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Water-Soluble Tripeptide Aβ (9−11) Forms Amyloid-Like Fibrils and Exhibits Neurotoxicity

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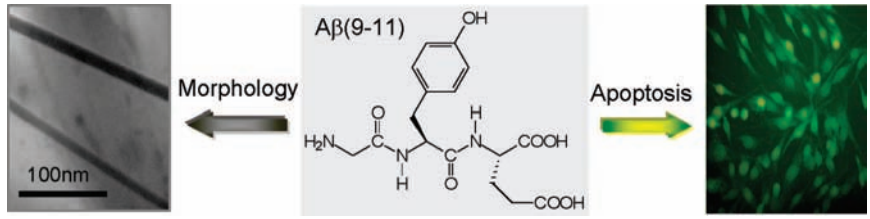
ABSTRACT



A water-soluble, hydrophilic tripeptide GYE, having sequence identity with the N-terminal segment of amyloid peptides Aβ(9-11), upon self-association exhibits amyloid-like fibrils and significant neurotoxicity towards the Neuro2A cell line. However, the tripeptides GFE and GWE, in which the centrally located tyrosine residue has been replaced by phenylalanine or tryptophan, fail to show amyloidogenic behavior and exhibit little or no neurotoxicity.

The aggregation of normally soluble proteins into well-ordered amyloid fibrils is associated with a number of diseases including Alzheimer's disease, Parkinson's disease, type II diabetes, prion-related diseases, and others.[1] Among these diseases, Alzheimer's disease (AD) is the most prevalent and progressive neurodegenerative disease associated with deposition of β-sheet-rich protein aggregates in specific regions of the human brain as amyloid fibrils,[2] which consist mainly of amyloid peptides like Aβ(1-40) and Aβ(1-42).[3] Aβ peptides are generated from highly regulated and sequential cleavage of the amyloid precursor protein (APP) by proteases designated as β- and γ-secretases and are readily detected in human CSF[4] as a range of isoforms between 38 and 43 amino acids in length. They normally exist as soluble random coils. However, in a diseased condition they misfold and form self-assembled oligomers which further self-associate to form amyloid fibrils.[5] The molecular structure of full-length Aβ fibrils is still not completely clear because of the difficulty of growing good quality crystals that can diffract well enough to obtain crystal structures. Low solubility and noncrystallinity of amyloid fibrils obtained from full-length Aβ peptide preclude the possibility of getting

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good crystals. Determination of the molecular basis of recognition and self-assembly process that govern amyloid fibril formation is therefore a very challenging task. So, short model peptides, which provide valuable information about the factors responsible for amyloid formation are of crucial importance.

Westermark and co-workers have carried out pioneering work on the use of model peptides for the study of amyloid fibril formation.[6] Serranno et al. have shown the residue-specific tendency for amyloid fibrilation in a series of model hexapeptides.[7] Gazit et al. have illustrated the self-aggregation of short peptide fragments into amyloid fibrils and established the role of an aromatic residue (phenylalanine) in amyloidosis.[8] Serpell et al. have described the self-association of an amyloid-forming 12-residue peptide in the solid state, and the structure illustrates the molecular arrangement of the amyloid fibril forming peptide in crystals showing a tentative model for side-chain packing within the amyloid fiber.[9] Mihara et al. have recently reported that a series of designed short peptides with various hydrophobicities based on the sequence of Aβ(14-23) can form amyloid-like fibrils effectively by using mature Aβ(1-42) fibrils as nuclei.[10] Recent studies have also demonstrated that the fundamental unit of amyloid-like fibrils is a steric zipper formed by two tightly interdigited β-sheets.[11]

Our group has been engaged in studying the self-assembly of short model peptides which form supramolecular β-sheets and amyloid-like fibrils upon self-association.[12] It has been found that C-terminal portion of the full length Aβ peptide has a definitive role in amyloid fibril formation.[13] So, there is a need to test whether any fragment from N-terminal hydrophilic region can from amyloid-like fibrils or not. Gazit and his co-workers have made a seminal contribution in the self-assembly of very short peptide unit Phe−Phe.[14] In this report, we present the self-assembly of the water soluble tripeptide having sequence identity with the N-terminal segment of Aβ peptide Aβ(9-11), GYE, peptide **1**, which forms intermolecularly hydrogen-bonded supramolecular β-sheet structure in crystals and in solution. It also forms straight, unbranched nanofibrils that exhibit amyloid-like behavior, and these fibrils are toxic towards the Neuro 2A cell line. We have also synthesized two mutant tripeptides in which the centrally positioned amino acid residue has been substituted by other proteinous aromatic amino acid phenaylalanine (Phe) or tryptophan (Trp) to examine whether these tripeptides can form amyloid-like fibrils and exhibit neurotoxicity. Figure 1 shows chemical structures of reported peptides **1**, **2**, and **3**.

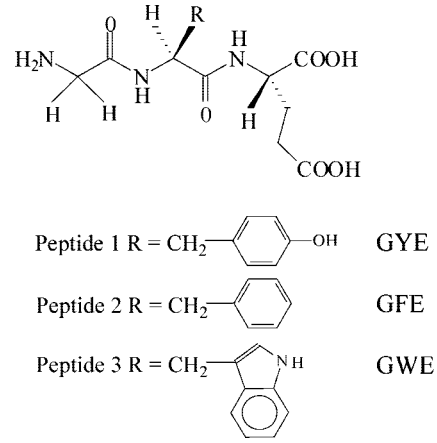

Figure 1. Chemical structures of peptides **1**, **2**, and **3**.

FT-IR spectroscopic studies were carried out to obtain structural information in the solid state and in solution. The FT-IR spectra of peptide **1** in the solid state shows a well-defined C═O stretching band (amide I) at 1637.5 cm^{-1} and NH-stretching band at 3280 cm^{-1}, typical of intermolecularly hydrogen-bonded β-sheet structure in the solid state (Figure S8).[15a] Moreover, peptide **1** shows a medium-intensity band at 1678 cm^{-1} indicating the formation of an antiparallel β-sheet structure in the solid state. N−H bending frequencies of this peptide appear at 1515 cm^{-1} suggesting also the formation of a β-sheet structure.[15b] On the other hand, peptides **2** and **3** show a sharp C═O stretching band at 1666.4 and 1670 cm^{-1}, respectively, which clearly indicates that peptides **2** and **3** do not form the β-sheet structure in the solid state.[16] FTIR spectra of solutions contaning peptides **1** and **3**, aged over 7 days, shows a sharp C═O stretching (amide I) band at 1617 and 1620 cm^{-1}, which indicates the H-bonded supramolecular β-sheet conformation in solution (Figure S9). On the other hand, a similarly aged solution of peptide **2** shows a characteristic C═O stretching band at 1653 cm^{-1}, indicating its random coil conformation.[17]

The conformational analysis of peptide **1** in solid state from FT-IR spectroscopy was further supported by a single-crystal X-ray diffraction study.[18] Colorless, needle-shaped

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single crystals of peptide **1**, suitable for X-ray analysis were obtained by slow evaporation at room temperature from water−methanol (95:5) solution (500 mg per 15 mL). The molecular conformation of peptide **1** shows that there are two independent molecules (A and B), present in the asymmetric unit (Figure S10) and upon self-association they from a complex quaternary supramolecular β-sheet structure (Figure S11). Backbone torsion angles (Table S2) of the two molecules present in the asymmetric unit fall mostly within the extended region of the Ramachandran plot.[22] Unfortunately, we were unable to grow crystals of peptides **2** and **3** using a wide range of solvents and thereby preventing the valuable insight on their organization in the solid state.

To test whether the aggregates formed by the peptides are amyloidogenic, we studied the morphologies of the aggregates by TEM. A solution (4×10^{-3} M) of peptide **1** in PBS buffer (10 mM) at pH 7.3 aged over 7 days at 37 °C gave a straight, unbranched, and rodlike nanofibrillar structure with diameters ranging from 10 to 15 nm, thus exhibiting a typical feature of amyloid-like fibrils (Figure 2). Under

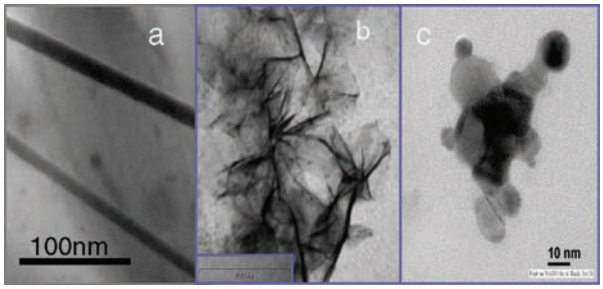

Figure 2. (a−c) TEM images of the aggregates formed by peptides **1**−**3** having been dissolved in PBS buffer (10 mM) at pH 7.3 and incubated at 37 °C for 7 days.

similar conditions, peptide **2** forms only branch fibrillar morphology and peptide **3** failed to show any fibrillar aggregates (straight or branched).

Further Congo Red (CR) and Thioflavin T (ThT) binding assays were performed to check whether these aggregates formed by peptides **1**, **2**, and **3** were amyloidogenic.

CR binds to Aβ fibrils to provide a diagnostic test for amyloid deposits, as the dye-stained plaques appears green in color under cross-polarized light due to birefringence.[23]

Fibrils of peptide **1** bind with CR and exhibit a typical green gold birefringence indicating its amyloid-like nature. By contrast, both peptides **2** and **3** fail to bind with CR under similar conditions (Figure 3).

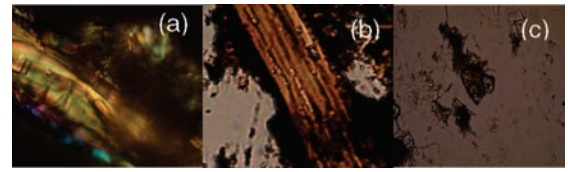

Figure 3. (a−c) CR binding assay of peptides **1**−**3**.

Along with CR, ThT is believed to specifically interact in some unknown way with the cross β-sheet structure of amyloid fibrils, and this gives rise to an excitation (absorption) maxima at 450 nm and enhanced emission at 482 nm as compared to excitation at 385 nm and emission at 445 nm for the free dye molecules of ThT.[24] The aged solution of peptide **1** interacts with ThT and gives rise to an emission at 482 nm (Figure 4) with much higher intensity compared

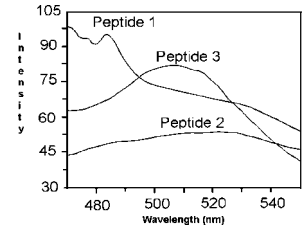

Figure 4. Peptide **1** binds with ThT and gives an emission band at 482 nm with much higher intensity than the mixture of ThT and peptide **2**/**3**.

to the emission maxima of the mixture of ThT and each of the peptides **2** and **3**. It clearly establishes that the aged solution of peptide **1** binds with ThT showing an amyloidogenic property.

Toxicity towards neuronal cells is one of the important characteristics of amyloid fibrils.[25] The toxicity of aged peptides solution on the viability of 96 well cultured neuronal cells (Neuro2A) were investigated by MTT assays as performed earlier.[26] It was found that only aggregates of peptide **1** induced both time- and dose-dependent neuronal cell death. On the other hand, peptide **2** leads only 15% cell

(18) Crystal data: $C_{16.5}H_{25.5}N_3O_{8.75}$, FW = 405.9, triclinic, space group *P*1, a = 9.4682(13) Å, b = 9.6899(15) Å, c = 12.0583(15) Å, α = 109.199(13)°, β = 99.525(11)°, γ = 102.145(12)°, Z = 2, d_{calcd} = 1.365 gm/cm^3, cryst size = 0.02 × 0.02 × 0.23 mm^3. Diffraction data were measured with Mo Kα (λ= 0.71073 Å) radiation at 150 K using an Oxford Diffraction X-Calibur CCD system. Data analysis was carried out with the Crysalis program.[19] The structure was solved by direct methods using the SHELXS-97[20] program. Refinement was carried out with a full matrix least squares method against F^2 using SHELXL-97.[21] The non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atoms were included in geometric positions and given thermal parameters equivalent to 1.2 times those of the atom to which they were attached. The final R values were R = 0.1043 and wR2 0.2931 for 3443 data points with $I > 2\sigma(I)$ for peptide **1**. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre with reference number CCDC 682269.

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death after 48 h of treatment and peptide **3** failed to show any significant neuronal toxicity (Figure 5).

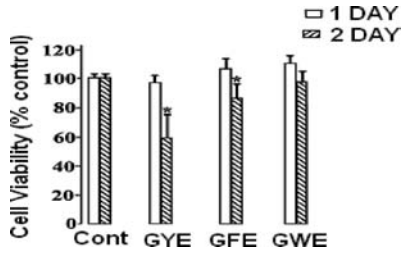

Figure 5. MTT assay for cell viability. Peptide **1** (GYE) shows significant neuronal toxicity and toxicity is reduced in peptide **2** (GFE), whereas peptide **3** (GWE) has insignificant neuronal toxicity. Here the asterisk indicates significant difference ($p < 0.0001$) from untreated control. Assays were performed using 5 mM concentration of each peptide.

Interestingly, the toxicity induced by peptide **1** increased with the dose of the compound (Figure 6) and at 0.014 M concentrations, almost all the cells died. Further characterization of the nature of cytotoxicity induced by peptide **1** using terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay suggested that the cell death was due to apoptosis (Table 1).

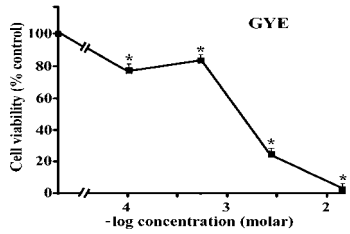

Figure 6. Dose response curve of peptide **1** (GYE) on cell viability determined by MTT assay. Neuro 2A cells were treated for 48 h in the presence of different concentration of peptide **1**. The asterisk indicates $p < 0.0001$ from untreated controls.

Table 1. Effect of Peptide **1** on Apoptosis of Neuro 2A Cells Using TUNEL Assay[a]

treatment	% of apoptotic cells
control	5.47 ± 1.17
peptide **1**	88.79 ± 2.80*

[a] Apoptotic cells were positive for both fluorescein−dUTP and propidium iodide. The percentage of apoptotic cells in each group were expressed as percentage of total number of cells which is represented as 100%. Three fields were selected randomly for each experiment, and both apoptotic and total number of cells were counted. Values are mean ± S.E.M. of three experiments. The asterisk indicates $p < 0.0001$ from untreated controls.

This study has demonstrated that a water-soluble tripeptide comprised of N-terminal hydrophilic region of Aβ peptide Aβ(9-11), GYE self-assembles to form supramolecular β-sheet structure and amyloid-like fibrils that exhibit toxicity towards Neuro2A cell lines. However, replacement of the centrally located amino acid residue (Tyrosine) by phenylalanine (Phe) or tryptophan (Trp) abolishes amyloidogenic behavior and toxicity of peptide **2** has been reduced whereas peptide **3** exhibit insignificant neurotoxocity. The result highlights on the self-aggregation behavior, formation of β-sheet structure, and amyloid-like nature of a small hydrophilic segment of Aβ peptide.

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Supporting Information Available: Synthetic and experimental procedures, ^{1}H NMR, mass, and FTIR spectra for all new compounds, crystal data for peptide **1** in CIF format, dose-dependent assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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