



Probing The Interaction Of Chlorogenic Acid With Human Gamma Globulin: A Fluorescence Quenching And Fret Based Study

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Abstract: This study investigates the interaction between Human Gamma Globulin (HGG) and Chlorogenic acid using UV–Visible and fluorescence spectroscopic techniques. UV–Vis absorption spectra indicated complex formation between the protein and ligand. Fluorescence emission spectra showed a gradual decrease in fluorescence intensity of HGG with increasing concentration of Chlorogenic acid. Stern–Volmer analysis suggested that the quenching mechanism follows static quenching. Binding constants and number of binding sites were calculated using double logarithmic plots. Furthermore, Fluorescence Resonance Energy Transfer (FRET) analysis was performed to estimate the binding distance between donor and acceptor molecules. The results reveal strong binding interaction between Human Gamma Globulin and Chlorogenic acid, forming a stable complex.

Keywords: Human Gamma Globulin, Chlorogenic acid, UV–Vis spectroscopy, Fluorescence quenching, FRET

1. INTRODUCTION

Proteins are essential biomolecules that perform a wide range of biological functions including immune response, molecular transport, enzymatic catalysis, and cellular regulation (Lakowicz, J. R. (2006)). Understanding the interaction between proteins and small bioactive molecules is important for studying biochemical mechanisms and drug–protein binding. Human Gamma Globulin (HGG), an important plasma protein belonging to the immunoglobulin family, plays a significant role in immune defense mechanisms. Investigation of the interaction between HGG and biologically active compounds can provide valuable information about protein structure, stability, and binding behaviour (Feng, R., et al. (2014)).

Polyphenols are naturally occurring compounds widely present in fruits, vegetables, cereals, and beverages such as coffee (Belay, A., et al. (2016)). These compounds exhibit several biological activities including antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Chlorogenic acid, an important phenolic acid formed from caffeic acid and quinic acid, is known for its strong antioxidant activity and health benefits (Li, S., et al. (2010)). The interaction of polyphenols with proteins may influence both protein structure and biological activity, making such studies important in biochemical and pharmaceutical research.

Spectroscopic techniques such as UV–Visible absorption spectroscopy and fluorescence spectroscopy are widely used to investigate protein–ligand interactions. UV–Visible spectroscopy provides information about structural changes and complex formation between proteins and ligands, while fluorescence spectroscopy is highly sensitive for studying conformational changes and quenching mechanisms (Chen, Z., et al. (2013)). Intrinsic fluorescence of proteins mainly arises from aromatic amino acids such as tryptophan and tyrosine, which serve as useful probes for monitoring molecular interactions.

In addition, Fluorescence Resonance Energy Transfer (FRET) is an effective method for studying molecular distances and energy transfer between donor and acceptor molecules. In the present study, the interaction between Human Gamma Globulin and Chlorogenic acid has been investigated using UV–Visible absorption spectroscopy and fluorescence spectroscopy. The fluorescence quenching mechanism, binding parameters, and energy transfer efficiency were analyzed to understand the molecular interaction between the protein and ligand.

2. MATERIALS AND METHODS

2.1. Experimental Materials

Human Gamma Globulin (HGG, IgG), a Y-shaped immune protein (~160 kDa), was used as the primary macromolecule. Chlorogenic Acid (CGA), a natural antioxidant ester of caffeic and quinic acid, served as the ligand. Both HGG (90% purity) and CGA were obtained from Sigma-Aldrich, Bangalore.

2.2. Sample Preparation

1 M Phosphate buffer (pH 7.4) was used to maintain physiological conditions. HGG stock (1×10^{-5} M) and CGA stock (10^{-4} M) were freshly prepared using triply distilled water. Verified via melting point analysis and UV-Vis spectral consistency across various excitation wavelengths.

2.3. Spectroscopic Techniques

UV-Vis Absorption was measured using a Shimadzu 1800 PC (range 200–500 nm) to study ground-state complex formation. Fluorescence Quenching was performed on a Shimadzu RF 5301 PC. Protein was excited at 280 nm; emission was recorded at 300–450 nm. (Harding, S. E., & Chowdhry, B. Z. (2001)).

Quenching mechanism was calculated using following Stern-Volmer Equation:

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

Binding Constant (K_a) and Binding Sites (n) was calculated using following Double-Log Equation:

$$\text{Log} \left(\frac{F_0 - F}{F} \right) = \text{log} K_a + n \text{log} [Q] \quad (2)$$

2.4. Energy Transfer (FRET):

Förster Resonance Energy Transfer (FRET) was used as a "spectroscopic ruler" to calculate the distance (r) between HGG (donor) and CGA (acceptor) based on the spectral overlap of protein emission and ligand absorption.

3. RESULT AND DISCUSSION

3.1. UV–Visible Spectroscopic Analysis of HGG–CGA Interaction:

This study examines the interaction between Human Gamma Globulin (HGG) and Chlorogenic acid (CGA) using UV–Visible spectroscopy. The absorption spectra were recorded in the range of 200–800 nm under physiological conditions. Pure HGG showed a characteristic peak at 278 nm due to aromatic amino acids.

With increasing CGA concentration, the absorption intensity increased and a hypochromic shift from 278 nm to 273 nm was observed. This indicates structural changes in HGG, such as protein unfolding and changes in the microenvironment of aromatic residues (Busti, P., et al. (2005)). The results suggest that CGA binds to HGG through non-covalent interactions, mainly hydrophobic forces, leading to the formation of an HGG–CGA complex.

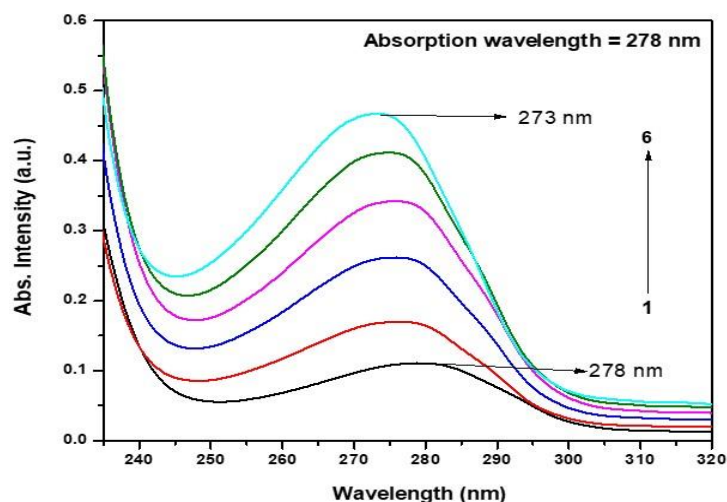


Fig. 1 UV-Vis absorption spectra of Human Gamma Globulin with different concentrations of Chlorogenic Acid (mol L^{-1}) (1) 0.0, (2) 0.2, (3) 0.4, (4) 0.6, (5) 0.8 & (6) 1.0.

3.2. Determination of Binding Constant of HGG–CGA Complex:

The binding interaction between Human Gamma Globulin (HGG) and Chlorogenic acid (CGA) was analyzed using UV–Vis absorption spectroscopy. The Benesi–Hildebrand method was applied to calculate the binding constant based on absorbance changes at 280 nm, which reflect alterations in the environment of aromatic amino acids. The interaction was assumed to follow a 1:1 stoichiometry between HGG and CGA. A double reciprocal plot of $1/(A - A_0)$ versus $1/[Q]$ (Fig.2) showed good linearity ($R^2 = 0.99$), confirming the formation of a stable complex.

The binding constant (K_b) was determined to be $4.26 \times 10^4 \text{ M}^{-1}$, indicating a strong interaction between HGG and CGA within the ideal range for protein–ligand binding (Table.1). The negative Gibbs free energy ($\Delta G = -22.32 \text{ kJ/mol}$) suggests that the binding process is spontaneous. Overall, the results confirm that CGA binds strongly to HGG through non-covalent interactions, likely dominated by hydrophobic forces, (Li, S., et al. (2010)) forming a stable HGG–CGA complex.

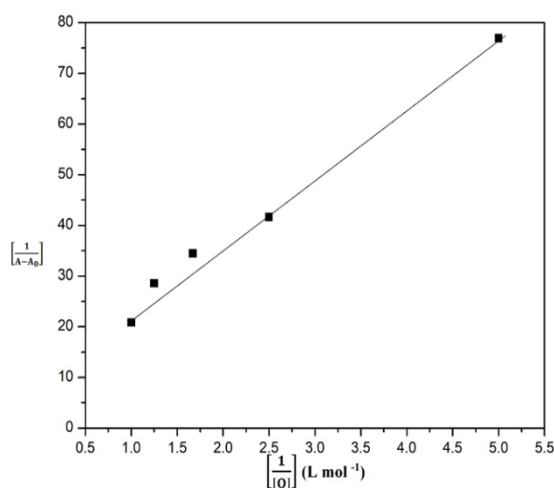


Fig. 2 Double reciprocal plot of $\frac{1}{A - A_0}$ versus $\frac{1}{[Q]}$ for HGG with CGA

Table 1 Binding constant of Human Gamma Globulin with Chlorogenic acid (CGA)

Quencher	Binding constant $K_g \times 10^4 \text{ (Lmol}^{-1}\text{)}$	R^a	ΔG_g
Chlorogenic acid (CGA)	4.26	0.99	-22.32

a $\rightarrow$ is the correlation coefficient.

3.3. Fluorescence Spectroscopic Study of HGG–CGA Interaction:

The interaction between Human Gamma Globulin (HGG) and Chlorogenic acid (CGA) was investigated using fluorescence spectroscopy, focusing on intrinsic fluorophores such as tryptophan, tyrosine, and phenylalanine residues (Cheng, D., et al. (2022)). Among these, tryptophan plays a dominant role due to its high sensitivity to changes in the local environment. HGG exhibited a strong fluorescence emission peak at 337 nm when excited at 278 nm, primarily due to tryptophan residues (Fig.3). Upon the gradual addition of CGA, a significant decrease in fluorescence intensity (quenching) was observed, indicating interaction between CGA and HGG. This quenching suggests changes in the microenvironment surrounding tryptophan residues, likely due to conformational alterations in the protein.

Additionally, a slight blue shift (3 nm) in the emission maximum was observed, indicating that CGA binds within a hydrophobic region of the protein. This results in increased hydrophobicity and displacement of water molecules from the binding site. Overall, the results confirm that CGA interacts strongly with HGG, causing structural changes and modifying the local environment of fluorescent amino acid residues.

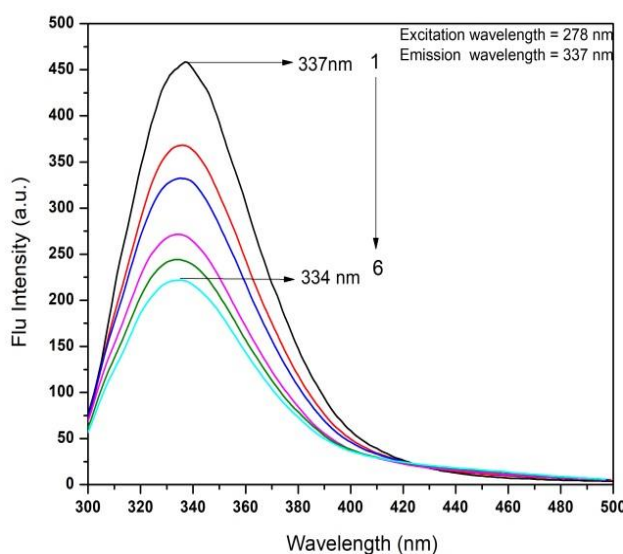


Fig. 3 Fluorescence spectra of Human Gamma Globulin with different concentrations of CGA (mol L^{-1}) (1) 0.0, (2) 0.2, (3) 0.4, (4) 0.6, (5) 0.8 & (6) 1.0.

3.4. Fluorescence Quenching Mechanism of HGG–CGA Interaction:

The fluorescence quenching mechanism of Human Gamma Globulin (HGG) by Chlorogenic acid (CGA) was analyzed using the Stern–Volmer approach. Fluorescence quenching occurs due to interactions between the fluorophore and quencher, either through dynamic (collisional) or static (complex formation) mechanisms (Fig.4). The Stern–Volmer plot (F_0/F vs $[Q]$) showed a strong linear relationship ($R^2 = 0.99$), indicating the presence of a single quenching mechanism. The Stern–Volmer quenching constant (K_{SV}) was found to be $10.92 \times 10^4 \text{ L mol}^{-1}$, and the bimolecular quenching constant (K_q) was $3.94 \times 10^{13} \text{ L mol}^{-1} \text{ s}^{-1}$ (Table.2)

Since the obtained K_q value is much higher than the typical maximum value for dynamic quenching ($\sim 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$), the quenching mechanism is identified as **static quenching** (Eftink, M. R., & Ghiron, C. A. (1976)). This indicates the formation of a stable ground-state complex between HGG and CGA rather than quenching through molecular collisions. Overall, the results confirm strong interaction between HGG and CGA, with quenching occurring mainly via complex formation, supporting the existence of a stable HGG–CGA complex.

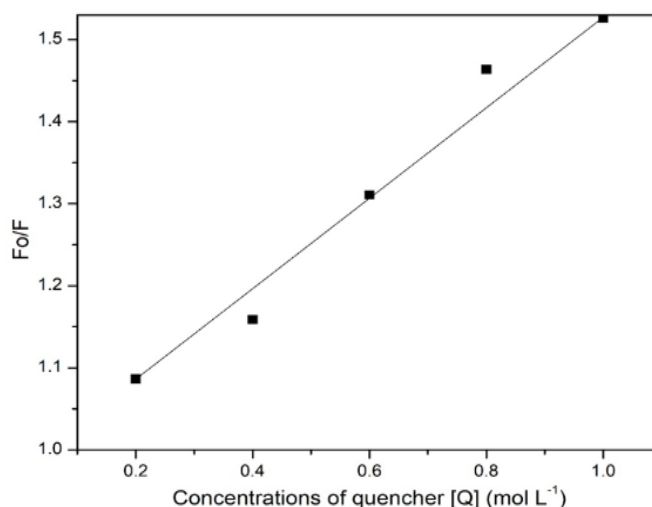


Fig. 4 The Stern Volmer plot of Human Gamma Globulin with CGA.

Table 2 Stern – Volmer (K_{SV}) and bimolecular quenching rate constant (K_q) of human Gamma Globulin with azo dyes

Quencher	$K_{SV} \times 10^4$ (L mol ⁻¹)	$K_q \times 10^{13}$ (L mol ⁻¹ S ⁻¹)	R^a	S.D ^b
Chlorogenic acid (CGA)	10.92	3.94	0.99	0.63

^a→ R is the correlation coefficient,

^b→ S.D is the Standard Deviation

3.5. Binding Parameters of HGG–CGA Interaction:

The binding parameters between Human Gamma Globulin (HGG) and Chlorogenic acid (CGA) were determined using fluorescence quenching analysis. A double logarithmic plot was used to calculate the binding constant (K_a) and the number of binding sites (n) (Bolel, P., et al. (2012)). The plot (Fig.5) showed good linearity ($R \approx 1.00$), confirming that the interaction follows a site-binding model. The binding constant was found to be $6.19 \times 10^5 \text{ L mol}^{-1}$, indicating strong binding affinity between HGG and CGA. The number of binding sites ($n \approx 1-1.4$) suggests the presence of a single primary binding site on the protein.

The negative free energy change ($\Delta G = -76.09 \text{ kJ/mol}$) indicates that the binding process is spontaneous (Table 3). Overall, the results confirm that CGA strongly binds to HGG at a single site through a stable and spontaneous interaction.

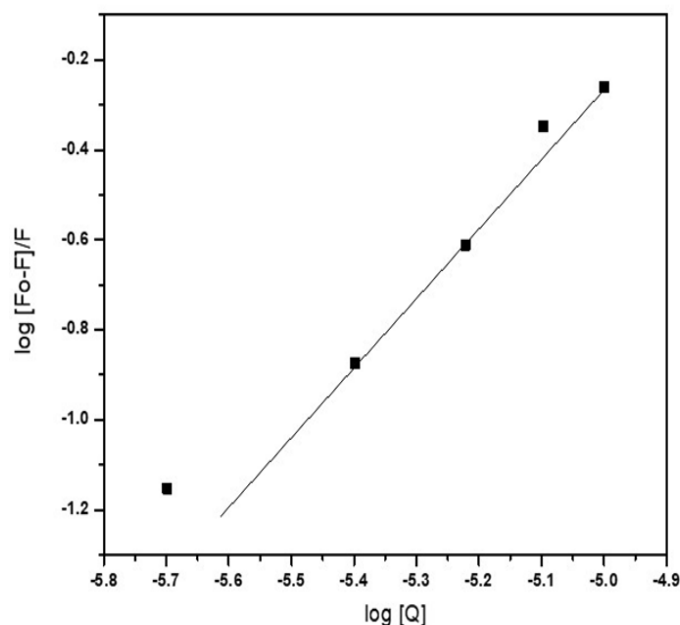


Fig. 5 Double log plot of Human Gamma Globulin with CGA

Table 3 Binding constant (K_a), and binding sites (n) values of Human Gamma Globulin with CGA

Quencher	K_a ($L\ mol^{-1}$)	n	R^a	S.D. ^b	ΔG_e
Chlorogenic acid (CGA)	6.19×10^5	1.40	1.00	2.41	-76.09

^a→ R is the correlation coefficient,

^b→ S.D is the Standard Deviation

3.6. FRET Analysis of HGG–CGA Interaction:

Fluorescence Resonance Energy Transfer (FRET) was used to study the interaction between Human Gamma Globulin (HGG) as the donor and Chlorogenic acid (CGA) as the acceptor. FRET analysis helps determine the distance between interacting molecules and provides insight into binding mechanisms. The overlap between the fluorescence emission spectrum of HGG and the absorption spectrum of CGA confirmed the possibility of energy transfer (Fig.6). The calculated FRET parameters showed an energy transfer efficiency (E) of **0.35**, Förster distance (R_0) of **2.36 nm**, and donor–acceptor distance (r) of **2.61 nm** (Table 4).

Since the distance ($r < 8\text{ nm}$) falls within the effective FRET range, (Lakowicz, J. R. (Ed.). (2006)) efficient energy transfer occurs between HGG and CGA. Additionally, the proximity between donor and acceptor supports the formation of a stable complex. The fact that r is close to R_0 further indicates a strong interaction and confirms a static quenching mechanism. Overall, FRET analysis demonstrates that CGA binds near the tryptophan residues of HGG, enabling energy transfer and confirming close molecular interaction and complex formation.

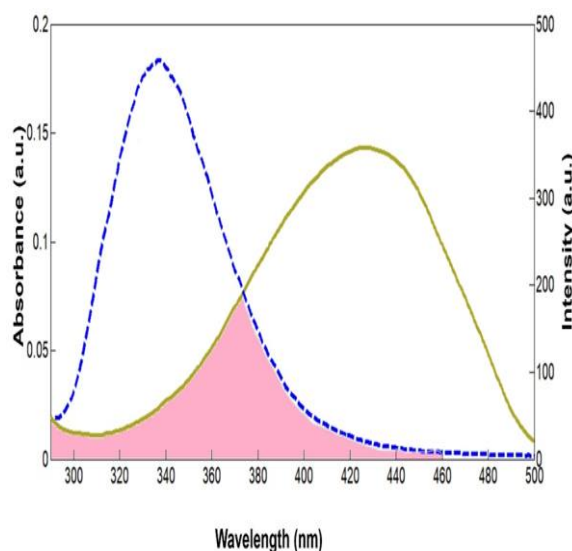


Fig. 6 The overlap of UV absorption spectra of CGA (solid line) with the fluorescence emission spectra of Human Gamma Globulin (dashed line)

Table 4 Förster energy transfer parameters of Human Gamma Globulin with Chlorogenic acid (CGA)

Quencher	Energy(E)	R_0 (nm)	J ($\text{cm}^{-3}\text{LM}^{-1}$) (10^{-15})	r (nm)
Chlorogenic acid (CGA)	0.35	2.36	8.04	2.61

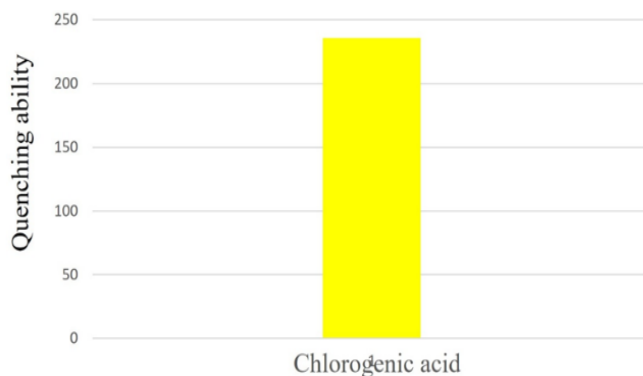
3.7 Overall Summary of HGG–CGA Interaction Study:

This research investigates the interaction between the phenolic compound Chlorogenic acid (CGA) and the protein Human Gamma Globulin (HGG), with potential applications in the food industry. The study combines UV–Vis absorption and fluorescence spectroscopy to understand the binding mechanism and structural changes in the protein (Marian, L., et al. (2018)). The quenching ability has been shown in Table 5 and Fig.7.

UV–Vis analysis confirmed the formation of the HGG–CGA complex and revealed conformational changes in the protein. Fluorescence studies showed a significant decrease in the intrinsic fluorescence of HGG upon addition of CGA, indicating strong interaction. The Stern–Volmer analysis demonstrated a linear relationship, confirming a single quenching mechanism. The high quenching constant (K_q) suggested that the process follows a **static quenching mechanism**, involving ground-state complex formation rather than dynamic collisions. Binding studies revealed a high binding constant ($K_a \approx 6.19 \times 10^5 \text{ L mol}^{-1}$) and approximately one binding site, indicating strong affinity and a 1:1 interaction between HGG and CGA (Table 6). FRET analysis further confirmed close proximity between CGA and tryptophan residues of HGG, supporting energy transfer and complex formation. Overall, CGA exhibits strong quenching ability, high binding affinity, and stable complex formation with HGG through non-covalent interactions. These findings highlight the potential application of protein–phenolic interactions in food and biochemical systems.

Table 5 Quenching Ability of the Quencher for HGG

Quenchers	Maximum Intensity	Minimum Intensity	Quenching Ability	$K_a \times 10^5$ (L mol ⁻¹)
Tartrazine	459	223	236	6.19

**Fig. 7** Quenching Ability of the Quencher for HGG**Table 6** Comparison table for all experimental results

Globulin	Phenolic compound	Quenching Constant $K_q \times 10^{13}$ (L mol ⁻¹ s ⁻¹)	Binding Constant $K_a \times 10^5$ (L mol ⁻¹)	Quenching Ability	Energy (FRET)E
HGG	CGA	3.94	6.19	236	35.42

From the above all results, it can be concluded that CGA shows a high quenching effect for the globulin and exhibits higher binding affinity.

4. CONCLUSION

This study investigated the influence of Phenolic compound, Chlorogenic acid (CGA) on globulin through a combination of multi-spectroscopic studies. Fluorescence spectrum analysis reveals that CGA quenched the fluorescence of HGG efficiently by static quenching mechanism, resulting from the formation of a ground-state complex with strong binding affinity. The comprehension of chemico-biological interactions is crucial for drug design, pharmacy, pharmacology, and biochemistry (Liu, C., et al. (2022)). This is achieved through the study of ligand binding to Globulins (Basu, A., & Suresh Kumar, G. (2016)). While derivatives of phenolic chemicals exhibit antiviral and anticarcinogenic properties, Protein evaluate their potential in the prevention of degenerative diseases and actively function as anticancer agents. These findings provide light on the relationship and process of phenolic chemicals' binding to amino acids and could serve as a helpful manual for future research in the food sector (Khan, M. S., et al. (2019) & Ying. L., Chao. W. and Guanghua. L., 2010).

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