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# Intrinsic tryptophan fluorescence and related energy transfer in Leghemoglobin isolated from *Arachis hypogea*

## [*Arachis hypoge*'dan izole edilmiş Leghemoglobin'lerde içsel Triptofan floresan ve floresana bağlı enerji transfer çalışmaları]*

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**ABSTRACT**

**Objective:** To explore the structural properties of heme containing leguminous protein Leghemoglobin (Lb) by means of intrinsic tryptophan (Trp) fluorescence and interaction with fluorescent dye 1-anilinonaphthalene-8-sulphonate (ANS). Hence to compare the melting temperature, quantum yield and energy transfer properties of Lb with other standard globular proteins.

**Method:** Intrinsic Trp fluorescence, extrinsic probe ANS, UV fluorescent signal were used to investigate Förster energy transfer in the proteins. Quantum yield and melting temperature ($T_m$) were determined to characterize Lb isolated from *Arachis hypogea*.

**Result**: Binding of ANS with the proteins Bovine serum albumin (BSA), lysozyme, Ovalbumin (Oval) and Cytochrome-C (Cyt-C) manifested differential enhancement of ANS fluorescence revealing energy transfer from Trp. Förster equation was used to estimate the efficiency of energy transfer from Trp to ANS. Estimated binding constants were different for 295 nm and 375 nm excitation suggesting the involvement of an additional pathway in Lb via Förster resonance energy transfer (FRET). This is reflected in higher $K_D$ value indicating lower binding affinity of ANS-Lb.

**Conclusion:** This is a pioneering endeavor to unfold the structural properties of Lb isolated from *Arachis hypogea*, since there is no report regarding the spectroscopic properties of this protein, which is of immense agricultural importance. Our work revealed a comparison of thermal stability of heme containing globular proteins which followed the order: Hb > Lb > Cyt-C. Quantum yield and the binding constant for Trp-ANS interaction of Lb were determined. Apparent distance for Trp-ANS energy transfer in Lb and other globular proteins were explored to follow the order:
Oval < Lb < BSA < Cyt-C < Lysozyme.

**Keywords**: Leghemoglobin, tryptophan, ANS, FRET, quantum yield

**Conflict of interest:** Authors have no conflict of interest.

**ÖZET**

**Amaç:** Bu projenin amacı heme taşıyan ve bir leguminous protein olan Leghemoglobin'in (Lb) içsel triptofan floresanı ve bu floresanın 1-anilinonaphthalene-8-sulphonate (ANS) floresanı ile olan etkileşimi incelenerek yapısal özelliklerini araştırmaktır. Bu amaç doğrultusunda Lb'nin erime ısısı, kuantum değeri ve enerji transfer özellikleri diğer globular proteinlerle karşılaştırılacaktır.

**Yöntem:** Bu çalışmada, proteinlerdeki Förster enerji transferinin hesaplanması için içsel triptofan floresanı, dışarıdan eklene ANS probu ve UV uyaranı kullanılmıştır. *Arachis hypoge'*dan izole edilen Lb proteinin karakterizasyonunda kuantum değeri ve erime ısısı tayin edilmiştir. were determined to characterize Lb isolated from *Arachis hypogea*.

**Bulgular**: ANS probunun BSA, lysozyme, ovalbumin (Oval) ve Sitokrom-C (Cyt-C) gibi proteinlerle etkileşmesi sonucunda içsel triptofan floresansına dayalı enerji transferinden dolayı ANS floresansında bir artış tespit edilebilmektedir. Förster denklemi kullanılarak içsel triptofan ve ANS arasındaki bu enerji transferinin nekadar kuvetli olduğu hesaplanabilmektedir. 295 nm ve 375 nm ile uyarılmadan sonra hesaplana bağlanma katsayıları farklılık göstermektedir. Bu sonuçlar Lb'nin farklı bir yolağına sahip olabileceğini göstermektedir. ANS nin Lb'ye yüksek bir $K_D$ dolayısı ile düşük bir bağlanma afinetisi ile bağlanması bu bulguyu desteklemektedir.

**Sonuç:** *Arachis hypogea*'dan izole edilen ve büyük tarımsal önem taşıyan Lb proteininin spektroskopik özelliklerinin çalışıldığı bu çalışma Lb proteinin yapısal verisi bulunmadığından büyük önem taşımaktadır. Çalışmamızda heme taşıyan globular proteinlerin termal dayanıklılık yönünden karşılaştırılmış ve sıralama Hb > Lb > Cyt-C şeklinde tespit edilmiştir. Lb protein için Trp-ANS arasındaki kuantum değeri ve bağlanma katsayıları belirlenmiştir. Trp-ANS için hesaplana Föster uzaklığı LB ve diğer hme taşıyan globular proteinler için tespit edilmiş ve şu şekilde sıralanmıştır: Oval < Lb < BSA < Cyt-C < Lysozyme.

**Anahtar kelimeler:** Leghemoglobin, triptofan, ANS, FRET, kuantum verimi

**Çıkar Çatışması:** Yazarların çıkar çatışması yoktur.

## Introduction

Leghemoglobin (Lb) is a monomeric oxygen transport protein with tertiary structure resembling that of myoglobin. It is synthesized during early symbiotic interaction between leguminous plant and rhizobia .Once Lb is expressed in the infection zone it enhances the bacterial differentiation by supporting oxidative phosphorylation. The function of Lb is to carry an ample supply of oxygen to the endosymbionts within the micro aerobic environment of the root nodule so that nitrogen fixation can be sustained. Several types of Lbs have been expressed in their nodules. Lb with lower oxygen affinities predominate in young nodule tissue and Lb with higher oxygen affinities predominate in older nodule tissue [1]. Lb expressed in the matured nitrogen fixing zone of the nodule helps in nitrogen fixation by lowering the oxygen affinity for the Nitrogenase enzyme [2]. Lb has a molecular weight of 15-17 kDa and the polypeptide chain pattern in the globin moiety of this heme containing protein is similar to myoglobin. Soil contains varieties of chemical compounds and different kinds of environmental stresses which are transmitted to affect nodule formation and its function in leguminous plants. Different stresses may induce conformational changes in the Lb structure consequently damaging its function, which in turn affect the nitrogen fixing and oxygen balancing property of the leguminous plant.

Two heme proteins, hemoglobin and myoglobin are well known as oxygen transport and oxygen Storage proteins in mammals but less is known about Lb, the heme containing plant protein found in the root nodules of legumes. Lb buffers the concentration of free oxygen in the cytoplasm of infected plant cells to ensure the proper function of root nodules [5]. Lb has a high affinity for oxygen (a $K_m$ of about 0.01 μM), about ten times higher than the β chain of human hemoglobin. This allows an oxygen concentration that is low enough to allow nitrogenase to function but high enough to provide the bacteria with oxygen for respiration. This monomeric Lb binds to and transport molecular oxygen maintaining a low oxygen environment within the root nodule. This permits the micro aerobic bacteria to convert atmospheric dinitrogen into ammonia, which is then assimilated by the plant for growth. So it is obvious that the structural properties and mechanism of oxygen binding to Lb is significant to understand its function relative to other heme proteins [3]. Interestingly, Lb and myoglobin have almost identical structure and similar non-covalent heme interactions. However, soybean Lb exhibits approximately 20-fold greater affinity for oxygen compared to myoglobin [2].

The physiological function of Lb has long been known, though very little is known regarding its structural properties. Of all the Lb's the soybean protein is by far the most well characterized [5-7] and a crystal structure for the nicotinate bound derivative is also available [8-9] As a more detailed study, cDNA sequence information for some Lb like Glycine Max, Medicago sativa, Lupinus luteus have been published (10). Electrospray mass spectrometry and Circular dichroism spectrometry was performed by a recombinant Lb expressed in E.Coli [12]. In this paper, we explored the fluorescence characteristics, quantum yield, ANS binding and the energy transfer between Trp and ANS molecule in Lb isolated from *Arachis hypogea*. Here, Tm for protein denaturation has also been evaluated for Lb and all the parameters have been compared to other standard globular proteins. As already been discussed, Lb is instrumental for $N_2$ fixation process in legumes and this demands major importance in agriculture. Thus our study unfolds the spectral properties of Lb isolated from *Arachis hypogea* which can be identified as good markers for binding or any type of interactions with Lb in the soil.

## Experimental

### *Plant growth*

Seeds of Arachis hypogea (ground nut legume, strain no AK 1224, JL 24) were planted at different location of light textured soils to facilitate nodule formation. After 25-30 days nodules were collected and stored in liquid nitrogen for 20min and preserved at -80 °C until the experiment was performed.

### *Isolation of leghemoglobin from root nodules of Arachis hypogea*

Nodules were homogenized in 0.1 M cold phosphate buffer, pH 6.8 at 4 °C [5]. The homogenate was mixed with polyvinylpyrrolidone in a ratio of 0.3 gm per gram of nodules to remove polyphenols [1]. The homogenate mixture was centrifuged at 10000 g for 20 min and fractionated with solid ammonium sulphate between 50 % to 80 % saturation, with the phosphate buffer, pH 6.8 at 4 °C. The precipitate was dissolved in a small volume of buffer and dialysed with stirring against 0.1 mM EDTA, pH 6.8, for 16 h [11]. Dialyzed material was then centrifuged at 25000 g for 15 min and loaded to a column of DEAE-cellulose equilibrated with 100 mM phosphate buffer at pH 6.8. The eluent fractions were collected with NaCl gradient mixture in phosphate buffer pH 6.8 and absorbance was measured at 403 nm [10]. Fractions having optical density value larger than 0.25 were pooled and concentrated in an ultrafiltration cell equipped with a UM 10 membrane.

### *Estimation of leghemoglobin*

Purified Lb was assayed by the pyridine hemochromogen method [13]. Equal volumes of 4.2 M pyridine in 0.2 M NaOH and Lb solution were mixed and the resulting hemochrome was reduced with a few crystals of sodium dithionite. Absorbance at 556 nm was measured against a reagent blank.

### *SDS-PAGE electrophoresis*

Purified and concentrated sample was analyzed in SDS-PAGE, using 5 % and 15 % of acrylamide for stack gel and main gel respectively [14]. Protein molecular weight marker (3.5-43.5 kDa) was used to estimate the molecular weight of Lb Protein and marker were stained with silver stain reagent.

### *Determination of melting temperature of the protein*

Tm is the characteristic signature of a protein directly associated with its tertiary structure. Currently protein Tm is determined by laboratory methods such as Differential Scanning Calorimetry, Circular Dichroism, Fourier transform infrared spectroscopy and several other methods. These methods are laborious and costly. Therefore, we propose a novel spectroscopy based method for predicting protein melting temperature. $T_m$ value of Hb, Lb, BSA, Lysozyme, Cyt-C and Oval were determined in Tm analysis software (module DLL, version 1.21) supported by UV-Visible Spectrophotometer (Shimadzu UV 2450). Protein samples were loaded in the 8 Micro Multi Cell quartz cuvette. Prior to loading, degassing of the samples was done for 10 min and sample filled cells were tightly stoppered with special silicon caps and $T_m$ value was determined by differential method.

This parameter has been identified as an important marker in the case control study universally. Absorbance was measured from 0 to 100 $^0$C at 280 nm and Tm value was calculated by the differential method.

### *Determination of protein and ANS concentration*

ANS ammonium salt was purchased from sigma Aldrich.

ANS was dissolved in the phosphate buffer (pH 6.8). Absorption spectrum was recorded by a Spectrophotometre and concentration of ANS was estimated by assuming the molar extinction coefficient value of 5000 $M^{-1}$ $cm^{-1}$ at 350 nm [15]. The concentrations of protein solutions were determined by the method of Folin- Lowry [16].

### *Study of energy transfer*

All fluorescence measurements were performed in a spectrofluorimeter using 5 nm excitation and 5 nm emission slit width. Excitation wave length at 295 nm was used to estimate the rate of energy transfer from proteins and 375 nm excited wave length was used to measure enhancement of ANS fluorescence.

The importance of energy transfer phenomenon in protein chemistry is to express the efficiency of transfer as the function of separation of the fluors and can therefore be used to measure inter-molecular distances. We designed such an experiment with Lb and ANS. The efficiency of transfer (E) is described by the following equation [17].

$E = 1 - F_{da} / F$

The relationship between the transfer efficiency and the distance between the donor and acceptor, $R$ is given by the equation:

$E = R_o^6 / (R_o^6 + R^6)$ or $R = R_o (1/E - 1)^{1/6}$

$R_0$ is the critical Förster distance related to each donor – acceptor pair. In this specific case $R_0$ is 28.6 Angstrom (A°) for Trp-ANS [28].

$R_o$ is the Förster distance, between donor and acceptor at which energy transfer is 50 % efficient. In other words, it is the distance where 50 % of excited donors are deactivated by FRET. At $R_o$, there is an equal probability for resonance energy transfer and the radiative emission of a photon. The magnitude of $R_o$ is dependent on the spectral properties of the donor and acceptor, bearing a specific value for a particular donor-acceptor pair. R is the distance between any donor-acceptor pair.

### *Determination of the Quantum Yield of Lb compared to other Proteins*

Fluorescence quantum yield is the most important characteristics of a fluorophore .The quantum yield is defined as the ratio of the number of photons emitted to the number of photons absorbed by the sample.

$Q = \text{Photons}_{emi} / \text{Photons}_{abs}$

The easiest way to estimate the quantum yield of a fluorophore is to compare it with a known standard. Determination of the quantum yield of a fluorophore is generally accomplished by comparing the wavelength integrated intensity of an unknown sample to that of a standard, using the formula [18].

$Q = Q_R I/I_R OD_R/ OD\ n^2/ n^2_R$

Where Q is the quantum yield, I is the integrated intensity, n is the refractive index, OD is the optical density. The subscript $_R$ refers to the reference flourophore of known quantum yield [19-20] The proteins were excited at 295 nm and for the measurement of relative quantum yield area of emission spectra was calculated (Hitachi Spectrofluorometer, Model No F-7000)

## Result and Discussion

### *Estimation of the Molecular weight of Lb*

Isolated and purified Lb from *Arachis hypogea* was analyzed in SDS-PAGE (Fig.1), when molecular weight of Leghemoglobin was estimated to be 14.5 kDa.

### *Absorption Spectrum of Lb from Arachis hypogea*

The absorption spectra of Lb has been provided in Fig.2 indicating three absorption peaks at 403.5 nm, 496 nm, 530 nm, and 560 nm respectively, which identifies the protein to be Ferric Lb [21]. The absorption property of Lb is also much similar with other heme containing globular proteins.

## *Melting Temperature as an Index of stability of Lb*

Tm is an intrinsic property associated with the conformational stability of a protein; it provides information about the physical stability of the molecule. Tm is directly related to the physical stability. The Tm value of freshly isolated and purified Lb was noted to be 67.7 °C. Tm values for BSA, Lysozyme, Cyt-C and Oval were found to be 68.2 °C , 51.2 °C , 66.7 °C and 59.0 °C respectively (Fig. 3) .The structure and functional properties of any protein is highly linked to each other. Tetrameric heme containing protein Hb has a $T_m$ value 71.4°C [22] which reveals Lb is much less thermo stable than Hb. Thermal stability of heme containing globular proteins was arranged in the order : Hb > Lb > Cyt-C. Considering all the globular proteins, the thermal stability order is as follows Hb > BSA > Lb > Cyt-C > Oval > Lysozyme.

## *ANS binding to leghemoglobin and other globular proteins*

The hydrophobic fluorescent probe ANS has been used extensively as an extrinsic fluorescent probe to monitor conformational changes in the biological macromolecules [23]. The ANS chromophores is known to have week fluorescence in water and other solvents, but bind to the hydrophobic regions of the proteins and display strong fluorescence emission property. ANS fluorescence spectrum is blue shifted and its intensity is dramatically increased in nonpolar solvents or when it binds to nonpolar sites of proteins [23-24]. The binding of ANS to Lb was accompanied with an enhanced fluorescence and a blue shift of the emission maximum from 520 nm to 480 nm (Fig. 4) was noticed.

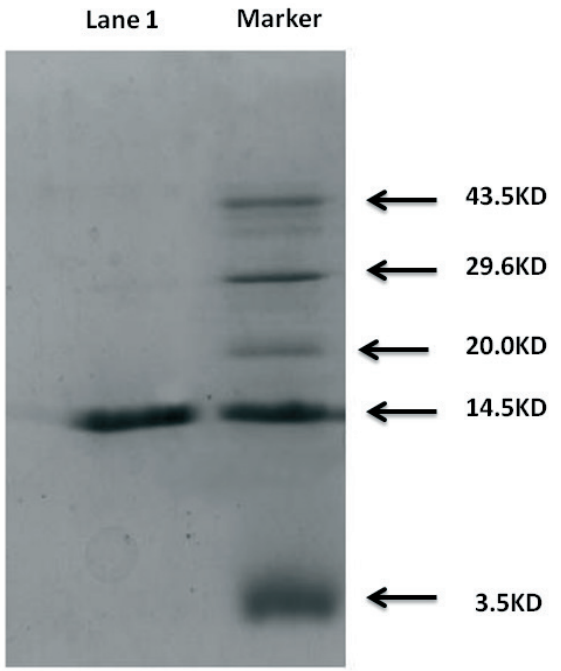


**Figure 1:** Molecular weight estimation of Leghemoglobin Proteins was separated by SDS- PAGE in 15% gel. The gel was stained with silver nitrate. The molecular weight of Lb was estimated to be 14.5 kDa**.**

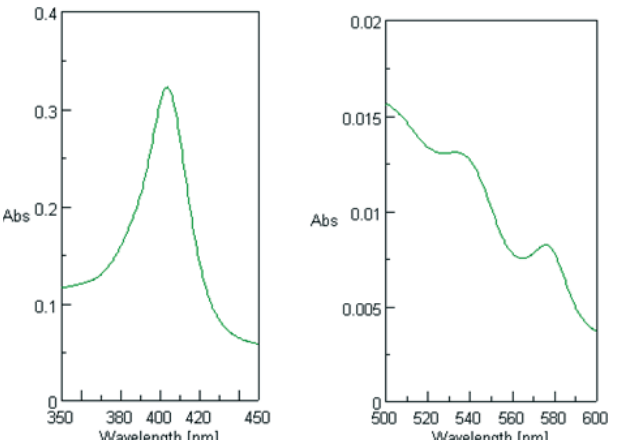


**Figure 2: A**bsorption and fluorescence spectra of leghemoglobin isolated from A***rachis hypogea***. Absorption spectra of purified Lb with Soret peak at 403 nm, 540 nm and 576 nm.

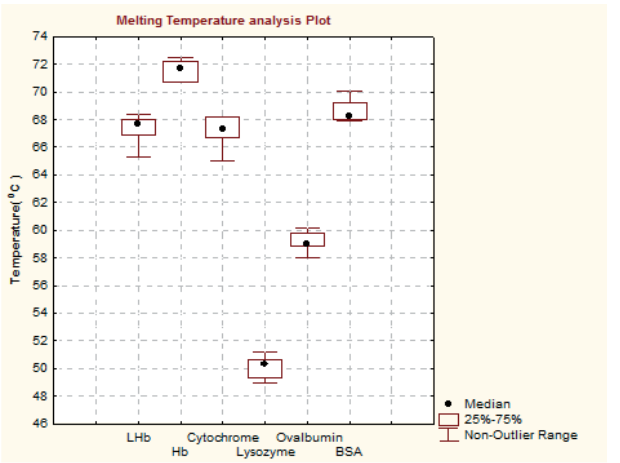


**Figure 3:** Box plot of Melting temperatures ($T_m$) in degree Celsius of leghemoglobin, Cytochrome-C, Lysozyme, Ovalbumin and BSA. Tm is directly related to the Conformational stability of protein, it provides information about the physical stability of the molecule. Higher the Tm value greater the physical stability [22], Tm value of freshly isolated and purified Lb is 67.70 $^0$C for BSA is 68.22 $^0$C, Lysozyme is 51.20 $^0$C, Cyt-C is 66.66 $^0$C and Oval is 59.00 $^0$C respectively.

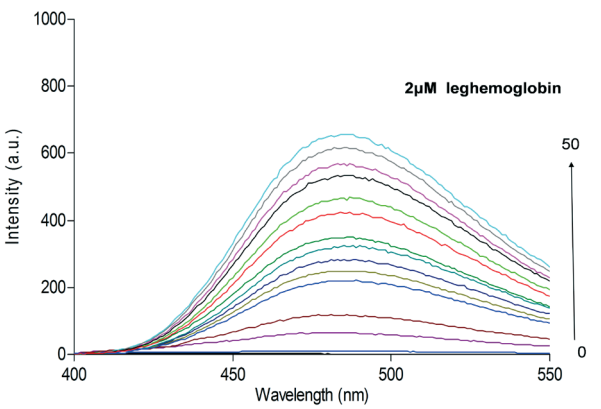


**Figure 4:** Enhancement of ANS fluorescence with leghemoglobin. The fluorescence emission spectra When 2 µM Lb was titrated with 0-50 µM ANS at pH 6.8 (0.1 M Phosphate buffer) at 22 $^0$C with excitation at 375 nm. Binding of ANS to Lb resulted in an enhanced ANS fluorescence and a blue shift in the Wavelength of maximum emission from 520 nm to 480 nm.

ANS probe was excited at 375 nm and corresponding emission was recorded at 480 nm so that no Trp residue was excited (Fig.4). Figure 4 indicates that no background emission was noted from 2 μM solution Lb in absence of ANS. Figure 5 represents the kinetics of ANS binding to Lb at 375nm excitation. ANS binds with Lb resulting ANS fluorescence enhancement and the data was fitted to the binding constant $K_D = 0.83 \times 10^{-1}$ $\mu M^{-1}$**.** It must be mentioned here, that, many factors such as the binding affinity for ANS, the number of ANS binding sites, and the structural character of ANS binding sites, might influence ANS fluorescence in combination, and any individual factor is not enough to account for enhanced fluorescence of proteins and concomitant energy transfer between Trp residues and ANS. Figure 6 represents the fluorescence from Lb in absence and presence of ANS using UV excitation at 295 nm .In this case satisfactory fit was observed with a binding constant of $K_D = 1.25 \times 10^{-1}$ $\mu M^{-1}$ (Fig. 7). Strikingly the binding constants are different for excitations at 295 nm and 375 nm. In case of 295 nm there is an additional pathway for ANS excitation by energy transfer from intrinsic Trp residue in Lb. At this wavelength, Trp residues of Lb are excited [25] , resulting in a broad fluorescence peak with the maximum emission at 336nm.Trp residues are relatively sensitive to the polar environment [26], their interaction with ANS display a red shift of 8nm and decrease in fluorescence emission maximum at 344 nm . Trp fluorescence is highly sensitive to its local environment, changes of emission maximum often occur in response to the conformational transition, substrate binding or denaturation of the protein.

## *Quantum yield of Lb in comparison to other globular proteins*

Trp fluorescence quantum yield observed in proteins is primarily due to different degrees of electron transfer from the indole ring to a local amide. The dependence on protein environment arises mainly due to the average local electric potential difference between the Trp ring and local backbone amide [27]**.** The quantum yield and emission maximum of different protein after ANS binding have been summarized in Table.1. One interesting characteristics of intrinsic fluorescence of Trp in these proteins is the low value of quantum yields, the basis of which is definitely the microenvironment of the residue. The light-absorbing part of Trp, effectively methylindole (3MI), shows little variability in quantum yield when dissolved in different solvents varying in polarity from pure hydrocarbon to water, being always near 0.3 [28]. Charged groups near the Trp can have profound effects on quantum yield, but location is critical. Negative (positive) charge decreases (increase) quantum yield, if it is closer to the indole ring than to the electron acceptor because these arrangements stabilize (destabilize) the charge transfer state. Thus revelation of low quantum yield of Trp in Lb also throws some light in the structure of this monomeric protein isolated from *Arachis hypogea*. This parameters is less compared to other globular proteins but greater than Cyt C.

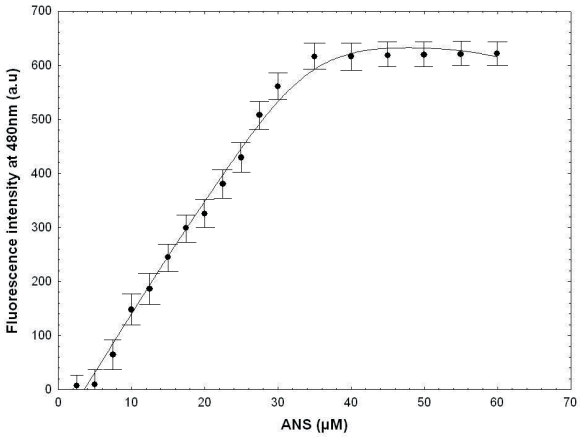


**Figure 5:** Kinetics of ANS binding to leghemoglobin observed with excitation at 375 nm.

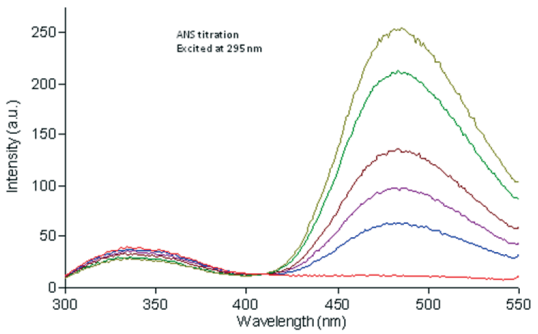


**Figure 6**: Emission spectra of Leghemoglobin titrated with ANS at 295 nm excitation. Progressive increase of ANS concentration (0-50 μM) results in a decrease in fluorescence intensity of Lb (336 nm) with a simultaneous increase of ANS fluorescence (480 nm) at pH 6.8 (0.1 M Phosphate buffer) at 22 $^0$C.

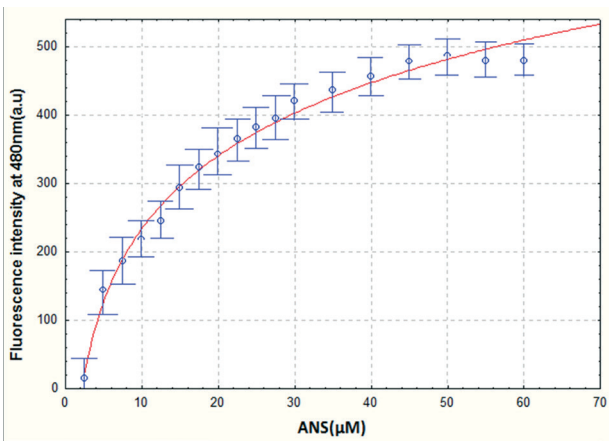


**Figure 7:** Kinetics of ANS binding to leghemoglobin observed with 295 nm excitation.

**Table 1.** Quantum yield of Leghemoglobin compared to other proteins

| Protein | Emission maximum(nm)* | Quantum yield+ |
|---|---|---|
| BSA | 342 | 0.030 |
| Ovalbumin | 334 | 0.027 |
| Lysozyme | 340 | 0.011 |
| Leghemoglobin | 336 | 0.003 |
| Cytochrome C | 342 | 0.001 |

* Emission wavelength of maximum fluorescence intensity and excitation wavelength of 280nm,
+ Quantum yield relative to Tryptophan as standard. [33]

## *Energy transfer between Trp residue and ANS in different globular proteins*

Progressive addition of ANS in titration experiments resulted in a decrease of Trp emission in Lb with a simultaneous increase of ANS fluorescence (Fig.6). The competing ANS absorption and re absorption cannot account for the magnitude of the quenching noted in protein fluorescence. The quenching of Trp emission by ANS can only be explained in the light of Förster resonance energy transfer (FRET) mechanism. In our experiment, efficiency of energy transfer and critical Förster distance[29] was utilized to calculate apparent Trp-ANS distance (Table.2) .Resonance energy transfer is the radiation less transmission of an energy quantum from its site of absorption to the site of its utilization in the molecule, or system of molecules, by resonance interaction between donor and acceptor over distances considerably greater than inter atomic, without conversion to thermal energy, and without the molecules coming into kinetic collision [30]. Förster (or Fluorescence) Resonance Energy Transfer (FRET) is unique in generating fluorescence signals sensitive to molecular conformation, association, and separation in the 10-100 Å range [10]. Resonance energy transfer in terms of a dipole-dipole interaction between donor and acceptor pair depends on $1/R^6$, where R is the apparent Förster distance between donor-acceptor.

FRET experiment was also performed for other globular proteins (Fig. 8) and in order of proximity of Trp and ANS, the globular proteins (Table.2) can be arranged as Oval < Lb < BSA < Cyt-C < Lysozyme (Table 2).Ap-

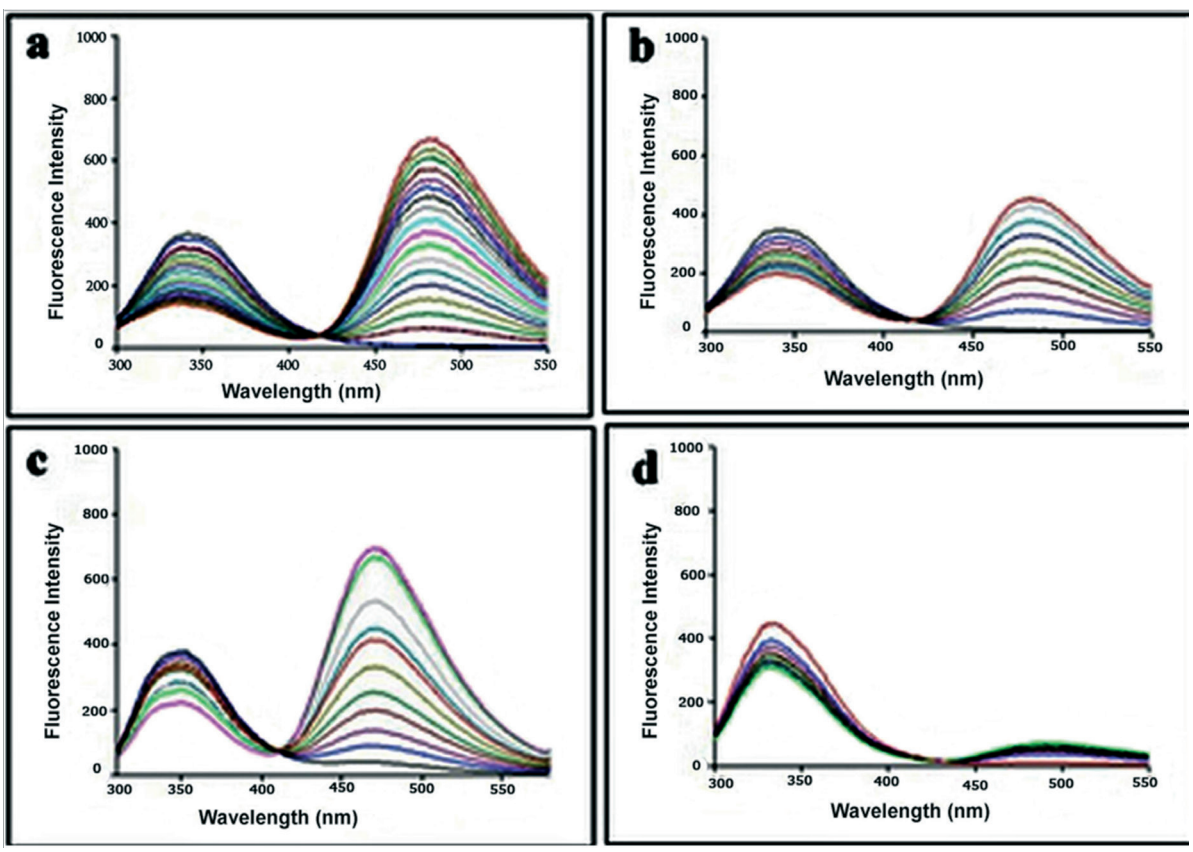


**Figure 8**: Emission spectra of (a) BSA (b) Cytochrome-C (c) Ovalbumin (d) Lysozyme titrated with ANS. Progressive increase of ANS concentration (μM) results in a decrease of Trp fluorescence (336 nm) in Lb with a simultaneous increase of ANS fluorescence (480 nm) at pH 6.8 (0.1 M Phosphate buffer) at 22 $^0$C.

**Table 2.** FRET study for Leghemoglobin and other proteins

| Protein | % of Energy Transfer | Apparent distance |
|---|---|---|
| Ovalbumin | **73%** | 24.47°A |
| Leghemoglobin | **70%** | 25.06 °A |
| BSA | **48%** | 26.09 °A |
| Cytochrome-C | **55%** | 27.79 °A |
| Lysozyme | **18%** | 36.9 °A |

parent distance for Lb was 25.06 Å, when efficiency of energy transfer is 70 %. It is interesting to mention here, that Lb possesses only one Trp in each molecule according to the data available till now. Similarly Cyt C and Oval also possess one Trp residue and thus apparent distance is 27.79 Å and 24.47 Å respectively. BSA possesses two Trp residues and the apparent Trp-ANS distance is 26.09 Å. But it is very interesting, that Lysozyme possesses six Trp residues and thus longer distance between Trp and ANS (36.9 Å) indicates the uncertainty of the location of the ANS binding site. BSA also shows a discrepancy regarding the distance between Trp-ANS location, which may be the result of more than one Trp residues in the protein.

It is obvious that, differential energy transfers observed for the proteins partially depend on differences in donor-acceptor distance. Resonance energy transfer is used to study macromolecular system like proteins, extent of energy transfer being influenced by the presence of donor-acceptor diffusion during donor lifetime. Theory of energy transfer is based on the concept of coupled oscillators with similar resonance frequency, like two swings on a common supporting beam [31]. Analytical calculations of energy transfer between multiple donors and acceptors are complex. Using a Monte Carlo calculation scheme, Corry et al demonstrated the efficiency of energy transfer for individual fluorophores randomly distributed in two and three dimensions, for linked pairs of donors and acceptors and pentameric structures of five linked fluorophores [32] when donor acceptor pairs are attached to the same host molecules, and when the hosts are at low concentration, the model reproduces Förster's well-known $1/r^6$ relationship which was well verified. This approach can be used to relate the efficiency of energy transfer to the distances between fluorophores, molecular concentrations, laser power, and donor/acceptor ratios in ensembles of molecules or when many fluorophores are attached to a single molecule such as in multimeric proteins.

We anticipate that these structural features will be useful to understand the further energy landscape and the stability of this agriculturally important monomeric protein.

## Conclusions

Groundnut *(Arachis hypogaea*) is endowed with nitrogen fixing ability through nodulation and rhizobia. Throughout the world ground nut is known for their nutrition and health benefits. The function of this heme containing protein Lb is to carry an ample supply of oxygen to the endosymbionts within the microaerobic environment of the root nodule so that nitrogen fixation can be sustained. Our work unfolded the thermal stability of heme containing globular proteins which was arranged in the following order: Hb > Lb > Cyt-C. The emission maximum and Quantum yield (Table1) of Lb and other proteins was also estimated. The binding constants for Trp-ANS interaction was different for 295 nm and 375 nm excitation suggesting that in case of 295 nm excitation , an additional pathway may be involved due to energy transfer from Trp residues. This is reflected in higher $^{K}{}_{D}$ value of ANS binding indicating lower binding affinity with Lb. Apparent distance for Trp-ANS binding in Lb and other globular proteins were observed to follow the order:

Oval < Lb < BSA < Cyt-C < Lysozyme. Different heme containing proteins like Hemoglobin, Myoglobin, Cyt-C are well studied and they have significant role in human welfare. Lb also plays a crucial role in leguminous plants. This study is significant from the perspective of structure-function understanding of Lb, which is of immense importance in agriculture.


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## Conflict of interest

Authors have no conflict of interest.

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