

# Comparative Studies on Interactions of Synthetic and Natural Compounds with Hen Egg-White Lysozyme

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**Abstract**—Amyloid aggregation of polypeptides is related to a growing number of pathologic states known as amyloid disorders. In recent years, blocking or reversing amyloid aggregation via the use of small compounds are considered as two useful approaches in hampering the development of these diseases. In this research, we have compared the ability of several manganese-salen derivatives, as synthetic compounds, and apigenin, as a natural flavonoid, to inhibit of hen egg-white lysozyme (HEWL) aggregation, as an *in vitro* model system.

Different spectroscopic analyses such as Thioflavin T (ThT) and Anilino-naphthalene-8-sulfonic acid (ANS) fluorescence, Congo red (CR) absorbance along with transmission electron microscopy were used in this work to monitor the HEWL aggregation kinetic and inhibition. Our results demonstrated that both type of compounds were capable to prevent the formation of lysozyme amyloid aggregation *in vitro*. In addition, our data indicated that synthetic compounds had higher activity to inhibit of the  $\beta$ -sheet structures relative to natural compound. Regarding the higher antioxidant activities of the salen derivatives, it can be concluded that in addition to aromatic rings of each of the compounds, the potent antioxidant properties of salen derivatives contributes to lower lysozyme fibril accumulation.

**Keywords**—Aggregation, anti-amyloidogenic, apigenin, hen egg white lysozyme, salen derivatives.

## I. INTRODUCTION

UP to now, more than 20 different proteins and peptides ( $\beta$ -amyloid, tau,  $\alpha$ -synuclein, huntingtin, amylin,  $\beta$ 2-microglobulin, lysozyme, etc.) have been found to form amyloid aggregates in humans [1]-[5]. It has recently been found that amyloid fibril formation is associated with not only disease-related proteins, but also with proteins not involved in any known amyloid diseases [1]-[5]. This has led to the suggestion that the ability to form amyloid aggregates is a generic property of polypeptide chains, and that most or indeed all peptides and proteins have the potential to form such structures *in vitro* under appropriate conditions [4]-[6]. Regarding these facts, the study of amyloid aggregation of disease-unassociated proteins could shed light on our understanding on the modes of aggregation and ways to disrupt them. Hen egg white lysozyme (HEWL), a monomeric protein composed of 129 amino acids with helix rich conformation, is one of the best known model proteins to study protein aggregation [7], [8]. Recent *in vitro* studies have

shown that under acidic pH and high temperature HEWL and wild-type human lysozyme undergo amyloid aggregation [8], [9]. In recent years some attention has been devoted to small molecule inhibitors of amyloid fibril formation. Small molecule inhibitor approach was initially based on the long known finding that molecules such as congo red (CR) and thioflavin T (ThT), having aromatic structures, interact specifically with amyloid fibrils and inhibit their formation [7]-[9].

EUK-8 and EUK-134 (Fig. 1) are two active members of salen-manganese complexes whose structures and catalytic activities have been described previously [10]. Both compounds have equivalent SOD activities, but EUK-134 possesses a higher CAT activity [10]. Apigenin (4,5,7-trihydroxyflavone) is a member of the flavone family and is found in many fruits, vegetables and nuts, including onion, orange and tea [9]. Many reports have shown that apigenin possesses various pharmacological effects such as antioxidative, antiviral, antitumor, and anti-inflammatory activities [9]. In this research, we have compared the ability of several manganese-salen derivatives, as synthetic compounds, and apigenin, as a natural flavonoid, to inhibit of hen egg-white lysozyme (HEWL) aggregation, as an *in vitro* model system.

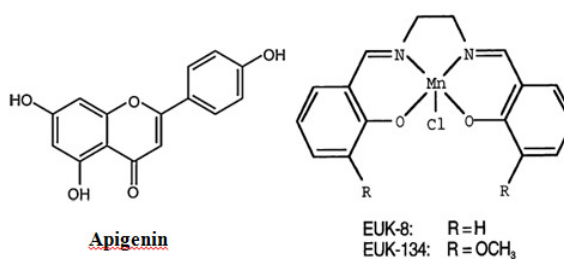


Fig. 1 Chemical structures of compounds

## II. MATERIAL AND METHODS

### A. Lysozyme Amyloid Fibrils Preparation

Lysozyme fibrils were prepared according to Arnaudov and de Vries method (30). HEWL sample solutions (1mM) were prepared in glycine-HCl buffer (50 mM, pH 2.4) containing 0.02% NaN<sub>3</sub>. To induce the production of the amyloid structure, HEWL solutions were incubated for 13 days at 57°C in a water bath without agitation. The formation of lysozyme aggregates was monitored by ThT fluorescence assay, Congo red binding assay, ANS fluorescence assay and transmission

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electron microscopy (TEM) [11].

### B. Anti-Aggregating Activities of Compounds

To study the anti-aggregating effects, all compounds at a molar ratio of 5:1 (drug to protein), were added to lysozyme solution (1 mM) prepared in glycine buffer and incubated for 13 days at 57°C. The mixtures were then assayed at indicated periods for the residual aggregates.

### C. Thioflavin T (ThT) Fluorescence Assay

Fibril formation was monitored by characteristic changes in ThT fluorescence intensity. Five  $\mu$ L of HEWL samples (1mM) was added to 445  $\mu$ L of 15  $\mu$ M ThT solution (from 1 mMThT stock solution in 10 mM sodium phosphate, 150 mMNaCl, pH 7.0, passed through a 0.45  $\mu$ m filter paper), mixed thoroughly, and incubated for 1 min. The fluorescence intensity was measured using a Cary Eclipse VARIAN fluorescence spectrophotometer (Mulgrave, Australia), with an excitation wavelength of 440 nm. Emission spectra were recorded at from 460 - 600 nm and the fluorescence intensity at 482 nm was employed for determination of the relative content of fibrils in the sample. Slits were adjusted to 5 and 10 nm for the excitation and emission, respectively.

### D. Congo Red (CR) Binding Assay

The presence of amyloid aggregates was followed by characteristic changes in the absorbance spectrum of CR (peak shifts from 492 nm to 512 nm and appearance of a large shoulder). Five microliter of each lysozyme well-mixed sample was added to 400  $\mu$ L of the Congo red solution (final concentration 10  $\mu$ M) consisting of 0.15 M NaCl and 5 mM potassium phosphate (pH 7.4) and incubated at room temperature for at least 30 min before detecting absorbance by a Shimadzu UV-visible spectrophotometer between 400 to 700 nm at 25°C.

### E. Anilinonaphthalene-8-sulfonic Acid (ANS) Fluorescence Assay

Five microliters of each HEWL sample solution (1mM) was mixed with 400  $\mu$ L ANS working solution (20  $\mu$ M), and then the mixture was incubated for 1 min at room temperature. ANS fluorescence intensity was recorded on a Cary Eclipse VARIAN fluorescence spectrophotometer by exciting each sample at 380 nm, and the emission was recorded between 420 and 600 nm. The fluorescence intensity between 470-490 nm was employed for determination of the hydrophobicity of fibrils in the different samples. Excitation and emission slits widths were both set at 5 nm.

### F. Transmission Electron Microscopy (TEM)

For TEM analyses, 5  $\mu$ L of each diluted sample (40-fold) was placed on a glow discharged 400-mesh carbon coated copper grid. After adsorption for 2 min, the sample was washed with distilled water and air-dried. Each grid was then stained with 2% (w/v) uranyl acetate for 2 min. Excess stain was removed, and the sample was again allowed to air-dry. Finally, each grid was viewed with a CEM 902A Zeiss microscope (Oberkochen, Germany).

### G. Statistical Analyses

All data are presented as means  $\pm$  SD. The mean values were calculated based on the data taken from at least three independent experiments using freshly prepared reagents. The statistical significances were achieved when  $P < 0.05$ .

## III. RESULTS AND DISCUSSION

Many studies have focused so far on understanding the molecular basis of fibril formation. Despite some progress in this area, there are still lots to be done to fully elucidate the mode and mechanism of fibrilization of proteins [1]-[5].

It is generally accepted that  $\beta$ -pleated sheets usually interact with each other and thus, leading to aggregation of proteins. If this is true, then it would be logical to attenuate protein aggregation by compounds having hydrophobic  $\beta$ -sheet domains [1]-[5].

In this research, we have compared the ability of several manganese-salen derivatives, as synthetic compounds, and apigenin, as a natural flavonoid, to inhibit of hen egg-white lysozyme (HEWL) aggregation, as an *in vitro* model system (Fig. 1). Due to the presence of two aromatic rings in their structures, we hypothesized that the aromatic groups of each compound might interact with the aromatic residues within the protein and stabilize their native form.

Regarding our hypothesis, we initially produced amyloid fibrils from HEWL (1 mM) by incubating the HEWL solution (pH 2.4) at 57°C for 13 days in the presence or the absence of each of the drugs. The lysozyme aggregates were confirmed by several different techniques such as ThT and ANS fluorescence assays, CR absorbance assay and transmission electron microscope. To evaluate the ability of each compound to influence the lysozyme aggregation, we investigated the effect of each compound at a drug o protein molar ratio of 5:1 on aggregation inhibition.

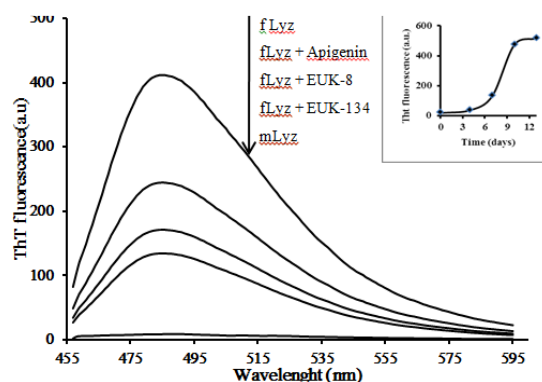


Fig. 2 Inhibition of HEWL aggregation and by EUK-8 and EUK-134 and Apigenin at drug to protein ratio (5:1; mM) based on ThT fluorescence emission. The inset shows the kinetics of lysozyme fibril growth monitored by ThT fluorescence

As shown in inset of Fig. 2, following 13 days incubation at 57°C, HEWL sample showed a significant increase in ThT fluorescence emission. The increase in the ThT fluorescence was obtained after a lag time of about three days. These results

were in agreement with nucleation-dependent polymerization model of most amyloidogenic proteins [9]. Simultaneous incubation of EUK-8 and/or EUK-134 compounds and apigenin at dose of 5:1 (drugs to protein; mM) with HEWL attenuated the ThT fluorescence intensity, relative to the control sample (Fig. 2). EUK-134 with an extra methoxy group at the ortho position had higher activity to inhibit the  $\beta$ -sheet structures relative to EUK-8. In addition, the potency of salen derivatives to inhibit of aggregation was higher than apigenin.

Amyloid formation was also evaluated using Congo red absorbance approach [7], [8]. As shown in Fig. 3 and its inset, with increasing incubation time, protein solutions containing amyloid fibrils exhibited a considerable increase in Congo red absorption at 540 nm accompanied with a red shift of the spectral maximum relative to native HEWL sample. In addition, a second shoulder peak at around 540 nm was observed which is indicative of a strong binding affinity between Congo red dye and lysozyme. Similar to our ThT fluorescence results, simultaneous incubation of compounds at dose of 5:1 (drug to protein, mM) with HEWL, resulted in both reduction of absorbance intensity and also a shift in  $\lambda$  max of fibrillar HEWL from 540 nm toward shorter wavelengths (a blue shift) (Fig. 3).

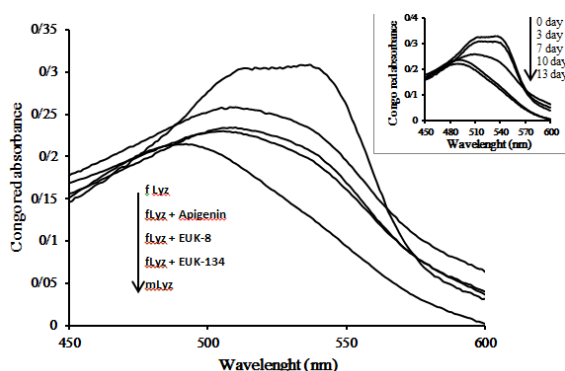


Fig. 3 Evaluation of the anti-aggregating activities of EUK-8 and EUK-134 and Apigenin at drug to protein ratio (5:1; mM) against HEWL, using Congo red spectroscopy analyses. The inset shows the kinetics of lysozyme fibril growth monitored by Congo red absorbance

Variation in surface hydrophobicity of HEWL in the presence of compounds was examined based on the ANS fluorescence emission spectrum following excitation at 380 nm. ANS, as a hydrophobic fluorescent probe, is widely used for documentation of hydrophobic patches on the surface of amyloid aggregates [7], [8].

Interaction of ANS with hydrophobic regions on proteins, leads to augmentation of fluorescence emission and also a blue shift in the emission maximum [8]. As shown in Fig. 4, the fluorescence intensity of native HEWL was remarkably low. This low intensity is due to the hidden hydrophobic sites within the native protein [8]. However, a marked increase in ANS fluorescence intensity was observed after fibrilization of HEWL for 13 days, suggesting the exposure of hydrophobic

patches on the surface of the protein (Fig. 4). Simultaneous incubation of HEWL with either of the drugs significantly lowered ANS fluorescence, as illustrated in Fig. 3, indicating the attenuation of exposed hydrophobic regions on the fibrils. Our results are in accordance with the other studies in which many compounds with ring structures are able to inhibit the amyloid aggregation effectively [12], [13].

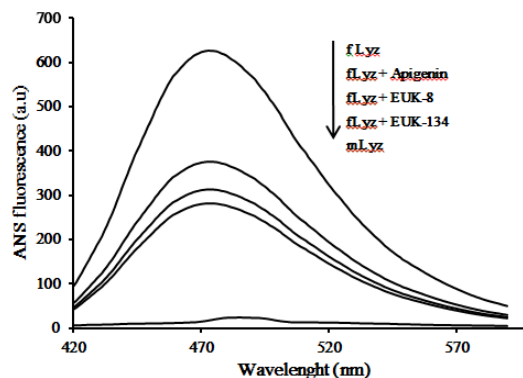


Fig. 4 Inhibition of HEWL aggregation by EUK-8 and EUK-134 and Apigenin at drug to protein ratio (5:1; mM) based on ANS fluorescence emission

The effects of compounds on lysozyme fibrillar aggregation were also evaluated by transmission electron microscopy. As it is evident from Fig. 5 (a), freshly prepared lysozyme sample is devoid of fibrillar structure. However, sample incubation at 57°C for 13 days, has led to the formation of fibrillar structures (Fig. 5 (b)). The addition of EUK-134 and apigenin at the drug to protein molar ratio of 5:1 led to lower extent of fibrillar structures accompanies with the amorphous aggregates, as documented in Figs. 5 (c) and (d), respectively (with reduction of ThT fluorescence intensity). These results clearly confirmed our spectroscopy observations.

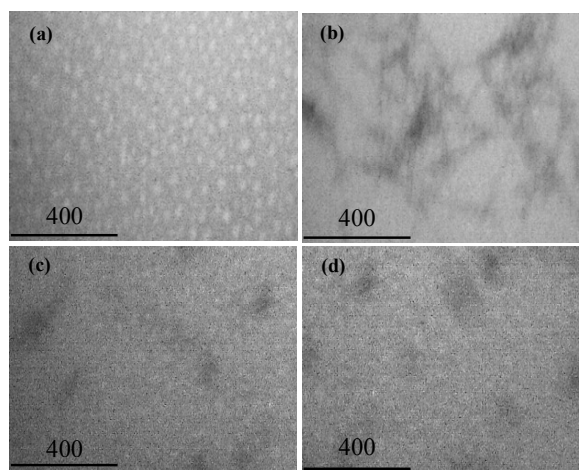


Fig. 5 TEM images of HEWL samples incubated in acidic environment (pH 2.4) at 57°C for 13 days in the (a) absence or (b) and (c) presence of EUK-134 and (d) Apigenin, respectively at a drug to protein molar ratio of 5:1

## IV. CONCLUSION

Regarding the established role of hydrophobic interactions in amyloid formation, logical strategies might be developed to prevent their formation and/or their dispersion. In that regard, compounds with hydrophobic structural zones might be effective. The presence of aromatic rings in salen derivatives and apigenin encouraged us to evaluate the inhibitory effects of these compounds, on HEWL aggregation, under high temperature and acidic condition. Our results demonstrated that both type of compounds (natural and synthetic) were capable to prevent the formation of lysozyme amyloid aggregation *in vitro*. In addition, our data indicated that synthetic compounds had higher activity to inhibit of the  $\beta$ -sheet structures relative to natural compound. Regarding the higher antioxidant activities of the salen derivatives, it can be concluded that in addition to structural properties of the compounds, the potent antioxidant properties of salen derivatives contributes to lower lysozyme fibril accumulation. Further elaborative work is required to further confirm this view.

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