Electronic Supplementary Information (ESI)

"Yohimbine as a Multifunctional Therapeutic Agent: Inhibition of Lysozyme Aggregation, Glycation and Antioxidant Properties"

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Preparation of Lyz fibril

In-vitro studies on the impact of Yohimbine on Lysozyme fibrillation were carried out to understand if the ligand inhibits or accelerates the fibrillation process. After understanding how Yoh interacts with Lyz, our focus turned to studying the impact of Yoh on protein fibrillation using spectroscopic and microscopic methods. When native Lyz is subjected to harsh conditions—low pH (pH 2.0), high temperature (65°C), and continuous agitation (~150 rpm) for a duration of 60 hours, it leads to partial unfolding, triggering the formation of fibrillar aggregates through intermediate oligomers. Beyond temperature and pH, factors such as ionic strength and Lyz concentrations have a substantial influence on the fibril formation process.[5] In our study, a Lyz protein solution at a concentration of 10 mg/mL in KCl-HCl buffer at pH 2.0 served as the basis. This solution underwent a 60-hour incubation at 65°C to simulate the protein fibrillation process. To assess the impact of Yoh on this fibrillation process, we introduced a concentration of 200 μM Yoh into the protein solution during incubation. The system of Lyz (1:0) and Lyz-Yoh (1:1) was set up for the aforementioned experiment.

Fluorescence Studies- Using Agilent Cary Eclipse spectrofluorometer, ANS and NR assay were carried out. 8-anilinonaphthalene-1-sulfonic acid (ANS) as an irrelevant fluorescent probe is widespread for detecting exposed hydrophobic regions in proteins.[1,2] In the native state of protein, hydrophobic amino acids are concealed within the folded conformation, resulting in negligible ANS fluorescence intensity. However, in fibrillated Lyz, there is a notable increase in fluorescence intensity due to the exposure of hydrophobic residues on the protein surface[3] with emission maxima at ~480 nm when excited at 350 nm.

Nile Red (NR) is a fluorescent dye commonly used for staining and identifying lipid-containing structures, but it can also be employed to identify amyloid fibrils in biological samples. When used in the context of amyloid fibrils, NR exhibits a change in its fluorescence properties upon binding to these structures, making it a useful tool for studying fibrillation processes in proteins.[4] Nile Red typically shows a red fluorescence in hydrophobic environments, which is where amyloid fibrils form. This shift in fluorescence can be used to detect the presence of these fibrils. The fluorescence intensities of Lyz were recorded by exciting the samples at 550 nm.

Dynamic Light Scattering (DLS) measurements were carried out in a Nano S Malvern instrument with a 632.8 nm laser, and the photons were collected at a 173 ° scattering angle.

Mid-IR FTIR measurements and analysis. All mid-IR measurements were performed in the attenuated total reflection (ATR) method, as it requires a minimum sample ($\sim 20-30\ \mu l$). *VERTEX 70v* FTIR spectrometer (Bruker Germany) with a *DLaTGS* detector was used for the study. The sample droplet was placed on the diamond crystal surface and covered with a cap. The sample compartment was always kept evacuated (vacuum level <1hPa) by a dry pump before each measurement. Each scan was done in the frequency range 1200-1800 cm-1 with a typical spectral resolution of 4 cm^{-1} over 64 scans on average. Here, we take water as a reference to exclude its positive contribution. The spectral analysis and peak deconvolutions were then executed using *OriginPro 8.5* software. The samples were native Lyz of 200 μM, fibril of 200 μM (1:0) and Lyz fibril-Yoh (1:1, 1:3).

Glycation studies preparation

Lyz (1:0) and Lyz-Yoh (1:1) were incubated for 30 days at 37°C in 0.1 M SP buffer (pH 7.4) along with and without 0.5 M concentration of reducing sugar: D-fructose with 1 mM NaN_3 was added to inhibit bacterial growth. The process was conducted under sterile conditions, and aliquots were taken at specific intervals and diluted with 10 mM SP buffer (pH 7.4) to achieve the desired Lyz concentration. The control sample was native Lyz incubated at pH 7.4 and 37°C without D-fructose under the same conditions as the sugar-treated samples. The samples were incubated for 30 days, and in the interval of 0, 7, 14, 21, 23, and 30 days, the growth of the glycation was monitored.

Fluorescence Emission Studies for AGEs Detection

Native and glycated Lyz samples, with and without Yoh, were excited at wavelengths of 295 nm and 370 nm, and the resulting emission intensities were measured within the ranges of 300–450 nm and 400–550 nm, respectively.

DPPH Assay for Radical Scavenging Activity

The DPPH assay was conducted to evaluate Yoh's free radical scavenging abilities. Solutions of Yoh were prepared at various concentrations (10, 50, 100, 300 and 500 μM). A 10 μM

DPPH (2,2-Diphenyl-1-picrylhydrazyl) solution was mixed in methanol. Each reaction mixture, consisting of 0.1 mL Yoh and 2.9 mL of DPPH solution, was incubated in the dark for 30 minutes at room temperature. The absorbance was then measured at 517 nm using a UV–vis spectrophotometer, with methanol as the blank. The DPPH solution alone served as the control. The antioxidant activity was calculated using the following equation:

$$\%\text{Radical Scavenging Activity} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100 \quad \text{(S1)}$$

where A_0 is the absorbance of the control (DPPH solution only), and A_1 is the absorbance of the reaction mixture.

HRTEM Measurements: High-resolution transmission electron microscopy (HRTEM) was performed using a TECNAI microscope operating at a voltage of 150 kv. A small volume of the prepared solution was drop-cast onto a copper (Cu) grid, which was allowed to air-dry before proceeding with the imaging process.

FESEM measurements: We perform field emission scanning electron microscopy (FESEM) measurements using the *FEI QUANTA FEG 250* microscope. A sample specimen was prepared using a properly cleaned silicon wafer substrate. A squared chip-like silicon wafer was dipped in Milli-Q water and a 90% ethanol mixture and sonicated for 10 mins. Then the sample was drop-cast on that substrate, and complete de-wetting was done by keeping it in a desiccator overnight. A 1nm thick conducting gold layer was deposited on the sample to get the information. Then, the collected morphologies were analysed by Image J. The system was Lyz fibril (1:0).

Molecular Docking Studies

Molecular docking studies were carried out using AutoDock 4.2 and AutoDock Tools-1.5.7 (ADT) to explore and comprehend the putative binding site and surrounding amino acid residues implicated in the interaction between Lysozyme (Lyz) and Yohimbine (Yoh). The best binding sites and orientations for the ligand were found employing the Lamarckian genetic algorithm (LGA). The docking method applied was the blind docking model, which looks for potential binding sites across the entire protein structure to produce a reliable and

thorough result. The docking parameters were set to 50 GA runs, a population size of 300, and a maximum evaluation of 2,500,000. The crystallographic coordinates of the Yoh ligand were obtained in .sdf format from PubChem with compound ID 8969, and OpenBabel was utilized to convert it to .pdbqt format. The PDB structure of Lyz with ID 1dpx was retrieved from the Protein Data Bank (PDB). Before docking, the protein structure's water molecules were removed, and Gasteiger charges were assigned to the protein atoms, with non-polar hydrogen atoms merged. Docking calculations were carried out on a grid with dimensions of 86 x 80 x 92 along the X, Y, and Z axes and a grid spacing of 0.392 Å. The torsional bonds of the Yoh ligand were rendered observable and rotatable for docking studies, while the protein structure remained rigid. To get insights into critical intermolecular chemical interactions, a structural analysis of the complex was carried out using Discovery Studio Visualizer 2021. By employing the following equations, the p*Ki* (negative decimal logarithm of the inhibition constant) was determined based on the Δ*G* parameter:

$$\Delta G = RT\left(LnKi_{pred}\right) \quad \text{(S2)}$$

$$Kipred = e^{\frac{\Delta G}{RT}} \quad \text{(S3)}$$

$$pKi = -\log(Ki_{pred}) \quad \text{(S4)}$$

where Δ*G* corresponds for the binding affinity (kcal mol^{-1}), R represents the gas constant, T is an ambient room temperature (298 K) and Ki_{pred} represents the anticipated inhibitory constant. Ligand efficiency (LE), a popular metric for discovering promising ligands by comparing average binding energy per atom values, was computed using the following formula:

$$LE = -\Delta G/N \quad \text{(S5)}$$

where LE signify ligand efficiency measured in kcal mol^{-1} non-H $atom^{-1}$, ΔG the binding affinity (kcal mol^{-1}) and N represents the number of non-hydrogen atoms in the ligand.

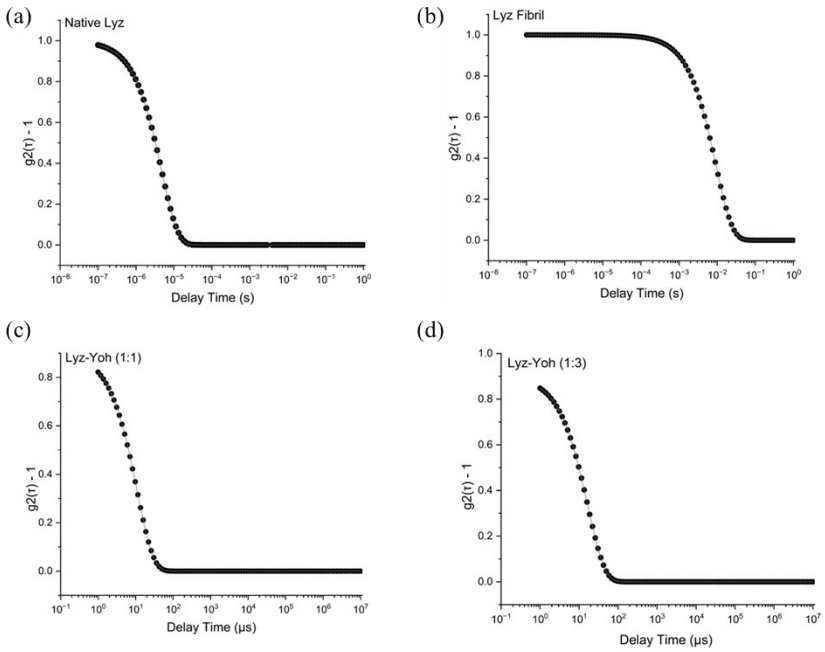

Figure S1. Intensity autocorrelation functions obtained from Dynamic Light Scattering (DLS) measurements. Experimental data are shown as scatter points, while the solid lines represent the corresponding cumulant fits.

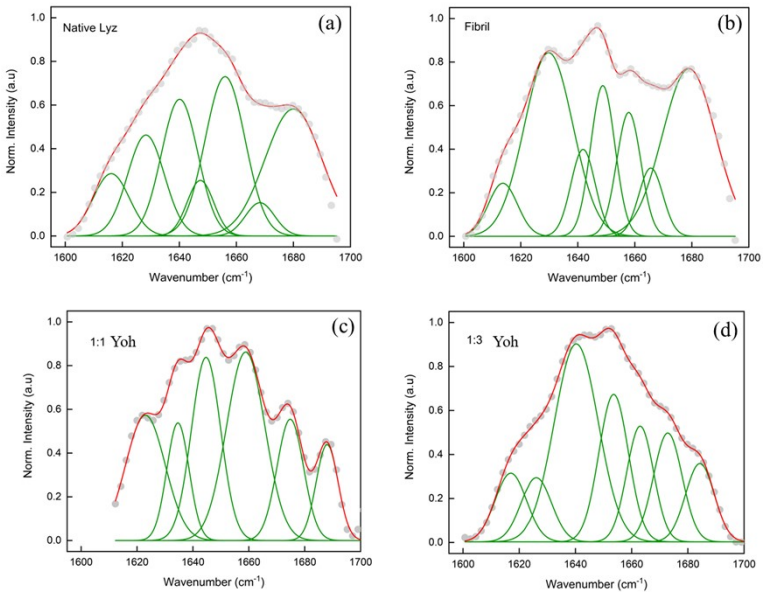

Figure S2. FTIR normalised intensity of Native Lyz (a) and Lyz fibril (b), Lyz fibril-Yoh,1:1 (c) and Lyz fibril-Yoh 1:3 (d).

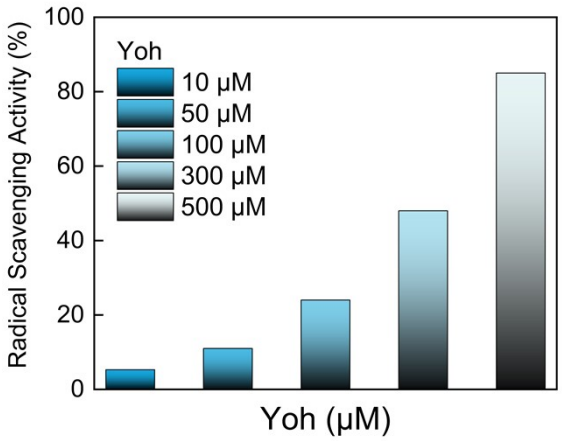

Figure S3. Radical scavenging activity assay with increasing Yoh concentration.