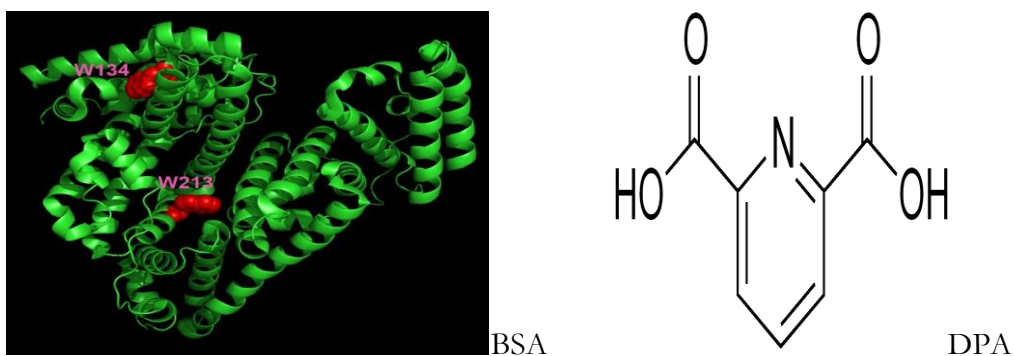


Fig. 1: Structure of BSA and DPA.

2.2. Instrumentation:

Fluorescence spectra and synchronous spectra were obtained on a Perkin Elmer LS50B spectrophotometer and 1.0 cm quartz cuvette; all spectra were taken over the range of 290-500 nm. The absorption spectra were recorded on a Thermo Insight UV-Vis spectrophotometer evolution 220, using a 1.0 cm quartz cell; the spectra were taken

over a wavelength range of 250 -400 nm

2.3. Method

Stock solutions: $2.00 \times 10^{-3} \text{ mol L}^{-1}$ of DPA and $1.00 \times 10^{-4} \text{ mol L}^{-1}$ BSA were prepared in pH phosphate buffer (PBS, pH 7.2). Lower concentrations of DPA and BSA were diluted with buffer from stock solutions. For the fluorescence spectra measurements, 200 μL aliquot of $1.00 \times 10^{-5} \text{ mol L}^{-1}$ BSA solution was added to 1800 μL of buffer solution. The BSA solution was titrated by successive additions of $2.00 \times 10^{-4} \text{ mol L}^{-1}$ or $2.00 \times 10^{-3} \text{ mol L}^{-1}$ DPA solution, and the DPA concentration varied from 0 to $2.00 \times 10^{-4} \text{ mol L}^{-1}$.

The fluorescence emission spectra of the solution were taken and recorded from 290-500 nm at an excitation wavelength of 280 or 295 nm using excitation and emission slits of 10 nm. All the emission spectra were recorded at the temperature of 295 K except the temperature studies at 308 K. Synchronous spectra were obtained from 270-350 nm at $\Delta\lambda=15 \text{ nm}$ and $\Delta\lambda=60 \text{ nm}$ with the excitation and emission slits at 10 nm. The UV-Vis absorption spectra of DPA solutions in the concentration range of 1-200 ($\times 10^{-6} \text{ mol L}^{-1}$), BSA of 10.0 or 1.00 ($\times 10^{-6} \text{ mol L}^{-1}$), and the mixture of BSA-DPA in various concentration ratios in buffer were recorded in the range of 250 to 400 nm with the double beam spectrometer.

2.4. Theory and Data Treatment

The kinetics of the intermolecular deactivation process can be examined using Stern-Volmer equation (Lakowicz, 2006)

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

F_0 and F are the fluorescence intensities without and with the quencher, respectively. k_q is the quencher rate constant, τ_0 is the life time of the emissive excited state of fluorescence molecular without the quencher present and $[Q]$ is the concentration of the quencher. K_{sv} is the dynamic quenching constant. k_q can be calculated with K_{sv} when τ_0 is known: $k_q = K_{sv}/\tau_0$. For static quenching, the apparent association constant K , is obtained from the modified Stern-Volmer (Linerweaver-Burk) equation to determine the apparent binding constant K (Lakowicz, 2006):

$$\left[\frac{F_0}{F_0 - F} \right] = 1 + \frac{1}{K[Q]} \quad (2)$$

The tryptophan residues (W134 and W213) are in two distinct environments, the residue under hydrophobic environment (W134) is not usually accessible to the polar or ionic compounds (He et al., 1992). The following modified Stern-Volmer equation, analogous to the Klotz equation (Klotz et al., 1946) is used to determine the binding constant and the fraction of initial fluorescence that is accessible to the quencher f (Lakowicz, 2006):

$$\left[\frac{F_0}{F_0 - F} \right] = \frac{1}{f} + \frac{1}{Kf[Q]} \quad (3)$$

Since there is a significant absorbance at 280 nm by DPA above 1×10^{-5} mol L^{-1} , the fluorescence was corrected with the equation (Anbazhagen et al., 2008; Yu et al., 2015; Islam et al., 2016):

$$F_{corr} = F e^{A/2} \quad (4)$$

Here, F is the fluorescence intensity measured at the corresponding concentration of the quencher; A is the absorbance of the quencher DPA at 280 nm. The correction is valid when absorbance of the quencher (A) is lower than 0.3, corresponding to DPA concentration below $8 \times 10^{-5} \text{ mol L}^{-1}$.

3. Results and discussion:

3.1. Absorbance characteristics

BSA absorbs in the UV range below 250 nm to 300 nm with the λ_{max} around 280 nm. DPA has an aromatic ring with an absorption peak at 270 nm. There is an overlap of absorption spectra of BSA and DPA at 280 nm. At DPA concentration higher than $1 \times 10^{-5} \text{ mol L}^{-1}$, the absorbance at 280 is very significant. Fig. 2a shows absorbance spectra of $1.0 \times 10^{-6} \text{ mol L}^{-1}$ BSA, $2.0 \times 10^{-5} \text{ mol L}^{-1}$ DPA, as well as BSA-DPA mixture in the same concentration. The absorbance of the mixture in the range is slightly lower than the additive of BSA and DPA absorbances of the same concentration. Similar spectra change is observed at higher concentrations of BSA, as shown in Fig. 2b, with $1.0 \times 10^{-5} \text{ mol L}^{-1}$ of BSA and $8.0 \times 10^{-5} \text{ mol L}^{-1}$ of DPA and their mixtures. These results indicate that there is weak interaction between BSA and DPA which altered the electronic energy levels of both compounds slightly.

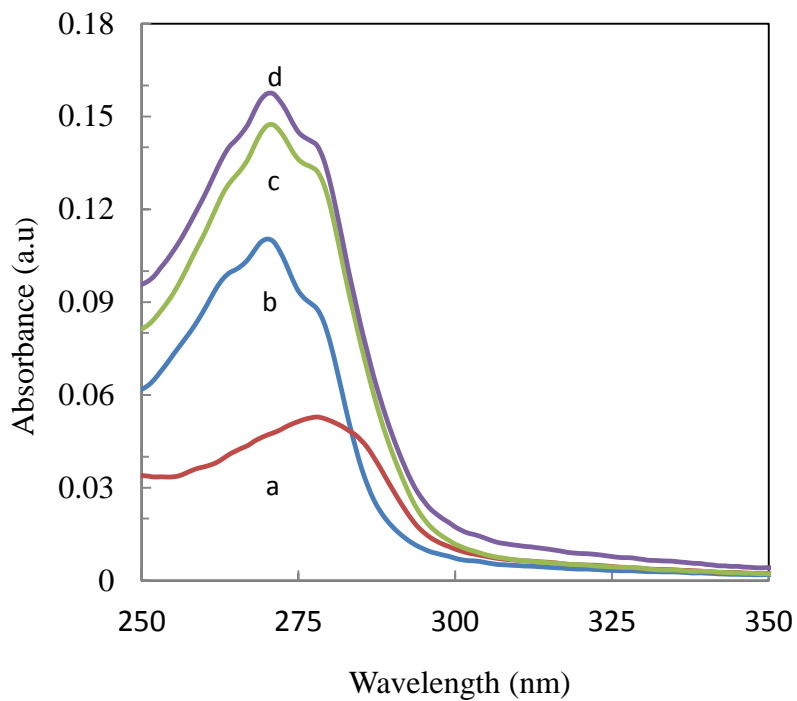


Fig. 2a: Absorbance spectra of (a) $2.0 \times 10^{-5} \text{ mol L}^{-1}$ DPA, (b) $1.0 \times 10^{-6} \text{ mol L}^{-1}$ BSA, (c) BSA($1.0 \times 10^{-6} \text{ mol L}^{-1}$) and DPA ($2.0 \times 10^{-5} \text{ mol L}^{-1}$), and (d) the additive of (a) and (b), at pH 7.2.

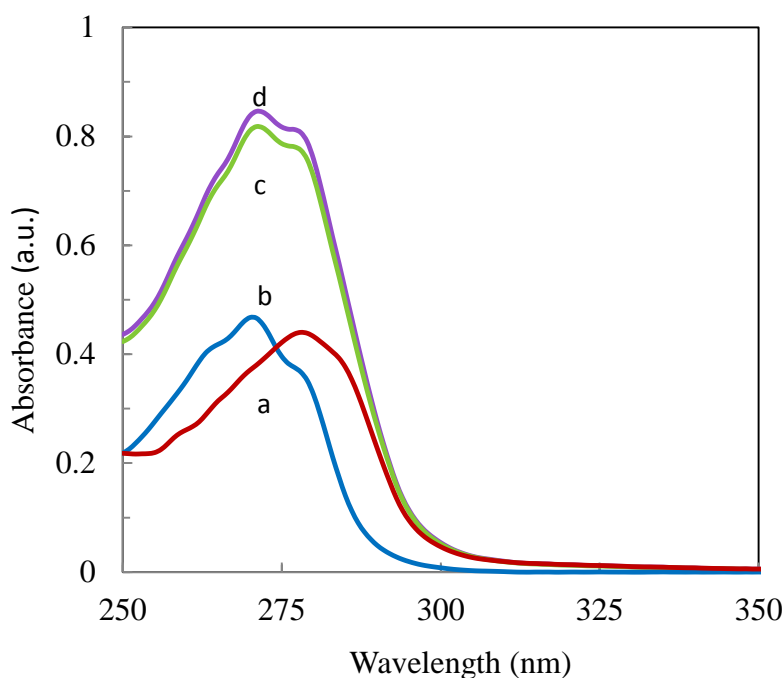


Fig. 2b: Absorbance spectra of (a) $8.0 \times 10^{-5} \text{ mol L}^{-1}$ DPA, (b) $1.0 \times 10^{-5} \text{ mol L}^{-1}$ BSA, (c) BSA ($1.0 \times 10^{-5} \text{ mol L}^{-1}$) and DPA ($8.0 \times 10^{-5} \text{ mol L}^{-1}$), and (d) the additive of (a) and (b), at pH 7.2.

3.2. Fluorescence characteristics of BSA

Tryptophan, tyrosine and phenylalanine all contribute to the fluorescence of BSA. These three residues have specific absorption and emission wavelengths and they differ in their quantum yields (see Table 1) (Lakowicz, 2006). Tryptophan, the most significant fluorescence emitter, will have an emission peak at lower wavelengths if it is buried within the hydrophobic inner (e.g. W134) regions of a protein. Tyrosine moieties will often transfer their energy to adjacent tryptophan amino acids, while ionized tyrosinate also demonstrates wavelengths similar to tryptophan, suggesting that for many proteins a good starting point for excitation and emission wavelengths are those

for tryptophan. Thus when the protein is excited at 280 nm, most of the fluorescence emissions are due to excitation of tryptophan residues with little emission from tyrosine and none from phenylalanine. At 295 nm, the emission is resulted from the excitation of tryptophan, since the phenylalanine and tyrosine has no absorbance at this wavelength.

Table 1: Parameters of the three aromatic residue of protein at 298 K (Chen, 1967; Lakowicz, et al., 1973).

| Resides | Absorption | | Fluorescence | | |
|---------------|-----------------|--|-----------------|---------------|----------------|
| | Wavelength (nm) | ϵ (L mol ⁻¹ cm ⁻¹) | Wavelength (nm) | Quantum Yield | Life time (ns) |
| Tryptophan | 280 | 5,600 | 348 | 0.14 | 2.6 |
| Tyrosine | 274 | 1,400 | 303 | 0.13 | 3.6 |
| Phenylalanine | 257 | 200 | 252 | 0.024 | 6.4 |

The fluorescence spectra of BSA were measured at pH of 7.2 at temperatures 295 K and 308 K with excitation wavelength of 280 nm and 295 nm. The maximum fluorescence at the two temperatures and two wavelengths is 350 nm, with a higher intensity at 295 K and 280 nm. A fluorescence emission spectrum of BSA at 295 K is shown in Fig. 3 (the top spectrum).

3.3. Quenching of Fluorescence

The fluorescence of BSA is quenched by DPA as shown in Fig. 3 at 295 K at excitation wavelength of 280 nm significantly. With the addition of various concentration of DPA, the fluorescence intensity of the BSA gradually decreased.

Further, at DPA concentrations below $1.0 \times 10^{-5} \text{ mol L}^{-1}$, the progress of quenching is rapid; however, it gradually slowed down after the concentration is beyond $2.0 \times 10^{-5} \text{ mol L}^{-1}$, this pattern is shown in the insert plotted as F_0/F at 345 nm.

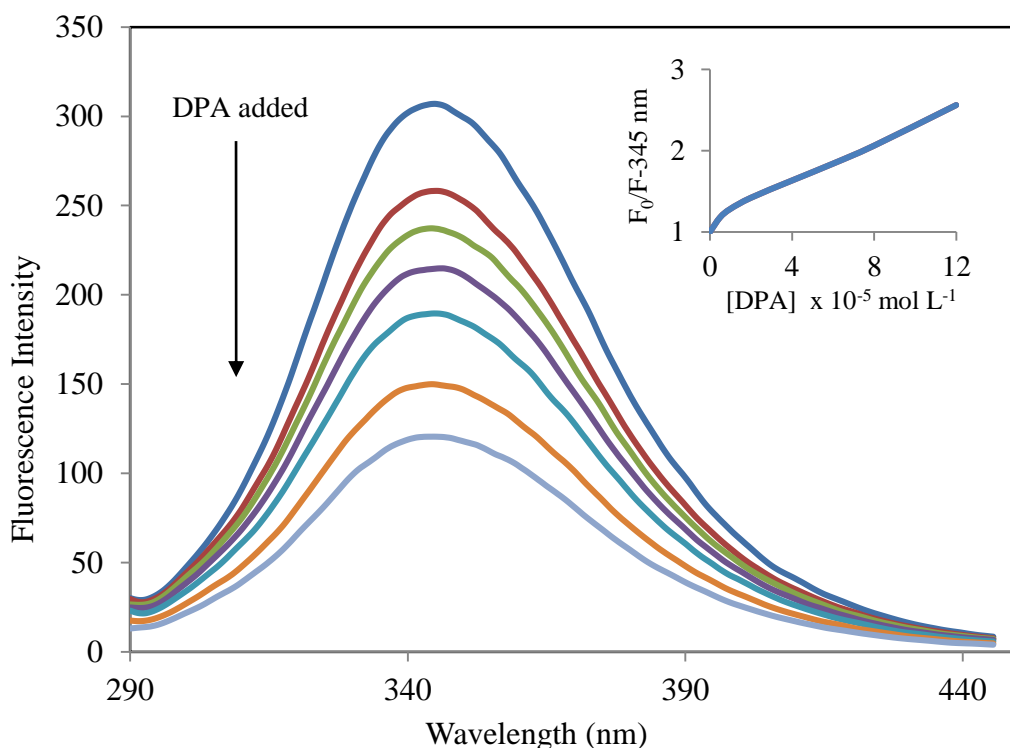


Fig. 3: Fluorescence spectra of $1.0 \times 10^{-6} \text{ mol L}^{-1}$ M BSA in absence of DPA and presence of DPA at pH 7.2 and 295 K. DPA concentrations from top to bottom: 0, 0.5, 1.0, 2.0, 4.0, 8.0 and 12 ($\times 10^{-5} \text{ mol L}^{-1}$). Insert: Stern-Volmer plot of the fluorescence intensity at 345 nm.

Similar quenching pattern is found at 295 nm excitation wavelength, with much reduced fluorescence quenching than that observed at 280 nm excitation at higher DPA concentrations. This difference reflects the reduced inner filter effect by DPA absorption and the absence of absorbance of tyrosine residue at 295 nm.

3.4. Quenching mechanism

For the quenching characteristics, two quenching processes are considered: collisional and static quenching. Their behaviors in various temperatures are strongly related to the quenching process. With increasing temperatures, it is assumed that there will be a large amount of collisional quenching due to large degree of collision, static quenching will be decreased because weak bonds will be dissociated (Lakowicz, 2006). With this in mind, Stern–Volmer plots at two different temperatures with corrected fluorescence (F_{corr}) using equation 4, the plot of (F_0/F_{corr}) vs. [DPA] is shown in Fig. 4 at low DPA concentrations at two temperatures. Fig. 4 also shows the plot with the F_0/F vs. [DPA] at 295 nm and 308 K without any absorbance correction.

At the same temperature, the corrected curve at 280 nm is only slightly higher than at 295 nm. The curved shape of the Stern-Volmer plot of the DPA quenching to BSA indicates that there are more than one binding sites (Sulkowska, 2002; Tian et al., 2004; Barbero et al., 2009; Rownicka-Zubik et al., 2013; Rub et al., 2014). In the present system, it includes the tyrosine and the two tryptophan sites in BSA. The similarity of the fluorescence quenching curves at excitation wavelength 280 nm and 295 nm at the same temperature confirms that the fluorescence is dominated by the tryptophan residues, and one of the tryptophan residues is not accessible due to the hydrophobic environment by ionic quencher DPA.

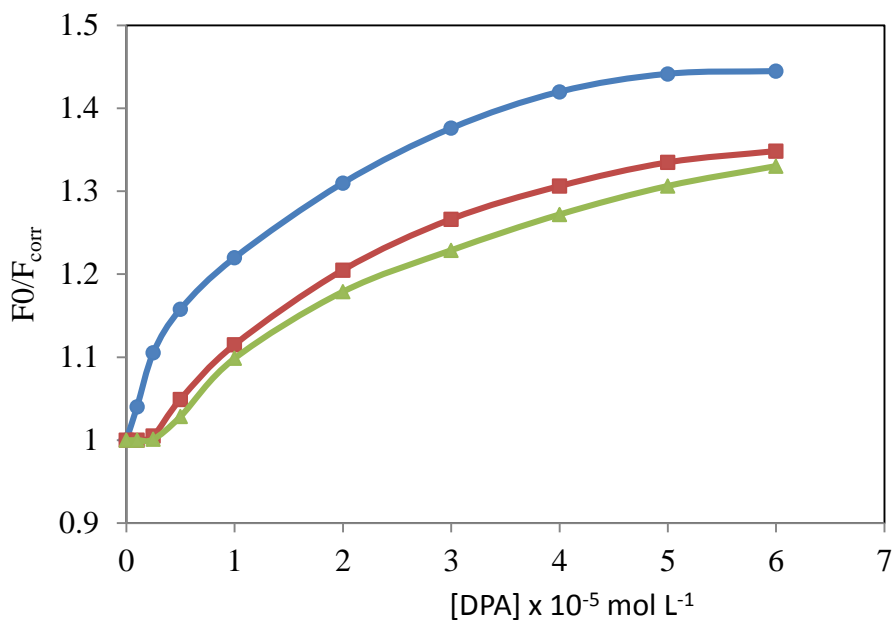


Fig. 4: Stern-Volmer plot of the BSA ($1.0 \times 10^{-6} \text{ mol L}^{-1}$, pH 7.2) with corrected fluorescence emission intensity at 345 nm by DPA at (●) 295 K, $E_x=280 \text{ nm}$, (■) 308 K, $E_x=280 \text{ nm}$ and (▲) 308 K, $E_x=295 \text{ nm}$.

At concentrations between 0-2.0 and 2.0-5.0 ($\times 10^{-5} \text{ mol L}^{-1}$) DPA, the average values of K_{sv} are obtained from the slopes of a linear regression. K_{sv} of the Stern-Volmer constant with the corrected fluorescence curve at 280 nm and the non-corrected curve at 295 nm are listed in Table 2. The fluorescence lifetime τ_0 of biopolymer is taken as $1.0 \times 10^{-8} \text{ s}$. The biomolecular quenching rate constant k_q was calculated and also listed in Table 2. As seen from Table 2, all k_q values are higher than that obtained for maximum scattering collision (dynamic) quenching of various quenchers with biopolymers ($2.0 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$), indicates that static quenching initiated by formation of a complex exists (Willis et al., 1991; Sulkowska, 2002; Papadopoulou et al., 2005; Rub et al., 2014) in the concentration range studied.

As shown in Table 2, at lower temperature, the K_{sv} values are higher than these

at higher temperature in the corresponding concentration range. The decrease of K_{sv} with the increase of temperature further confirms that static quenching exists and the binding between BSA and DPA decreases with temperature. At higher concentrations (2.0 - 5.0 ($\times 10^{-5}$ mol L⁻¹)), the K_{sv} values are the same at both temperatures, showing that the accessible binding sites are the same.

Table 2: Quenching parameters of DPA quenching to 1.0×10^{-6} molL⁻¹ BSA at Temperatures 295 K and 308 K calculated with F correction.

| T K | Ex nm | [DPA] x 10 ⁻⁵ molL ⁻¹ | K _{sv} L/mol | k _q , L/(mol S) |
|--------|----------|--|--------------------------|-------------------------------|
| 295 | 280 | 0.5-2.0 | 17000 | 1.7 x10 ¹² |
| 308 | 280 | 0.5-2.0 | 11000 | 1.1 x10 ¹² |
| 295 | 280 | 2.0-5.0 | 4400 | 4.4 x10 ¹¹ |
| 308 | 280 | 2.0-5.0 | 4400 | 4.4x10 ¹¹ |
| 308 | 295 | 0.5-2.0 | 8800 | 8.8x10 ¹¹ |
| 308 | 295 | 2.0-5.0 | 3800 | 3.8x10 ¹¹ |

3.5. Synchronous fluorescence spectra and Conformation of BSA:

Synchronous fluorescence spectra were employed to investigate the protein conformational changes. If the $\Delta\lambda$ between the excitation wavelength and emission wavelength is stabilized at 15 nm and 60 nm, the synchronous fluorescence spectra give the characteristic information of tyrosine or tryptophan residues respectively (Anbazhagan et al., 2008; Yu et al., 2015). Fig. 5 (a and b) showed that their emission intensities decreased with increasing concentration of DPA, in addition, the quenching

of the fluorescence intensity of tryptophan is stronger than that of tyrosine. From Fig. 5a ($\Delta\lambda = 15$ nm) and 5b ($\Delta\lambda = 60$ nm), there are shifts in both the emission wavelengths of both tyrosine and tryptophan residues, with the intensity decreased more significant at the tryptophan residue. This indicates that DPA binds to both sites and caused the conformational change of both sites, and it binds stronger to the tryptophan residues.

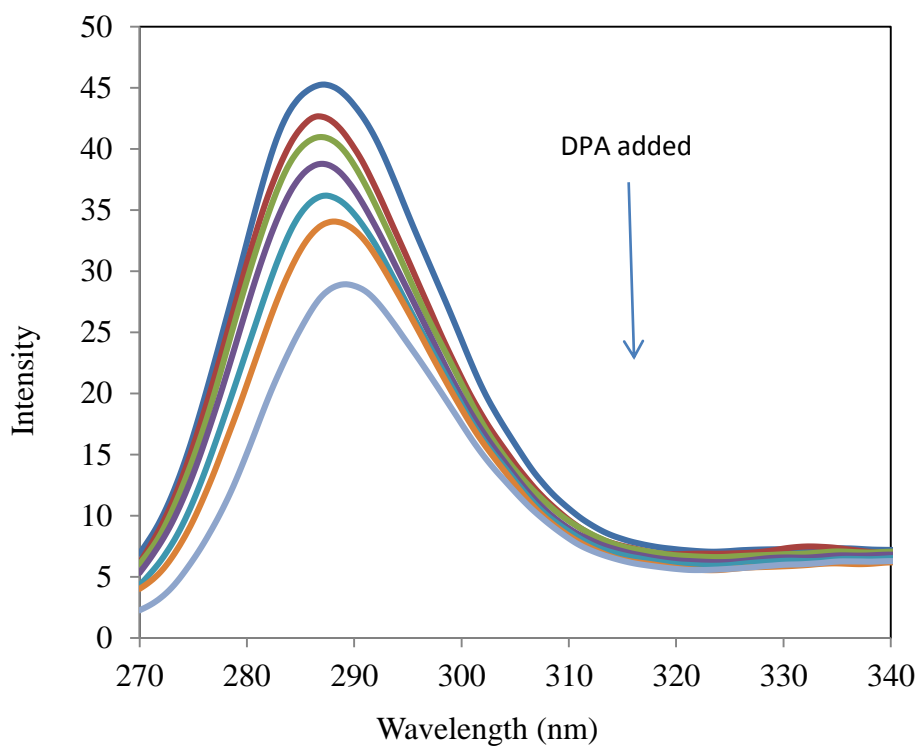


Fig. 5a. Synchronous Fluorescence spectra of 1.0 mol L^{-1} BSA in the absence and in the presence of DPA at $\Delta\lambda = 15$ nm at 295 K and pH 7.2. DPA concentrations from top to bottom: 0, 0.5, 1.0, 2.0, 4.0, 8.0 and 12 ($\times 10^{-5} \text{ mol L}^{-1}$)

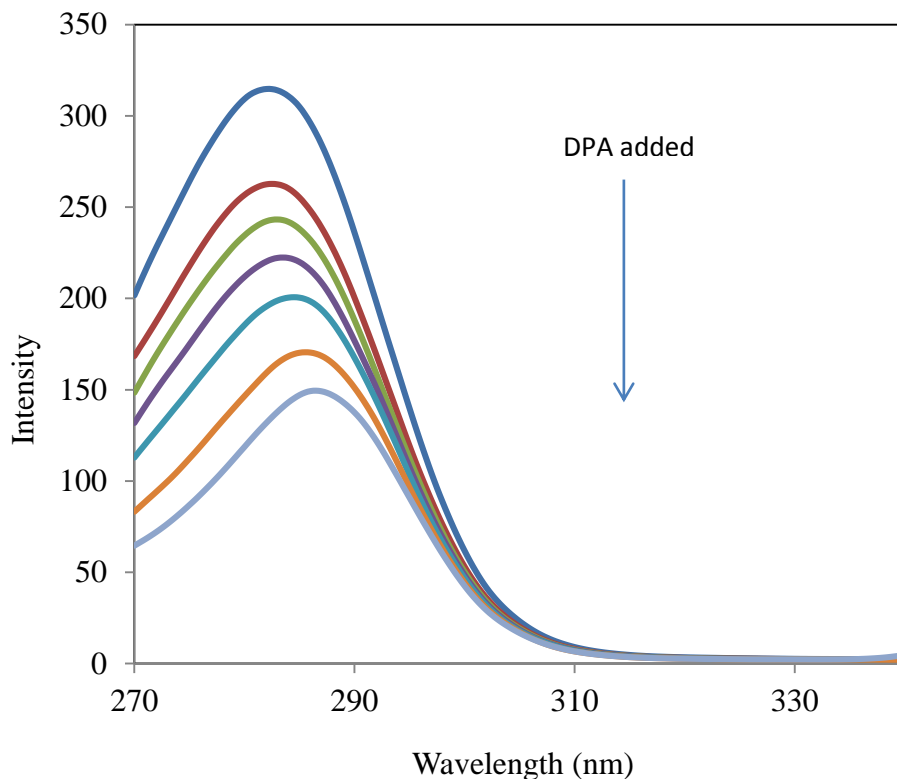


Fig. 5b. Synchronous Fluorescence spectra of 1.0 mol L^{-1} BSA in the absence and in the presence of DPA at $\Delta\lambda=60 \text{ nm}$ at 295 K and $\text{pH } 7.2$. DPA concentrations from top to bottom: 0, 0.5, 1.0, 2.0, 4.0, 8.0 and $12 \times 10^{-5} \text{ mol L}^{-1}$.

However, there are differences in the conformation change for the two sites at different concentrations of DPA. The wavelength shifts of the synchronous fluorescence a spectrum was plotted vs. the DPA concentrations (see Fig. 6). At low concentrations of DPA, there was a blue shift for the tyrosine residue ($\Delta\lambda=15 \text{ nm}$), at a higher DPA concentration, there was a red shift. As the BSA concentration increases, the wavelength shift reversing point of the DPA concentration is increased, which may indicate binding of DPA at the tyrosine residue, possibly $\pi-\pi$ interactions between the aromatic rings on BSA and DPA, that resulted an increase in hydrophobic environment

below $1.5 \times 10^{-5} \text{ mol L}^{-1}$ DPA.

At higher DPA concentration, the further decrease of the intensity of the tyrosine site indicates continued interaction of DPA with BSA, however, the site is experiencing an increase of polar environment. Data not shown on the graph, at 308K, at low DPA concentrations, the blue wavelength shifts of the synchronous fluorescence spectra at $\Delta\lambda=15 \text{ nm}$ was only from 288.0 to 287.0 nm before it red shifted back, indicating a smaller degree of binding and conformational change of the tyrosine site.

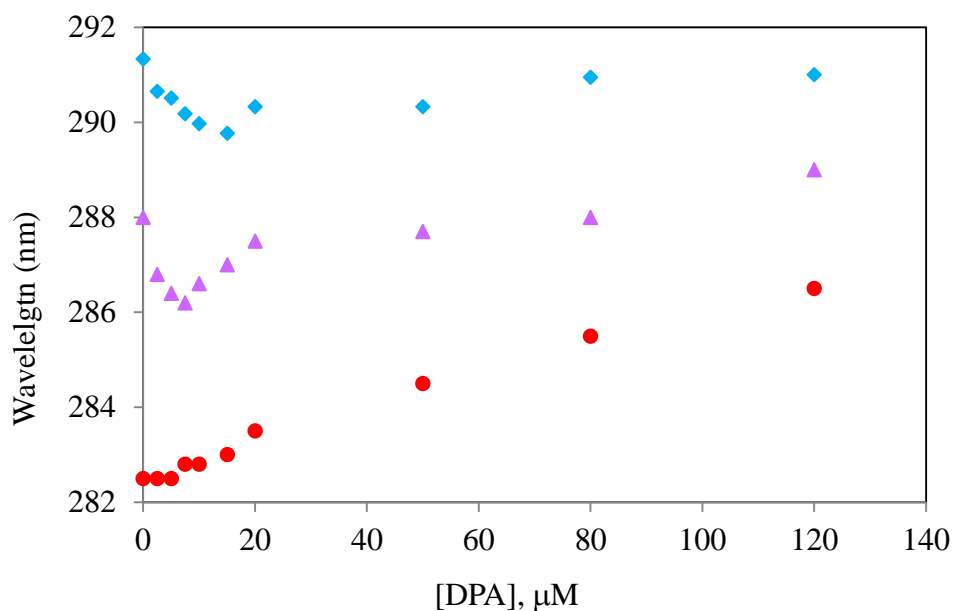


Fig. 6: Wavelength shifts of the Synchronous spectra at various DPA concentrations. (\blacklozenge) $1.0 \times 10^{-5} \text{ mol L}^{-1}$ BSA, $\Delta\lambda = 15 \text{ nm}$ (\blacktriangle) $1.0 \times 10^{-6} \text{ mol L}^{-1}$ BSA, $\Delta\lambda = 15 \text{ nm}$ and (\bullet) $1.0 \times 10^{-6} \text{ mol L}^{-1}$ BSA, $\Delta\lambda = 60 \text{ nm}$.

On the other hand, the peak shifts of the synchronous fluorescence spectra at $\Delta\lambda=60$ with BSA concentration 1 to 10 ($\times 10^{-6} \text{ mol L}^{-1}$) in the presence of various

concentrations of DPA are all red shifted at all DPA concentrations and temperatures studied. The shifts at 1×10^{-6} mol L⁻¹ BSA is shown in Fig.6. The tryptophan residues only experience increasing polar environment change at all DPA concentrations. This can be explained with the fact that at neutral pH, the two carboxyl groups are deprotonated due to their large pKa values (2.16 and 4.76) (Lide, 2006), the double charged anion that is close to BSA will increase the polarity of the environment.

3.6. Binding Parameters

The apparent association constant K , was estimated with the modified Stern-Volmer equation with the corrected fluorescence. The $F_0/(F_0-F_{cor})$ vs $1/[DPA]$ plots give linear lines with intercepts all around 2.7 (see Fig. 7). It can be assumed that one of the tryptophan is inaccessible in this concentration range. Using equation 4, the fraction can be calculated with the intercept of the curve: $f = 1/\text{intercept}$, K was calculated from the slope ($K = \text{Intercept}/\text{slope}$). The results are listed in Table 3. The binding constants are moderately large and decreased with increasing temperature, which are comparable to many of the aromatic acids quenching to BSA (Tian et al., 2004; Barbero et al., 2009; Rownicka-Zubik et al., 2013; Rub et al., 2014; Yu et al., 2015; Islam et al., 2016). Of the two binding sites, only less than 40% of them are accessible to DPA quenching. These values are smaller than most reported values (binding number: 1 or higher). The difference may be due to fact that many papers did not account for the absorption of their ligands at 280 nm, thus the quenching efficiency appears large, so as the calculated binding number. Here if the original observed fluorescence values are used, the fraction can be as large as 0.8, corresponding to binding number of 1.6 per BSA molecule. As an anion at physiological pH, DPA is expected to have electrostatic interaction with many positive ionic sites (e.g. arginine, lysine and histidine) on BSA (Ross et al., 1981; Matulis et al., 1999; Wang et al., 2011), while these sites do not fluorescence, these binding sites will not show with the

fluorescence quenching data.

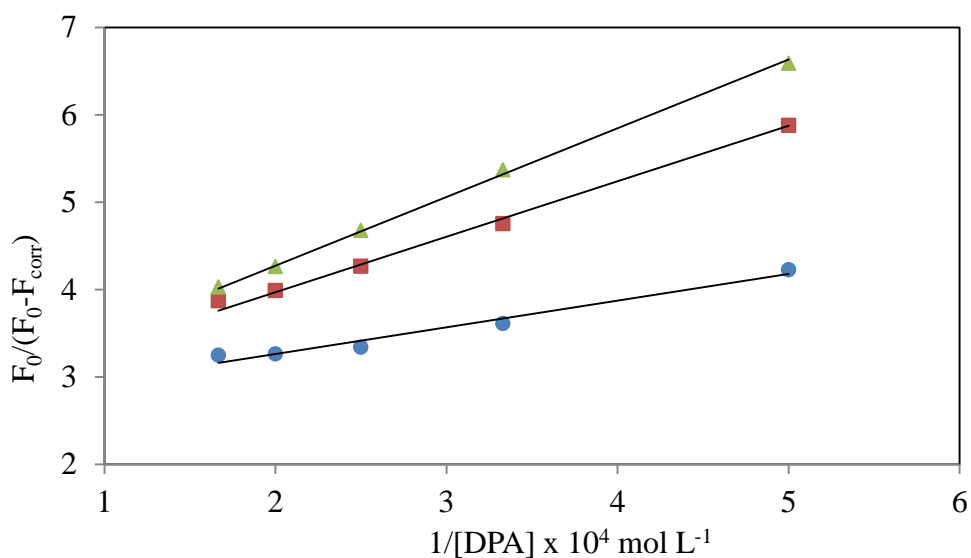


Fig. 7: Modified Stern-Volmer plot for the BSA-DPA system at (●) 295 K, $E_x=280$ nm, (■) 308 K, $E_x=280$ nm and (▲) 308 K, $E_x=295$ nm.

Table 3: Binding Parameters of BSA (1.0×10^{-6} mol L $^{-1}$) and DPA at Temperatures 295 K and 308 K calculated with F correction.

| T K | E_x (nm) | Fraction quenched | Binding constant | ΔG , KJ/mol | ΔH KJ/mol | ΔS J/(mol K) |
|--------|---------------|----------------------|---------------------|------------------------|----------------------|-------------------------|
| 295 | 280 | 0.37 | 8.9×10^4 | -28 | -38 | -34 |
| 308 | 280 | 0.39 | 4.0×10^4 | -27 | | |
| 308 | 295 | 0.38 | 3.6×10^4 | -27 | | |

3.7. Nature of the binding or interaction Mode:

The interaction of BSA and DPA may involve hydrophobic forces, electrostatic forces, Van der Waals, Hydrogen bonds and other interactions. From the

enthalpy change (ΔH) and entropy (ΔS), the mode of interaction can be assessed. It is suggested that if $\Delta H > 0$ and $\Delta S > 0$, hydrophobic interaction occurs, and if $\Delta H < 0$ and $\Delta S < 0$, Van der Waals and Hydrogen bonds dominates, and if $\Delta H < 0$ and $\Delta S > 0$, is due to electrostatic forces (Ross et al., 1981). Using the following thermodynamic equations, the free energy change ΔG and enthalpy change ΔH are calculated with:

$$\Delta G = -RT \ln K \quad (5)$$

$$\ln \frac{K_2}{K_1} = \frac{\Delta H}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \quad (6)$$

Where R is the gas constant and K is the association constant. With $\Delta G = \Delta H - T\Delta S$, ΔS values can be calculated.

The calculated thermodynamic values are listed in Table 3. The negative ΔG value clearly defines a spontaneous reaction. The highly negative value for ΔH shows a very strong exothermic process. The ΔS has negative value but small, indicating an unfavorable entropy contribution to the binding. The negative values of ΔH and ΔS suggest Van der Waals and Hydrogen bonding dominates the interaction between BSA and DPA. Giving the structure of DPA and tryptophan and tyrosine, it is reasonable to expect π - π interactions between the aromatic rings on BSA and DPA exists. This agrees well with the many studies that BSA interacts with a range of neutral to anionic aromatic compounds through hydrophobic interactions (Papadopoulou et al., 2005; Barbero et al., 2009; Rownicka-Zubik et al., 2013; Rub et al., 2014; Yu et al., 2015; Islam et al., 2016). Hydrogen bonding can be expected between the DPA COO^- groups with OH group on the tyrosine residue as shown in previous work (Willis et al., 1991). These binding forces will allow the aromatic carboxylate anion DPA to bind W213 and

Tyrosine, thus change the local chemical environment around these sites, consequently, causes fluorescence spectral changes.

The negative value of ΔH also indicates that DPA has electrostatic interaction with BSA. Other methods have shown electrostatic interaction exists between the many positive sites of BSA interacting with small inorganic anions, although these interactions are much smaller (small ΔG , ΔH) compare to the aromatic lipophilic anions (Teresi et al., 1948; Barbero et al., 2009; Rownicka-Zubik et al., 2013; Rub et al., 2014; Ross et al., 1981; Wang et al., 2011; Medda et al., 2012). Thus electrostatic interaction between BSA and DPA cannot be excluded.

4. Conclusion

This work presents a new study on the quenching of BSA fluorescence intensity by DPA and the analysis of the interaction of BSA-DPA. The quenching of BSA fluorescence intensity by DPA is both dynamic and static due to forming BSA-BPA complex at the tyrosine and tryptophan sites. The conformation of BSA changed during its interaction with DPA. The binding constant is moderate and comparable to many of the polar aromatic acids and only about 40% of the fluorescence binding sites are available to the ionic DPA quenching. This work will enhance the understanding of the interaction strength and mechanism between BSA and DPA.

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