Interaction of flavokawain B with lysozyme: A photophysical and molecular simulation study

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A B S T R A C T

Interaction of flavokawain B (FB), a therapeutic flavonoid with lysozyme (LYZ), was studied using various spectroscopic and molecular simulation techniques. The association constant, \( K_a \) of the binding reaction was determined to be \( 2.79 \pm 0.16 \times 10^4 \text{M}^{-1} \) at 25°C based on fluorescence quenching titration results. Thermodynamic analysis of the binding data obtained at different temperatures along with molecular docking results suggested the involvement of hydrophobic and van der Waals forces, as well as hydrogen bonding in FB-LYZ interaction. The binding reaction between FB and LYZ was found to affect the microenvironment around protein fluorophores (Tyr and Trp) as revealed by intrinsic and three-dimensional fluorescence results. A comparison of the LYZ thermograms, obtained by far-UV CD spectroscopy in the absence and the presence of FB, suggested improved protein thermal stability upon complexation with FB. Presence of metal ions was found to affect FB-LYZ interaction. Molecular docking predicted the formation of two hydrogen bonds between the oxygen atoms of FB and amino acid residues of LYZ (Asn-59 and Trp-63), located in the vicinity of the active site, in addition to various non-polar contacts. Molecular dynamics studies showed that the complex reached equilibrium during simulation, indicating the stability of the FB-LYZ complex.

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1. Introduction

Flavonoids, a class of natural compounds, are essential components of plant cells and play important roles in plant physiology [1,2]. In addition to their biological importance in plants, flavonoids have been extensively investigated for their pharmacological significance. This includes among others, antioxidant, anticancer, antiinflammatory, antimicrobial, and immunostimulatory activities [1–3].

Flavokawain B (FB) (Fig. 1), primarily found in kava plants [4,5], is a member of the chalcone subclass of flavonoids and has been shown to exhibit a number of pharmacological properties [4–7]. It is well known that deposition, transportation, metabolism and efficacy of a therapeutic compound are strongly influenced by its binding to proteins [1,2]. The interaction between a drug and a protein might increase its solubility, reduce its toxicity, prolong its half-life and delay its elimination from the body [1,2]. On the other hand, ligand–protein interactions involving lysozyme (LYZ) have been shown to induce significant conformational changes in the protein, which have been demonstrated to affect its function [8–10].

In our previous report [11], we have described the interaction of FB with human serum albumin, the major transport protein in the human circulation. Apart from albumin, LYZ, which is commonly known for its antimicrobial action, has also been reported to act as a ligand transporter [12–14]. It is abundantly distributed in many secretory fluids such as saliva, tears and mucus as well as in cellular lysosomes, serum, synovial fluids and lymphatic tissues [15–18]. On top of its antimicrobial activity, LYZ also possesses other pharmacological functions including anti-inflammatory, antiviral, antiseptic and antihistaminic properties [19,20]. Furthermore, LYZ has long been utilized as a model protein in the study of protein structure, function, dynamics and ligand interaction owing to its ubiquity, high stability and small size [18]. Its three Trp residues (Trp-62, Trp-63 and Trp-108) are all situated near the
The fluorescence intensity values were treated for the inner filter effect correction according to the method described by Lakowicz [23]

\[ F_{\text{corr}} = F_{\text{obs}} e^{-\frac{A_{\text{obs}}}{A_{\text{em}}}} \]  

(1)

where \( F_{\text{corr}} \) and \( F_{\text{obs}} \) represent the corrected and the observed fluorescence intensity values, respectively. \( A_{\text{obs}} \) and \( A_{\text{em}} \) refer to the difference in the absorbance values of the protein samples at the excitation and emission wavelengths, respectively, upon addition of the ligand.

Values of the association constant \( K_a \) for FB–LYZ system were obtained after treating the fluorescence quenching titration data according to Bi et al. [24], using the following equation:

\[ \log (F_0 - F)/F = n \log K_a - n \log [1/(1 + (F_0 - F)(P_f)/F_0)] \]  

(2)

where \( F_0 \) and \( F \) are the fluorescence intensity values in the absence and the presence of the quencher, respectively; \( n \) is the Hill coefficient, while \([L]_1\) and \([P_f]\) represent the total concentration of the ligand and the protein, respectively.

In order to ascertain the quenching mechanism involved in the FB–LYZ system, the titration data obtained from the fluorescence quenching experiments at three different temperatures were analyzed according to the Stern–Volmer equation [23]

\[ F_0/F = K_{SV} [Q] + 1 = k_q \tau_0 [Q] + 1 \]  

(3)

where \( K_{SV} \) is the Stern–Volmer constant and \([Q]\) is the quencher concentration. A value of 1.8 \times 10^{-9} s was used for \( \tau_0 \), the fluorescence lifetime of LYZ in the absence of the quencher to compute the value of the bimolecular quenching constant, \( k_q \) [10].

The thermodynamic parameters (\( \Delta H \), enthalpy change and \( \Delta S \), entropy change) for FB–LYZ interaction were calculated from the van’t Hoff equation

\[ \ln K_a = -\Delta H/RT + \Delta S/R \]  

(4)

where \( K_a \) is the association constant at temperature \( T \) and \( R \) is the gas constant (8.3145 J mol^{-1} K^{-1}).

The value of the free energy change \( \Delta G \) was subsequently obtained by fitting the \( \Delta H \) and \( \Delta S \) values into the following equation:

\[ \Delta G = \Delta H - T \Delta S \]  

(5)

2.4. Binding analysis

The interaction between FB and LYZ was studied using fluorescence spectroscopy. Titration of LYZ (4 mM) with increasing FB concentrations (0–32 μM) was performed at three different temperatures, viz. 25, 35 and 45 °C according to the experimental procedure described earlier [11]. The fluorescence measurements were performed on a Jasco FP-6500 spectrophuometer using a 1 cm path length quartz cuvette, placed in a thermostatically-controlled cell holder. The fluorescence spectra of the protein solutions were recorded in the wavelength range 300–400 nm upon excitation at 280 nm, using excitation and emission bandwidths of 10 nm each. These solutions were also tested for absorption measurements for inner filter effect correction. Absorbance values were recorded on a PerkinElmer Lambda 25 double beam spectrophotometer, using quartz cuvettes of 1 cm path length.

2.5. Thermal stability studies

Thermal stability of LYZ in the absence and the presence of FB was checked using circular dichroism (CD) spectroscopy. The measurements were performed using a Jasco J-815 spectropolarimeter, equipped with a Jasco PTC-420S/15 temperature controller under constant nitrogen flow, using quartz cuvette of 1 mm path length. CD values at 222 nm of 6 mM LYZ in 60 mM sodium phosphate buffer pH 7.4 in the absence and the presence of 12 and 30 μM FB were recorded in the temperature range 25–90 °C. The FB–LYZ mixtures were incubated for 1 h at 25 °C in order to achieve equilibrium prior to CD measurements. At each temperature, the samples were allowed an equilibration period of 3 min before the readings were recorded.
2.6. Three-dimensional fluorescence

Three-dimensional (3-D) fluorescence spectroscopy was performed to study microenvironmental perturbation around protein’s fluorophores upon FB binding. Three-dimensional fluorescence spectra of LYZ (4 μM) in the absence and the presence of FB (16 μM) were obtained by monitoring the emission spectra in the wavelength range 220–500 nm, while the excitation wavelength range was set as 220–360 nm with 10 nm increments. The total number of scans was 15.

2.7. Effect of metal ions on FB–LYZ interaction

The influence of metal ions on the binding of FB to LYZ was studied by determining the $K_a$ value of the interaction in the presence of Cu$^{2+}$, Mg$^{2+}$, Fe$^{2+}$, Ca$^{2+}$ and Zn$^{2+}$ at three different metal ion concentrations, i.e., 40, 160 and 334 μM. Incubation mixtures containing chloride salts of the selected metal ions and LYZ were incubated for 12 h prior to titration with FB. The experiments were performed at 25°C under buffered (60 mM sodium phosphate buffer, pH 7.4) and non-buffered (water) conditions to evaluate the effect of buffer on $K_a$ values.

2.8. Molecular docking

Molecular docking, visualization and rendering simulations were performed using AutoDock 4.2 [25] and AutoDockTools 1.5.4 (ADT) [26] at the Academic Grid Malaysia Infrastructure. The structure of FB was drawn using ACD/ChemSketch Freeware (Advanced Chemistry Development Inc. Ontario, Canada), 3-D optimized and exported as a mol file. Geometry optimization of FB was refined with the VegaZZ 2.08 [27] batch processing MOPAC script and the structure was converted and stored as a mol2 file. For the docking study, the FB non-polar hydrogens were merged and the rotatable bonds were defined. The crystal structure of LYZ (PDB code: 1LYZ, 2.0 Å resolution [28]) was obtained from the Protein Data Bank [29]. Water molecules were removed from the structure and the atomic coordinates of the protein chain of 1LYZ were stored in a separate file and used as input for ADT. Polar hydrogens were then added to the protein structure using ADT’s Hydrogen module and Kollman united atom partial charges were assigned.

During the docking of FB to LYZ, the protein structure was fixed at the initial input and set to be rigid while all torsional bonds of the ligand were set to be flexible. A grid map of 70 × 70 × 70 Å grid points with a 0.375 Å grid space was generated using the Auto-grid4 program. A blind docking simulation was conducted with the grid box centered at the coordinates $x = -0.486, y = 20.576$ and $z = 19.266$ of the protein structure. Lamarckian genetic algorithm with local search (GA-LS) was used as the search engine for a total of 100 runs. For each run, a population of 150 individuals with 27,000 generations and 250,000 energy evaluations were employed. Operator weights for crossover, mutation and elitism were set to be 0.8, 0.02 and 1.0, respectively. For local search, default parameters were used. The resulting conformations were exported as a mol2 file. Geometry optimization of FB was refined and used as input for ADT. Polar hydrogens were then added to the protein structure using ADT’s Hydrogen module and Kollman united atom partial charges were assigned.

Table 1

<table>
<thead>
<tr>
<th>$T$ (°C)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_{sv}$ (M$^{-1}$)</th>
<th>$\Delta S$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>$(2.79 \pm 0.16) \times 10^4$</td>
<td>$(2.51 \pm 0.19) \times 10^4$</td>
<td>19.68</td>
<td>$-19.48$</td>
<td>$-25.34$</td>
</tr>
<tr>
<td>35</td>
<td>$(2.13 \pm 0.13) \times 10^4$</td>
<td>$(1.92 \pm 0.10) \times 10^4$</td>
<td>19.68</td>
<td>$-19.48$</td>
<td>$-25.54$</td>
</tr>
<tr>
<td>45</td>
<td>$(1.54 \pm 0.12) \times 10^4$</td>
<td>$(1.43 \pm 0.14) \times 10^4$</td>
<td>19.68</td>
<td>$-19.48$</td>
<td>$-25.74$</td>
</tr>
</tbody>
</table>

Fig. 2. Fluorescence quenching titration of LYZ with increasing FB concentrations, studied in 60 mM sodium phosphate buffer, pH 7.4. [LYZ] = 4 μM, [FB] = 0–32 μM with 2 μM intervals (1–17), $A_{ex} = 280$ nm, $T = 25$ °C. The dotted line represents the fluorescence spectrum of 16 μM FB. Inset shows the decrease in the relative fluorescence intensity of LYZ at 342 nm (FI342 nm) with increasing FB/LYZ molar ratios.

Fig. 3. Analysis of the fluorescence quenching data of FB–LYZ system. (A) Logarithmic plots of $F_0/F$ against $1/([F_0]-[F_0]-[F][P_1]/[F_0])$ and (B) Stern–Volmer plots obtained at different temperatures. Inset of (A) shows the van’t Hoff plot for FB–LYZ interaction.
clustered using a root-mean-square deviation (rmsd) of 2.0 Å and the clusters were ranked in order of increasing binding free energy of the lowest binding energy conformation in each cluster.

2.9. Molecular dynamics

Molecular dynamics (MD) simulations were performed with the NAMD program [30] using the CHARMM36 force field [31]. Topology and parameter files for the ligand were created using the ParamChem 0.9.7.1 webserver with CGenFF 2b8 force field [32,33]. The Particle Mesh Ewald (PME) [34] method was used to treat long-range electrostatic interactions and the SHAKE algorithm [35] was applied to constrain all bonds. The complex was surrounded by a periodic box of TIP3P [36] water molecules with a margin of 20 Å for all dimensions. At the initial stage, energy minimization was performed for 2500 steps, followed by at 1 and 4 ns for equilibration and production phases, respectively. The simulations were carried out at 310 K, with the pressure set to 1 atm during the equilibration phase. The simulation time step was fixed at 2 fs, and coordinates were saved every 0.5 ps. Molecular visualization

![Image](image1.png)

**Fig. 4.** Thermal stability profiles of LYZ and FB-LYZ complexes (2:1 and 5:1), monitored by CD at 222 nm values in 60 mM sodium phosphate buffer, pH 7.4. [LYZ] = 6 μM, [FB] = 12 and 30 μM, T = 25–90°C.

![Image](image2.png)

**Fig. 5.** Three-dimensional fluorescence spectral projections and corresponding contour maps of LYZ (A and A') and 4:1 FB-LYZ complex (B and B'), recorded in 60 mM sodium phosphate buffer, pH 7.4. [LYZ] = 4 μM, [FB] = 16 μM, T = 25°C.

<table>
<thead>
<tr>
<th>System</th>
<th>Peak</th>
<th>Peak position (λ&lt;sub&gt;ex&lt;/sub&gt;/λ&lt;sub&gt;em&lt;/sub&gt;, nm/nm)</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYZ a</td>
<td>230/350</td>
<td>16.84 – 235.44</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>280/342</td>
<td>65.19</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>230/340</td>
<td>467.36</td>
<td></td>
</tr>
<tr>
<td>4:1 FB-LYZ a</td>
<td>230/350</td>
<td>20.60 – 331.39</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>250/500</td>
<td>135.24</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>280/343</td>
<td>340.01</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>230/341</td>
<td>148.42</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>290/431</td>
<td>300.49</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Characteristics of three-dimensional fluorescence spectra of native LYZ and its complex with FB.
and analyses were performed using the UCSF Chimera [37] and VMD [38] software packages.

2.10. Statistical analysis

All fluorescence experiments were performed at least three times and the results represented the average values. Statistically significant differences between the $K_q$ values were assessed using two-way ANOVA with 95% confidence limits ($p < 0.05$).

3. Results and discussion

3.1. FB–LYZ interaction

3.1.1. Binding affinity

Fig. 2 shows intrinsic fluorescence spectra of LYZ in the wavelength range 300–400 nm upon excitation at 280 nm, obtained in the absence and the presence of increasing FB concentrations at 25 °C. Appearance of the emission maxima at 342 nm was suggestive of the presence of Trp residues [23]. Addition of FB to the protein solution produced a progressive decrease in the LYZ fluorescence intensity. About 46% reduction in the fluorescence intensity was observed at the highest (32 μM) FB concentration (inset of Fig. 2). It should be noted that the fluorescence signal of free FB was negligible at this wavelength and only appeared to be significant beyond 375 nm. Significant quenching in the fluorescence intensity of LYZ upon FB addition was indicative of the interaction of FB with LYZ [23,39].

The fluorescence quenching titration data were analyzed according to Eq. (2) to determine the association constant, $K_a$. Fig. 3A shows linear plot between log $(F_0 - F) / F$ and log $[1/([LZ] - (F_0 - F) / [Fl])$ and the value of $K_a$ was obtained from the y-axis intercept. Unlike other popular methods that have been used to analyze fluorescence quenching data, such as the modified Stern–Volmer plot and the double logarithmic plot of log $(F_0 - F)$ vs. log Q; the method used in the present study based on Eq. (2) is free from any assumptions to be made regarding free or bound concentrations of the ligand [24]. As can be seen from Table 1, a value of 2.79 ± 0.16 × 10^4 M^-1 was obtained for $K_a$, suggesting intermediate affinity between FB and LYZ. The binding affinity of many ligand–protein complexes has been shown falling within the range, 1 × 10^1–15 × 10^4 M^-1 [1]. A moderate value of $K_a$ is advantageous as it allows reversible association between ligand and the protein, thereby facilitating efficient transportation of the ligand and smooth release at its target site [1].

3.1.2. Quenching mechanism

Fluorescence quenching refers to any process that involves a decrease in the fluorescence intensity of a sample and may arise from several molecular phenomena including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation and collisional quenching [23]. In the case of interactions involving a fluorescent macromolecule and a small ligand, fluorescence quenching may occur mainly as a result of either collisional or static quenching. Whereas the former results from collisions between the fluorophore and the quencher, the latter mechanism of quenching involves complex formation between the fluorescent compound and the quencher [23]. These two modes of quenching can be differentiated by their dependence on temperature. Higher temperatures give rise to faster diffusion and thus, a higher extent of collisional quenching. Conversely, reversibly-bound complexes are destabilized at higher temperatures, leading to decrease in static quenching [23].

In order to determine the quenching mechanism involved in FB–LYZ system, titration of LYZ with increasing FB concentrations was also performed at two other temperatures viz. 35 and 45 °C. Fluorescence quenching titration data, obtained at three different temperatures, were treated according to Eq. (3) and the resulting Stern–Volmer plots are shown in Fig. 3B. The values of the Stern–Volmer constant, $K_{SV}$ at these temperatures were obtained from the slopes of the plots and are listed in Table 1. An inverse correlation between the $K_{SV}$ values and the temperature can be clearly seen from Table 1, implying that the quenching of LYZ fluorescence by FB followed the static quenching mechanism involving the complexation of FB and LYZ. This was further supported by the considerably larger values of the bimolecular quenching constant, $k_q$, calculated using Eq. (3), which were found to be 1.39 ± 0.11 × 10^13, 1.07 ± 0.06 × 10^13 and 0.79 ± 0.08 × 10^13 M^-1 s^-1 at 25, 35 and 45 °C, respectively. On the other hand, the upper limit of $k_q$ value for a collisional quenching process has been suggested to be 2 × 10^10 M^-1 s^-1 [23].

Treatment of the fluorescence quenching titration data obtained at 35 and 45 °C according to Eq. (2) yielded straight line plots (Fig. 3A), and the values of $K_a$ thus obtained, are included in

### Table 3

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>$K_a$ (× 10^4 M^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mM sodium phosphate buffer, pH 7.4</td>
<td>Water</td>
</tr>
<tr>
<td>40 μM</td>
<td>160 μM</td>
</tr>
<tr>
<td>$-$</td>
<td>2.79 ± 0.16</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>2.54 ± 0.14</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>2.60 ± 0.13</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>2.58 ± 0.17</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>2.57 ± 0.15</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>2.53 ± 0.20</td>
</tr>
</tbody>
</table>

Fig. 6. Cluster analysis of the docking results of FB–LYZ system. The results represent a total of 100 docking runs.
the thermodynamic data for the particular binding reaction. Thus, be facilitated by several intermolecular forces such as hydrogen

3.1.3. Forces involved

Δ

negative sign of Δ

The interaction between a ligand and a biomacromolecule may

be facilitated by several intermolecular forces such as hydrogen bonding, electrostatic interaction, hydrophobic and van der Waals forces [40]. The involvement of these forces can be evaluated from the thermodynamic data for the particular binding reaction. Thus, by fitting the Kₐ values obtained at different temperatures into Eq. (4), values of ΔS and ΔH for FB–LYZ interaction were determined from the van’t Hoff plot ( inset of Fig. 3A), while the values of ΔG at different temperatures were obtained using Eq. (5). These values of thermodynamic parameters are also listed in Table 1. The spontaneity of the binding reaction was evident from the negative values of ΔG. The positive ΔS value can be ascribed to the destruction of water molecules that were previously arranged in an orderly manner around the protein and the ligand in their free forms, upon ligand–protein complex formation as a result of hydrophobic interactions between the two components [40,41]. This seems justifiable in view of the presence of benzenoid character as well as nonpolar moieties in the FB molecule. Absence of any ionizable group in the ligand structure coupled with a significant ΔH value obtained for FB–LYZ interaction excluded the involvement of electrostatic interactions in the complex formation, which are normally characterized by very small enthalpic changes [41]. The negative sign of ΔH value, however, may account for the participation of hydrogen bonding and/or van der Waals forces in the FB–LYZ interaction [41,42]. This was supported by the presence of functional groups in FB capable of forming hydrogen bonds, along with our molecular docking results, as discussed in Section 3.5. Taken together, evaluation of the energetics of FB–LYZ interaction suggested the involvement of hydrophobic and van der Waals forces together with hydrogen bonding in the complexation.

3.2. FB-induced thermal stabilization of LYZ

Any change in the thermal stability of a protein in the presence of a ligand points towards complexation between the ligand and the protein. In many instances, increased thermal stability of proteins has been observed in the presence of ligands [43–45]. This phenomenon results due to perturbation of the equilibrium between the native and the denatured protein species during thermal unfolding, which is coupled to the ligand binding equilib-rium [43–45]. In order to study this phenomenon in the FB–LYZ system, CD values at 222 nm (CD_{222 nm}) were used to probe the loss in the α-helical content of the protein with increasing temperature both in the absence and the presence of the ligand. FB, Fig. 4 shows thermal denaturation profiles of LYZ, obtained in the absence and the presence of two different FB concentrations (12 and 30 μM), in the temperature range 25–90 °C. The transition curves of free LYZ as well as its complex with FB exhibited a two-state denaturation behavior. At temperatures below 50 °C, the CD_{222 nm} value of the native protein remained unchanged, showed a slow decrease up to 65 °C and a marked reduction in the CD_{222 nm} value within 65–85 °C, indicating major loss in the protein’s helicity before levelling off. As can be seen from the figure, presence of FB in the incubation mixture significantly reduced the extent of the loss in the helicity at temperatures >75 °C. At the highest temperature (90 °C), CD_{222 nm} value increased from −58 (for native LYZ) to −64 and −68 for 2:1 and 5:1 FB–LYZ complexes, respectively. These results clearly suggested higher thermal stability of LYZ in the presence of FB.

3.3. FB-induced microenvironmental changes around protein fluorophores

Three-dimensional (3-D) fluorescence spectroscopy was employed to study microenvironmental perturbations around protein fluorophores upon FB binding. The 3-D fluorescence spectra and the corresponding contour maps of free LYZ (A and A’) and 4:1 FB–LYZ system (B and B’) are shown in Fig. 5, while the characteristics of these spectra in terms of the peak position and the intensity are given in Table 2. Peaks a (λ_{em}=λ_{ex}) and b (2λ_{ex}=λ_{em}) referred to the Rayleigh and the second-order scattering peaks, respectively [23]. On the other hand, peaks 1 (λ_{ex}=280 nm) and 2 (λ_{ex}=230 nm) represented spectral characteristics of the protein’s fluorophores (Trp and Tyr). Peak 3 (λ_{ex}=290 nm) in the 3-D fluorescence spectra of the FB–LYZ system corresponded to the fluorescence contribution of FB [11]. The significant decrease in the intensity of peaks 1 and 2 (27% and 32%, respectively) as observed in the FB–LYZ system clearly suggested that binding of FB had a strong influence on the microenvironment of LYZ fluorophores.

3.4. Metal ion interference in FB–LYZ interaction

Metal ions in the human system are vital to normal human physiology [46]. Although most of these metal ions are protein-bound, small concentrations of these metal ions are also present in free form in various body fluids including plasma [46]. Previous reports have demonstrated the interaction between LYZ and several metal ions such as Cu^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+} [47–49].
Hence, presence of free metal ions in the body might affect the binding of LYZ to other molecules including FB. Table 3 presents values of the binding constant for FB–LYZ interaction, obtained in the absence and the presence of five common metal ions, namely, Cu$^{2+}$, Mg$^{2+}$, Fe$^{2+}$, Ca$^{2+}$ and Zn$^{2+}$ at three different concentrations. As evident from the table, presence of the metal ions at a concentration of 40 μM (10:1 metal ion/LYZ molar ratio) had little effect on the $K_a$ value. This concentration was higher than the average concentration of most metal ions (except Mg$^{2+}$ and Ca$^{2+}$) present in the plasma and other body fluids [50]. On the other hand, a significant decrease in the $K_a$ value was observed with all metal ions at a concentration of 334 μM. Even at 160 μM concentration, all metal ions except Fe$^{2+}$ were found to significantly reduce the $K_a$ value of FB–LYZ interaction (Table 3). These results suggested interference of these metal ions to the binding of FB to LYZ, which could alter the pharmacokinetics of FB under in vivo conditions.

Since the buffer used in these experiments contained significant amounts of Na$^+$ ions, which might affect the interaction of these metal ions with LYZ, $K_a$ values were also determined in a buffer-free medium (ultrapure water). A comparison of the $K_a$ values with those obtained in the presence of 60 mM sodium phosphate buffer pH 7.4 clearly indicated a certain degree of influence of the buffer on the FB–LYZ interaction. An earlier report has also shown a similar buffer effect on LYZ using tris(hydroxymethyl)aminomethane buffer [51].

3.5. FB–LYZ molecular docking

Molecular docking studies of the interaction between FB and LYZ were performed using the AutoDock software package to predict the preferred FB binding site on the protein. The constructed 3-D structure of FB was geometrically optimized prior to docking to a high resolution X-ray crystal structure of LYZ (PDB code: 1LYZ). Cluster analysis of the FB–LYZ blind docking using a rmsd tolerance of 2.0 Å identified a total of 14 multimember conformational clusters, obtained from 100 docking runs (Fig. 6). The highest populated cluster was also found to be the lowest on the energy scale ($-21.56$ kJ mol$^{-1}$), and hence the most stable, with 39 structural configurations.

The binding model showing the most favorable binding energetics was then selected for the binding orientation analysis. As can be seen from Fig. 7, the binding site of FB was identified to be located along the cleft between the two domains of LYZ. Incidentally, the active site of LYZ is also located in this deep cleft with Glu-35 and Asp-52 being the key active site residues [52]. The
FB binding pocket (defined as amino acid residues within 5 Å from the ligand) was lined by the following 16 amino acid residues: Glu-35, Asp-52, Gln-57, Ile-58, Asn-59, Trp-62, Trp-63, Ile-98, Val-99, Asp-101, Gly-102, Asn-103, Ala-107, Trp-108, Val-109 and Ala-110. In addition, the binding model predicted two hydrogen bonds involving the hydrogen atoms of Asn-59 and Trp-63 and the oxygen atoms of the methoxy and carbonyl groups of FB, respectively (details are summarized in Table 4). The two aromatic rings of FB are held in place within the cleft through van der Waals forces, particularly through face and edge stacking interactions with the side chains of the aromatic amino acid residues of LYZ (Fig. 7). The shortest distances calculated from the benzene ring and the 2-hydroxyl-4,6-dimethoxyphenyl group of FB to the aromatic side chains of Trp-63 and Trp-108 were 4.60 Å and 3.58 Å, respectively. Despite the presence of Trp-62 in the vicinity of the FB benzene ring, a π–π stacking interaction between them seems to be highly unlikely on account of the large distance (5.98 Å), separating the aromatic groups. Aside from the aromatic residues, the binding site was found to consist of several hydrophobic, polar as well as ionic residues in the proximity of the bound ligand. Therefore, hydrogen bonding, van der Waals forces and hydrophobic interactions are believed to contribute in stabilizing the FB–LYZ complex. These results were in agreement with the thermodynamic data obtained above.

3.6. FB–LYZ molecular dynamics simulations

Molecular dynamics simulations of FB–LYZ system were performed to analyze the conformational stability of the docked complex. As shown in Fig. 8A, the rmsd values of free LYZ (green) and its complex with FB (blue) were almost identical, indicating the introduction of FB had little influence on the flexibility of the protein throughout the simulation period. Analysis of the MD data revealed the total energy of the ligand–protein complex was nearly uniform after the minimization procedure (Fig. 8B), concluding that the complex was stable during simulation. The rmsd of the superimposed initial (t = 0 ns) and final (t = 5 ns) structures of the complex (Fig. 8C) was found to be 1.02 Å.

As mentioned earlier, the binding site of FB was found to be near the active site of LYZ. While the 2-hydroxyl-4,6-dimethoxyphenyl group of FB was buried deep inside the cleft, the benzene ring was located rather, on the entrance to the active site. Interestingly, the propenone connecting these substructures permitted some rotational freedom to the benzene ring (Fig. 8C), leading to irregularities in the rmsd of the ligand (Fig. 8A).

4. Conclusions

In summary, the interaction between FB and LYZ was characterized based on spectroscopic techniques and was supported by molecular simulation results. FB–LYZ complex showed moderate binding affinity and involved hydrogen bonding, hydrophobic and van der Waals forces. The binding produced changes in the microenvironment around protein’s fluorophores but increased its thermal stability. Presence of metal ions at higher concentrations led to a significant decrease in the association constant of FB–LYZ interaction. Analysis of the binding orientation identified the binding locus of FB on LYZ in the vicinity of the protein’s active site, and MD simulation results showed the stability of the complex formed. It is expected that the work presented here will be helpful in understanding the transport of flavonoid-based drugs by LYZ.

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