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Exploring the non-covalent binding behaviours of 7-hydroxyflavone and 3-hydroxyflavone with hen egg white lysozyme: Multi-spectroscopic and molecular docking perspectives

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ABSTRACT

The interactions of bio-active flavonoids, 7-hydroxyflavone (7HF) and 3-hydroxyflavone (3HF) with hen egg white lysozyme (HEWL) have been established using differential spectroscopic techniques along with the help of molecular docking method. The characteristic dual fluorescence of 3HF due to the excited intramolecular state proton transfer (ESIPT) process is altered markedly upon binding with HEWL. Both the flavonoids quenched the intrinsic fluorescence of HEWL through static quenching mechanism while the binding affinity of 7HF was found to be greater than 3HF under experimental conditions. The binding constant (K_b) values were estimated to be in the order of $10^4\,M^{-1}$ and decreased with the rise in temperature. The contributions of the thermodynamic parameters (ΔH° and ΔS°) revealed that hydrophobic forces along with hydrogen bonding played a crucial role in the interaction of HEWL with 7HF and 3HF respectively and this finding was aptly supported by the molecular docking studies. The donor (HEWL) to acceptors (7HF and 3HF) binding distances were calculated using the Föster's theory. The phenomena of blue shifting of the emission maxima of the residues indicated the increase in hydrophobicity around the Trp micro-environment upon addition of the flavonoids was observed from synchronous and 3D fluorescence measurements whereas REES study indicated the decrease in mobility of the Trp residues upon addition of the ligands. The CD, FTIR and thermal melting studies indicated the alteration in the structural stability of HEWL on ligand binding and it was found that the % α-helical content decreased on complexation with 7HF and 3HF respectively as compared to native state. The flavonoids were found to inhibit the enzymatic activity of HEWL. The molecular docking results and accessible surface area (*ASA*) calculations revealed that the flavonoids bind within the active site of HEWL. The negative ΔG° values obtained from experimental and molecular docking studies indicate the spontaneity of the interaction processes.

1. Introduction

Lysozyme is a small monomeric globular protein (M.W~14.7 kDa) containing 129 amino acid residues with six tryptophan (Trp 28, 62, 63, 108, 111 and 108) and three tyrosine (Tyr 20, 23 and 53) residues. The presence of four disulphide bridges that links the amino acid framework is responsible for conferring extra stability to the protein [1]. The substrate binding site (active site) of lysozyme consists of a deep crevice that splits it between two domains linked by an α-helix, one domain primarily consists of α-helical region while β-Sheet conformations dominates the other [2]. The crystal structure of lysozyme revealed that Trp 62, Trp 63 and Trp 108 are present at close proximity of the substrate binding site and plays a deciding role in terms of substrate/inhibitor binding and in stabilizing the structure. Out of the six Trp residues, Trp 62 and Trp 108 have been recognized as most dominant fluorophores in the overall geometry [3]. Lysozyme is abundantly found in various tissues and number of secretions e.g., saliva, tears, human milk, mucus and also in the cytoplasmic granules of the polymorphonuclear neutrophils [4,5]. It exhibits antiviral, antiseptic, antihistaminic, anti-inflammatory and antineoplastic properties [6,7]. It is also helpful in food preservation [8] and has been used as an anti-microbial agent for food packaging [9] due to which the food and drug administration (FDA) has included lysozyme under "generally recognized as safe" (GRAS) category. A very important attribute of lysozyme is to carry drug molecules to the target receptors as lysozyme is known to bind many exogenous and endogenous compounds reversibly which is important for the realization of the drug transportation and metabolism processes as well as to understand the concept of

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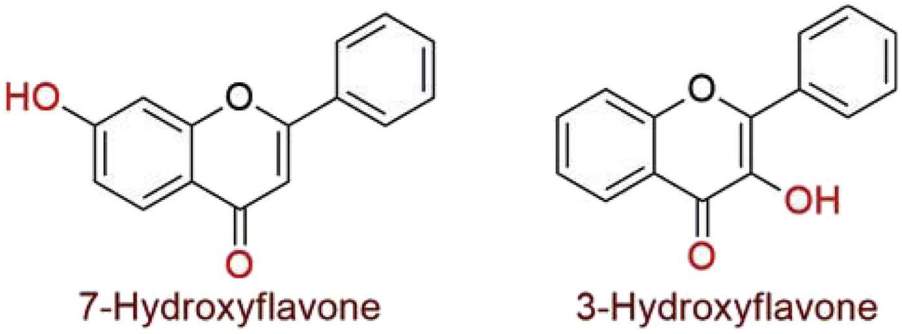

Fig. 1. Chemical structures of 7-hydroxyflavone and 3-Hydroxyflavone.

structure–affinity relationship in the protein–ligand interactions [10,11]. Hen egg white lysozyme (HEWL) has been utilized as a model protein for studying protein ligand interactions as it shares around 60% sequence homology with human lysozyme [2].

The effective usages of dietary polyphenols in the pharmaceutical industry due to their high potency and low systemic toxicity have been a matter of interest to different research groups worldwide. Flavonoids, a set of naturally occurring biologically active polyphenols present in various plants and fruits have received much attention due to their broad range of pharmaceutical and biological applications e.g., anti-oxidant, anti-virial, anti-cancer, anti-tumor and anti-inflammation which reduces the risk of diseases like cancer, cardiovascular and stroke etc. [12–14]. The bioavailability and bioactivity of flavonoids within the body depends on their distribution and transportation to different tissues. The multifarious biological properties of dietary flavonoids render them as compounds of primary importance for investigating their interactions with a range of carrier proteins in order to get insights into the transportation and metabolic processes of the flavonoids at the molecular level.

7-hydroxyflavone (7HF) and 3-hydroxyflavone (3HF) (Fig. 1) are naturally occurring compounds having great medicinal properties [15,16]. Recent reports have indicated the usefulness of 7HF as a fluorescent probe [17], as an binder with proteins [18] and DNA [19], its application as antioxidative [20] and antimutagenic agents [21]. Recently 3HF has been found to possess antioxidative and anti-antihemolytic [22] properties. Another report stressed on the anti-diabetic effect of 3HF in high fructose fed insulin resistant rats [23]. The effectiveness of 7HF and 3HF in therapeutic applications could be due to their capability to interact with the proteins and enzymes. Hence such information may provide crucial insights into their transportation, distribution and metabolism to the required sites within the body at molecular level.

Further the proposed manuscript, reports the binding of HEWL with 7HF and 3HF using UV-vis, steady-state fluorescence, time-resolved fluorescence, circular dichroism (CD), Fourier-transform infrared spectroscopy (FT-IR) and molecular docking methods. The nature of quenching mechanism involved, the excited state intramolecular proton transfer (ESIPT) process, binding constant (K_b), number of binding sites (n) for the HEWL-7HF and HEWL-3HF complexes were analyzed with UV–vis and fluorescence quenching studies. Synchronous fluorescence, red edge excitation shift (REES) and three dimensional (3D) fluorescence measurements were carried out to monitor the change in the microenvironment around the Trp residues within HEWL. The inhibitory effect of 7HF and 3HF on the enzymatic activity of HEWL was also investigated. CD and FT-IR were carried out to check the alteration in secondary structural components of HEWL. Thermal studies on HEWL and HEWL-7HF/3HF were carried out to monitor the stability of HEWL in the presence of the ligands. Molecular docking studies were performed to validate the experimental findings and to locate the probable binding site of 7HF and 3HF in HEWL. This type of interaction between small drug like molecules with the carrier proteins finds considerable applications in the fields of pharmacology and pharmacokinetics for drug delivery and drug transportation.

2. Experimental Section

2.1. Materials

Lysozyme from chicken egg white (L6876), 3HF (H4280), 7HF (H4350) and *Micrococcus lysodeikticus* (*M. lytus)* ATCC No. 4698 powder (M3770) were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Tris buffer (AR grade) was obtained from Sisco Research Laboratory (SRL), India. Double distilled water was used for the preparation of solutions wherever required. All the experiments were carried out in 20 mM Tris-HCl buffer of pH 7.4. Stock solution of HEWL was prepared in the said buffer and concentration was estimated spectrophotometrically using $\varepsilon_{280} = 37{,}646\ M^{-1}\ cm^{-1}$ at 280 nm [24]. Stock solutions of 7HF and 3HF were prepared in absolute ethanol due to their low aqueous solubility and the final experimental concentration of both the flavonoids were kept at 10^{-6} M such that the ethanol concentration remained < 3% (v/v) which almost has no effect on the protein structure.

2.2. Methods

2.2.1. Absorption Spectral Measurements

All the UV–Vis absorption studies were executed in a PerkinElmer Lambda 35 spectrophotometer using Tris-HCl buffer as references. For ground state complexation, the concentration of HEWL, 7HF and 3HF was kept at 20 μM and the HEWL-7HF/3HF complex was prepared at 1:1 molar ratios and incubated for 2 h prior to the experiment at room temperature. Further for the determination of ground state association constant (K_a), a 3 mL solution of 25 μM 7HF/3HF was successively titrated with HEWL solution (0–20 μM). The spectra were recorded in the range of 250–500 nm.

2.2.2. Steady State Fluorescence Measurements

The steady state fluorescence studies were carried out using a 1.0 cm quartz cuvette on a Fluoromax-4 spectrofluorometer (Horiba Jobin Vyon, Japan) fitted with Newport temperature controller (Model 350 B, California, USA). The emission profiles of HEWL in the absence and presence of 3HF and 7HF with HEWL were monitored at three different temperatures (293, 300 and 307 K) in the range of 305–500 nm using λ_{ex} = 295 nm keeping slit widths as 2/2 nm. A 3 mL solution of HEWL (2 μM) was titrated with the successive addition of 3HF/7HF (0–26 μM) at three temperatures. Both the polyphenols showed no fluorescence property in the HEWL emission range at λ_{ex} = 295 nm. All the spectra were corrected with the corresponding blank data.

For the ESIPT process, a solution of 20 μM 7HF and 3HF were successively titrated with HEWL (0–16.4 μM) at room temperature and the emission spectra were recorded upon excitation at 340 nm using a slit width of 2/2 nm. The REES effect was determined by exciting 5 μM HEWL and its 1:5 complexes with 7HF and 3HF at 295 nm and 305 nm respectively using a slit width of 5/5 nm.

The synchronous fluorescence measurements were carried out in the