Spectroscopic and Molecular Docking Investigation on the Noncovalent Interaction of Lysozyme with Saffron Constituent “Safranal”

Mohd. Sajid Ali* and Hamad A. Al-Lohedan

ABSTRACT: Owing to the various beneficial properties of the popular spice saffron, the interaction of safranal, a secondary metabolite of the former, with hen egg white lysozyme was investigated. The formation of a complex was evidenced by UV–visible spectroscopy. Fluorescence quenching experiments were also performed to understand the binding mechanism and to evaluate the forces involved in binding. The strong absorption of safranal in the range of excitation and emission wavelengths of lysozyme fluorescence required the correction of the inner filter effect for fluorescence spectra to obtain the apparent extent of binding. There was a considerable difference between the observed spectra and corrected spectra, and a similar observation was found in the case of synchronous fluorescence spectra. From the analysis of quenching data, it was found that the mechanism involved in quenching was static with 1:1 binding between them. The interaction was found to be driven, mainly, by hydrophobic forces and hydrogen bonding. Safranal had negligible impact on the secondary structure of lysozyme. The interaction was also studied by molecular docking, and the results were in good agreement with the results obtained experimentally. The binding site of safranal was in the big hydrophobic cavity of lysozyme. The amino acids involved in the interaction were Asp52, Ile58, Gln57, Asn59, Trp62, Trp63, Trp108, Ile98, Asp101, and Ala107.

1. INTRODUCTION

Molecular interactions of small molecules, such as drugs, natural products, and complex molecules, with large biomolecules, such as proteins and nucleic acids, in solutions play an important role in understanding the mechanism of binding and the forces involved in it. Although the interactions that take place inside the biological system are more complex, one can get an idea of the specific interaction taking place between a ligand and a biomolecule.2,3 These in vitro molecular interactions are also helpful in drug designing and discovery.

Lysozyme is a pervasive globular protein, which possesses a number of applications and enzymatic activities. The existence of lysozyme is found in several secretions, which include tears, mucus, saliva, and human milk. Lysozyme also exists in egg white protein. The principal function of lysozyme is its antibacterial activity in which it hydrolyzes and breaks the peptidoglycan cell membrane of Gram-positive bacteria. Apart from the antibacterial properties, it is known to have various other beneficial characteristics, such as anticancer, antiseptic, antiviral, and anti-inflammatory properties.4 Lysozyme can also be used as a food preservative agent to protect food from microbes.5–6 Lysozyme binds, reversibly, a number of endogenous and exogenous7–9 ligands and is known to be applicable in drug delivery systems, where it binds with drugs and is used as a carrier.10,11 This striking property of lysozyme is an imperative feature for understanding the mechanism of transportation and metabolism of small molecules and drugs.12,13

Studies of interactions of various natural products, such as juglone,14 cinnamic acid,15 naringin palmitate,16 chelerythrine,17 ginsenosides,18 6-hydroxyflavone,19 curcumin,20 and sanguinarine,21 with lysozyme have been found in the literature. Li et al. have investigated the interaction of lysozyme with various dietary anti-oxidants and concluded that all of the studied compounds interacted very strongly with lysozyme, which may increase their half-life in vivo and lead to unwanted side effects.22 Hence, it is very important to know the extent of binding of exogenous ligands with lysozyme.

Recently, we have studied the interaction and antimyloidogenic properties of safranal (C10H14O; 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) with human and bovine serum albumins and found that the former inhibited the temperature-induced amyloid formation of both albumins.23–25 Safranal is a major saffron constituent covering 70% of the entire volatile substances and is also responsible for the

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It is known to have several medicinal properties, such as anti-oxidant, anti-diabetic, and anticancer activities. It has also been found to show inhibitory effects against the fibrillation of lysozyme. However, the mechanism of binding of safranal and its effect on the conformation of lysozyme is unknown. Saffron is a popular spice, which is used in many food preparations. It also has cultural values at several places and is used as a traditional household medicine to cure a number of diseases. If consumed, it is very likely that its secondary metabolites would interact with the important carrier proteins. Therefore, we have planned this study to investigate the interaction of an important saffron constituent, i.e., safranal, with hen egg white lysozyme (which has around 60% sequence homology with its human counterpart) using experimental and molecular docking simulation methods.

2. RESULTS AND DISCUSSION

2.1. Spectroscopic Evaluation of the Lysozyme–Saffanal Interaction. We have used UV–visible, intrinsic, extrinsic, and synchronous fluorescence, Rayleigh light scattering (RLS), and circular dichroism (CD) spectroscopies to estimate the binding mechanism of lysozyme and safranal, which are given in the following subsections.

2.1.1. UV–Visible Absorption Study of the Lysozyme–Saffanal Interaction. Safranal has strong absorption in the range of 200–400 nm with two distinct peaks at around 205 and 320 nm, which are due to the π-π* and n-π* transitions, respectively (Figure 1A). In the case of lysozyme, the strong absorption around 210 nm is ascribed to the protein backbone and refers to the secondary structure of the protein. Three amino acids (tryptophan, tyrosine, and phenylalanine) present in the globular structure of lysozyme also show an absorption band at 278 nm. The change in the UV–visible spectrum of a protein upon the addition of a ligand can give a preliminary idea about the complex formation between them along with the conformational alteration of the former. The difference UV–visible spectra of lysozyme in the presence of various amounts of safranal are given in Figure 1B. Addition of safranal to lysozyme slightly decreased the absorbance but did not cause any significant change in the location of the peak belonging to the protein backbone; hence, it can be deduced that safranal does not affect the secondary structure of lysozyme. There is a small hypochromic shift in the absorption peak at 278 nm, probably due to the π-π stacking interaction between the aromatic ring of safranal and the phenyl rings of amino acid residues.

2.1.2. Intrinsic Fluorescence Measurements. Fluorescence spectroscopy was employed to evaluate the fluorescence quenching profiles of lysozyme and safranal binding. The intrinsic fluorescence property of lysozyme is due to the presence of tryptophan and tyrosine residues among which tryptophan is the major contributor to the fluorescence emission. Although phenylalanine is also a fluorescent amino acid, its quantum yield is negligible as compared to those of tryptophan and tyrosine. When the protein solution is excited at 280 nm both tryptophan and tyrosine fluoresce, whereas at an excitation wavelength of 295 nm, the fluorescence intensity is solely due to the tryptophan residue. The observed fluorescence spectra of lysozyme in the presence of various amounts of safranal at 25 °C and at λex of 280 and 295 nm are given in Figure 2A and 2B, respectively. The fluorescence emission spectrum of pure lysozyme exhibits the maximum emission at around 340 nm at both excitation wavelengths. It can be seen from the figure that sequential addition of safranal results in robust fluorescence quenching of lysozyme at both wavelengths. However, this is a case of an inner filter effect because safranal has a significant absorption from 200 to 400 nm, which covers both excitation wavelengths along with the most significant emission spectral range of lysozyme (290–400 nm). The fluorescence quenching spectra are, thus, corrected for the inner filter effect using eq S1.

The corrected fluorescence spectra of lysozyme in the absence and presence of various amounts of safranal at 25 °C are given in Figure 3A for λex = 280 nm and in Figure 3B for λex = 295 nm. The corrected fluorescence spectra of safranal at both excitation wavelengths are also shown in the corresponding figures, which show that safranal has negligible quantum yield under the experimental conditions as compared to lysozyme, although, as described above, due to the large absorption of light by the former in this range, its contribution to the inner filter effect cannot be ruled out. Relative fluorescence intensities (RFI) of lysozyme at excitation wavelengths of 280 and 295 nm in the presence of several concentrations of safranal at 25 °C are plotted in Figure 4A,B for observed and corrected data, respectively. A comparison of Figures 2 and 3 at the corresponding λex shows a huge difference between the observed spectra and corrected spectra. The RFIs at both excitation wavelengths for observed and corrected data are also compared in Figure 4. It is evident that RFIs of observed data show a nonlinear trend and have noteworthy differences at the two wavelengths but there was

![Figure 1](https://example.com/figure1.png)
only a negligible change in the RFIs of corrected data at a particular concentration of safranal for both excitation wavelengths. Herein, we can conclude that the inner filter effect is an important feature and its correction should be taken into account before analyzing the fluorescence parameters.

Since there was a trivial difference between the RFIs at both excitation wavelengths for the corrected fluorescence data, we selected 295 nm for further studies and to evaluate various quenching and binding parameters.

2.1.3. Analysis of Fluorescence Quenching Parameters.

Fluorescence quenching of a fluorophore by a ligand is generally classified into dynamic quenching and static quenching. Dynamic quenching or collisional quenching is a result of the deactivation of the excited-state fluorophore by the quencher. In the case of static quenching, the formation of a ground-state complex between the fluorophore and quencher takes place. Dynamic and static quenching have opposite dependencies on temperature and can be identified with the help of temperature variation. While a temperature increase is favorable for dynamic quenching, static quenching increases on decreasing the temperature. Hence, for understanding the quenching mechanism, studies were also carried out at 35 and 45 °C, for which the respective observed spectra at the excitation wavelength of 295 nm are given in Figures S1 and S2 and the corrected spectra are given in Figures S3 and S4. For analytical purposes, use of corrected data is an obvious choice. Fluorescence quenching can be analyzed with the help of the well-known Stern–Volmer equation.

Figure 2. Observed fluorescence emission spectra of lysozyme at 25 °C in the presence of various amounts of safranal for (A) λex = 280 nm and (B) λex = 295 nm. The concentration of lysozyme was 10 μM, whereas safranal concentrations for (1−9) were 0, 7.5, 15, 22.5, 30, 37.5, 45, 52.5, and 60 μM.

Figure 3. Corrected fluorescence emission spectra of lysozyme at 25 °C in the presence of various amounts of safranal for (A) λex = 280 nm and (B) λex = 295 nm. The concentration of lysozyme was 10 μM, whereas safranal concentrations for (1−9) were 0, 7.5, 15, 22.5, 30, 37.5, 45, 52.5, and 60 μM. Plot 10 in both (A) and (B) belongs to only 30 μM safranal.

Figure 4. RFI of lysozyme at 25 °C in the presence of various amounts of safranal from (A) observed and (B) corrected data. The concentration of lysozyme was 10 μM.

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\[
\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q[Q]
\]

(1)

where \( F_0 \) is the emission intensity of lysozyme in the absence of safranal and \( F \) is the emission intensity in the presence of safranal. \( K_{SV} \) is the Stern–Volmer constant and \([Q]\) is the concentration of the quencher, which is safranal in the present case. \( k_q \) is the bimolecular quenching constant and \( \tau_0 \) is the average lifetime of the fluorophore in the absence of the quencher, whose value is \( 5.9 \times 10^{-9} \) s.\(^{40}\)

The values of Stern–Volmer constants \((K_{SV})\) can be obtained through the plots of \( F_0/F \) versus \([Q]\), which are displayed in Figure 5A at several temperatures. The values of diffusion-controlled limit reflects the involvement of static quenching.\(^{38}\) For the lysozyme and safranal interaction, the value of \( k_q \) at 25 °C, given in Table 1, is more than 100-fold as compared to the maximum diffusion-limited rate, which is a resilient indication of the involvement of static quenching. Additionally, as has been stated above that static quenching has an inverse temperature dependence, the involvement of static quenching has also been confirmed with the decrease in \( K_{SV} \) on increasing the temperature (Table 1).

### 2.1.4. Evaluation of Binding and Thermodynamic Parameters.

The binding or association constant \((K_b)\) and the number of binding sites \((n)\) are also very important characteristics of the protein–ligand interaction. A modified Stern–Volmer equation (eq 2) can be used to calculate these parameters from the fluorescence quenching data.\(^{42}\)

\[
\log \frac{F_0 - F}{F} = \log K_b + n \log [Q]
\]

(2)

From the linear regressions of the plot of \( \log(F_0 - F/F) \) versus \( \log[Q] \), which are given in Figure 5B, the values of \( K_b \) and \( n \) can be obtained (Table 1). The binding constant was found to be on the order of \( 10^4 \), which shows a strong interaction between lysozyme and safranal. An increase in temperature also caused a decrease in binding constant. The value of \( n \) was found to be more than one, which shows that the interaction of safranal with lysozyme is cooperative in nature.\(^{43}\)

The binding between a protein and a ligand generally takes place via noncovalent interactions, which generally involve hydrogen bonding, hydrophobic forces, and electrostatic interactions. The contribution of these interactions can be understood by evaluating the thermodynamic parameters (enthalpy change (\( \Delta H \)), entropy change (\( \Delta S \)), and free energy change (\( \Delta G \))) using the renowned van’t Hoff equations, which are given in eqs S2 and S3. The van’t Hoff plot for the lysozyme—safranal interaction is given in the inset of Figure 5B, and the values of thermodynamic parameters obtained from the van’t Hoff plot and equations are given in Table 1. The negative values of \( \Delta G \) show that the binding between safranal and lysozyme is energetically favorable, whereas the positive value of \( \Delta S \) and negative value of \( \Delta H \) suggest the dominance of hydrophobic forces and hydrogen bonding.\(^{44}\) These findings are in excellent agreement with the results obtained through molecular docking, which are given in the corresponding section.

### 2.1.5. Synchronous Fluorescence Spectroscopy.

The binding of lysozyme and safranal was further studied by synchronous fluorescence spectroscopy, which was introduced by Lloyd in 1971.\(^{45}\) It is also a very common tool to understand the conformational changes in proteins and can separate the fluorescence of tryptophan and tyrosine residues.\(^{15,48}\) When \( \Delta \lambda = \lambda_{em} - \lambda_{ex} = 15 \) nm, the fluorescence is due to the tyrosine residue, whereas \( \Delta \lambda = 60 \) nm gives the fluorescence spectrum of pure tryptophan. The observed

![Figure 5. Plots of \( F_0/F \) versus [safranal] (A) and plots of log\((F_0 - F/F) \) versus log[safranal] (B) at various temperatures. The inset in (B) is the van’t Hoff plot of lysozyme interaction with safranal. The concentration of lysozyme was 10 \( \mu \)M.](https://dx.doi.org/10.1021/acsomega.9b04291)


### Table 1. Stern–Volmer Quenching Constants, Binding Parameters, and Thermodynamic Parameters for the Interaction of Lysozyme with Safranal at Various Temperatures

| Temperature (°C) | Stern–Volmer quenching constants | Binding parameters | Thermodynamic parameters |
|------------------|----------------------------------|--------------------|-------------------------|
|                  | \( 10^3 K_{SV} \) (mol\(^{-1}\)) | \( 10^3 k_q \) (mol\(^{-1}\) s\(^{-1}\)) | \( R^2 \) | \( n \) | \( 10^3 K_b \) (mol\(^{-1}\)) | \( R^2 \) | \( \Delta G \) (kJ mol\(^{-1}\)) | \( \Delta H \) (kJ mol\(^{-1}\)) | \( \Delta S \) (J mol\(^{-1}\) K\(^{-1}\)) |
| 25               | 7.9 ± 0.18                      | 1.3 ± 0.03         | 0.9972                  | 1.08 | 1.7 ± 0.04 | 0.9880 | -24.1 ± 0.2 | -19.1 ± 0.3 | 16.9 ± 0.2 |  
| 35               | 6.2 ± 0.12                      | 1.1 ± 0.03         | 0.994                   | 1.07 | 1.3 ± 0.03 | 0.9988 | -24.3 ± 0.3 |  
| 45               | 4.2 ± 0.08                      | 0.7 ± 0.02         | 0.9979                  | 1.07 | 1.1 ± 0.03 | 0.9974 | -24.4 ± 0.2 |  

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synchronous fluorescence spectra of lysozyme quenching by safranal are given in Figure S5, and like other fluorescence data, we have also corrected the synchronous fluorescence data with the inner filter effect, which are given in Figure 6A. A comparison of both observed and corrected data shows that the inner filter effect also has a huge impact on synchronous fluorescence spectra. As can be seen from Figure 6A, tyrosine has a very little or negligible contribution, while the major part of the interaction is contributed by the tryptophan residue. Thus, it can be claimed that the interaction takes place near the tryptophan residues.

2.1.6. Förster Resonance Energy Transfer. The molecular distance between a protein and a ligand can be obtained using Förster resonance energy transfer (FRET), according to which a successful energy transfer from the donor to acceptor relies upon (i) the orientations of the dipoles of the donor and acceptor, (ii) the overlap between the fluorescence spectrum of the donor and the absorption spectrum of the acceptor (Figure 6B), and (iii) the distance between the former and latter, which must be less than 8 nm.69 According to Förster’s theory, the energy transfer efficiency (E) can be given as

\[ E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \]  

where \( r \) is the distance between the acceptor (safranal) and donor (lysozyme) and \( R_0 \) is the critical distance when the transfer efficiency is 50% which can be calculated as

\[ R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \]  

where \( N \) is the refractive index of the medium, \( k^2 \) is the orientation factor, and \( \Phi \) is the quantum yield of the donor. The spectral overlap integral \( (J) \) between the donor emission spectrum and the acceptor absorbance spectrum can be given as

\[ J = \frac{\sum F(\lambda)\epsilon(\lambda)\Delta\lambda}{\sum F(\lambda)\Delta\lambda} \]

where \( F(\lambda) \) is the fluorescence intensity of the donor and \( \epsilon(\lambda) \) is the molar extinction coefficient of the acceptor. The values of \( k^2, N, \) and \( \Phi \) were given as 2/3, 1.336, and 0.15. The values of \( E, R_0^6, \) and \( r^6 \) were found to be 0.112, 3.1, and 4.4, respectively. The value of \( r \) is considerably smaller than 8 nm and \( 0.5R_0 < r < 1.5R_0 \) which is reminiscent of energy transfer from lysozyme to safranal. A very small distance between the donor and accepter also suggests the involvement of static quenching.50,51

2.1.7. ANS Binding Assay. ANS is a hydrophobic dye, which is used as an extrinsic probe to investigate hydrophobic interactions.52 An ANS displacement study can be used to investigate the involvement of hydrophobic interactions between a ligand and lysozyme.53 When dissolved in water or buffer, ANS shows a minimal fluorescence, which increases considerably on mixing it with a protein solution due to its binding with the hydrophobic groups of the protein.53,54 ANS is also known to bind with lysozyme, which results in an enhancement of its fluorescence quantum yield.55 In the absence of lysozyme, the quantum yield of ANS is very low (Figure 7A), which increases significantly in the presence of the former owing to the binding of the latter with the hydrophobic groups. The sequential addition of safranal causes a reduction in the fluorescence intensity of ANS, which means that there is a competition between ANS and safranal for the hydrophobic groups due to which some of the ANS are displaced from the binding site, leading to the decrease in fluorescence intensity.56 These results are in good agreement with the molecular docking simulations, which also show that the principal forces involved in the binding are hydrophobic forces.

2.1.8. Secondary Structural Analysis. CD spectroscopic studies were also performed to estimate the secondary structure of lysozyme in the absence and presence of safranal (Figure 7B). Far-UV CD is a straightforward technique to understand the secondary structural changes in a protein; the negative peaks at 208 and 222 nm are accredited to the \( \alpha \)-helical contents of the protein, whereas a single negative peak at 218 nm is characteristic of the \( \beta \)-sheets.57,58 The far-UV CD profile of pure lysozyme shows the characteristics of the \( \alpha \)-helical protein (Figure 7B), whereas pure safranal does not show any CD signal in the far-UV CD range studied in this work.

Equations S4 and S5 were used to calculate the % \( \alpha \)-helicity of the protein. Lysozyme holds around 40% of \( \alpha \)-helical content,59,60 and the experimental value in the present case is 39.4%, which is very close to the literature value. When an equimolar amount of safranal is added to lysozyme, there is no significant change in the \( \alpha \)-helicity (39.1%) of the protein. Further, a high concentration of safranal (30 \( \mu \)M) is also not very much effective in causing a secondary structural change in lysozyme with an \( \alpha \)-helicity of 37.4%. Thus, it can be inferred...
that safranal does not affect the secondary structure of lysozyme.

2.1.9. Rayleigh Light Scattering. RLS measurement is also a very good method to gain qualitative information about the change in the size of the protein as a result of unfolding and aggregation.\textsuperscript{61,62} RLS\textsubscript{350} was obtained with simultaneous excitation and emission of the protein at 350 nm, but, as we know that safranal has sufficient absorbance at 350 nm,\textsuperscript{23} the observed data were corrected for the inner filter effect, and for the sake of comparison, RLS\textsubscript{650} (for which simultaneous excitation and emission were performed at 650 nm) was also studied because at 650 nm there was no absorption of safranal, and hence, the inner filter effect did not exist at this wavelength.\textsuperscript{24,25} The respective plots of RLS\textsubscript{350} and RLS\textsubscript{650} are given in Figure 7C, D, which shows that there is no significant change in the RLS intensities of lysozyme in the presence of safranal because in the case of unfolding of the protein, a multifold increase in the RLS intensities is observed.\textsuperscript{61}

2.2. In Silico Investigation of Lysozyme and Safranal Binding. Molecular docking was also performed to investigate the location of safranal inside lysozyme.\textsuperscript{65} There are a lot of PDB structures available in the RCSB protein databank (https://www.rcsb.org/); thus, to check the binding accuracy, we docked safranal on five different structures of lysozyme, obtained by various depositors at various resolutions. The structures named 2LYZ,\textsuperscript{64} and 2YDG\textsuperscript{65} were reported to be obtained through the X-ray diffraction method with 2 Å resolution, while 1DPX,\textsuperscript{66} 2VB1,\textsuperscript{67} and 3WUN\textsuperscript{68} were obtained at 1.65, 0.65, and 2.4 Å, respectively. Initially, blind docking was performed, followed by docking, which involved only the big hydrophobic cavity of lysozyme, and interestingly, the docked poses with the least energy were identical in both cases. The docked poses of safranal and lysozyme having the least energy, for each PDB entry of latter selected in the current study, are given in Figure 8. It is interesting to note that the most stable conformer in the case of each PDB structure has the same binding site and most of the amino acids involved in the binding are common.

The dominant forces involved in the binding were hydrophobic forces with a small contribution of hydrogen bonding. The various amino acids involved in the binding for the several PDB structures of lysozyme were among Asp52, Ile58, Gln57, Asn59, Trp62, Trp63, Trp108, Ile98, Asp101, and Ala107 (Figure 9). The involvement of three Trp residues in the binding is also in line with the fluorescence quenching observed experimentally. Further, the results are also in good agreement with those observed with ANS dye binding assay, which deduced the involvement of hydrophobic forces in the binding between lysozyme and safranal.

![Figure 7](https://dx.doi.org/10.1021/acsomega.9b04291)
The flexibility of safranal molecules also played an important role in the binding. This can be understood by comparing the changes in the spatial orientation of the atoms, especially the oxygen of the carbonyl group (Figure S6), along with the bond lengths of various bonds in the case of free safranal and bound safranal, which are given in Table S1.

3. CONCLUSIONS

Owing to the important characteristics of lysozyme in various therapies and its binding with various important compounds, its interaction with safranal, which is one of the principal constituent of saffron, was studied in vitro using several experimental methods, such as fluorescence, UV-visible, and circular dichroism spectrophotometries, and an in silico method using molecular docking simulations. The inner filter effect of safranal has a huge impact on the experimental fluorescence data of lysozyme; hence, it was corrected before analyzing various binding and quenching parameters. There was 1:1 cooperative binding between lysozyme and safranal. The mechanism of fluorescence quenching was of static type, and the binding was found to be energetically favorable. The main forces involved in binding were hydrophobic forces and hydrogen bonding, which were also confirmed by molecular docking. Safranal did not affect the secondary structure of lysozyme, and the protein’s α-helicity remained almost unchanged in the presence of the former. The binding site of safranal in the lysozyme was the big hydrophobic cavity, which contains Asp52, Ile58, Gln57, Asn59, Trp62, Trp63, Trp108, Ile98, Asp101 and Ala107 amino acids.

Table 2. Noncovalent Interactions between Various PDB Entries of Lysozyme with Safranal Obtained through Molecular Docking

| PDB ID | docking energy (kJ/M) | residues involving H-bonds | residues involving hydrophobic interactions |
|--------|-----------------------|----------------------------|---------------------------------------------|
| 2LYZ   | -20.06                | Gln57, Asn59, Trp62, Trp63, Ile98, Ala107 | |
| 1DPX   | -20.9                 | Trp63                      | Asp52, Gln57, Gln57, Asn59, Trp63, Ile98, Ala107, Trp108 |
| 2YDG   | -20.9                 | Trp63                      | Gln57, Ile58, Asn59, Trp63, Ile98, Ala107, Trp108 |
| 2VBI   | -19.65                | Trp63                      | Asn59, Trp62, Trp63 |
| 3WUN   | -19.65                | Asn59                      | Asp52, Gln57, Asn59, Trp62, Trp63, Ala107 |

Figure 8. Docking poses of safranal bound to various PDB structures of lysozyme.
4. MATERIALS AND METHODS

4.1. Materials. Lysozyme from hen egg white (L4919, ≥98%) and safranal (W338907, ≥95%) were bought from Sigma. A stock solution of lysozyme was made in 20 mM pH 7.4 Tris HCl (Sigma, 10812846001, >99%) buffer and its concentration was estimated by UV−visible spectroscopy using a molar extinction coefficient of 38,940 M$^{-1}$ cm$^{-1}$ and a protein concentration of 10 μM was used throughout the study.

UV−visible absorption studies were performed using 1 cm quartz cuvettes in the range from 200 to 500 nm. Intrinsic fluorescence measurements were carried out by exciting the protein at 280 and 295 nm, and the respective emissions were recorded between 290–500 nm and 300–500 nm. Synchronous fluorescence spectroscopy was studied at two different wavelength intervals ($\Delta \lambda$), i.e., 15 and 60 nm. Extrinsic fluorescence measurements using the 8-anilinonaphthalene-1-sulfonic acid (ANS) dye were performed by exciting the protein solution at 380 nm and recording the emission spectrum from 400 to 600 nm. For resonance light scattering (RLS), the emission was recorded at the excitation wavelength, and two wavelengths (350 and 650 nm) were selected for this purpose. For studying circular dichroism (CD) spectroscopy in the far-UV range (200–250 nm), a quartz cuvette of 0.5 cm was used and the baseline for an equal amount of safranal in buffer was corrected before every measurement.

For computational studies, the geometry of safranal was optimized (Figure 1A) using ORCA software by generating an ORCA input file in Avogadro software; after that, it was used for molecular docking. In silico measurements were carried out using Autodock vina and visualized using discovery studio visualizer, Ligplot+, and Pymol. More detailed experimental methods are given in the Supporting Information.

### ASSOCIATED CONTENT

**Supporting Information**
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04291.

Detailed methodology, equation used for inner filter effect correction (eq S1), van’t Hoff equations (eqs S2 and S3), equations used in the analysis of CD data (eqs S4 and S5), observed fluorescence emission spectra of lysozyme at 35 °C in the presence of various amounts of safranal at $\lambda_{ex} = 295$ nm (Figure S1), observed fluorescence emission spectra of lysozyme at 45 °C in...
the presence of various amounts of safranal at $\lambda_{ex} = 295$ nm (Figure S2), corrected fluorescence emission spectra of lysozyme at 35 °C in the presence of various amounts of safranal at $\lambda_{ex} = 295$ nm (Figure S3), corrected fluorescence emission spectra of lysozyme at 45 °C in the presence of various amounts of safranal at $\lambda_{ex} = 295$ nm (Figure S4), observed synchronous fluorescence spectra of lysozyme at 25 °C in the presence of various amounts of safranal at $\Delta\lambda = 15$ nm (solid lines) and 60 nm (dotted lines) (Figure S5), three-dimensional view of the C–C and C–O bond lengths of safranal obtained through molecular docking (Figure S6), the C–C and C–O bond lengths of safranal obtained through molecular docking of safranal with various crystallographic structures of lysozyme (Table S1) (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**
Mohd. Sajid Ali – Surfactant Research Chair, Department of Chemistry, King Saud University, Riyadh 11451, Saudi Arabia; orcid.org/0000-0003-0191-9708; Email: smsajdali@gmail.com

**Author**
Hamad A. Al-Lohedan – Surfactant Research Chair, Department of Chemistry, King Saud University, Riyadh 11451, Saudi Arabia

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.9b04291

**Notes**
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