Molecular recognition of two bioactive coumarin derivatives 7-hydroxycoumarin and 4-methyl-7-hydroxycoumarin by hen egg white lysozyme: Exploring the binding mechanism, thermodynamic parameters and structural changes using multispectroscopic and computational approaches

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ABSTRACT
Multispectroscopic and computational methods of exploring the interaction between a carrier protein and therapeutic compounds provide a preliminary investigation into establishing the efficacy of such compounds. Here, two coumarin derivatives, 7-hydroxycoumarin (7-HC) and 4-methyl-7-hydroxycoumarin (4-Me-7-HC), were selected to carry out numerous biophysical interaction studies with a model carrier protein, hen egg white lysozyme (HEWL). Fluorescence spectroscopy studies conducted between HEWL and 7-HC/4-Me-7-HC revealed the binding constants \( K_b \) were in the range of \( 10^4 \) M\(^{-1} \), indicating a moderate nature of binding. The quenching mechanism observed during complexation process was an unusual static quenching due to the effect of temperature on the rate constant. Thermodynamic parameters revealed a positive \( \Delta H \) and \( \Delta S \) for HEWL-7-HC/4-Me-7-HC, indicating hydrophobic forces played a dominant role in the interaction process. FRET studies suggested a possible non-radiative energy transfer from the donor (HEWL) to the acceptor (coumarins). Molecular docking studies revealed the interaction of 7-HC/4-Me-7-HC with intrinsic fluorophores, Trp63 and Trp108, Trp108 being an essential residue for binding as proven by molecular dynamic (MD) simulation. MD simulation studies also indicated conformational stability gained by HEWL upon interaction with 7-HC and 4-Me-7-HC. The microenvironment surrounding the Trp residues showed a significant Stokes’ shift on carrying out 3-D fluorescence. CD studies revealed a decrease in the alpha helical content of HEWL upon interacting with the ligands. Enzymatic assay conducted for HEWL in the presence of 7-HC/4-Me-7-HC saw an increase in the activity of HEWL, suggesting a change in structural conformation and stability of the protein, altering its activity.

1. Introduction
Ever since the discovery of lysozyme by Fleming in the year 1922 (Tan & Tatsumura, 2015), this protein has surfaced as a model for various binding study investigations. Popularly known for its lytic activity to kill bacterial cells by hydrolyzing the β-glicosidic bond between N-acetylmuramic acid (NAG) and N-acetylglicosamine (NAM) of the peptidoglycan cell wall (Strynadka & James, 1991), lysozyme is a low weight protein, ∼14.3 kDa, made of a single polypeptide chain of 129 amino acid residues (Barford & Stuart, 2012). Under physiological conditions, lysozyme folds into a compact globular structure that is highly stable due to four disulphide bonds present, formed between eight cysteine residues (Blake et al., 1965). A deep crevice present in lysozyme, indicating the catalytic site of the protein, separates the molecule into two domains linked together by an α-helix (Strynadka & James, 1991). One domain is predominated by β-sheets, whereas the second domain comprises more α-helices (Strynadka & James, 1991). Three tryptophan residues, Trp62, Trp63, and Trp108, are located in the catalytic site of lysozyme. Trp62 and Trp108 are known to be the dominant intrinsic fluorophores, as proven by steady-state fluorescence studies (Badley & Teale, 1969). In addition to its well-established enzymatic activity, lysozyme is also known to display numerous physiological activities. Found in granules of neutrophils and macrophages, bodily fluids, and secretions such as tears, saliva, blood, urine, cerebrospinal fluid, serum, honey, milk, and hen egg white (Callewaert & Michiels, 2010; Lelouard et al., 2010), lysozyme is also known for its anti-inflammatory, immune-modulatory, anti-cancer, anti-viral, anti-histaminic, anti-septic properties (Khan et al., 2019;
Ogundele, 1998; Parrot & Nicot, 1963). Being a low molecular weight protein, lysozyme also plays a significant role in reversibly binding and transporting various endogenous and exogenous molecules throughout the biological system (Ali & Al-Lohedan, 2020; Millan et al., 2017). It is popularly known for its ability to act as a vehicle for drug-delivery, where it binds to small drug molecules and delivers it to the target tissue (Paramaguru et al., 2010).

Studies on this protein-drug interaction can further help uncover the structural features of lysozyme to a great extent, a prerequisite in understanding its affinity towards drug molecules. Of late, interaction studies between many naturally occurring compounds and lysozyme are coming to the forefront to unveil the structure-affinity relationship affiliated to protein-ligand binding. Natural compounds found in plants have been playing a central role in the medical and pharmaceutical fields, with more than 50% of currently studied drug candidates are based on plant origin or their corresponding derivatives (Jash & Suresh Kumar, 2014). Their therapeutic and pharmacological properties are now being extensively explored as modern drug-like molecules for the treatment of various ailments and diseases. A group of naturally occurring compounds, well distributed among numerous plant families, are coumarins. Coumarin compounds and their derivatives have been highly investigated as potential drug-like molecules due to their therapeutic properties such as anti-cancer, anti-oxidant, anti-coagulant, anti-viral, anti-inflammatory, anti-neurodegenerative, anti-microbial, etc. (Bubols et al., 2013; Hoult & Payà, 1996; Matos et al., 2013, Matos et al., 2015; Yu et al., 2003). In addition, their low toxicity, high bioavailability, broad-spectrum, high abundance, and least side effects have elevated their value as potential candidates in drug discovery (Wang, Lu, et al., 2009). Investigation into their binding affinities with the carrier proteins such as serum albumins have been studied by various research groups to determine their distribution, metabolism, and overall biological activity (Cao et al., 2018; Garg et al., 2013; Liu, Tian, He, et al., 2004; Liu, Tian, Tian, et al., 2004; Liu et al., 2008; Xiao et al., 2009; Yue et al., 2018).

Protein-ligand interaction studies give a critical insight to understand the bio-affinity of a ligand towards a protein induced by conformational changes in the protein structure. Valuable input regarding the pharmacokinetic and pharmacodynamic behavior of the ligand in the protein environment can also be obtained. A well-established and concrete understanding of such protein-ligand interactions has a cascading effect on the therapeutic efficacy of the ligand as a potential drug candidate when brought under drug trial investigations. Multispectroscopic approach coupled with in silico techniques can help address the above-cited concerns.

With this objective in mind, the proposed study is focused on understanding the interaction occurring between lysozyme and two natural plant origin coumarin derivatives, 7-hydroxycoumarin (7-HC) and 4-methyl-7-hydroxycoumarin (4-Me-7-HC), which belong to the class of simple coumarins. The lysozyme used in this study is derived from hen egg white which shares 60% sequence homology to its human counterpart (Das, Pahari, et al., 2019), with their tertiary structure being similar (Blake & Swan, 1971). This validates hen egg white lysozyme (HEWL) as a model protein to be used in studying its interaction with varied ligand molecules. 7-HC (Figure 1 (a)), commonly known as umbelliferone, is a pharmacologically active compound and due to the simplicity in its structure, it is a parent molecule for other complex coumarin derivatives. Umbelliferone has been extensively studied for its therapeutic properties related to human well-being. It is known to display anti-oxidant properties where studies have revealed its ability to scavenge hydroxyl radicals and also inhibit the formation of superoxide anions (Kanimozhi et al., 2011; Mazimba, 2017). Anti-cancer activity of umbelliferone has also been reported by Kielbus and group against laryngeal cancer cells by reducing their migration and viability (Kielbus et al., 2013). Umbelliferone at all concentrations ranging from 0 μM to 50 μM also exhibits anti-tumor activity against liver hepatocellular cell lines (Yu et al., 2015). Other properties of umbelliferone include anti-inflammatory, anti-diabetic, anti-molluscicidal, etc (Mazimba, 2017). 4-methyl-7-hydroxycoumarin (Figure 1 (c)), a derivative of umbelliferone, has been reported to display moderate scavenging properties against reactive oxygen species (ROS) and slight inhibitory activity against xanthine oxidase (XO), an important source of free radicals which can cause gout and oxidative damage to tissues (Lin et al., 2008). Studies have also reported it to possess anti-skin cancer properties (Bhattacharyya et al., 2009).

In this study, various biophysical techniques were used to explore the binding interactions and affinities of 7-HC and 4-Me-7-HC ligands with HEWL. The binding constant ($K_b$), the mechanism of quenching, number of binding sites ($n$), thermodynamic parameters for HEWL-7-HC and HEWL-4-Me-7-HC systems were determined by conducting steady-state fluorescence and UV-vis spectroscopic studies. Synchronous and 3-D fluorescence measurements were carried out to ascertain the occurrence of microenvironmental changes around the tryptophan (Trp) residues. Alterations in the secondary structure of HEWL on binding to the two coumarin compounds were investigated using circular dichroism (CD) measurements. Enzymatic activity of HEWL upon binding with 7-HC and 4-Me-7-HC ligands was also determined.
Subsequently, molecular docking and molecular simulations were used to further establish and gain a deeper understanding of the binding site of HEWL upon interacting with 7-HC and 4-Me-7-HC, the conformational flexibilities, the type, and stability of interactions that govern such binding mechanism.

2. Materials and methods

2.1. Materials and sample preparation

Coumarin derivatives, 7-hydroxycoumarin (H0236) and 4-methyl 7-hydroxycoumarin (M0453) were procured from TCI Chemicals. Hen egg white lysozyme (≥98% purity, Molecular weight= 14.3 kDa (L6876) and Micrococcus lysodeikticus ATCC No. 4698 powder (M3770) were purchased from Sigma-Aldrich Chemicals Co. Other analytical grade reagents were procured from SRL India. No further purification was performed for the chemicals utilized in this study. A 20 mM stock solution of lyso- phosphate buffer solution of pH 7.4 prepared in double-dis-

2.2. Instrumentations

The steady-state fluorescence spectra were recorded using a Horiba Jobin Yvon spectrofluorometer Fluoromax-4 from Japan, provided with a Newport temperature controller, model 350 B, California, USA. 3-D fluorescence was carried out on Cary Eclipse Spectrofluorimeter, from Agilent technologies, USA. Double beam PerkinElmer Lambda 35 UV-vis spectrophotometer, consisting of two quartz cuvettes of 1 cm path length, was used to record absorption spectra. JASCO-J1500 CD spectrophotometer was used to carry out circular dichroism (CD) measurements.

2.3. Steady-state fluorescence spectral measurements

A range of concentration (0–16.39 μM) of ligand (7-HC or 4-Me-7-HC) was titrated against 3 μM of HEWL in a 1 cm path length quartz cuvette at three different temperatures (288, 298, and 308 K) at an excitation wavelength of 295 nm. This excitation wavelength excited the intrinsic Trp fluorophore, and the emission spectrum was recorded in the range of 305–500 nm. The blank spectrum was used to generate the correct emission spectrum, and inner filter effect correction was also considered for required samples (Lakowicz, 2006). The slit width used was fixed at 2/2 nm for both excitation and emission.

Synchronous fluorescence spectra were recorded on Fluoromax-4 spectrofluorometer for HEWL with the successive addition of the ligands (0 to 16.39 μM of 7-HC/4-Me-7-HC) at wavelength interval of Δλ = 15 nm for tyrosine resi-
dues and Δλ = 60 nm for tryptophan residues, respectively (Vo-Dinh, 1978). The 3-D fluorescence spectral measurements of 3 μM HEWL and its complexes, HEWL-7-HC and HEWL-4- Me-7-HC (protein:ligand ratio of 1:4 has been used) were carried out by recording the excitation and the emission spectra in the wavelength ranges of 200–380 nm (increment of 5 nm) and 250–500 nm (increment of 1 nm), respectively.

Förster’s Resonance Energy Transfer (FRET) theory was used to calculate the binding distance (r) between the donor (HEWL) and acceptor (7-HC/4-Me-7-HC) and for the said purpose spectral measurements were carried out for equal concentrations of HEWL and HEWL-7-HC/4-Me-7-HC complexes (3 μM) using an excitation wavelength of 295 nm (Förster, 1948).

2.4. Inner filter effect correction

The fluorescence emission spectra were corrected for the absorption of the emitted light and/or reabsorption of the emitted light by the ligands present in the solution to reduce the inner filter effect with the help of the following equation (Chi & Liu, 2011).

\[ F_{corr} = F_{obs} 10^{(A_{ex} + A_{em})/2} \]  

where \( F_{corr} \) and \( F_{obs} \) are the corrected and observed fluorescence intensities, respectively, \( A_{ex} \) and \( A_{em} \) are the absorption values of 7-HC and 4-Me-7-HC at the excitation and emission wavelengths, respectively. The corrected fluorescence intensities were used for the estimation of different parameters further.

2.5. UV-vis studies

Ground state complexation studies were conducted using absorption spectrophotometer. Absorption spectrum of 20 μM HEWL in the 20 mM phosphate buffer (pH 7.4) was recorded in the wavelength range of 200–700 nm, at room temperature. Similarly, the absorption spectra of HEWL-7-HC/ 4-Me-7-HC complexes (in a concentration ratio of 1:1) were also recorded in the same wavelength region at room temperature on the same instrument.

2.6. Circular dichroism measurements

Far UV CD spectra of 20 μM HEWL and HEWL-7-HC/4-Me-7-HC complexes (in 1:2 and 1:5 molar ratios) were recorded in the wavelength range of 190–240 nm. Secondary structural alteration of the native protein and protein in the presence of ligands were analyzed using DICHROWEB, an online server (Whitmore & Wallace, 2004).
2.7. Molecular docking studies

Molecular docking studies were executed for HEWL with 7-HC and 4-Me-7-HC ligands to determine the binding affinity of the ligands towards the protein and to visualize the interactions that occur between the residues present in the active site of HEWL and the ligands. Autodock vina and Autodock Tools-1.5.6 (ADT) (Sanner, 1999) were used to carry out docking studies. 3-D structures of 7-HC and 4-Me-7-HC were downloaded as .mol files from PubChem. The ligands were further optimized using B3LYP level and 6-311 G (d,p) (Kanaani et al., 2014) as the basis set of Gaussian 09 programme (Frisch, 2009). HEWL 3-D crystal structure (PDB ID: 6lyz) (Berman et al., 2000) was downloaded from Protein Data Bank (https://www.rcsb.org/). The protein was prepared using ADT from which water molecules were removed, polar hydrogens added, and assigned Gasteiger charges as per the Lamarckian Genetic Algorithm (LG) requirement. Keeping the protein rigid, all torsional bonds of the ligands were released and the number of rotatable bonds was defined before docking. A grid size of $40 \times 40 \times 40$ with a spacing of 1 Å and a grid box centered at x, y, and z coordinates, $-0.471, 20.566$ and 19.245, respectively was prepared around the protein. Autodock vina was then used to dock HEWL with 7-HC and 4-Me-7-HC separately. Nine independent docked poses were generated for each protein-ligand complex each with its own binding energy ($\Delta G$) and clustered on the basis of RMSD values. The docked pose with the lowest binding energy was considered and visualized using PyMOL (DeLano, 2004) and Discovery Studio Visualizer Software (Biovia, 2019). An online server was exploited to determine the change in accessible area (ASA) of HEWL upon binding with 7-HC and 4-Me-7-HC (http://clb.cf.ocha.ac.jp/bitool/ASA/).

2.8. Molecular dynamics simulation

Two different MD simulations of HEWL-7-HC and HEWL-4-Me-7-HC complexes in an aqueous medium were performed by taking the minimum energy conformers generated from Auto Dock Vina as the initial structures. Another three sets of separate MD simulations in an aqueous medium were performed with free HEWL, free 7-HC, and free 4-Me-7-HC to compare the results as obtained from their complexed states. The complex molecules and the free HEWL protein were immersed in a cubic cell of box length ~78 Å contained water molecules, whereas a cubic cell of ~30 Å box length was used to solvate the free ligands 7-HC and 4-Me-7-HC. An appropriate number of counter ions was added to neutralize the systems. CHARMM36 all-atom force field and parameters (Huang et al., 2017) were used to model protein, whereas CGenFF (Vanommeslaeghe et al., 2010) was used to model the ligand molecules. Modified TIP3P water model as consistent with the CHARMM36 force field was used in order to model water molecules (Berendsen et al., 1981). At first, the systems were minimized by using conjugate gradient energy minimization method to eliminate the initial stress. We have used GROMACS 4.6.5 code (Hess et al., 2008; Van Der Spoel et al., 2005) to perform the simulations. After minimization, all the systems were equilibrated for 2 ns under NVT ensemble condition followed by another 2 ns equilibration under NPT ensemble condition. Thereafter, a long 200 ns production trajectory under NVT condition was generated for all the systems. During simulations, the temperature of the system was controlled by using Nosé-Hoover thermostat (Hoover, 1985) with the coupling constant 1.0 ps$^{-1}$. Parrinello-Rahman barostat (Parrinello & Rahman, 1981) pressure controller with isotropic coupling constant 2 ps was used to control system’s pressure. Periodic boundary conditions (PBC) were used in each simulation in order to minimize the boundary effects. Long range electrostatic interaction was handled by using Particle mesh Ewald (PME) method (Abou-Zied & Al-Shihi, 2008). We have taken 14 Å cut off radius for the van der Waals interactions and neighbor list.

2.9. Enzymatic assay

Enzymatic activity was determined spectrophotometrically for 10 μM HEWL in the absence and presence of 7-HC and 4-Me-7-HC (1:5 and 1:10 molar concentrations) against Micrococcus lysodeikticus (M. lytus). The methodology was carried out by referring to preexisting literature (Li et al., 2010; Liang et al., 2013; Singha Roy & Ghosh, 2016).

Figure 2. (a) UV-vis absorption spectra of HEWL, 7-HC, and HEWL-7-HC complex. (b) UV-vis absorption spectra of HEWL, 4-Me-7-HC, and HEWL-4-Me-7-HC complex. 

$[\text{HEWL}] = [\text{7-HC}] = [\text{4-Me-7-HC}] = 20 \mu M$ each.
3. Results and discussion

3.1. UV-vis absorption studies

Changes in the structural conformation of HEWL in the absence and presence of coumarins can best be understood by carrying out UV-vis absorption studies due to their interaction with specific aromatic Trp and Tyr amino acid residues. Figure 2 indicates the absorption spectra of HEWL (native), 7-HC (free unbound), 4-Me-7-HC (free unbound), and their complexes. At 280 nm it could be observed that the absorption spectrum of HEWL in the presence of coumarins was greatly enhanced ([HEWL]$_{7HC}$, [HEWL]$_{4-Me-7-HC}$). This indicated an interaction was occurring between HEWL and the coumarin derivatives. However, when the free unbound coumarins were subtracted from the complex, indicated by ([HEWL]$_{7-HC}$ - 7-HC) and [HEWL + 4-Me-7-HC - 4-Me-7-HC]), the spectral lines did not overlap with the native HEWL spectrum. The observed result suggested a ground state complex formation, which ultimately could indicate a static quenching mechanism was taking place between HEWL-7-HC/4-Me-7-HC.

3.2. Fluorescence quenching studies

A deeper insight into understanding protein-ligand interaction can be obtained through steady-state fluorescence quenching studies. The binding mode, binding affinity, thermodynamic parameters, type of interactions, etc., between the protein and ligand(s), can be determined at a molecular level using fluorescence quenching data. HEWL consists of two dominant fluorophores (Trp62 and Trp108) in the active site and any structural alteration in the region of these residues due to ligand binding can have an overall effect on the fluorescence emission spectra of the protein. Thus, fluorescence studies on protein-ligand binding provide a considerable amount of knowledge about protein dynamics and structure. The fluorescence spectra of HEWL in the presence of an increasing concentration of 7-HC and 4-Me-7-HC (0–16.39 μM) are illustrated in the Figure 3 (a) and (b), respectively. Intrinsic fluorophores (Trp residues) of HEWL display fluorescence emission at 344 nm when excited at a wavelength of 295 nm. With increasing concentrations of 7-HC and 4-Me-7-HC, the intrinsic fluorescence of Trp residues of HEWL on the formation of the individual complexes (HEWL-7-HC and HEWL-4-Me-7-HC) were seen to be quenched, and a slight red shift in the emission spectrum was also observed in both the cases. The quenching of the fluorophores induced due to ligand binding and interaction is frequently associated with a decrease in quantum yield of fluorescence of the fluorophores, which could be due to a number of reasons such as energy transfer, excited-state reaction, molecular rearrangement, ground state complex formation, and dynamic quenching (Wang, Zhang, et al., 2009). In order for quenching to occur, a molecular contact between the ligand and the protein fluorophore needs to be established. This article investigates the type of quenching mechanism by determining certain variables at three different temperatures.

As 7-HC and 4-Me-7-HC are highly fluorescent molecules with a high absorbance at the excitation and emission wavelengths of HEWL, it became a necessity to consider the inner filter effect of the coumarin derivatives in order to reach a definite conclusion on the quenching mechanism. Using Eq. 1, the corrected fluorescence intensities were used to

![Figure 3. Modulation in the Trp fluorescence upon successive addition of (a) 7-HC and (b) 4-Me-7-HC. Stern-Volmer plots for (c) HEWL-7-HC complexes and (d) HEWL-4-Me-7-HC interaction at three different temperatures. (Inset: linear range of Stern-Volmer plot).](image-url)
determine the quenching data and mechanism involved during HEWL-7-HC/4-Me-7-HC complexation process.

The quenching mechanism observed in protein-ligand binding was evaluated using the Stern-Volmer equation.

\[
\frac{F_0}{F_{corr}} = 1 + K_S Q = 1 + K_S [Q]
\]

(2)

where \( F_0 \), \( F_{corr} \), \( K_S \), \( Q \), and \( t_0 \) is the intrinsic fluorescence of the protein in the absence of the quencher, the intrinsic fluorescence of the protein in the presence of the quencher, the Stern-Volmer quenching constant, the concentration of the quencher, the bimolecular quenching constant and the average lifetime of the fluorophore in the excited state (1.64 ns) (Das, Santra, et al., 2019), respectively (Lakowicz, 2006).

On plotting \( F_0/F \) versus \( [Q] \), the Stern-Volmer curve obtained would be linear if the quenching mechanism is a single mode of quenching action, i.e. static or dynamic. However, an upward curve would indicate a combined type of quenching mechanism (static and dynamic both). Figure 3 (c) and (d) depict the Stern-Volmer plots for the quenching action of 7-HC and 4-Me-7-HC on Trp residues of HEWL at 288, 298, and 308 \( K \), respectively. The data obtained from the Stern-Volmer plots for HEWL-7-HC and HEWL-4-Me-7-HC complexes is given in the Table S1. In both cases for HEWL-7-HC and HEWL-4-Me-7-HC systems, a non-linear Stern-Volmer curve with an upward slope was obtained at three temperatures which suggested both static and dynamic quenching mechanism may exist. Modified Stern-Volmer equations (Eqs. 3 and 4) were used to analyze the actual quenching mechanism observed in both the complexes.

\[
\frac{F_0}{F_{corr}} = (1 + K_D [Q])(1 + K_S [Q])
\]

(3)

\[
\frac{F_0 - F_{corr}}{F_{corr}} = \frac{1}{[Q]} = (K_S + K_D) + K_SK_D [Q]
\]

(4)

where \( K_S \) is the static quenching constant and \( K_D \) is the dynamic quenching constant. The \( K_S \) and \( K_D \) values determined from the regression plot of \( \{F_0 - F_{corr}\}/F_{corr} \) versus \( [Q] \) (Figure S1) were found to be imaginary, which signified a single form of quenching mechanism was present for both complexes. The linear range from the Stern-Volmer plot was then used to determine the \( K_{sv} \) value for HEWL-7-HC and HEWL-4-Me-7-HC complexes. The \( K_{sv} \) values for both complexes were seen to increase with the increase in temperature which indicated a dynamic quenching mechanism. However, the \( k_q (k_q = K_{sv}/t_0) \) values (Table S1) determined were seen to be greater than \( 2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \) in aqueous medium, which indicated a static mechanism of quenching occurring during both complex formations.

In order to explain these contradictory findings, Arrhenius’ theory was considered for a better understanding. According to this theory, the rate constant is dependent on temperature, and in the case of static quenching, the effect of temperature plays an important role (Hazra & Suresh Kumar, 2014; Tian et al., 2010). As earlier stated, the \( k_q \) value for dynamic quenching is limited to \( 2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \) in an aqueous medium due to the limitations encountered by the probability of collision, the number of excited molecules and diffusion. However, no such limitation is rendered on static quenching, which suggested that the rate constant of static quenching mechanism is more affected by temperature compared to dynamic quenching. Generally, in static quenching process, an increase in temperature leads to a reduction in quenching efficiency, i.e. \( k_q \) value decreases, due to instability of the ground state complex. In this case, it is assumed that since higher temperature leads to a decrease in the viscosity of the solvent, it ultimately results in an increased number of collisions between the coumarin derivatives and HEWL. Thus, the fluorescence quenching mechanism is probably dynamic quenching and the \( K_s \) decreases with the increase in temperature (Hazra & Suresh Kumar, 2014; Manna & Chakravorti, 2013; Roufegarinejad et al., 2018; Tian et al., 2012). On the other hand, according to Arrhenius’ theory, the value of \( K_{sv} \) should increase with the increase in temperature (Hazra & Suresh Kumar, 2014). If the increase in \( K_{sv} \) value resulting from the increase in temperature is more than the decrease in collisions in the unstable complex, this would result in an overall increase in \( K_{sv} \) value with the increase in temperature (Hazra & Suresh Kumar, 2014; Tong et al., 2012). Thus, the increase in \( K_{sv} \) and \( k_q \) values with increase in temperature for both complexes indicated the quenching mechanism to be static in nature, but an “unusual or untypical”, (Hazra & Suresh Kumar, 2014; Tian et al., 2012) quenching mechanism.

Using the Arrhenius equation (Eq. 5), the activation energy (\( E_a \)) of the quenching process was determined to comprehend the impact of the increase in temperature on the quenching constant (Martin, 2008).

\[
\ln \left( \frac{K_{sv}}{t_0} \right) = \ln (k_q) = - \frac{E_a}{RT} + \ln A
\]

(5)

where \( E_a \) is the activation energy of the quenching process and \( A \) is the pre exponential factor. On plotting \( \ln K_{sv} \) versus \( 1/T \) (Figure S2), the value of \( E_a \) can be determined from the slope of the plot. The \( E_a \) value determined for the quenching process of HEWL-7-HC and HEWL-4-Me-7-HC was found to be +12.940 ± 4.179 kJ mol⁻¹ and +10.953 ± 1.849 kJ mol⁻¹, respectively. These \( E_a \) values were found to be higher than \( E_a \) values reported by other research groups for interactions between other ligands and carrier proteins (Hazra & Suresh Kumar, 2014; Roufegarinejad et al., 2018; Tian et al., 2012). These findings provided information on the effect of temperature on the \( K_{sv} \) and \( k_q \) values which could be inferred as a static quenching mechanism occurring in both complexes, keeping in mind an unusual type of static quenching.

3.3. Binding parameters of HEWL-ligand complexes

The binding constant \( (K_b) \) and the number of binding sites \( (n) \) of HEWL-7-HC and HEWL-4-Me-7-HC complexes were determined separately using their corresponding fluorescence quenching data with the help of the following equation.

\[
\log \frac{F_0 - F_{corr}}{F_{corr}} = n \log K_b - n \log \left( \frac{1}{\{Q\} - \frac{1}{p_k (F_0 - F_{corr})}} \right)
\]

(6)
Figure 4. Double log binding plot of $\log \left( \frac{F_0 - F_{corr}}{F_{corr}} \right)$ vs $\log \left[ \frac{1/[Q]}{[P]/[F_0 - F_{corr}/F_0]} \right]$ for (a) HEWL-7-HC complex and (b) its corresponding van’t Hoff plot. (c) HEWL-4-Me-7-HC complex and (d) its respective van’t Hoff plot at three temperatures 288, 298, and 308 K. (e) Bar graph representation of a comparison of thermodynamic parameters of HEWL-7-HC and HEWL-4-Me-7-HC complexes.

Table 1. Binding and thermodynamic parameters of HEWL-7-HC and HEWL-4-Me-7-HC complexes at three temperatures.

| Complexes       | Temp (K) | $K_b$ $\times 10^4$ M$^{-1}$ | $n$  | $\Delta H$ (kJ mol$^{-1}$)   | $\Delta S$ (J mol$^{-1}$ K$^{-1}$) | $\Delta G$ (kJ mol$^{-1}$) |
|-----------------|----------|-------------------------------|------|-------------------------------|----------------------------------|--------------------------|
| HEWL-7-HC       | 288      | 3.832 ± 0.330                 | 1.263| $+(13.981 ± 0.873)$           | $+(136.170 ± 2.632)$             | $-25.234 ± 0.199$         |
|                 | 298      | 4.547 ± 0.462                 | 1.358|                               |                                   | $-26.596 ± 0.188$         |
|                 | 308      | 5.586 ± 0.370                 | 1.360|                               |                                   | $-27.958 ± 0.180$         |
| HEWL-4-Me-7-HC  | 288      | 3.375 ± 0.059                 | 1.254| $+(14.301 ± 1.371)$           | $+(136.040 ± 4.652)$             | $-24.877 ± 0.064$         |
|                 | 298      | 3.669 ± 0.148                 | 1.272|                               |                                   | $-26.238 ± 0.059$         |
|                 | 308      | 4.992 ± 0.165                 | 1.401|                               |                                   | $-27.599 ± 0.084$         |

Figure 5. Spectral overlap of HEWL emission spectra with the absorption profiles of (a) 7-HC. (b) 4-Me-7-HC.
where \([P_i]\) is the total protein concentration. The linear plots for \(\log (F_0 - F_{corr})/F_{corr}\) against \(\log[1/\langle Q \rangle] - [P_i](F_0 - F_{corr})\) for HEWL-7-HC and HEWL-4-Me-7-HC complexes are depicted in Figure 4 (a) and (c), respectively and their respective \(K_b\) and \(n\) have been summarized in Table 1. It could be seen for both complexes that the \(K_b\) value was in the order of \(10^4\, \text{M}^{-1}\) which is considered a moderate form of protein-ligand interaction when compared to other protein-ligand bindings which have \(K_b\) values in the order of \(10^6 - 10^8\, \text{M}^{-1}\) (Kragh-Hansen, 1990). This moderate form of interaction existing between HEWL and the coumarin derivatives is indicative of a reversible form of binding which can facilitate their transport and release in the target tissue. The data also revealed that the \(K_b\) value for HEWL-7-HC and HEWL-4-Me-7-HC complexes increased with an increase in temperature. This suggested the HEWL and coumarin derivatives formed stable complexes at higher temperatures. The number of binding sites \(n\) for both complexes was seen to be slightly greater than 1 and increased with an increase in temperature. This indicated more than one binding site was present within HEWL or because of higher concentration of coumarin derivatives present around the Trp fluorophores resulting in the upward curvature of the Stern-Volmer plot as witnessed in Figure 3 (c) and (d).

### 3.4. Thermodynamic parameters of HEWL-7-HC and HEWL-4-Me-7-HC complexes

From the fluorescence quenching studies discussed above, it became evident that an interaction between HEWL and the coumarin derivatives was occurring, resulting in a complex formation. Gaining insight on this interaction governing the complexation process becomes of utmost importance, which can be best understood by correctly analyzing thermodynamic parameters. The signs and magnitudes of thermodynamic parameters such as \(\Delta H\) (enthalpy change) and \(\Delta S\) (entropy change) provide information on the kind of interactions (hydrogen bond, hydrophobic and electrostatic interactions, van der Waals forces) existing between the protein and ligand (Ross & Subramanian, 1981). The van’t Hoff equation (Eq. 7) was used to calculate \(\Delta H\) and \(\Delta S\) of HEWL-7-HC and HEWL-4-Me-7-HC and the corresponding \(\Delta G\) (free energy change) was determined from Eq. 8

\[
\log K_b = \frac{-\Delta H}{2.303RT} + \frac{\Delta S}{2.303R} \quad (7)
\]

\[
\Delta G = \Delta H - T\Delta S \quad (8)
\]

Figure 4 (b) and (d) depict the van’t Hoff plot (\(\log K_b\) versus \(1/T\)) for HEWL-7-HC and HEWL-4-Me-7-HC complexes, and their respective thermodynamic parameters are tabulated in the Table 1. The data showed that the \(\Delta S\) for both complexes was positive (\(\Delta S > 0\)), suggesting that the complexation process was entropically favorable. The \(\Delta H\) value was also positive (\(\Delta H > 0\)) for both complexes, indicating the reaction was endothermic in nature, thereby adversely affecting the complexation process’s spontaneity. The results obtained for \(\Delta S\) and \(\Delta H\) indicated the predominance of hydrophobic interaction between HEWL and the coumarin derivatives. \(\Delta G\) value calculated using Eq. 8 was found to be negative (\(\Delta G < 0\)), indicating the interaction between HEWL and coumarin derivatives was spontaneous, the process being entropically driven. The energy level for HEWL-7-HC/4-Me-7-HC would go up due to the positive values of \(\Delta H\), making the complexes unstable. However, since the value of \(\Delta S\) for both complexes was found to be highly positive, the contribution from \(\Delta S\) supersedes (Figure 4 (e)) the endothermic value of \(\Delta H\), ultimately leading to the spontaneity of the process and stabilization of the complexes.

### 3.5. Energy transfer studies

Förster’s resonance energy transfer (FRET) theory explains the efficient energy transfer from a donor to an acceptor molecule. In order for this to occur, specific criteria must be fulfilled. Firstly, the absorption spectra of the acceptor molecule must overlap with the emission spectra of the donor molecule; secondly, the donor and acceptor transition dipole orientations must be almost parallel, and finally, the binding distance \(r\) between the donor and the acceptor molecules must be in between 2 nm and 7 nm (Margineanu et al., 2016; Thiel, 2001). HEWL consists of dominant fluorophores, Trp62 and Trp108, which have earlier been established as essential residues which play a role in interaction with ligands. An efficient energy transfer was witnessed from the donor (HEWL) to the acceptor (coumarin derivatives) due to a significant spectral overlap between the emission spectrum of HEWL and the absorption spectrum of the coumarin derivatives, which are depicted in Figure 5. The binding distance \(r\) between HEWL-7-HC and HEWL-4-Me-7-HC was determined from Förster’s theory using the following equations (Förster, 1948; Lakowicz, 2006).

\[
E = 1 - \frac{F_{corr}}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (9)
\]

\[
R_0 = 9.78 \times 10^3 \left| \kappa^2 N^{-4} Q_0 J(\lambda) \right|^{1/6} \quad (10)
\]

\[
J(\lambda) = \int \frac{F_0(\lambda)\kappa(\lambda)\lambda^4 d\lambda}{F_0(\lambda)d\lambda} \quad (11)
\]

where \(E\) is the efficiency of energy transfer between donor and acceptor molecule, \(R_0\) is the critical distance between donor and acceptor molecule when energy is 50% transferred, \(N\) is the refractive index of the medium, \(Q_0\) is the fluorescence quantum yield of the donor, \(\kappa^2\) is the orientation factor of the donor and acceptor dipoles, \(J(\lambda)\) is the spectral overlap, \(F_0(\lambda)\) is the normalized fluorescence intensity of the fluorophore donor in the region of \(\lambda\) and \(\Delta \lambda\), \(\kappa(\lambda)\) is the molar absorptivity coefficient of the acceptor at the same wavelength.

Using already established values of \(\kappa^2 = 2/3, N = 1.336, Q_0 = 0.118\) for interaction between HEWL and small ligands, the above parameters were determined. Table S2 represents the values obtained for \(E, R_0, J(\lambda)\), and \(r\). The binding distances \(r\) for HEWL-7-HC and HEWL-4-Me-7-HC systems were found to be 4.52 nm and 4.21 nm, respectively, which were less than 7 nm. Since the \(r\) values were found to be within the 0.5 \(R_0 < r < 2R_0\) range, this indicated non-radiative efficient
energy transfer was occurring between HEWL and the coumarin derivatives and this data also indicated the presence of static quenching mechanism (Lakowicz, 2006).

3.6. Synchronous fluorescence studies (SFS)

Interaction of a ligand in the catalytic site of a protein molecule can lead to several microenvironmental changes around the vicinity of the fluorophores of the protein. These changes can be detected by carrying out synchronous fluorescence studies as introduced by Lloyd (1971). Information related to alteration in the microenvironment around Tyr and Trp residues can be determined by keeping the wavelength interval (Δλ = λ_em – λ_ex) fixed at Δλ = 15 nm and Δλ = 60 nm, respectively (Panja & Halder, 2016). Figure S3 depict the synchronous fluorescence spectra of HEWL with increasing concentration of 7-HC/4-Me-7-HC with fixed

![Figure 6](image_url)

Figure 6. 3-D fluorescence spectral profile showing the contour plots (on top) of (a) HEWL and its 1:4 complexes with (c) 7-HC and (e) 4-Me-7-HC ligands.

![Figure 7](image_url)

Figure 7. CD spectral profile of HEWL and its complexes with (a) 7-HC and (b) 4-Me-7-HC.

Table 2. Percentage change of secondary structures of HEWL in absence and presence of 7-HC and 4-Me-7-HC.

| Complex             | % α-helix | % β sheets | % Turns | % Unordered |
|---------------------|-----------|------------|---------|-------------|
| HEWL                | 35.50 ± 0.15 | 14.63 ± 0.12 | 22.66 ± 0.29 | 27.10 ± 0.20 |
| HEWL-7-HC (1:2)     | 33.66 ± 0.37 | 14.80 ± 0.25 | 22.73 ± 0.18 | 27.90 ± 0.11 |
| HEWL-7-HC (1:5)     | 31.63 ± 0.65 | 16.03 ± 0.17 | 23.63 ± 0.17 | 28.90 ± 0.15 |
| HEWL-4-Me-7-HC (1:2)| 34.00 ± 0.35 | 15.10 ± 0.15 | 22.93 ± 0.13 | 27.63 ± 0.08 |
| HEWL-4-Me-7-HC (1:5)| 30.60 ± 1.10 | 17.40 ± 0.79 | 23.96 ± 0.23 | 29.23 ± 0.16 |
$\Delta \lambda = 15 \text{ nm (for Tyr)}$ and $\Delta \lambda = 60 \text{ nm (for Trp)}$. Results obtained revealed a reduction in fluorescence intensity of HEWL in the presence of an increasing concentration of 7-HC and 4-Me-7-HC, which was inferred as an interaction taking place between HEWL and the coumarins. However, no significant Stoke’s shift in the fluorescence spectra at both wavelength intervals, $\Delta \lambda = 15 \text{ nm}$ and $\Delta \lambda = 60 \text{ nm}$, was observed for both complexes (Table S3).

### 3.7. 3-Dimensional fluorescence studies

3-dimensional (3-D) fluorescence is another tool used to investigate structural changes in the protein upon ligand binding and to understand any alterations, if any, in the microenvironment of the protein in the presence of coumarin derivatives. Individual spectra for HEWL in the absence and presence of 7-HC and 4-Me-7-HC have been depicted in Figure 6.

Peak $a \left( \lambda_{\text{ex}} = \lambda_{\text{em}} \right)$ and peak $b \left( 2 \lambda_{\text{ex}} = \lambda_{\text{em}} \right)$ corresponds to the first and second order Rayleigh scattering peaks, respectively (Bortolotti et al., 2016). Two other distinct peaks (peak 1 and peak 2) for the 3-D fluorescence spectrum of HEWL were also clearly visible. Peak 1 ($\lambda_{\text{ex}}/\lambda_{\text{em}}: \sim 275/342 \text{ nm}$) signifies the spectral behavior of the intrinsic aromatic amino acid fluorophores, Trp and Tyr, found in HEWL. Peak 2 ($\lambda_{\text{ex}}/\lambda_{\text{em}}: \sim 230/342$) has been credited to the excitation of a higher excited state of Trp and Tyr residues of HEWL (Bortolotti et al., 2016). As can be seen in the figure, the intensity of peak 1 and peak 2 decreased upon the binding of HEWL to both 7-HC and 4-Me-7-HC. A significant increase in Stoke’s shift was seen in peak 1 on the binding of 7-HC to HEWL ($\sim 9 \text{ nm}$) and 4-Me-7-HC to HEWL ($\sim 3 \text{ nm}$), which indicated a structural alteration was occurring in HEWL with the aromatic amino acid residues (Trp and Tyr) being exposed to a more polar environment (Table S4). This shift in the emission spectrum of both complexes towards longer wavelength was also observed on carrying out fluorescence quenching studies. Peak 2 witnessed an increased Stoke’s shift in the case of HEWL-7-HC complexation ($\sim 7 \text{ nm}$) but no significant shift was observed in the case of HEWL-4-Me-7-HC binding (Table S4).

### 3.8. Circular dichroism (CD) studies

Circular dichroism (CD) is a method utilized in order to study structural changes in the secondary and tertiary structure of a protein in the presence of a ligand. The far UV region was considered in order to see the conformational alterations in the secondary structure of HEWL in the absence and presence of 7-HC and 4-Me-7-HC. Two bands of negative ellipticity at 208 and 222 nm of native HEWL (Ikeda et al., 1972) were visualized in the CD spectrum. These bands are characteristic of the $\alpha$-helices of HEWL. Figure 7 represents the CD spectra of HEWL in the absence and presence of 7-HC and 4-Me-7-HC. As can be seen in the figure, the intensity of both bands decreased upon the binding of HEWL to both 7-HC and 4-Me-7-HC. The percentage change of secondary structures of HEWL in the presence of 7-HC and 4-Me-7-HC is illustrated in Table 2.
3.9. Molecular docking results

Molecular docking is a valuable tool used to visualize protein-ligand interactions and analyze the type of interactions involved to stabilize the complex. In this study, molecular docking was carried out between HEWL/7-HC and HEWL/4-Me-7-HC to corroborate further and understand the type of binding and interactions observed in the previous abovementioned studies. Figure 1 (b) and (d) represent the optimized structures of 7-HC and 4-Me-7-HC ligands, respectively. Nine docked conformations results were obtained from docking studies for both HEWL-7-HC and HEWL-4-Me-7-HC complexes, and their characteristic parameters have been summarized in Table S5. The docked poses with the least binding energy (Figure 8 (a) and (c)) for both complexes were considered for further analysis. In the case of HEWL-7-HC/HEWL-4-Me-7-HC docking data, docking experiments showed the binding energy of the best-docked conformers were close to the $\Delta G$ values determined from fluorescence quenching studies (Table 1). Figure 8 (b) and (d) represents the surrounding amino acid residues (Table S5) near the binding site of 7-HC and 4-Me-7-HC, namely, Ala107, Leu56, Trp108, Gln57, Asp52, Asn46, Ile58, Asn59, Trp62, Trp63, and Ile98 along with the surface hydrophobicity of the binding site. Table 3 depicts the binding distances of the amino acid residues from the coumarin derivatives along with the change in accessible surface area. Also, the presence of the ligands near the Trp fluorophores could have resulted in their quenching, as proven by fluorescence studies.

In order to determine the kind of interactions existing between the amino acid residues and the coumarin derivatives, discovery studio visualizer was used. On comparing the 2-D plot of the docked pose (Figure 9 (a) and (b)) and the hydrophobic surface near the 7-HC/4-Me7-HC binding site (Figure 8 (b) and (d)), it was evident that a major portion of these coumarins occupied a slight hydrophobic environment and underwent hydrophobic interactions with Ala107 ($\pi$-alkyl) and Ile98 ($\pi$-alkyl) (in the case of 7-HC) and Ala107 ($\pi$-alkyl), Trp63 ($\pi$-alkyl), and Ile98 ($\pi$-alkyl) (in the case of 4-Me-7-HC). The coumarin derivatives also did lie in the solvent-exposed sides (indicated in blue), to a certain extent, enabling the formation of hydrogen bonds. Trp108, and Asn59 residues formed hydrogen bonds with 7-HC and 4-Me-7-HC, thereby stabilizing the complex. Other forces of interaction were also seen to be involved during the complexation process. Trp63, Leu56, Gln57, Asp52, and Asn46 of HEWL formed van der Waals type of interaction with 7-HC. Leu56, Gln57, Asp52, and Asn46 of HEWL displayed van der Waals interaction with 4-Me-7-HC. Therefore, it could be inferred that the occurrence of more than one type of interaction in the case of HEWL-7-HC and HEWL-4-Me-7-HC should not be neglected.

In the presence of a ligand, the protein undergoes a significant structural conformation where the packing of the amino acid residues inside the protein alters. Determining the changes in accessible surface area ($\Delta A$) of the protein provides valuable information with regards to the goodness of the packing of amino acids of the protein on interaction with a ligand (Das et al., 2018). The amino acids that undergo $\sim$10 Å$^2$ change in accessible area from ligand-free to ligand-bound state are considered residues that actively participate during the protein-ligand interaction process (Das, Santra, et al., 2019). The following relation can be used to determine the change in accessible area.

$$\Delta A = A_{\text{free}} - A_{\text{complex}}$$  \hspace{1cm} (12)

where $A_{\text{free}}$ and $A_{\text{complex}}$ represent the native protein

![Figure 9. 2-D interaction plots of (a) HEWL-7-HC complex and (b) HEWL-4-Me-7-HC complex.](image)
and protein-bound ligand complex, respectively. The value of ASA for the free HEWL was determined to be 6613.12 Å², whereas the ASA of HEWL bound 7-HC and HEWL bound 4-Me-7-HC was determined to be 6467.26 Å² and 6452.04 Å², respectively. This reduction in total ASA value in both complexes indicated the presence of specific interactions occurring on the binding of coumarin derivatives to HEWL. The values for change in ASA for the amino acids of HEWL on interaction with 7-HC and 4-Me-7-HC is depicted in Table 3, where it was observed that most of the residues lost ~10 Å² ASA on forming a complex with the coumarins. This change in ASA due to the formation of interactions ultimately leading to a change in the microenvironment of the protein has also been discussed in the experimental findings.

3.10. Molecular simulation analysis

Figure 10 and Figure 11 showed the simulated snapshots of the HEWL-7-HC and HEWL-4-Me-7-HC complexes, respectively, at a regular interval of 100 ns along with the initial starting structures. Further, the superimposed configurations of the complexes are displayed to visualize the conformational flexibilities of the complexes.

Both the figures demonstrate that the ligand molecule occupies an almost similar position of the protein’s cleft region as compared to the respective initial starting configuration. To quantify the apparent flexibility of the protein and ligand relative to their initial starting conformation and to quantify the influence of complexation on the flexibilities of these molecules, we have computed Root Mean Square Deviation (RMSD) between the initial and simulated structure of HEWL, 7-HC, and 4-Me-7-HC, separately from the trajectories generated for the free and complexed forms of the molecules. In the left panel of Figure 12, the time evolution of RMSDs for the 7-HC and HEWL in their complexed forms are shown, whereas that for 4-Me-7-HC and HEWL are shown in the right panel of the Figure 12. The RMSDs of the respective molecules as obtained from the separate simulations performed with the free forms of the molecules are added in the figures for comparison. Apparent flexibility of both the ligand molecules and the protein at their free or complexed forms is evident from the figures. However, while comparing the flexibility of the molecules in the complexed form with that to the respective free form, in general, the reduction in flexibility at the complexed state was observed. This was particularly true for the protein molecule. Our calculation showed that the average RMSD (<RMSD>) of free 7-HC, ~0.3 Å does not deviate much while forming HEWL-7-HC complex; <RMSD> ~ 0.32 Å. However, as seen from the figure, the conformational flexibility decreases for the free HEWL; <RMSD> ~ 1.9 Å as a result of the formation of HEWL-7-HC complex, <RMSD> ~ 1.1 Å by 1.7 times. On the other hand, the conformational flexibility of free 4-Me-7-HC (<RMSD> ~ 0.24 Å) remains similar to that when it formed a complex with HEWL (<RMSD> ~ 0.30 Å). HEWL, while bound to 4-Me-7-HC, was observed to have <RMSD> ~ 1.3 Å, which is ~1.5 times smaller than the <RMSD> of free HEWL.

To recognize the role of the residues of HEWL that are present in the cleft region of the protein (identified by the molecular docking experiment) in the binding process, we
have quantified the residue-wise <RMSD> values as well as Root-mean-square-fluctuation (RMSF) values. The calculations were performed using the equilibrated trajectories of the free protein and its complexed forms. Table 4 displayed the results. Mostly, reduction in flexibility/fluctuation of the protein’s cleft region residues when bound with the ligand is evident from the table. This is especially true when HEWL binds with 7-HC, as compared to 4-Me-7-HC. The <RMSD> indicates that major depletion in flexibility occurred for Trp108 and Asp52 when the protein formed complex, specifically with 7-HC. The contribution of Trp108 was further observable from the reduction of the respective RMSF value. Identification of Trp108 as an important residue to facilitate the binding is in accordance to the experimental observation.

To obtain the relative binding affinities of HEWL towards 7-HC and 4-Me-7-HC, we have quantified the binding free energy of the HEWL with 7-HC and 4-Me-7-HC from the simulated trajectories by using Molecular Mechanics-Poisson Boltzmann-surface area (MMPBSA) method (Homeyer & Gohlke, 2012). g_mmpbsa module of GROMACS (Kumari et al., 2014) was used for such calculation. According to this method, the binding free energy ($\Delta G_{bind}$) can be computed by the equation, $\Delta G_{bind} = G_{complex} - (G_{protein} - G_{ligand})$. Where $G_{protein}$, $G_{ligand}$, $G_{complex}$ represents the free energies of the free protein (HEWL), free ligand (7-HC/4-Me-7-HC), and the protein-ligand (HEWL-7-HC/HEWL-4-Me-7-HC) complex, respectively. The free energy ($G$) of each molecule (protein/ligand/complex) was obtained by $G_{molecule} = <E_{MM}> - TS + <G_{solv}>$, where $<E_{MM}>$ is the average molecular mechanics energy in vacuum, TS is the entropic contribution, and $<G_{solv}>$ is the free energy of solvation. The entropic contribution was omitted in this study. The solvation free energy was the summation of polar and non-polar contribution of the solvation free energy. The computed $\Delta G_{bind}$ as obtained using this methodology for HEWL-7-HC and HEWL-4-Me-7-HC was found to be $-26.68$ kJ mol$^{-1}$ and $-30.85$ kJ mol$^{-1}$, respectively. This suggests that both the complex formation process is favorable. This is in well agreement with the molecular docking and experimental evidence as discussed above.

### Table 4. Average RMSD and RMSF values of the residues present at the cleft region of the HEWL in its free and in two different complexed forms as obtained from the equilibrated simulated trajectories.

| Residues | Free HEWL <RMSD> [Å] | HEWL-7-HC <RMSD> [Å] | HEWL-4-Me-7-HC <RMSD> [Å] | Free HEWL RMSF [Å] | HEWL-7-HC RMSF [Å] | HEWL-4-Me-7-HC RMSF [Å] |
|----------|------------------------|------------------------|---------------------------|------------------|-------------------|---------------------|
| Asn46    | 0.112                  | 0.113                  | 0.115                     | 0.648            | 0.624             | 0.680               |
| Asp52    | 1.040                  | 0.459                  | 0.471                     | 0.399            | 0.410             | 0.435               |
| Leu56    | 0.133                  | 0.146                  | 0.132                     | 0.558            | 0.573             | 0.587               |
| Gln57    | 0.186                  | 0.198                  | 0.191                     | 0.148            | 0.171             | 0.147               |
| Ile58    | 0.444                  | 0.379                  | 0.353                     | 0.275            | 0.240             | 0.222               |
| Asn59    | 0.263                  | 0.194                  | 0.206                     | 0.141            | 0.130             | 0.133               |
| Trp63    | 0.246                  | 0.235                  | 0.346                     | 0.153            | 0.155             | 0.176               |
| Ile98    | 0.226                  | 0.217                  | 0.281                     | 0.127            | 0.118             | 0.148               |
| Ala107   | 0.122                  | 0.114                  | 0.119                     | 0.116            | 0.103             | 0.090               |
| Trp108   | 0.504                  | 0.017                  | 0.089                     | 0.426            | 0.372             | 0.249               |

3.11. Enzymatic activity of HEWL in the presence of 7-HC and 4-Me-7-HC

Enzymatic activity is greatly influenced by the surrounding environment, such as temperature, pH, and the presence of small molecules that act as enzyme modulators. Enzyme modulators have the ability to activate or inhibit the activity...
of a particular enzyme. 7-HC and 4-Me-7-HC were determined for their role as enzyme modulators on the enzyme activity of HEWL against M. lytus. The following equation was used to determine the enzyme activity of HEWL in the absence and presence of 7-HC and 4-Me-7-HC.

\[
\frac{\Delta E_{450}}{0.001 \times E_w} = U/mg
\]

where \(\Delta E_{450}\) is the absorbance change per unit at 450 nm and \(E_w\) is the mass of HEWL in the reaction mixture, U is one unit of enzyme activity which is defined as the reduction in absorbance of a species at 450 nm by 0.001 (Chakraborti et al., 2010; Das, Pahari, et al., 2019).

Figure 13 illustrates the enzyme activity of HEWL in the presence of 7-HC and 4-Me-7-HC. The finding revealed that the enzyme activity of HEWL in the presence of 7-HC and 4-Me-7-HC increased. This could suggest that the coumarins derivatives acted as an inducer for HEWL by altering the structural conformation and stability of the protein. From previous experiments carried out, such as molecular docking, 3-D fluorescence, and CD studies, it was evident that both 7-HC and 4-Me-7-HC could bind to the active site of HEWL, resulting in a structural alteration affecting the overall stability of the protein. The decreased RMSD values obtained through MD studies indicated an alteration in the stability of HEWL due to 7-HC/4-Me-7-HC binding, which was assumed to also occur during activity studies. A similar kind of result was observed on a study carried out between HEWL and 5-fluorouracil, as reported by Millan et al (2018).

4. Conclusion

The interaction between a model carrier protein, HEWL, and two pharmacologically active coumarin derivatives, 7-HC and 4-Me-7-HC, were investigated using multispectroscopic and computational methods, and the results have been reported herewith. On conducting UV-visible spectroscopy, an interaction between HEWL and 7-HC/4-Me-7-HC was observed, and, in addition, a ground state complexation was occurring, indicating a static quenching mechanism. Information perceived from steady-state fluorescence saw a reduction in the intrinsic fluorescence of the Trp residues suggesting the binding of 7-HC/4Me-7-HC near the vicinity of these residues. On determining the quenching mechanism the Trp residues were subjected to due to ligand binding, an “unusual static quenching” mechanism was observed as indicated by the increase in the quenching rate constant with the rise in temperature. The binding constant, \(K_b\), for both complexes, was found to be in the order of \(10^3\) M\(^{-1}\) which revealed a moderate form of affinity of the ligands for HEWL. This could suggest a reversible form of protein-ligand binding facilitating their transport and release at the target site. The \(K_b\) value was also found to be higher for HEWL-7-HC than for HEWL-4Me-7-HC when compared at three different temperatures, 288, 298, and 308 K. On determining the signs and magnitudes of the thermodynamic parameters, \(\Delta H\) and \(\Delta S\), hydrophobic interactions were the dominant forces existing between HEWL-7-HC and HEWL-4-Me-7-HC such that the complexation process were entropically driven. From FRET studies, the probability of non-radiative energy transfer was assumed to occur from the donor (HEWL) to the acceptors (7-HC/4-Me-7-HC) due to obtaining a binding distance of less than 7 nm for both complexes. Synchronous fluorescence revealed a decrease in the fluorescence intensity of the Trp residues due to ligand binding, but no significant Stoke’s shift was observed. However, when 3-D fluorescence was carried out, a Stoke’s shift towards a longer wavelength was observed for HEWL-7-HC and HEWL-4-Me-7-HC, indicating the Trp residues was occupying a more polar environment due to their interaction with the coumarin derivatives. CD studies monitoring structural alterations in the secondary structure of HEWL due to its binding with 7-HC and 4-Me-7-HC saw a reduction in the percentage of \(\alpha\)-helical content of HEWL. To further verify the binding of the coumarin derivatives to the active site of HEWL, molecular docking studies conducted proved so. 7-HC and 4-Me-7-HC could interact with important catalytic residues of HEWL such as Ala107, Leu56, Trp108, Gln57, Asp52, Asn59, Ile58, Asn59, Trp62, Trp63, and Ile98. The role of hydrophobic forces during the complex formation seen through docking studies further cemented what was revealed through experimental findings. In addition, hydrogen bonds, van der Waal’s forces, and other forces were also seen to be involved, thereby eliminating the existence of a single non-covalent force model. Through MD studies, the protein complexed with 7-HC/4-Me-7-HC was found to be more stable than the ligand-free protein. Also, the dominant intrinsic fluorophore, Trp108, was identified to be an important residue to facilitate the binding process. The \(\Delta G\) values computed through MD studies for both complexes were

![Figure 13](image-url)
also comparable to those obtained through molecular docking and experimental findings, indicating the complexation processes for HEWL-7-HC and HEWL-4-Me-7-HC occurred spontaneously. The change in structural conformation and effect on the stability of HEWL due to binding with 7-HC and 4-Me-7-HC was also suggested to have an impact on the enzyme activity of HEWL. The above findings provide a gainful insight into the biochemical association of the HEWL with two therapeutically important coumarin derivatives. The in vitro work carried out for the above study establishes a foundation for the buildup of other important in vivo experiments that could be conducted to verify further and validate the pharmaceutical efficacy of 7-HC and 4-Me-7-HC.

Acknowledgements

ASR is thankful to Science and Engineering Research Board (SERB) (Gov’t of India, File No. CRG/2019/000852) for funding. The authors are grateful to the Department of Bioscience and Bioengineering, IIT Guwahati for the computational facility created under the grant no. SB/FT/CS-065/2012 and EMR/2017/001325. RG thanks NIT Rourkela for providing a scholarship.

Disclosure statement

No potential conflict of interest was reported by the authors.

Authors contribution

Sona Lyndem: Conceptualization, Investigation, Writing-Original draft
Rabibul Gazi: Software, Formal analysis, Writing-Original draft.
Vinay Kumar Belwal: Investigation
Madhurima Jana: Supervision, Software, Validation, Writing-Review & Editing, Funding acquisition.
Atanu Singha Roy: Supervision, Project administration, Validation, Writing - Review & Editing, Funding acquisition.

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