A natural heptameric peptide exhibits multifaceted inhibitory role in the fibrillation pathway of amyloid-beta

Running title: *Anti-amyloidogenic natural peptide against Aβ fibrillation*

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Abstract

Background and Purpose

Alzheimer's disease (AD) pathogenesis involves amyloid-β (Aβ) aggregation where the intermediate oligomers are considered the prime toxic species. Here, we aimed to identify an effective peptide sequence from a medicinal plant-derived enzyme having anti-amyloidogenic properties against Aβ.

Experimental Approach

LC-MS/MS followed by computational analysis identified the active peptide (termed here as P_{active}). Visualization techniques along with biophysical and biochemical approaches were used to determine the anti-amyloidogenic potency of the purified enzyme and peptides identified from the enzyme. Cytotoxicity was measured on SHSY-5Y cell lines. Interaction studies were done with bio-layer interferometry (BLI) and bio-stability of the peptide was assessed by NMR. P_{active} induced conformational alterations of Aβ monomer and oligomers was determined with DSC and NMR.

Key Results

A small heptameric peptide (P_{active}) identified form a medicinal plant-derived fibrinolytic enzyme proved to be a multifunctional inhibitor against Aβ aggregation. The results suggested that P_{active} arrests Aβ molecules in non-toxic off-pathway oligomers that can no longer participate in the cytotoxic fibrillation pathway. Mechanistically, P_{active} binding induces conformational alterations in the Aβ molecule, thus modulating its hydrophobicity, one of the key players in inducing aggregation.

Conclusions and Implications

The study identified a peptide P_{active} (GFLLHQK) of natural origin that displays potential anti-amyloidogenic properties against Aβ aggregation. The bio-stability of P_{active} in human blood

serum as well as its non-toxic nature makes it a promising therapeutic candidate against Alzheimer's, for which no disease-modifying treatments are available till date.

Keywords: Alzheimer disease; Amyloid-β; Aggregation; Peptide inhibitor; Off-pathway oligomers; Cytotoxicity; Conformational alteration.

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; EDPs, Enzym-derived peptides; ThT, Thioflavin T; CR, Congo red; ANS, 8-Anilinonaphthalene-1-sulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEC-HPLC, size exclusion HPLC; AFM, Atomic force microscopy; TEM, transmission electron microscopy; CD, Circular Dichroism; BLI, Bio-layer interferometry; DSC, Differential scanning calorimetry.

What is already known

- AD is the most common neurodegenerative disease with no available disease modifying therapies.
- Aβ fibrillation is the hallmark of AD where oligomers are considered primary toxic agents.

What this study adds

- A nontoxic, bio-stable, natural hepta-peptide (P_{active}) exhibits remarkable potential against Aβ fibrillation and cytotoxicity.
- P_{active} binding induces conformational alterations in Aβ, modifies its hydrophobicity, thus reduces aggregation propensity.

Clinical significance

- Impairing the fibrillation process by sequestering Aβ in non-toxic oligomers is an attractive therapeutic approach.

- The study offers a prospective anti-amyloidogenic peptide candidate for future drug designing against AD.

1 INTRODUCTION

Aggregation of Aβ and consequent neurodegeneration in the brain are the neuropathological hallmarks of AD (Alzheimer *et al.*, 1995; Ross *et al.*, 2004; Irvine *et al.*, 2008). Following primary nucleation, Aβ aggregation proceeds via formation of intermediate oligomers and protofibrils which then intertwine into mature fibrils and eventually form plaques (Lee *et al.*, 2011). Early detection of AD being inefficient, preventive measures cannot be implemented at the onset, while currently available treatments include limited non-specific approaches that only help to alleviate the symptoms, without interfering with the root cause of disease progression (Perrin *et al.*, 2009; Graham *et al.*, 2017). Elucidation of the aggregation pathway highlighting oligomers as the drivers of neurodegeneration (Lesne *et al.*, 2006; Sakono *et al.*, 2010; Benilova *et al.*, 2012), has shifted the research focus towards the development of agents that will inhibit the neurotoxic effects of these species. Hence, treatment of AD requires a multifunctional molecule that will not only serve as an anti-aggregation and fibril destabilization agent but also attenuate oligomer toxicity.

Aristolochia indica, a medicinal plant of the Indian subcontinent is used for the ailment of various diseases, with reports indicating its potential against certain amyloids (Bhattacharjee *et al.*, 2013; Bhattacharjee *et al.*, 2015).

Here, we report a fibrinolytic enzyme isolated from the roots of *A. indica* having potential Aβ aggregation-inhibition as well as disaggregation properties. Interestingly, the efficacy of the inactivated enzyme eliminated the possibility of proteolytic activity behind the anti-amyloid property. Further, enzyme-induced changes in hydrophobicity of Aβ suggested the involvement of specific interacting portions of the enzyme in the process. However, the size of

the isolated enzyme (13.6 kDa) poses limitation to its use as a drug molecule. Instead of proteins (comparatively larger in size), small peptides are currently being considered possible drug molecules against AD due to their relatively easier delivery along with other advantages of increased stability and lower chance of interaction with the immune system (Ladner *et al.*, 2004; Berthoumieu *et al.*, 2015; Oller-Salvia *et al.*, 2016; Baig *et al.*, 2018a; Baig *et al.*, 2018b; Ribaric, 2018).

Hence, we aimed to bring down the study to peptide level instead of reporting the enzyme as the inhibitor molecule. LC-MS/MS analysis followed by computational screening of the proteolytically digested enzyme identified two sequences. Chemically synthesized version of one of the peptides (termed here as P_{active}) displayed remarkable anti-amyloidogenicity against Aβ, sequestering toxic oligomers and fibrils in non-toxic off-pathway forms. In contrast, the other peptide (termed as P_1) enhanced the aggregation process. Further investigation to elucidate the mechanism revealed P_{active}-induced modifications of hydrophobicity along with conformational alterations of Aβ. We have also shown that P_{active} is not only non-toxic but also reasonably stable in human blood serum suggesting that the peptide can be a potential candidate for designing therapeutics against AD.

2 METHODS

2.1 Extraction and isolation

For extraction, roots (100 mg/ml) were kept overnight in sodium phosphate buffer at 4˚C. Roots were cut, macerated and centrifuged. The supernatant served as the extract.

Isolation of the fibrinolytic enzyme was achieved with substrate affinity chromatography (fibrinogen-coupled Sepharose CL-6B, Sigma-Aldrich, USA) followed by SDS-PAGE. Further purification involved size exclusion HPLC (SE-HPLC, Waters) with Waters Protein-Pak 300 column pre-equilibrated with 10 mM sodium phosphate buffer containing 0.1 M NaCl

operated at 0.8 ml/min. HPLC was performed on Waters 600 HPLC system with Waters 2487 Dual λ Absorbance detector. A calibration curve was constructed using marker proteins and a linear dependence of log Mw (molecular weight) versus retention time was observed (R^2 = 0.9755 where 'R^2' is the regression coefficient). The approximate molecular weight of the enzyme was calculated from the corresponding retention time that revealed a highest (major) peak corresponding to 13.6 kDa (Fig. S1, indicated by area within dashed lines). Purification was further confirmed with fibrinogen zymography (Leber *et al.*, 1997).

2.2 Enzyme inactivation by thermal denaturation

Inactivation of the proteolytic activity of the enzyme was done by heat denaturation at 100°C for 2 h in a water bath and verified with fibrinogen zymography (Leber *et al.*, 1997).

2.3 Tryptic digestion of the inactive enzyme

The inactivated enzyme was digested overnight at 37°C with trypsin and chymotrypsin (enzyme:trypsin/chymotrypsin - 50:1 wt/wt) followed by separation with Sephadex G-10 gel filtration column (GE Healthcare Life Sciences). The peptide pool was characterized by Q-TOF ESI XENO XS mass spectrometry to identify the molecular weights of the digested separated fragments (abbreviated here as EDPs, enzyme-digested peptides) as well as to identify for any residual undigested enzyme.

2.4 Zymography

Zymography was performed with the purified active and inactive enzyme in 10% PAGE co-polymerized with fibrinogen (10 mg/ml). After electrophoresis, SDS was removed by incubating the gel in 2.5% Triton X-100 for 1 h at 25°C under gentle shaking, followed by overnight incubation in 50 mM Tris-HCl (pH - 7.5), 0.02 mM $CaCl_2$ at 37°C. The gels were stained with coomassie blue and destained, which revealed sites of proteolysis as white bands against dark blue background (Leber *et al.*, 1997).

2.5 LC-MS/MS analysis of EDPs

Three different sets of the EDPs were analysed using ThermoFisher Scientific LTQ-Orbitrap Mass Spectrometer with integrated Easy nLC-1000 operating at a 40 mins acetonitrile/water elution gradient conducted at 300 nl/min. MS/MS data were searched with Mascot Matrix Science Server (Perkins *et al.*, 1999) using the following parameters –

Type of search – MS/MS ion search, Database – SwissProt, Taxonomy – all entries, Enzyme - Trypsin/Chymotrypsin/TrypChymo, Mass value - monoisotropic, Protein mass – unrestricted, Peptide mass tolerance - ± 10 ppm, Fragment mass tolerance - ± 0.5 Da, Max missed cleavage – 1/2.

Analysis of the three sets of the EDPs identified four peptide sequences with molecular weight <1kDa (Table S1). Upon computational screening, only two sequences of the four were predicted to bind $A\beta_{42}$ (PDB: 1IYT, (Crescenzi *et al.*, 2002) model 1) with high propensity, as indicated by p-values from PepSite 2.2.20 (Trabuco *et al.*, 2012) web server (Table S1).

2.6 Solid phase synthesis and RP-HPLC purification of the identified peptides (P_1 and P_{active})

The identified peptides were manually synthesized in solid phase (Chakraborty *et al.*, 2017) on a 0.05 mmol scale using Rink amide MBHA resin (mesh size - 0.58) and Fmoc-protected amino acids. Peptides were cleaved from the resin using a cocktail of trifluoroacetic acid (TFA) (81%), phenol (5%), thioanisole (5%), 1, 2-ethanedithiol (2.5%), dimethyl sulphide (2.5%) and water (3%), followed by precipitation with chilled ether and washed four times with the same. The precipitate was then dried overnight and dissolved in water.

The crude peptides were then purified using a C-18 (ThermoFisher) reverse-phase HPLC (RP-HPLC) column with a 60 mins linear gradient of water/acetonitrile containing 0.1% TFA conducted at 1 ml/min. Pure peptides were then lyophilized and stored at -20°C until further use. The molecular weight and purity of the synthesized peptides was verified with MALDI Mass spectroscopy with 4800 MALDI TOF/TOF analyzer (Applied Biosystems MDA SCIEX) and ^{19}F NMR spectroscopy.

For further experiments, the HPLC purified lyophilized peptides were re-dissolved in desired buffer (PBS) and evaluated for their efficiency in disaggregating and inhibiting Aβ aggregation.

2.7 *$A\beta_{40}$ and $A\beta_{42}$ aggregation inhibition and destabilization assay*

Aβ peptides were dissolved in HFIP to 1.0 mg/ml ($A\beta_{40}$) and 5.0 mg/ml ($A\beta_{42}$). Thereafter, the solutions were lyophilized by vacuum-freeze drying and finally stored at -20°C. Immediately prior to use, the HFIP-treated Aβ was re-dissolved in DMSO to make the final concentration of 5 mM and sonicated for 10 mins to remove any pre-existing aggregates (Barghorn *et al.*, 2005). For fibrillation, $A\beta_{40}$ solution was diluted with PBS (pH - 7.5) containing 0.2% SDS leading to final $A\beta_{40}$ concentration of 100 µM and incubated at 37°C for 7 days (Barghorn *et al.*, 2005). $A\beta_{42}$ fibrils were formed by incubating $A\beta_{42}$ (100 µM) in PBS at 37°C for 48 h under shaking conditions (Aran Terol *et al.*, 2015).

For inhibition experiments, monomeric Aβ (100µM) was incubated in presence of active/inactive enzyme or EDPs (2.5µg/ml) or the identified peptides ($A\beta_{40}$:Peptide - 10:1; $A\beta_{42}$:Peptide - 2:1) under above mentioned aggregation conditions for different time periods. For disaggregation assay, preformed fibrils (10µM) were treated with the active/inactive enzyme or EDPs (2.5 µg/ml) or the identified peptides ($A\beta_{40}$:Peptide - 10:1; $A\beta_{42}$:Peptide - 2:1) under similar aggregation conditions for different time periods.

2.8 $A\beta_{40}$ and $A\beta_{42}$ oligomerization

$A\beta_{40}$ oligomers were obtained by incubating the monomers under pre-described aggregation conditions at a final concentration of 100 µM for 6 h (Barghorn *et al.*, 2005). $A\beta_{42}$ oligomers (100 µM) were formed by diluting the peptide solution in PBS and 0.2% SDS. The samples were incubated overnight at 37°C, followed by a further dilution with PBS. The diluted sample was re-incubated for another 24 h (Saleem *et al.*, 2017).

2.9 Atomic force microscopy (AFM)

Imaging was done with AAC mode AFM using a Pico plus 5500 ILM AFM (Agilent Technologies, USA) with a piezo-scanner having a maximum range of 9 µm. Micro fabricated silicon cantilevers of 225 µm length with a nominal spring force constant of 21-98 N/m were used from Nano sensors, USA. Cantilever oscillation frequency was tuned into resonance frequency. The cantilever resonance frequency was 150-300 kHz. The images (256 by 256 pixels) were captured with a scan size ranging between 0.8 and 9 µm at the scan speed rate of 1 lines/S. The images were processed using PicoView 1.12 software (Agilent Technologies, USA).

2.10 Transmission electron microscopy (TEM)

Samples were absorbed onto glow-discharged carbon-coated copper grid (300-mesh), followed by staining with 1% uranyl acetate. Imaging was done using a TECHNAI G2 transmission electron microscope system with an accelerating voltage of 120 kV.

2.11 Thioflavin T (ThT) binding assay

Assay mixtures containing treated/untreated $A\beta_{40}$ (5 µM) were added to ThT (20 µM) and detected immediately with Hitachi F-7000 fluorescence spectrometer (ex: 450 nm; em: 480-600 nm; ex/em slit width of 10 nm). The fluorescence intensity of the solution without $A\beta_{40}$ was subtracted as background from each reading with $A\beta_{40}$.

2.12 Confocal spectroscopy

Confocal slides were prepared by placing 10 µl of the treated/untreated samples, followed by staining with filtered stain solutions (0.5 µM Congo red for 30 secs; 20 µM ThT for 5 mins). The slides were washed thrice and air dried under dark. Imaging was done with Olympus Fluoview FV10i confocal microscope.

2.13 Circular Dichroism (CD) spectroscopy

Far-UV CD (range - 195-250 nm) spectra of treated-untreated $A\beta_{40}$ solutions were recorded with a J-815 spectrometer (JASCO, Japan) using a quartz cell with 1 mm path length at room

temperature with 1 nm bandwidth, at a scanning speed of 100 nm/min. The spectra of solutions without Aβ were subtracted as background from the CD signals with Aβ to identify for Aβ-specific changes. All spectra are average of three consecutive scans. The quantitative changes in the secondary structure, particularly β-conformation due to aggregation and destabilization of preformed aggregates was estimated by monitoring the change in the degree of ellipticity (Bruggink *et al.*, 2012).

2.14 Cell cytotoxicity and viability assay

SHSY-5Y human neuroblastoma cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, 1% Penstrap, and 0.2% sodium bicarbonate at 37°C under 5% CO_2. Cells were grown till they reached 70-80% confluency followed by trypsinization with 0.25% trypsin.

For cell viability assays, cells were seeded into 96-well plates at a density of 10^4/well in DMEM-serum media and allowed to grow. Cells were treated with 5 μM $A\beta_{42}$ oligomers in absence or presence of the inhibitors at a final concentration 2.5 μg/ml in DMEM serum-deprived media for 4 h followed by serum replenishment for 44 h. Cytotoxicity was assessed after 48 h by monitoring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) reagent for 4 h at 570 nm.

To assess the cytotoxicity of P_{active}, MTT cell viability assay was performed on SHSY-5Y cells. Cells were treated with different concentrations of P_{active} (up to 1mM) in serum derived media for 24 h followed by assessment of cell viability. Average for three replicates was used for each treatment as well as the control. Experiments were repeated in triplicates and survivality was expressed as percentage of control/untreated cells.

2.15 ANS binding assay

$A\beta_{40}$ with or without the enzyme or P_{active} at different concentrations were mixed with 8-Anilinonaphthalene-1-sulfonic acid (ANS, 20 μM, ex: 360 nm; em: 400-700 nm, ex/em slit-10

nm) and fluorescence measurements were done using Hitachi F-7000 fluorescence spectrometer equipped with a thermostatic cell holder and magnetic stirrer. The fluorescence intensity of the solution without Aβ was subtracted as background from each reading with Aβ.

2.16 Bio-layer interferometry (BLI)

Interaction studies were performed with P_{active} and $A\beta_{42}$ (monomers and oligomers) in real time with bio-layer interferometry (Ciccone *et al.*, 2018; Lessard *et al.*, 2018) with an Octet RED 96e (Pall ForteBio, CA, USA). Experiments were performed at 37°C in PBS with a constant agitation at 1000 rpm. $A\beta_{42}$ (monomers and oligomers) at different dilutions in PBS were loaded onto 96-well black microtiter plates. For biotinylation, P_{active} was incubated with 10-fold excess biotin in PBS for 1 h at room temperature. Excess biotin was removed and the biotinylated P_{active} was then used for the experiments.

Streptavidin biosensors were hydrated in PBS for 10 mins and then loaded with biotinylated P_{active} (100 µg/ml), which were then transferred to fresh PBS for baseline measurements. These preloaded sensors were then associated with $A\beta_{42}$ monomers or oligomers for 5 mins and finally moved back to PBS for dissociation for 10 mins. The Kd values of interaction were determined by full local fitting of the binding curves for each ligand dilutions and fitted by applying a 2:1 interaction model using ForteBio Data Analysis HT software (CA, USA), version 11.1.0.25

2.17 Differential scanning calorimetry

Calorimetric measurements were performed with $A\beta_{42}$ oligomers as control and compared with P_{active} treated oligomers (24 h) on a MicroCal VP-differential scanning calorimeter (DSC) (MicroCal, Malvern Instruments, U.K). DSC scans were conducted between 25-95°C with a constant scan rate of 1.5°C min^{-1}. Instrumental baselines were obtained with PBS and buffer scans were repeated until reproducible. On cooling, the sample cells were rinsed and loaded with $A\beta_{42}$ oligomers or P_{active} treated oligomers. Excess heat capacities as a function of

temperature were measured and the DSC thermograms of excess heat capacity versus temperature were analysed with Origin 7.0 software. The area under the experimental heat capacity (C_{p°) curves was used to determine the calorimetric transition enthalpy (ΔH_{cal}) given by the equation

$$\Delta \boldsymbol{H_{cal}} = \int \boldsymbol{C_{p^\circ}}\, \boldsymbol{dT}$$

Where T is the absolute scale temperature in kelvin. This calorimetrically determined enthalpy is model dependent and thus unrelated to the nature of the transition. The temperature at which excess heat capacity is at a maximum defines the transition temperature (T_m).

2.18 Bio-stability Assay of P_{active} against degradation by human serum enzymes

The stability of P_{active} against degradation in human blood serum (HBS) was assessed over a time period of 24 h with NMR spectroscopy with same number of scans to monitor peak intensity and correlated with peptide concentration over the time. HBS was centrifuged to remove the lipid components and the supernatant was incubated with P_{active} (1 mM) at 37°C. Stability was analysed by comparing the 1D NMR spectra at different time points (0, 2, 5, 12, 16, and 24 h).

2.19 NMR spectroscopy

All NMR spectra were acquired with BRUKER 600 MHz equipped with 5 mm QCI room temperature probe at 298K using standard BRUKER pulseprogram. Concentrations used for both free $A\beta_{42}$ and P_{active} were 300 µM and 600 µM respectively as stock. To monitor peak shift or conformational changes, $A\beta_{42}$ was titrated with P_{active} at 1:0.1 ratio on the assumption of formation of 1:1 complex. A series of 1D spectra for both free $A\beta_{42}$ and P_{active} as well as the complex was acquired for 128 scans. Standardization was done with TSP.

^{19}F NMR spectra of the HPLC purified peptides was recorded with JEOL 400 MHz at 298K. Externally added TFA served as standard for comparison.

2.20 Docking studies

Docking studies were done with $A\beta_{42}$ monomer (PDB ID: 1IYT (Crescenzi *et al.*, 2002), model 1) and the identified heptameric peptide P_{active}, to identify the binding energy and generate a conformation for MD simulation. The structure of P_{active} for docking studies was generated with PEP-FOLD3 (Thevenet *et al.*, 2012; Shen *et al.*, 2014; Lamiable *et al.*, 2016), a de novo approach that helps in peptide structure prediction from the amino acid sequence. Haddock 2.2 (van Zundert *et al.*, 2016) web server was used for docking studies which requires only the structure and residue list of the molecules to be docked.

2.21 MD simulation

Molecular dynamics simulation studies were performed with the free $A\beta_{42}$ and $A\beta_{42}$-P_{active} complex at pH 7.4, temperature 37°C and pressure of 1 bar with AMBER99sb-ildn force field (Lindorff-Larsen *et al.*, 2010) using GROMACS 5.1.5 software package (Hess *et al.*, 2008). To correlate with the physiological conditions, pH of all complexes were adjusted by protonating required residues on the basis of pKa values, obtained from the PROPKA framework of PDB2PQR web server (http://nbcr-222.ucsd.edu/pdb2pqr_2.0.0/). Solvation was done by TIP3P water model followed by neutralizing the overall charge with 137 mM NaCl within a cubic box with cut-off distances of 1 nm between the complex surfaces and the edges of the box. A 1.2 nm cut-off was used for non-bonded interactions. The particle mesh Ewald method was used for long range force calculations. The energy minimized systems were equilibrated in an NVT followed by an NPT condition by restraining positions for 1 ns. Final simulation was carried out for up to 0.4 µs without position restrain and trajectories were stored every 50 ps interval. Data corresponding to all trajectories were analysed by different GROMACS tools.

All-atom Principal Component Analysis (PCA) was done by calculating covariance matrix of the atomic fluctuations. Diagonalization of this matrix gives a set of eigenvectors and eigenvalues, which describe different modes of fluctuations of the protein. The eigenvectors

corresponding to the largest eigenvalues i.e. "principal components" was analyzed by g_covar and g_anaeig tools provided by GROMACS based on the 0.4 μs trajectory.

2.21 Statistical analysis

All experiments were performed in triplicates and all reported values represent the mean ± standard deviation (SD) (n=3). All microscopic images are representative of multiple fields.

2.22 Materials

Aβ peptides were purchased from American Peptide Company, biotin and all cell culture reagents were purchased from ThermoFisher Scientific. Other major reagents were from Sigma-Aldrich, USA. All other products were products of analytical grade. All buffers used in the experiments were adjusted to pH – 7.5. The SHSY-5Y cells were received as a kind gift from Dr. S. N. Bhattacharyya (CSIR-IICB).

3 RESULTS

3.1 Anti-amyloid property of the purified enzyme against $A\beta_{40}$

Enzyme purification involved chromatographic separation (Fig. S1A) followed by SE-HPLC (Fig. S1D) which was confirmed with fibrinogen zymography (Fig. S1B). Aggregation experiments were performed in presence of the isolated fibrinolytic enzyme and its inhibitory property was assessed by comparing the aggregation states of the treated versus the untreated control. Untreated $A\beta_{40}$ solutions form extensive network of long branched fibrils within 7 days (Fig. 1A & S2A). On the contrary, co-incubation of $A\beta_{40}$ with the enzyme under similar aggregation conditions revealed a mixture of mostly non-fibrillating multimeric species accompanying few short unbranched fibrils but no extensive fibrillar networks (Fig. 1B & S2B) even after extended time up to 30 days (Fig. S3B). Further, the ability of the enzyme to destabilize Aβ fibrils (Fig. 1E & S2E) was monitored over a time period by treating preformed aggregates with the enzyme. Treatment resulted in loss of pre-existing fibrils and conversion

to amorphous aggregates or multimeric species within 7days (Fig. 1F & S2F). Long term treatment (for 30 days) in presence of the enzyme revealed similar multimeric species with few broken fibrils suggesting that the structures formed upon treatment are stable and hence do not further fibrillate even when kept under aggregation inducing conditions, whereas, under similar conditions untreated Aβ eventually give rise to plaques (after 30 days) (Fig. S3I & S3J).

3.2 Relevance of proteolytic activity of enzyme in its anti-amyloid property

To evaluate the relevance of enzymatic activity of the fibrinolytic protein, inhibition and destabilization experiments were performed with the inactivated (thermal denaturation) enzyme. Inactivity was confirmed by zymography, an electrophoretic method for measuring proteolysis (Leber *et al.*, 1997). Proteolytic activity of the inactivated enzyme was compared with that of the active enzyme in SDS gel impregnated with substrate, fibrinogen. While the active enzyme, by virtue of its proteolytic activity, shows clear bands (due to proteolysis of fibrinogen) against blue background (post coomassie staining of fibrinogen) when SDS was replaced with titron X-100 and the gel was subjected to reaction buffer, but that was missing for the inactive one, thus confirming the loss of proteolytic activity post denaturation (smearing effect is due to sample overloading, overloading was done to confirm no residual activity post denaturation, Fig. S1C).

Interestingly, inhibition and dissolution experiments with the inactivated enzyme showed that it retains comparable fibrillation inhibition (Fig. 1C & S2C) as well as the fibril degradation property (Fig. 1G & S2G & S3K). Therefore, the aggregation modification potency is not a property associated with its enzymatic activity but rather may involve interaction with a particular segment of the enzyme which is equally available in both active and inactive forms.

This along with the size barrier of the enzyme prompted us to identify the specific interacting stretches responsible for the anti-amyloid property. For this, the inactivated enzyme

was subjected to tryptic digestion followed by separation (by gel filtration) of generated peptides (enzyme-derived peptides, abbreviated as EDPs) within 1.5 kDa (as confirmed by ESI, Fig. S1E). Interestingly, the EDPs exhibited equal efficiency for fibrillation-inhibition (Fig. 1D & S2D) as well as fibril disaggregation (Fig. 1H & S2H, S3L), which further supported the claim that interaction of Aβ with specific portions of the enzyme is likely important for the anti-amyloidogenic property.

3.3 Modification of β-content and amyloid nature

Alteration in β-content and amyloid nature of Aβ following treatments with active, inactive enzymes and EDPs was assessed by comparing their ThT binding affinity and congo red (CR) staining. ThT binding assay was performed to compare the aggregation states of the treated samples with respect to the untreated aggregates. Association of ThT with β-sheet rich structures results in enhanced fluorescence (Li *et al.*, 2009) which was observed for the untreated aggregate within 24 h. With increased incubation (up to 30 days) there was a gradual increase in the fluorescence intensity indicating strong fibrillation (Fig. 2I). Conversely, kinetics of ThT binding for Aβ when followed in presence of either the active or inactivated enzyme or the EDPs confirmed that the rate of aggregation was dramatically slower than in their absence (Fig. 2I). Similarly, treatment of pre-formed aggregates showed lower ThT binding capacity thus signifying reduced β load post-treatment (Fig. 2J). Confocal imaging of ThT-stained samples identified the presence of β-sheet rich structures in the control (Fig. 2A & 2E) while treatment revealed structures with much lowered ThT binding efficiencies, as indicated by the reduced fluorescence intensities (Fig. 2B-D & 2F-H).

Subsequently, CR (Li *et al.*, 2009) staining was performed with both untreated and treated (active/inactive enzyme and EDPs) aggregates to identify their amyloid-like characteristics. Confocal imaging of untreated $A\beta_{40}$ revealed CR-positive structures (Fig. S4A & S4E),

confirming their amyloid character, whereas treatment revealed reduced intensity of CR staining compared to untreated controls indicating lesser amyloidogenicity of the treated samples (Fig. S4B-D & S4F-H).

3.4 The inhibitors reduce oligomer-induced cytotoxicity

The role of oligomeric species behind neurodegeneration (Lesne *et al.*, 2006; Sakono *et al.*, 2010; Benilova *et al.*, 2012) prompted us to evaluate the potency of the candidates (active/inactive enzyme and EDPs) to prevent fibrillation of preformed $A\beta_{40}$ and $A\beta_{42}$ oligomers. While untreated $A\beta_{40}$ oligomers revealed fibrils within 6 h under aggregation conditions (Fig. S5A-D), no significant fibrillation was observed in treated samples even after 24 h (Fig. S5E-P). Similarly, $A\beta_{42}$ oligomers (Fig. 3A) also formed extensive fibrillar network (Fig. 3B), while treated samples revealed mainly multimeric species (Fig. 3C-E). Additionally, the ability of the candidates (active/inactive enzyme and EDPs) to ameliorate $A\beta_{42}$ oligomer induced cytotoxicity on SHSY-5Y human neuroblastoma cells was evaluated with MTT cell viability assay. Cell treatment with $A\beta_{42}$ oligomers alone, resulted in ~76% reduction in survivality (only ~25% viable cells) whereas treatment with active/inactive enzyme or EDPs improved cell health, as suggested by increased survivality (~60-70% viable cells; Fig. 3F).

3.5 LC-MS/MS guided identification of the active peptide fragment

Proteins of the size of isolated enzyme are not suitable therapeutic agents. Moreover, comparable efficiency of anti-amyloidogenic activity of active, inactive enzymes and EDPs suggested that anti-amyloidogenic property of the enzyme is not associated with its enzymatic activity. On the contrary, the results indicated that interaction of a particular segment of the enzyme (available in both active and inactive forms) with Aβ might be instrumental for its anti-

amyloidogenic property. This observation prompted us to identify specific stretches responsible for the interactions with Aβ.

To this end, the EDPs were subjected to repeated mass spectrometric analysis (LC-MS/MS) followed by analysis of fragments generated by trypsin and chymotrypsin digestion with available database (Mascot Matrix Science Server (Perkins *et al.*, 1999)) that led to the identification of eight common peptides from which those with <1 kDa molecular weight (four fragments) were considered for further evaluation. The binding ability of these four fragments to $A\beta_{42}$ monomer (PDB ID: 1IYT (Crescenzi *et al.*, 2002), model 1) was evaluated using the online web server PepSite 2.2.20 (Trabuco *et al.*, 2012). This helps in the identification of peptide binding regions on the surface of the protein, when provided with the peptide sequence query and the protein structure either in the form of PDB ID or by uploading the structure in PDB format. Of the four sequences, the peptides that bound $A\beta_{42}$ with highly significant p-values (P_1 and P_{active}) were considered for further studies (Table S1) (The sequences of the four identified peptide fragments along with their binding p-values, as provided by the web server are listed in Table S1. The peptides that were synthesized and evaluated for their anti-amyloidogenic properties against Aβ are highlighted in bold letters.)

The identified sequences were further chemically synthesized based on solid phase peptide synthesis and purified with RP-HPLC (Fig. S6A & S6B). Purity of the peptides was confirmed with mass spectrometry (Fig. S6C & S6D) and the absence of trace amount of TFA as used to cleave from solid resin support was verified with ^{19}F NMR spectroscopy (Fig. S6E & S6F).

3.6 Evaluation of the anti-amyloidogenic property of the identified peptides

Aggregation inhibition and disaggregation experiments were performed with the two synthetic peptides (P_1 and P_{active}) to assess their ability to interfere with $A\beta_{40}$ aggregation. While P_{active} showed potent aggregation inhibition (Fig. 4A) along with its ability to remodel pre-existing

fibrils into small soluble oligomers (Fig. 4B), P_1 actually aggravated the aggregation tendency, as revealed by enhanced fibrillation in AFM (Fig. S7A & S7B). ThT binding assay also revealed reduced fluorescence in presence of P_{active}, indicating reduction in β load (Fig. 4C), while presence of P_1 resulted in enhanced aggregation of Aβ as indicated by even higher fluorescence in P_1-treated samples compared to control (Fig. S7C). The contradictory behaviour of the two peptides on Aβ aggregation confirmed their sole involvement in pure form in the processes.

Further studies were done with P_{active} only to evaluate its potential against $A\beta_{42}$, the more aggregation prone species, which again revealed its equally efficient $A\beta_{42}$ fibrillation inhibition property along with successful degradation of existing fibril networks (Fig. 5A-C).

3.7 P_{active}-mediated alterations in Aβ secondary structure

In order to probe the P_{active}-mediated changes in the secondary structure of $A\beta_{40}$, CD spectra of $A\beta_{40}$ monomer as well as aggregates pre-incubated in presence or absence of P_{active} under aggregation conditions were analysed. The initial secondary structure of $A\beta_{40}$ displayed a random coil with a major negative peak around 200 nm (Fig. 4D). Upon aggregation, a stronger positive band was observed around 195 nm, with a higher negative value around 216-218 nm indicating conformational change with disappearance of α-helix and increase in β-sheet content (Fig. 4D). However, treatment resulted in significant change in ellipticity values around 216-218 nm, pointing towards decrement of β-sheet content. $A\beta_{40}$ monomers when pre-treated with P_{active} under aggregation inducing conditions revealed structures that closely resembled monomer itself (Fig. 4D). Alterations in the secondary structure of Aβ aggregates monitored in presence of the enzyme (active/inactive) or the EDPs indicated towards similar decrease in β-content as observed by change in ellipticity around 216-218 nm (Fig. S8A & S8B).

3.8 Modification of Aβ surface hydrophobicity due to P_{active} binding

Aβ is an amphipathic molecule with a central hydrophobic region that plays a major role in aggregation. Hydrophobic modifications in the Aβ molecule due to P_{active} interaction was followed with ANS binding assay. The extrinsic probe ANS interaction with solvent exposed hydrophobic clusters of proteins results in enhanced fluorescence intensities (Bolognesi *et al.*, 2010). Here, successive increase in concentration of Aβ alone enhanced ANS fluorescence, indicating its occupancy of accessible hydrophobic cores (Fig. S8C). In contrast, when gradually increasing concentration of P_{active} was added to Aβ, ANS failed to bind the hydrophobic patches which was reflected by gradual reduction in fluorescence with increasing P_{active} concentrations (Fig.4E). Similarly addition of increasing concentration of the enzyme to Aβ reduced ANS fluorescence intensities along with a blue shift in maxima (Fig. S8D).

3.9 P_{active}-$A\beta_{42}$ interaction and conformational alteration

Bio-affinities of P_{active} to $A\beta_{42}$ (monomers and oligomers) was followed in real time with BLI. Aβ (monomers and oligomers) binding constants to P_{active} immobilized on BLI biosensors was determined over a range of concentrations at 37°C that indicated P_{active} could bind to both monomers and oligomers with reasonably strong affinity (Monomer -Kd ~1.11E-07M, Oligomer -Kd~1.88E-05M) (Fig. 5D & 5E).

Interaction between $A\beta_{42}$ monomer and P_{active} was further followed with NMR studies. 1D NMR spectrum of $A\beta_{42}$-P_{active} complex clearly showed notable differences in peak distribution in amino regions compared to both the constituent apo forms, indicating global rearrangement of $A\beta_{42}$ conformation upon complex formation (Fig. 6A). Further, docking followed by MD simulation with $A\beta_{42}$-P_{active} complex (docking structures - $A\beta_{42}$ – PDB ID: 1IYT (Crescenzi *et al.*, 2002) model 1; P_{active}– PEP-FOLD3 generated structure; Fig. S9) also supported the same. Throughout the 0.4 µs of MD trajectory of the complex, P_{active} bound $A\beta_{42}$ undergoes significant conformational changes, which led to conformational alteration of tyrosine 10

(Y10) making it feasible to be exposed towards solvent surface from the preferred orientation of fibrillation (Fig. 6B).

It is clear that peptide-treated oligomers are non-toxic in nature. Hence there must be a subtle structural reorganization upon treatment. To compare differences in oligomers upon peptide treatment, DSC was performed with control $A\beta_{42}$ oligomers as well as P_{active} treated $A\beta_{42}$ oligomers. The DSC thermogram of the toxic untreated control oligomers showed two endothermic peaks at 55.26 and 72.17°C (Fig. 6C). The occurrence of two peaks reflects the presence of a mixture of two oligomer populations with different heat capacities. In contrast, treatment of these oligomers with P_{active} for 24 h resulted in a thermally homogenous single population with T_m – 68.61°C (Fig. 6D). Interestingly, the species formed upon treatment are distinctly different from the toxic oligomers, as is clearly indicated by the different melting temperature and heat capacity values thus confirming structural reorganization.

3.10 Bio-stability of P_{active} against degradation by human serum enzymes

The stability of the proposed peptide P_{active} against degradation in human blood serum (HBS) was evaluated at 37°C with NMR spectroscopy. Incubation of P_{active} with HBS confirmed no visible degradation up to 24 h as indicated by no significant change in the backbone N-H region spectra (Fig.7). Precisely, conformational alterations were observed from 0h to 2h as suggested by the peak shift but the connectivity pattern between amino acids remained same along with their intensity with respect to same number of scans, used for data acquisition. Thus, the high bio-stability of P_{active} makes it suitable as a future drug candidate. Moreover, P_{active} was also found to be non-toxic towards SHSY-5Y cells up to a concentration of 1 mM (Fig.4F) where cell viability upon treatment is comparable to control untreated cells.

4 DISCUSSIONS

AD currently afflicts the largest percentage of elderly population with the only available treatments including symptomatic interventions with drugs that can help to palliate the symptoms without solving the underlying problems (Perrin *et al.*, 2009; Galimberti *et al.*, 2011; Yiannopoulou *et al.*, 2013; Graham *et al.*, 2017). Thus, particular attention has been devoted to seek inhibitors that will efficiently prevent Aβ aggregation. Furthermore, it has been known that amyloid plaques including fibrils begin to form even before disease symptoms start to develop (Perrin *et al.*, 2009). Therefore, an attractive therapeutic strategy for AD would be to find agents that can prevent further aggregation of Aβ as well as destabilize preformed fibrils.

This study reported effective anti-amyloidogenic potency of a peptide sequence identified from a fibrinolytic enzyme isolated from the roots of the medicinal plant *A. indica*. The isolated enzyme was found efficient both as an inhibitor and destabilization agent against Aβ aggregation. Importantly, though oligomers are known to exert the major cytotoxicity (Lesne *et al.*, 2006; Sakono *et al.*, 2010; Benilova *et al.*, 2012), enzyme treatment could successfully abate cytotoxic effects of these oligomers in human neuroblastoma cells (SHSY-5Y) suggesting that the enzyme-arrested, non-fibrillating oligomeric forms are much less toxic as compared to the cytotoxicity exerted by the on-pathway oligomeric intermediates of Aβ fibrillation.

Further, the anti-amyloidogenic property of the inactive enzyme suggested that the fibril destabilization and inhibition potency is not a property associated with the proteolytic activity of the enzyme. Instead, it may involve interaction of the Aβ peptide with certain segment of the enzyme molecule. ANS binding studies with Aβ in presence of the enzyme also indicated towards hydrophobic modifications of the Aβ molecule, which further substantiate the idea of involvement of specific interactions, possibly hydrophobic.

Attempts to elucidate the interacting segment of the enzyme identified two sequences, of which a single heptameric sequence (P_{active}) could minimize both $A\beta_{40}$ and $A\beta_{42}$ aggregation

successfully while the other (P_1) exacerbated the aggregation process (thus acted as a negative control). Aβ aggregation is preceded by the appearance of β-sheet rich structures which is recognized as a drop in CD signal at 218 nm (Bruggink *et al.*, 2012). Conversely, P_{active} mediated structural modifications indicated reduction in β-sheet rich structures, a finding that is also in accord with the simultaneous decrease in the ThT binding capacity of the P_{active}-treated aggregates. Bio-layer interferometric assays with immobilized P_{active} confirmed its strong bio-affinities to both $A\beta_{42}$ monomers as well as toxic oligomers. Increasing concentration of the monomer showed an exponential increase in association to P_{active}, with its binding towards saturation but the binding equilibrium had its tendency to dominate towards association, rather than complete dissociation, indicating stable complex formation. Oligomer and P_{active} binding kinetics revealed a dose-dependent induction of negative BLI signal. This may occur due to the binding induced conformational changes in Aβ oligomers as well as due to the binding of larger oligomers to BLI sensors which overwhelms the positive nm shift resulting in a negative value (Bornhop *et al.*, 2016). Conformational modifications of the oligomers through redistribution of interactions upon P_{active} treatment, as confirmed by DSC where the heat absorbed per mole of oligomers is perceptibly different, that revealed a distinct homogenous population in the treated sample compared to the mixed population observed in the control. Conclusively, it can be argued that the toxicity of control oligomers was suppressed in terms of P_{active} mediated conformational rewiring.

Additionally, interaction of P_{active} with Aβ monomers hampered ANS binding to Aβ suggesting reduced propensity of Aβ to expose hydrophobic clusters either by binding to or masking hydrophobic cores, or disrupting hydrophobic interactions between Aβ molecules or by conformational alterations, thus making the hydrophobic patches less accessible to ANS. Hydrophobicity along with aromatic packing is known to drive the process of aggregation of Aβ peptide (Kim *et al.*, 2006; Cukalevski *et al.*, 2012). Thus blocking of hydrophobic pockets

by P_{active} binding to Aβ may be a possible cause of disaggregation or inhibition of fibrillation. NMR interaction studies with P_{active} and Aβ monomers confirmed P_{active} mediated global conformational rearrangement of Aβ upon complex formation which was supported by the MD simulation trajectory over 0.4 μs. Hence, P_{active} induces conformational alterations in the Aβ molecule (both monomer and oligomers) along with its property to modify Aβ hydrophobicity making the hydrophobic patches less accessible, thus reducing its aggregation propensity and toxicity.

Though peptides offer a powerful tool for drug designing but the major impediments in the process are related to their high susceptibility to serum enzymes that compromises with their therapeutic efficiency (F.Powell, 1993; Sawyer, 2017). Interestingly, the identified peptide, P_{active}, remained stable in human blood serum with no visible degradation for 24 h. The use of naturally occurring peptides against Aβ aggregation increases the significance of the study because of their lower immunogenicity and smaller size that offer them better advantages as therapeutics compared to larger molecules. Hence, this could be the stepping stone in identifying and designing small peptides that with further relevant experimental assessment could be considered as a prospective drug capable of inhibiting further progression as well as decrease the severity of the disease.

AUTHOR CONTRIBUTIONS

JS and RB conceived the project. RB and SB designed and performed the experiments with consultation with BP. RB, SB and JS analysed the data and wrote the paper. RB and SB contributed equally.

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FIGURE LEGENDS

Figure 1 $A\beta_{40}$ aggregation modification. (A-D) AFM visualization of $A\beta_{40}$ aggregation inhibition by active/inactive enzyme and EDPs. While untreated Aβ (A) reveals extensive fibrillation, treatment with inhibitors (B-D) show fibrillation inhibition and formation of oligomers. (E-H) Fibril destabilization efficiency of the inhibitors. (E) Untreated preformed fibril revealing enhanced aggregation. (F-H) Treated preformed fibrils reveal degradation of fibrillar networks to oligomers. All experiments performed under aggregation conditions 7 days.

Figure 2 Thioflavin T binding of treated and untreated $A\beta_{40}$. Confocal images of ThT stained (A) untreated and (B-D) treated $A\beta_{40}$ monomer after 7 days incubation under aggregation inducing conditions revealing fibrils with strong ThT fluorescence in the untreated control while reduced ThT binding in the treated samples. Staining of (E) Aggregate revealed ThT bound fibrils. (F-H) Treatment of preformed aggregates indicated broken fibrils with reduced intensity of ThT stain post treatment (7 days). (I) Time-dependent kinetics of $A\beta_{40}$ fibrillation inhibition by the candidates. (J) Time-dependent destabilization of preformed aggregates in presence of the inhibitor candidates. Kinetics was measured by comparing the

ThT fluorescence at any given time. Error bars represent the average of three replicate experiments.

Figure 3 Modification of toxicity induced by $A\beta_{42}$ oligomers. (A) Control oligomers. (B) Untreated oligomers showing fibrillation. (C-E) Treated oligomers - active enzyme, inactive enzyme & EDPs respectively, incubated under aggregation conditions for 24 h. (F) Cell viability and toxicity assay in absence or presence of the inhibitors. (a) Control cells, (b) $A\beta_{42}$ oligomer treated, (c) oligomer + active enzyme, (d) oligomer + inactive enzyme, (e) oligomer + EDPs. Inhibitor treatment could successfully ameliorate oligomer toxicity and increase cell viability.

Figure 4 Effect of P_{active} on $A\beta_{40}$ aggregation. (A) Inhibition of $A\beta_{40}$ aggregation, and (B) destabilization of preformed aggregates by synthetic P_{active}. (C) ThT binding assay comparing the aggregation states of $A\beta_{40}$ in absence and presence of P_{active}. (D) CD spectra indicating changes in secondary structure before and after treatment. All experiments performed under aggregation conditions for 7 days. (E) ANS binding assay with $A\beta_{40}$ in presence of different concentrations of P_{active}. (I) Cell viability assay with different concentrations of P_{active} showing no significant cytotoxicity up to 1mM.

Figure 5 P_{active}–mediated modification of $A\beta_{42}$ aggregation.(A-C) $A\beta_{42}$ aggregation inhibition and fibril destabilization by P_{active} (48 h) - (A) untreated control showing fibrillation (B) P_{active} treated monomer inhibiting fibrillation (C) Degradation of preformed fibrils by P_{active}. (D & E) BLI binding affinity of P_{active} to (D) $A\beta_{42}$ monomers and (E) $A\beta_{42}$ oligomers.

Figure 6 P_{active}–mediated conformational alteration of $A\beta_{42}$ monomers and oligomers. (A) 1D NMR spectra of N-H regions of free $A\beta_{42}$ monomer, P_{active} and complex revealing chemical shift due to complex formation. (B) Mapping MD simulation trajectory of the complex from 0.4 µs (P_{active}, cyan in line representation) where transition of $A\beta_{42}$ monomer from initial point to 0.4 µs is shown in different colours, corresponding to their representative conformations and indicated by arrows. DSC thermograms of (C) Control $A\beta_{42}$ oligomers and (D) P_{active} treated $A\beta_{42}$ oligomers with significant differences, confirming the formation of conformationally altered species. The T_m and ΔH values are provide in the respective insets.

Figure 7 Bio-stability of P_{active} in human blood serum. 1D NMR spectra (N-H region) of P_{active} in human blood serum assessed over a period of 24 h incubated at 37°C showing no significant change in the backbone or degradation, confirming its stability.