the FEBS Journal

Inhibition of amyloid fibrillation of lysozyme by indole derivatives – possible mechanism of action

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Keywords
amyloid inhibition; docking; indole derivative; lysozyme; organic solvent

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(Received 21 August 2007, revised 10 October 2007, accepted 22 October 2007)

doi:10.1111/j.1742-4658.2007.06158.x

Amyloid aggregation of polypeptides is related to a growing number of pathologic states known as amyloid disorders. There is a great deal of interest in developing small molecule inhibitors of the amyloidogenic processes. In the present article, the inhibitory effects of some indole derivatives on amyloid fibrillation of hen egg white lysozyme (HEWL) are reported. Acidic pH and high temperatures were used to drive HEWL towards amyloid formation. A variety of techniques, ranging from thioflavin T fluorescence and Congo red absorbance assays to far-UV CD and transmission electron microscopy, were employed to characterize the HEWL fibrillation process. Among the indole derivatives tested, indole 3-acetic acid, indole 3-carbinol and tryptophol had the most inhibitory effects on amyloid formation, indole and indole 3-propionic acid gave some inhibition, and indole aldehyde and tryptophan showed no significant inhibition. Although indoles did not protect the HEWL native state from conformational changes, they were effective in diminishing HEWL amyloid fibril formation, delaying both the nucleation and elongation phases. Disaggregation of previously formed HEWL amyloid fibrils was also enhanced by indole 3-acetic acid. Various medium conditions, such as the presence of different anions and alcoholic cosolvents, were explored to gain an insight into possible mechanisms. These observations, taken together, suggest that the indole ring is likely to play the main role in inhibition and that the side chain hydroxyl group may contribute positively, in contrast to the side chain carbonyl and intervening methylene groups.

Amyloid deposits are associated with chronic neuronal and systemic pathologies, including Alzheimer's, Parkinson's and Huntington's diseases, transmissible spongiform encephalopathy, and type II diabetes [1–3]. Whole proteins and protein fragments that form the amyloid structures have microscopically observable fibrillar shapes, formed of a cross β-sheet structure, which is the result of a conformational change of the precursor protein [4–8]. Amyloid-like fibrils are also formed *in vitro* from the self-assembly of nonpathogenic proteins under suitable conditions. These proteins range from small peptides (e.g. amyloid β-peptide, amylin and insulin), to natively unfolded proteins (e.g. α-synuclein) and natively folded monomeric proteins (e.g. lysozyme and β_2-microglobulin), or even protein assemblies (e.g. transthyretin). As there is no sequence similarity between these structurally diverse proteins, it has been suggested that amyloid formation may be a generic property of polypeptide chains [9,10]. Given the exceptional importance of this phenomenon,

Abbreviations
ANS, 8-anilinonaphthalene-1-sulfonate; HEWL, hen egg white lysozyme; IAA, indole 3-acetic acid; I3C, indole 3-carbinol; I3P, indole 3-propionic acid; TEM, transmission electron microscopy; ThT, thioflavin T.

many efforts have been devoted to obtaining a better understanding of fibril formation mechanisms and, in parallel, finding methods for intervention. Observations on common features of amyloid structure formation have suggested the possibility of the existence of common inhibitors. Accordingly, there have been some reports on the inhibitory effects of small organic compounds on fibril formation by different proteins. Some of these inhibitors have been observed to effect conversion to oligomeric intermediates, which are considered to be the most harmful forms of these proteins for living cells [11–14]. They can also disaggregate previously formed filaments [14–17]. Moreover, they may stabilize the native form of aggregation-prone proteins by favoring the transformation kinetics towards native stable oligomeric states [18–20], and may possess antioxidant properties, thereby counteracting the toxic effects of active oxidants [21–23]. Usually, these molecules are effective at very low concentrations, and some, such as indole derivatives, are currently used in other related conditions [24–26], making them interesting leading structure candidates in amyloid-linked diseases [13,23,27]. There are still many obscure points regarding the detailed mechanisms of action of these compounds and the conditions under which they are functional. Because of the sequence dependency of protein fibrillation [28,29], and the fact that hydrophobic and stackable residues may participate in the early stages of this process [30,31], it has been suggested that disruption of hydrophobic and ð-stacking interactions of aromatic residues may be involved [30–34]. Indole derivatives can bind to proteins, and the nature of the derivative is considered to be important in this regard. For example, the presence of hydroxyl groups positioned at the C3 or C4 of indole is an important factor in relation to inhibition of amyloid fibril formation in amyloid β-peptide [13], and the electron-withdrawing fluorine contributes positively [27]. In the present study, the effects of indole derivatives on hen egg white lysozyme (HEWL) have been investigated. HEWL has a well-defined structure and may form amyloid fibrils under specific conditions [35]. Different HEWL intermediate states formed during the amyloid fibrillation process [36,37], as well as the actual fibrils, cause cell death [37]. Indoles have been reported in previous studies to bind HEWL and inhibit its catalytic activity [38,39]. Here, we show that some indole derivatives may influence HEWL fibril formation.

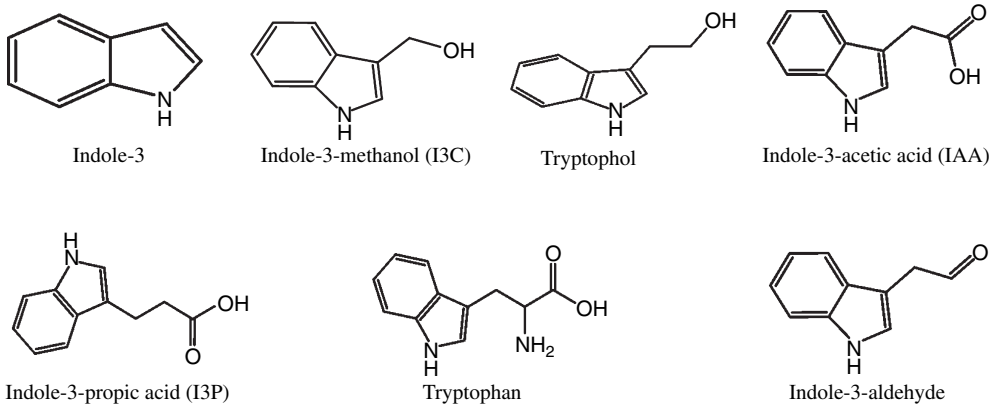
Results and Discussion

It is now widely accepted that the ability of proteins to form amyloid aggregates is an intrinsic characteristic of these macromolecules. The process is associated with a number of important human pathologic conditions, and therefore has been the subject of extensive research. Accordingly, inhibition or reversal of such events has been suggested to provide a possible preventive mechanism related to amyloid diseases [11–13]. Human lysozyme variants have been shown to form massive amyloid structures associated with amyloid disorder, a severe human pathologic condition. Both wild-type human lysozyme and HEWL have been shown to undergo amyloid aggregation under specific *in vitro* conditions [35,40]. As the structure and folding mechanisms of lysozyme have been well characterized, it provides a useful model for the study of amyloid aggregation.

To drive HEWL towards amyloid fibril formation, the protein was incubated at acidic pH and high temperature. Formation of amyloid fibrils was then verified through some specific methods of amyloid detection. For instance, thioflavin T (ThT) was shown to reach high fluorescence emission intensities, the absorbance spectrum of Congo red, which is another well-known dye for amyloid detection, also exhibited a characteristic enhancement and red-shift up to 40 nm, and finally, the transmission electron microscopy (TEM) image of HEWL samples incubated in this condition confirmed the formation of long, unbranched fibrils.

Effect of indole derivatives on fibrillation of HEWL

In recent years, various small molecule inhibitors of amyloid fibrillogenesis have been introduced [11–22]. The indole derivatives, which comprise widely studied class of such inhibitors, have been shown to inhibit some amyloid-forming systems [13,23,27]. To determine whether these compounds affect the process of HEWL amyloid formation, some of them (Scheme 1) were added to the incubation medium. Of the indoles tested, indole 3-propionic acid (I3C), tryptophole and indole 3-acetic acid (IAA) were most effective in inhibiting amyloid fibril formation by HEWL, as suggested by the observed decrease of both ThT fluorescence intensity and Congo red absorbance changes after 48 h of incubation (Fig. 1A,B). The effect of these compounds on the kinetics of HEWL amyloid formation was also investigated through monitoring maximal ThT emission intensity over the course of 7 days. As indicated in Fig. 2, the amyloid formation process was found to obey the characteristic nucleation-dependent pattern, with three distinct phases: initial nucleation; elongation; and equilibration. At a protein



Scheme 1. Structure of indole derivatives.

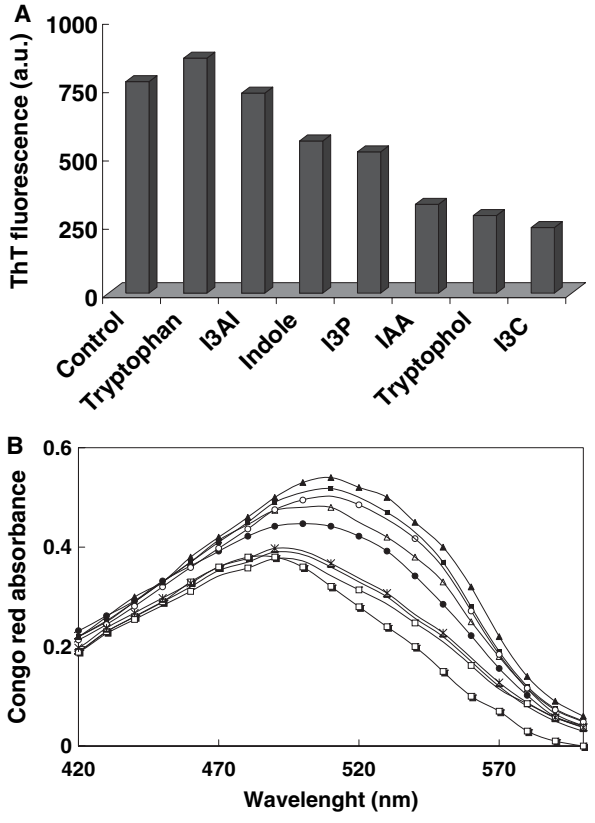


Fig. 1. Effects of various indole derivatives on HEWL amyloid fibrillation. This was measured at pH 2.5 and 57 °C by monitoring changes in ThT emission enhancement (A) or Congo red absorbance spectra (B) after 48 h. The indole derivatives examined were tryptophan (▲), indole 3-carbaldehyde (○), indole (△), I3P (●), IAA (_), tryptophol (✱) and I3C (□), at 0.4 mM; the protein concentration was 2 mg·mL^{-1}. Congo red absorbance alone and in the presence of HEWL is indicated by ❑ and ■, respectively. The control was that measured in the absence of any indole derivatives. For further details, see Scheme 1 and Experimental procedures.

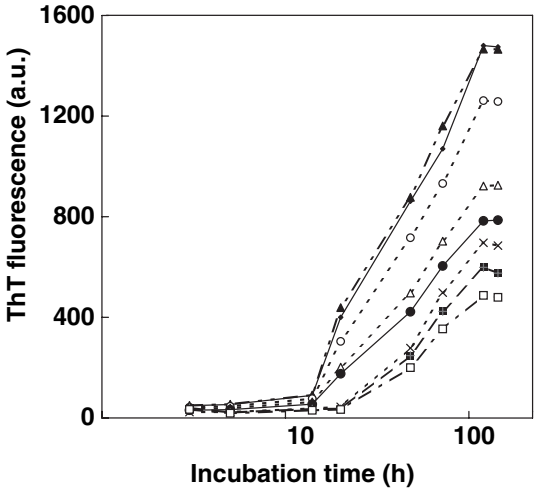


Fig. 2. Kinetics of HEWL fibrillation in the presence of different indole derivatives. This was followed by monitoring changes in ThT fluorescence intensity after 1 week of incubation at pH 2.5 and 57 °C. The indole derivatives examined were tryptophan (▲), indole 3-carbaldehyde (○), indole (△), I3P (●), IAA (◇), tryptophol or indole 3-ethanol (■), and I3C (□). HEWL in the absence of any derivatives is indicated by ◆. The protein concentration was kept at 2 mg·mL^{-1}. For further details, see Scheme 1 and Experimental procedures.

concentration of 2 mg·mL^{-1}, the duration of the nucleation phase was about 15 h, and the system reached its final equilibration phase after about 100 h. As demonstrated by the nature of the curves presented in Fig. 2, all of the known stages of HEWL fibrillation, namely nucleation, elongation and equilibration, seem to be affected by the presence of IAA, I3C and tryptophole, with the final ThT fluorescence being diminished by more than 50%. L-Tryptophan showed no significant inhibitory effect. The indole derivatives were effective

in the order I3C > tryptophol > IAA > indole 3-propionic acid (I3P) > indole > indole 3-aldehyde, as suggested by ThT intensity measurements.

On the basis of these observations, it may be proposed that the indole ring plays the primary inhibitory role and that the side chain groups are responsible for enhancing or diminishing its behavior. The hydroxyl group in the side chain seems to be superior to the carboxyl group with regard to inhibition, and the distance of the functional –OH group from the indole ring may also be relevant. Finally, the lower potency of I3P and tryptophol (with one more methylene group relative to IAA and I3C) may be taken to suggest that hydrophobic interactions involving the side chain do not make a positive contribution to the inhibitory potency of indole derivatives.

Figure 3 shows TEM images of HEWL, incubated for 2 and 7 days at acidic pH and high temperature, in the absence and presence of IAA or I3C. As illustrated in Fig. 3A,D, in the absence of indoles, long thin fibrils about 15–30 nm in diameter appear after 2 days, and thicker fibers of about 100 nm are gradually formed at later stages, probably due to lateral association of preformed thin fibrils. On the other hand, in the presence of IAA (Fig. 3B,E) and I3C (Fig. 3C,F), the formation of fibrillar structures is prominently inhibited and delayed, as indicated by the extensively modified TEM patterns. The thinner HEWL fibrils formed after 1 week in the presence of IAA and I3C may be taken to suggest that these indole derivatives are likely to influence not only the early stages of HEWL fibrillation but also the lateral association of fibrils occurring at later stages. It remains to be seen whether the granular particles observed in TEM images generated upon incubation with indole derivatives constitute certain types of protein assemblies.

Recent evidence suggests that amyloid oligomers, which represent intermediates in the fibril formation process, may be primarily responsible for amyloid pathogenesis, rather than the mature fibrils that accumulate as large aggregates [41]. Accordingly, it is of great importance to determine whether the types of structure formed in the presence of indoles are more toxic products or not.

Possible mechanism of the inhibitory effect of indole derivatives

It is widely believed that in the amyloid-forming systems, the amyloid fibrillation process competes with alternative reactions such as amorphous aggregation or formation of stable off-pathway oligomers [42]. With the various events occurring in the course of amyloid formation of lysozyme (destabilization of the

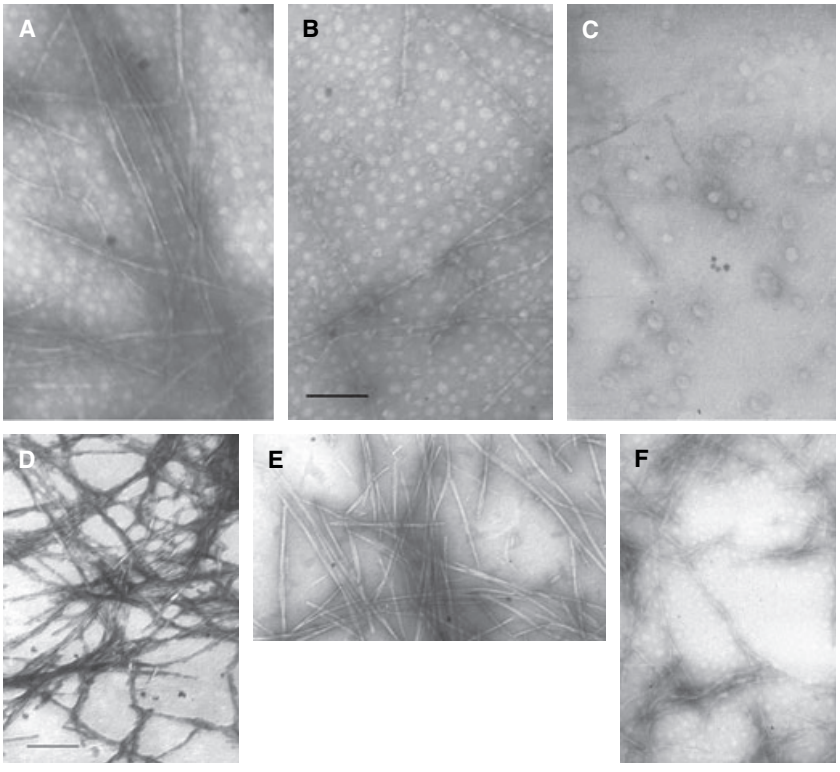
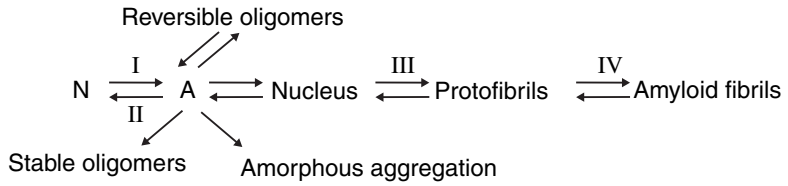


Fig. 3. Overview of HEWL fibrillation by TEM. TEM images of HEWL samples incubated at pH 2.5 and 57 °C for 48 h (A–C) or 1 week (D–F). In (B) and (E), HEWL samples had been treated with 400 μM IAA, and in (C) and (F), HEWL samples had been treated with 400 μM I3C. Scale bars represent 250 nm.



Scheme 2. Schematic representation of various forms of lysozyme aggregation.

native state, nucleation, elongation and lateral accumulation of fibrils), it appears likely that indoles may influence one or more of the steps involved, by delaying the forward reaction and/or promoting the backward reaction (Scheme 2).

Partial unfolding of native protein structures is a critical step in the amyloid aggregation process. Some inhibitors are known to stabilize native protein structures against changes preceding amyloid formation [19,20]. In order to investigate the binding site of the ligands and further explain their behavior, the docking module of the MOE program was used, and a putative binding site was found in the vicinity of the HEWL active site. As shown in Fig. 4, this binding site is delineated on one side by hydrophobic residues (Trp62, Trp63, Ile98, Ala107, and Trp108). On the other side, Glu35 and Asp52, which have critical roles in the catalytic activity of the enzyme [43], are positioned at the cleft entrance. Docking results show that indole interacts mainly with the tryptophan residues and the other hydrophobic residues via Van der Waals interactions, while the presence of a derivative on the indole ring positions the ligand side chain toward the acidic residues and allows hydrogen bonding in addition to van der Waals interactions (see one possible binding mode of carbinol in Fig. 4).

Some indole derivatives are known to bind native HEWL [39]. Owing to the presence of a binding site in native HEWL for indole derivatives, it is reasonable to propose that these compounds exert their antiamyloid effects through possible preferential binding to the native state, hence protecting it from conformational changes.

To investigate whether secondary structural changes of HEWL brought about by the denaturing conditions could be protected by indoles, far-UV CD spectra of the protein were obtained in the absence and presence of I3C and IAA. As indicated in Fig. 5, the far-UV CD spectrum of HEWL, after incubation under the above-mentioned conditions, changes from having peaks at 208 and 222 nm, characteristic of the α-helical structure of the native protein, to having a deep peak at 217 nm, as expected for intermolecular β-structures. Neither I3C nor IAA afforded complete protection, although the characteristic change of the spectrum, namely the appearance of a large negative peak around 217 nm, was delayed to some later stages in the presence of IAA and especially I3C. In the case of I3C, the far-UV CD spectrum remained rich in α-helical structure for longer, and the maximal alteration of CD spectra in favor of β-structure formation was postponed to about 60 h (data not shown).

Regarding possible tertiary structural changes that may take place prior to fibrillation, it is a well-established fact that low pH induces formation of molten globule-like structures in HEWL, characterized by

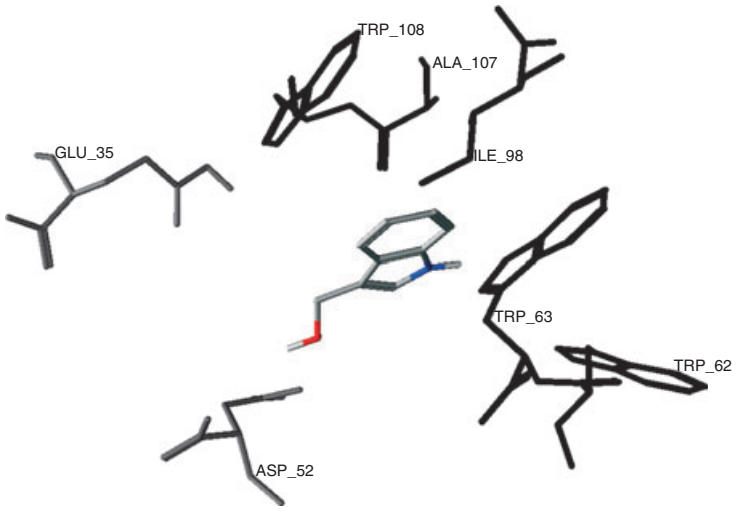


Fig. 4. A docking pose obtained for carbinol. This was obtained in the putative binding site of HEWL indole-derived ligands. The indole moiety of carbinol interacts with hydrophobic residues, while the side chain is positioned towards one catalytic residue. Only the essential hydrogens of carbinol are shown. The acidic residues are represented in a lighter shade.

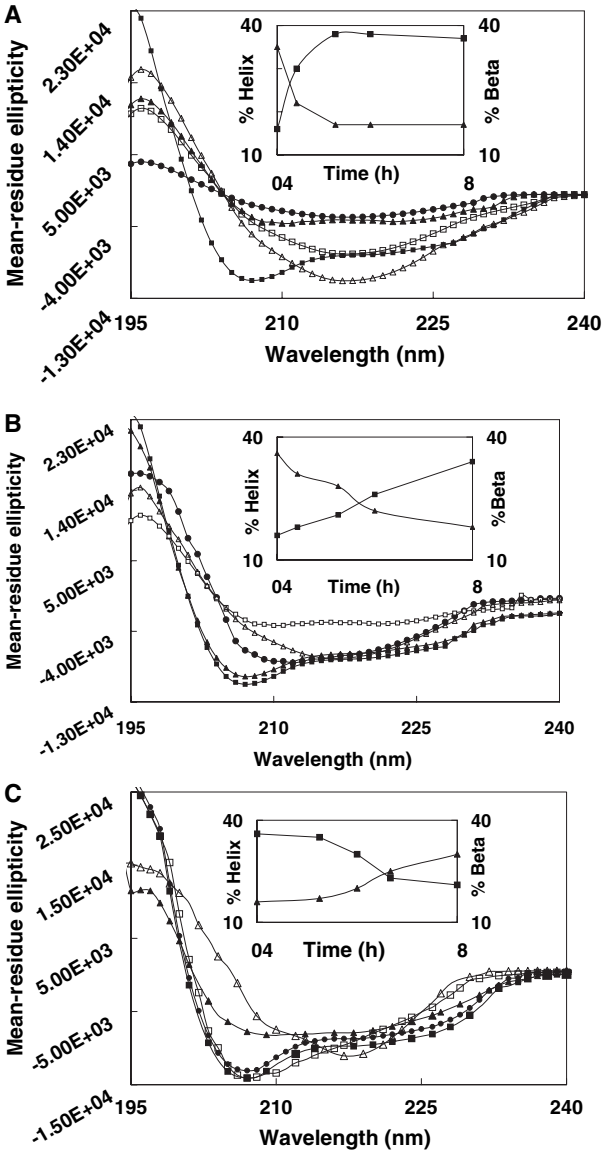


Fig. 5. Far-UV CD spectra. The spectra represent HEWL incubation for periods of 0 h (■), 5 h (▲), 15 h (●), 24 h (□) and 48 h (△) in the absence (A) or presence (B) of 400 μM IAA. In (C), the spectra of HEWL incubated with 400 μM I3C, obtained after 0 h (■),15 h (●), 24 h (□), 36 h (▲) and 48 h (△), are presented. The inset illustrates changes occurring in HEWL secondary structure, showing the contents of α-helix (▲) and β-sheet (■), during the fibrillation process. The units of the *y*-axis are degree·cm^2·dmol^{-1}.

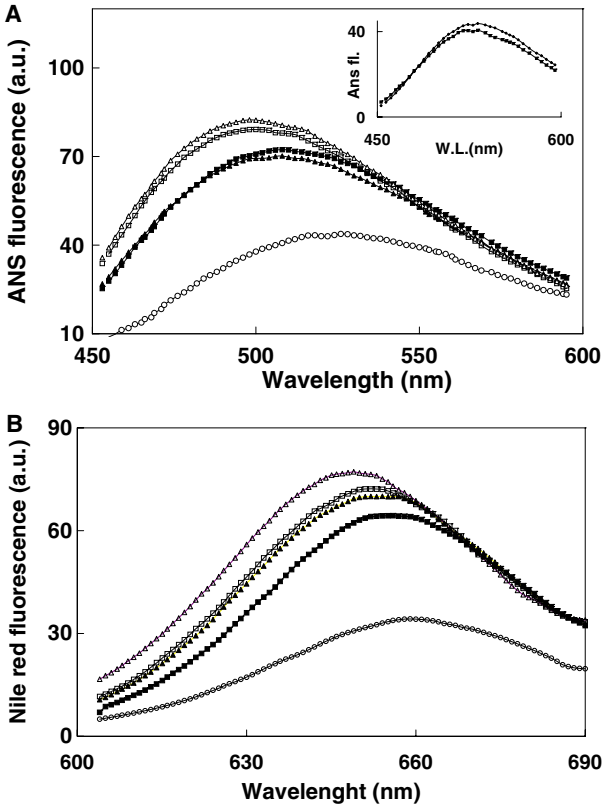


Fig. 6. Effect of IAA on the surface hydrophobicity of HEWL. Changes in ANS (A) or Nile red (B) emission spectra obtained in the presence of HEWL when incubated at pH 2.5 and 57 °C with 0 μM (□), 100 μM (▲), 400 μM (□) and 800 μM (△) IAA. Background ANS and Nile red fluorescence recorded in the absence of the protein are also presented (○). The inset shows the changes in the ANS fluorescence emission spectrum after treatment with native HEWL. Further details are given in Experimental procedures.

exposure of relatively mobile hydrophobic patches on the protein surface [44]. Fluorescence measurements involving 8-anilinonaphthalene-sulfonate (ANS) and Nile red, two hydrophobic reporter probes, revealed exposure of large hydrophobic patches on the surface of the protein molecule in the amyloid aggregation-prone state (Fig. 6). As shown in Fig. 6, the presence of IAA did not prevent exposure of hydrophobic patches but instead accentuated it. Taking these findings together, it seems quite unlikely that indole derivatives inhibit HEWL amyloid fibril formation by protecting the native protein from structural changes.

If, as shown by the observations discussed above, indoles cannot protect HEWL against secondary and tertiary structural changes, then what is the mechanism of their action?

As the relative inhibitory effect of IAA on amyloid formation clearly decreases when the total HEWL concentration is increased (Fig. 7), IAA is likely to have its influence on a step involving an association reaction. If IAA acted on a uni–uni reaction (with single reactant and product molecules) in the HEWL amyloid fibrilization process, its inhibitory effect would be expected to be independent of HEWL concentration.

Promotion of the formation of the large amorphous aggregates cannot be the mechanism of action, simply

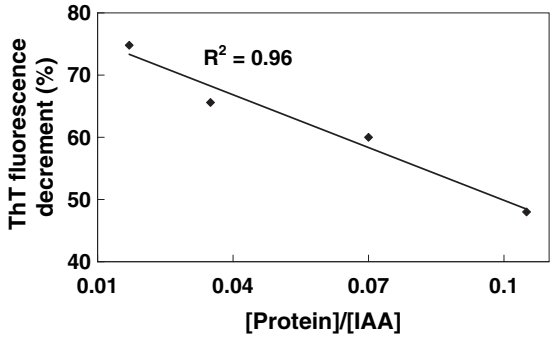


Fig. 7. The influence of total protein concentration on the inhibitory effect of IAA on HEWL fibrillation. This was measured by monitoring the ThT fluorescence emission decrement observed after 48 h of incubation in the presence of 400 μM IAA, as compared to that found in its absence.

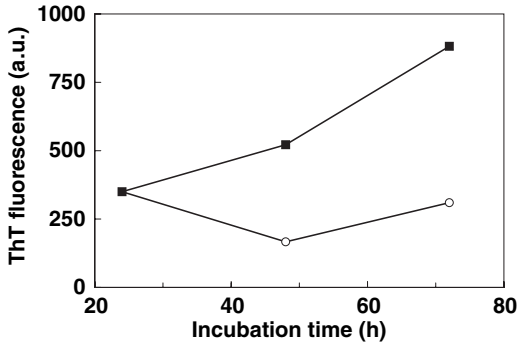


Fig. 8. Effect of IAA on HEWL amyloid fibrils added after the nucleation step. IAA at 400 μM (○) or the equivalent volume of buffer (■) were added to HEWL samples incubated for 20 h, at pH 2.5 and 57 °C. ThT fluorescence changes were then monitored over an approximately 3 day incubation in the same condition.

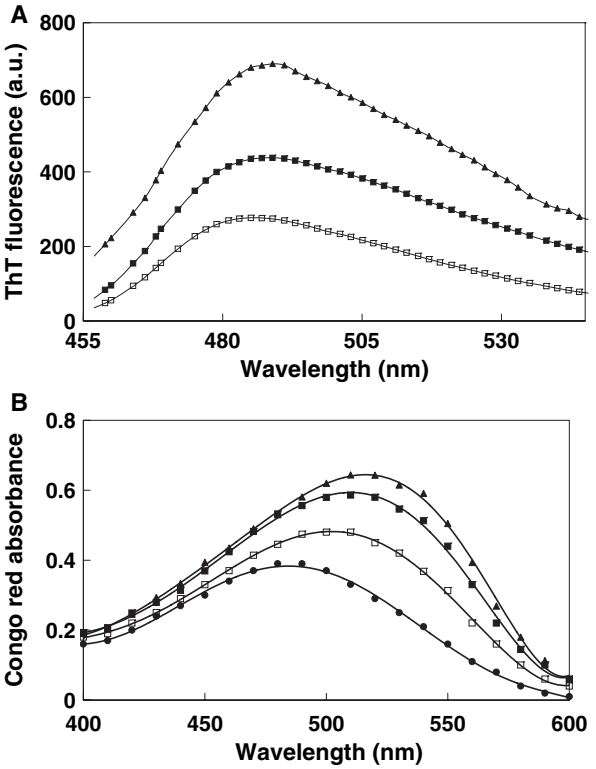


Fig. 9. Effect of I3C on preformed HEWL amyloid fibrils. ThT fluorescence emission (A) or Congo red absorbance (B) measured in the presence of preformed HEWL fibril suspensions, before (▲) and after (□) addition of 400 μM I3C or an equivalent volume of buffer (■) to HEWL fibril suspensions and 20 h of incubation at pH 2.5 and 25 °C with no agitation. The absorbance spectrum of free Congo red is also provided in (B) (●).

because no clear turbidity appeared when indole compounds were present in the system. The fact that the nucleation phase of amyloid formation was prolonged by the indole derivatives (Fig. 2) suggested the possibility of these compounds acting as inhibitors at this stage of the process. However, indoles were active against HEWL amyloid formation even when they were added after the nucleation phase (Fig. 8), making this proposition unlikely.

In the course of these observations, it appeared likely to us that inhibition of HEWL amyloid fibrillation was occurring with a concomitant enhancement of disaggregation. To test this possibility, the HEWL samples that had been incubated for 3 days in the amyloidogenic conditions were cooled to 25 °C, a temperature at which the disaggregation process was expected to proceed. As shown in Fig. 9, ThT emission intensity was significantly decreased after 20 h of incubation at this lower temperature, in accordance with previous observations [15,33]. Addition of I3C was found to significantly increase the instability of fibrils, enhancing the drop in ThT emission and Congo red absorbance (Fig. 9A,B). Similar results were obtained when IAA was added (data not shown). These findings may suggest that indoles destabilize the late amyloid fibrillar state of HEWL and accelerate its disaggregation. As a significant drop of ThT fluorescence intensity was also observed when IAA was added after the nucleation phase (Fig. 8), the indoles appear to be effective in destabilizing the early stages of the amyloid fibrillation process.

Effects of alcoholic cosolvents and salts on inhibition by indoles

The possible role of hydrophobic and electrostatic interactions in the inhibition of amyloid fibrillation afforded by indoles was further assessed by addition of alcoholic cosolvents (10% v/v) to the amyloid-forming

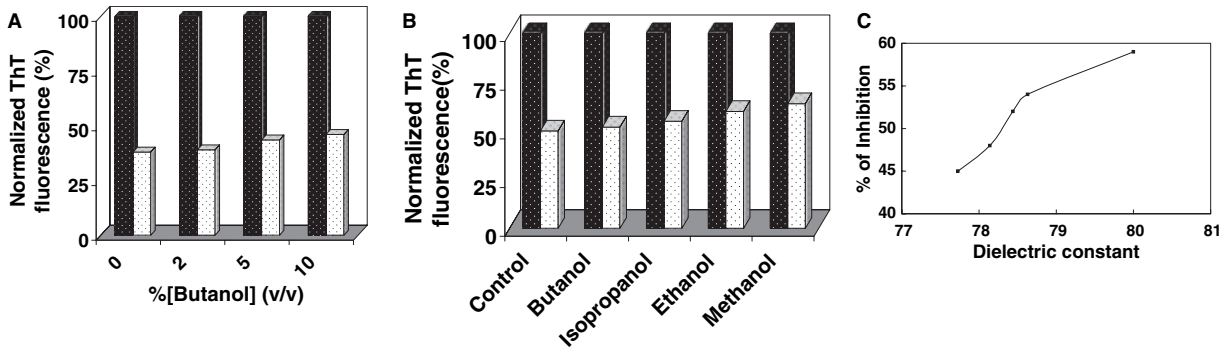


Fig. 10. Effect of the presence of alcohols on the inhibitory effect of I3C and IAA. (A) Normalized ThT emission of HEWL samples incubated for 48 h in the absence (■) or presence (▨) of I3C at various v/v concentrations of butanol. (B) Normalized ThT emission of samples incubated for 48 h in the absence (■) or presence (▨) of IAA at 10% v/v concentration of various organic solvents. (C) Presentation of the effects observed in (B), in terms of changes in the dielectric constant of the medium.

system, thereby modifying the dielectric coefficient of the medium. The final ThT fluorescence intensities observed in the presence of HEWL, alcoholic cosolvent and the indoles, and those obtained in the absence of the indoles, were compared. As shown in Fig. 10A, inhibition exerted by I3C was diminished by butanol in a concentration-dependent manner, so that at 10% butanol, the amount of I3C-mediated inhibition was decreased by about 12%. Other cosolvents, including methanol, ethanol, and isopropanol, were also found to be effective in lowering the inhibitory influence of indoles (Fig. 10B). Lowering the dielectric constant by addition of the alcohols seemed to be the main determining factor, irrespective of the type of alcohol used. Accordingly, methanol and isopropanol behaved identically when added at amounts such that the dielectric constant was set at the same value (data not shown). The percentage of IAA-mediated inhibition of HEWL fibrillation was found to gradually decrease when the dielectric constant of the medium was slightly diminished through addition of various cosolvents (Fig. 10C). As a decrease in dielectric constant would be expected to strengthen electrostatic interactions and simultaneously weaken hydrophobic interactions, the observed effects of alcohols (Fig. 10A–C) may be taken to suggest that hydrophobic interactions favor the inhibition mechanism of indoles, whereas electrostatic interactions are likely to have an opposite effect.

The effect of various anions on inhibition by IAA was also examined. Of the anions studied, SCN^-, and even more remarkably, SO_4^{2-} weakened the IAA inhibitory effect, but Cl^- was ineffective (Fig. 11). This finding cannot be attributed to the simple Debye–Huckel screening effect, as a clear dependence on anion type is observed. Furthermore, it cannot be caused by the

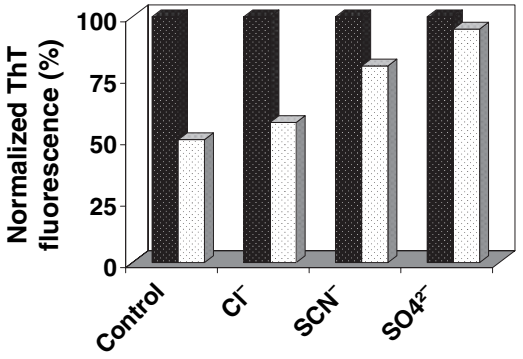


Fig. 11. Effect of salts on the inhibitory effect of IAA. Normalized ThT emission of samples incubated for 48 h in the absence (■) or presence (▨) of IAA. Concentrations used were: 20 μM for Na_2SO_4, 75 μM for NaSCN, and 150 μM for NaCl.

anion impact on the solvent structure, as both the kosmotropic (SO_4^{2-}) and the chaotropic (SCN^-) anions brought about changes in the same direction. Alternatively, this observation may be explained on the basis of specific binding affinities of anions for various HEWL species.

Conclusion

In the present study, the inhibitory effects of several indole derivatives on amyloid fibrillation of HEWL are reported. It is suggested that the indole ring is likely to play the main role, with the side chain hydroxyl group contributing positively, in contrast to the side chain carbonyl and intervening methylene groups. It remains to be seen whether the species generated upon incubation with indole derivatives are less or more toxic to the living cell than the amyloid fibrils formed in the absence of such inhibitors.

The results presented may be useful for gaining a deeper insight into possible mechanisms of amyloid fibrillation inhibition by indole derivatives and may provide useful guidelines in relation to screening for novel inhibitors.

Experimental procedures

Materials

HEWL (EC 3.2.1.17), ThT, Congo red, Nile red and indole derivatives, including IAA, I3P, indole 3-carbinol, L-tryptophan, *N*-acetyl-L-tryptophan and tryptophol, were purchased from Sigma (St Louis, MO, USA). ANS, indole, indole 3-aldehyde and all salts and organic solvents were obtained from Merck (Darmstadt, Germany). Protein concentration was determined spectrophotometrically at 280 nm, using an extinction coefficient of 2.65 $L \cdot g^{-1} \cdot cm^{-1}$. Stock solutions of all indole derivatives were prepared at 40 mM, using a 1% (v/v) dimethylsulfoxide/water mixture as solvent. The final concentration of indole derivatives was fixed at 0.4 mM.

Amyloid preparation

Lysozyme was dissolved at 2 $mg \cdot mL^{-1}$ in 0.1 M glycine buffer (pH 2.5), and then incubated at 57 °C for the specified durations while it was being stirred gently by Teflon magnetic bars.

Amyloid-specific dye-binding assays

All fluorescence experiments were carried out on a Cary Eclipse VARIAN fluorescence spectrophotometer (Mulgrave, Australia) at room temperature. To investigate whether HEWL was converted to the amyloid fibrils, 10 μL of HEWL samples (2 $mg \cdot mL^{-1}$) were added to 900 μL of 13 μM ThT solution (from 2.5 mM ThT stock solution in 10 mM sodium phosphate, 150 mM NaCl, pH 7.0, passed through a 0.45 μm filter paper), mixed thoroughly, and incubated for 5 min. Fluorescence emission spectra were then taken using excitation at 440 nm. The excitation and emission slit widths were set as 5 nm and 10 nm, respectively [45].

Congo red absorbance assays

Congo red was dissolved at 7 $mg \cdot mL^{-1}$ in a buffer consisting of 0.15 M NaCl and 5 mM potassium phosphate (pH 7.4). The Congo red solution was then filtered using a center-glass N4 filter. Five microliters of well-mixed incubation sample was added to 300 μL of the Congo red solution and incubated for 30 min. Absorbance spectra were recorded (400–600 nm) using a Shimadzu UV–visible spectrophotometer (Kyoto, Japan) [46].

CD

CD spectra in the far-UV region (190–260 nm) were obtained on an AVIV 215 spectropolarimeter (Aviv Associates, Lakewood, NJ, USA), using a 1 mm path cell at room temperature. The protein concentration was 0.1 $mg \cdot mL^{-1}$.

TEM

Ten microliters of HEWL samples were withdrawn and put on a copper 400 mesh grid, which had been covered with carbon-coated formvar film. After 2 min, excess fluid was drawn out using a paper filter, and 1% uranyl acetate added. After another 2 min, excess dye was removed. Finally, the grids were viewed with a CEM 902A Zeiss microscope (Oberkochen, Germany).

ANS fluorescence assays

A stock solution of ANS was prepared at 0.1 M, using pure ethanol as the solvent. The final concentrations of ANS and protein were 100 μM and 0.02 $mg \cdot mL^{-1}$, respectively. The excitation wavelength was 365 nm and emission spectra were obtained at 450–600 nm. Excitation and emission slit widths were both set at 5 nm.

Nile red fluorescence assays

The experimental procedure was as reported earlier [47]. The excitation wavelength was 550 nm and emission spectra were recorded between 600 and 700 nm. Excitation and emission slit widths were both set at 5 nm.

Docking studies

The software used was MOE 2006.08 from the Chemical Computing Group (Quebec, Canada). The Docking module of the software was used, with use of the Alpha triangle placement method and the affinity dG scoring function. The picture presented was generated using the ligand interaction computing module of MOE.

Acknowledgements

This work was supported by a grant from the Iranian National Science Foundation (INSF). We thank the Laboratory of Microscopy of College of Science, University of Tehran. Technical assistance, kindly provided by Ms A. Ghasemi, is gratefully acknowledged.

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