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Similar Characteristics of Fibrillar form of β-Amyloid Peptide Fractions from Mice Brain affected by Systemic Amyloidosis

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ABSTRACT

Enhanced expression of amyloid β-peptide (Aβ) and deposition is the main causative factor in Alzheimer's disease (AD). Factors that lead to the genesis of accumulation and toxicity of Aβs are yet to be identified. While studying the effect of systemic amyloid on the functions of the mice brain, it was accidentally found that the mice brains contain accumulated Aβs, which are extractable with hexafluroisopropanol (HFIP) solvent. By purifying with semi preparative HPLC on HFIP extracts, two major fractions containing mixture of Aβs with variable composition were observed. We have characterized these mixtures by electron microscopic and spectroscopic methods. Our results indicate that, the accumulated Aβ fibrils have similar morphological and conformational characteristics as that of Aβs of AD brains.

Keywords: *Systemic Amyloidosis; β-amyloid; Alzheimer's Disease; Circular Dichroism; FT Infra red Spectroscopy; Transmission Electron Microscopy; Congo red.*

1. Introduction

Alzheimer's disease (AD) is one of the most common chronic neurodegenerative diseases. It is characterized by progressive loss of memory and dementia. The ultimate cause of dementia is the loss of neurons and the synaptic connections. The insults that result in this loss remain to be determined. The innate immune response and resulting neuro inflammation may be important in the neuronal damage in AD. These responses include the activation of microglia, astroctytes, and the complement system, as well as increased cytokine expression and acute-phase protein responses (Akiyama et al., 2000 and Weiner and Selkoe, 2002).

The excessive accumulation amyloid β-protein (Aβ) in the brain is the causative factor for genetic as well as sporadic cases of AD (Hardy and Selkoe., 2002 and Seubert et al., 1992). These deposits share the same etiological properties and biophysical characteristics like other amyloidogenic proteins (Selkoe, 1991). Amyloid beta peptide is central to the pathology of Alzheimer's disease, because it is neurotoxic-directly by inducing, oxidant stress, and indirectly by activating microglia (Yan et al., 1996). The long, hyperaggregatable Aβ42 peptide is apparently essential for initiating the formation of senile plaques (Iwatsubo et al., 1994; Lemere et al., 1996), an invariant feature of Alzheimer's disease. Aggregation of the Aβ into toxic supramolecular structure is important in the pathophsyiology of AD. Though colossal amount of work is being carried out in the field of

aggregation biochemistry of Aβs, inducing the accumulation of Aβs in the animal model has been a elusive goal till date. Earlier reports indicate that the Aβ accumulation is possible by intracerebral infusion of Aβ peptide aggregate containing tissues but produced very little success. Inoculations of Aβ fibrils containing animal brain samples took 6-7 years period for the development of amyloid like aggregate in the marmosets (Kane et al., 2000). Mice transgenic containing human β-amyloid precursor protein (APP) gene is known to express excess human APP in the brain. These mice develop Aβ amyloids in the period of 5 months. In these animals, the amyloid plaques are entirely due to the over expression of APP, the condition, which is far from the reality in the sporadic cases of AD, where the Aβ accumulation is due to either up regulation of some endopeptidases inhibitors or down regulated expression of proteases (Iwata et al., 2000), which are involved in the degradation of Aβs. In the present work, we describe a mouse model that developed accumulated Aβs in the brain in the chronic condition of systemic amyloidosis induced by the regular casein injection (Botto et al., 1997). In this work, the two Aβ mixtures of amyloid aggregates were shown to have similar morphological, congophilic and spectroscopic characteristic as that of amyloid extracted in AD brain.

2. Results

2.1. Semi preparative analysis of Aβs by RP-HPLC

We found that mice affected with systemic amyloidosis also has high amount of Aβs in the brain as in the case of transgenic mice**.** However, it is extractable in HFIP as in the Aβs of AD brains.

The HFIP extract of the mice with systemic amyloidosis was further purified by RP-HPLC method (Fig. 1). At preparative scale 1mg/ml concentration, two broad peaks consisting of Aβ with different compositions were observed. However, when the eluents by RP-HPLC were analyzed, it was found that they resolved into fragments of Aβs as in the extracts of AD brain.

The molecular constituent of each fragment was characterized by analytical RP-HPLC (figure not shown). The results are given in the table I.

2.2. MALDI-TOF analysis of Aβs

The MALDI-TOF spectrum of the fraction I is given in the Fig. 2a. In the fraction II along with the peaks Aβ41, Aβ43, and Aβ54, additional peak appears for Aβ17-45 in Fig. 2b. The details of the molecular components of the two major fractions are given in the table I.

2.3. Transmission Electron Microscopic analysis of Aβs

The ultra structures of the fibrils were analyzed using electron microscopic pictures (Fig. 3a and b). The average length, periodicity and diameter of the fibrils with both the fraction were compared.

2.4. Circular Dichroic analysis of Aβs

The CD spectra are given in the Fig. 4a and b. The two CD spectra of both the fractions are characterized by red-shifted minimum around 228 nm. The FTIR spectra of hydrated thin films prepared from the samples of the protofibrils of the two fractions are depicted in the Fig. 5a and b.

2.5. Congo red stain of Aβs

The dispersed fibrillar solution was then analyzed with UV-Visible spectroscopy. The absorption maxima of Congo red dye shifted from 485 to 510 nm and the intensity of the absorption increased by two folds (figure not shown). Congo red treated, aggregated fibrils produced a bright, golden-yellow-color when examined using cross-polarized light (Fig. 6a and b). This staining pattern is found *in vivo* in deposits formed in wide variety of amyloidosis.

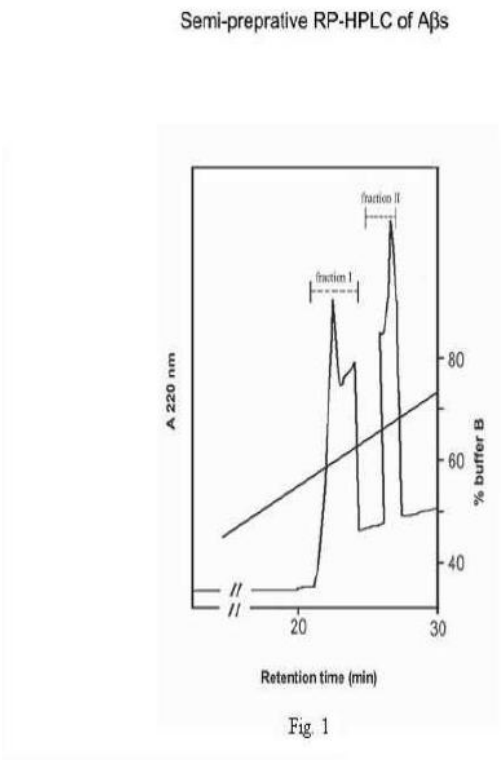

Fig. 1. The Aβs in HFIP extract of mice brain with systemic amyloidodsis is purified by RPHPLC using acidic elution buffer. The extract were dissolved in 70% formic acid, 20µl of sample was loaded (1 mg/ml) on to the Spherisorb-ODS2 25 cm × 4.6 mm, 5 µm, C18 silica column and eluted with 0.1% trifluoroacetic acid (TFA) in water (buffer A) and 0.1% TFA in acetonitrile (ACN) (buffer B). Samples were eluted with a linear gradient of buffer B at a flow rate at 1 ml/min while monitoring UV absorbance at 220 nm. The process is repeated several times to get appropriate concentration.

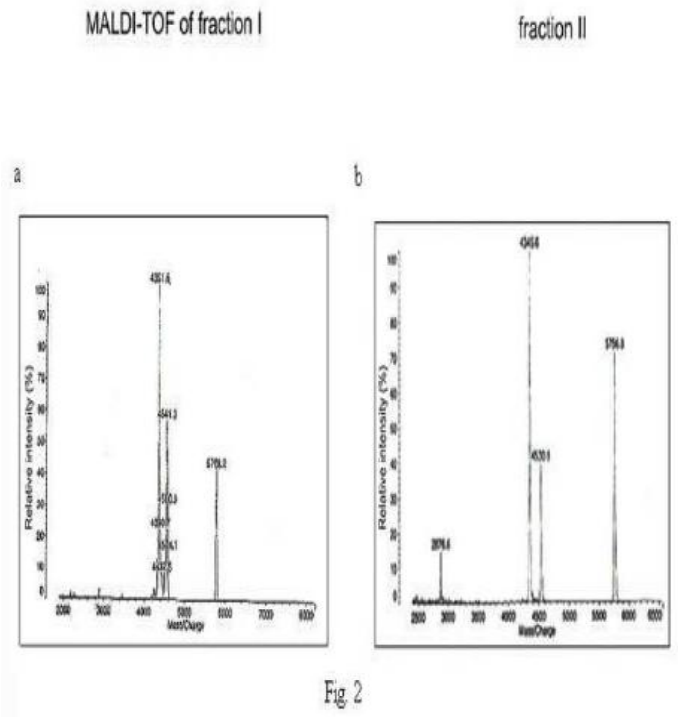

Fig. 2. MALDI-TOF spectrum of Aβ fragments in fraction I (a) and II (b).

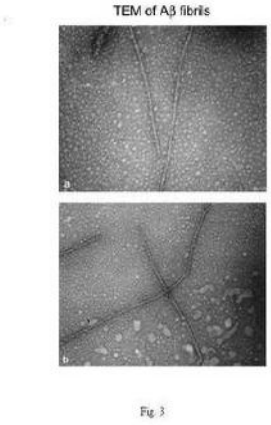

Fig. 3. Electron micrograph of negative stained preparation of fibrils aggregated Aβs of (a) fraction I and (b) fraction II. The peptide was stained as given in materials and methods with aqueous 1.5% uranyl acetate. Magnification: × 100000.

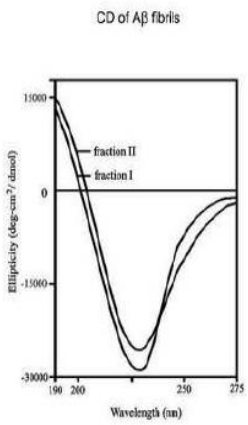

Fig. 4

Fig. 4. Circular dichroism spectrum in water for the fraction II (b) and fraction I (a). The molar elipticity was calculated by taking number average molecular weight.

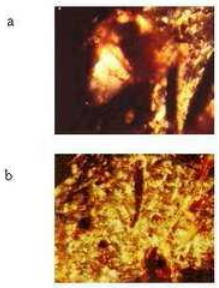

Fig. 6

Fig. 6. Cross-polarized micrograph of Congo red stained fibrils (a) fraction I and (b) fraction II. Magnification: × 40.

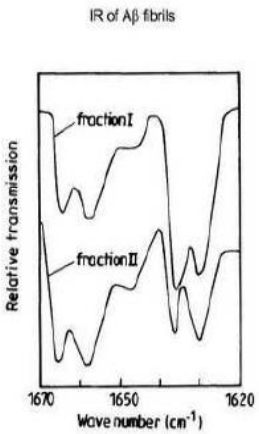

Fig. 5

Fig. 5. IR spectrum of fibrils prepared from fraction II (b) and I (a).

Table 1: A· peptide composition of HFIP extract of mice brain separated into two fractions.

Fraction I:

Abeta peptides	Observed Mass	Calculated Mass	Percentage (%)
$Abeta_{41}$	4351.6	4345.2	60
$Abeta_{43}$	4541.3	4517.2	20
$Abeta_{54}$	5768.2	5755.1	20

Fraction II:

Abeta peptides	Observed Mass	Calculated Mass	Percentage (%)
$Abeta_{17-45}$	2876.6	2889.6	10
$Abeta_{41}$	4345.6	4345.2	40
$Abeta_{43}$	4530.6	4517.2	20
$Abeta_{54}$	5756.8	5755.1	30

3. Discussion

The Mice transgenic for the human β-amyloid precursor protein (βAPP) genes are characterized by neuritic alteration, astrogliosis and microglial activation and deposition cerebrovascular amyloids. These models also have neuronal loss as seen in the AD patients. However, one of the characteristics of amyloid plaques which is present in AD patients is their ability to be resistive

to SDS (Lee et al., 1998 and Naslund et al., 1996), urea and guanidine chloride. The plaques found in the transgenic mice are highly soluble in SDS solution (Kuo et al., 2000). We found that mice affected with systemic amyloidosis also has high amount of Aβs in the brain as in the case of transgenic mice. In this work, we analyzed the fibril morphology and spectroscopic characterization of Aβs as two major mixtures. The observed mass 4351.6 is assigned to Aβ41, and the peak due to mass 4541.3 indicates presence of Aβ43. In addition to this, a peak due to Aβ54 appears with molecular mass of 5768.2 indicating the presence of Aβ54 In the fraction I, the ratio of the peptide fragments of Aβ41, Aβ43, and Aβ54 is 3:1:1. In the fraction II, the ratios of these three major peaks are 2:1:1.5. Since these two peaks are distinct in their composition, it was decided to analyze them as a mixture. It should be noted that most of the amyloid fragments occur as a mixture in the *in vivo* condition.

Both the fractions formed fibrils on incubation as in case of synthetic amyloid fragments (Walsh et al., 1999). The average length, periodicity and diameter of the fibrils with both the fraction were compared. Both the fibrils assumed non branched filament structure with first fraction having thicker width of 12 nm when compared to the second fraction with thinner width of 7 nm. The helical structure, which is observed in the fraction I, is absent in the faction II. Other than the fibrils, globular form of protofibrils in the fractions II and I was also observed. We have also compared the dimensions of the protofibrils using electron microscopic data. As far as the globular structure consisting of the protofibrils is concerned, the seeds (or the protomers) are smaller in II than I. This kind of structural heterogeneity has already been observed in the fibrils of synthetic Aβ42 (human).

Circular dichroic spectra for the two fractions were carried out in the form of protofibrils. Conditions for protofibrils were maintained as per the published experimental protocol (Nichols et al., 2002). Both the fractions exhibit similar CD spectra with minimum differences indicating that their protofibrilar conformation is rich in β-sheet.

Fourier transform infrared spectroscopy (FTIR) has been used to probe the peptide aggregate structure and the amide I band (1600-1700 cm-1) has been used to estimate protein secondary structure content (Fezoui et al., 2000). For the

fraction I the fibrils showed low-frequency β-sheet two types of structure (1630 cm-1 and 1636 cm-1) (Lansbury et al., 1992), with the small amount of unordered structure (1649 cm-1). The higher wave number two peaks (1659 cm-1 and 1666 cm-1), are due to helical turn and β-turn structures, respectively. For the fraction II the fibrils showed a little different low frequency spectrum indicating the presence of β-sheet (1629 cm-1 and 1637 cm-1), with the small amount of unordered structure (1649 cm-1). As in the case of fraction I, higher wavelength peaks around 1658 cm-1 and 1666 cm-1 indicating the presence helical turn and β-turn conformation were observed. The protofibrils having predominant β-sheet structure with same unordered helical, turn and β-turn conformation was already been modified by previous workers in the field in synthetic Aβ protofibrils.

To determine whether the matured fibrils have the Congo red binding properties characteristic of amyloid, dye-binding studies (Kane et al., 2000) were performed. Incubation of fractions I and II in phosphate puffer of pH 7.4 (5mM sodium phosphate) with Congo red solution resulted in absorption of the dye from the solution. This staining pattern is found *in vivo* in deposits formed in wide variety of amyloidosis. When the fibril aggregates were sufficiently large, a characteristic segmented, golden yellow pattern of birefringence was also observed. These data demonstrate that both the fractions formed amyloid fibrils that were characteristics Congo red binding as other known amyloid fibrils.

In this study we have shown that the presence of SDS insoluble and HFIP extractable Aβs in the mice brain affected with systemic amyloidosis. Our results indicate that the fibrils derived from these animals, share similar morphological and spectroscopic characteristics as those fibrils from AD brain.

4. Experimental Methods

All experiments were carried out in accordance with the guidelines of the Animal Care and Animal Ethics committee of Central Leather Research Institute.

4.1. Production of systemic amyloidosis.

Colony-bred adult male Swiss White mice (30-35 gm) were used. Animals were selected randomly and caged in groups of five at room temperature (25-35 °C) and supplied with food (Commercial pelleted animal feed marketed by M/s. Hindustan Lever, Bombay, India under the name "Gold Mohur rat feed") and water was provided ad libitum (control n=5, test n=5). Mice were given 0.5 mL of 10% Vitamin free Casein (1CN Pharmaceuticals, Cleveland, OH, USA) as subcutaneous injection (Botto et al., 1997) for 66 days (5 days a week) to induce systemic amyloidosis. Control animals were treated with saline for the same period. Animals were decapitated without anesthesia (group n=5). The systemic amyloidosis was confirmed by ladder formation in the liver. Details of these experiments are given elsewhere (Botto et al., 1997). Immediately after mice were decapitated, brain cerebral cortex were removed on an ice-chilled plate, weighed and stored at -70 °C.

4.2. RP-HPLC

The hexafluoroisopropanol (HFIP) extract (Naslund et al., 1996) containing Aβ peptides 20 µL (1mg/mL) were dissolved in 70% formic acid and separated using acidic RP-HPLC buffer systems on a waters HPLC (Waters, Milford, Massachusetts, USA), equipped with an Shimadzu SPD 10A UV-Visible detector (Shimadzu, Kyoto, Japan). Separations were performed using Spherisorb-ODS2 25 cm × 4.6 mm, 5 µm, C18 (Waters, Milford, Massachusetts, USA) silica column. The process was repeated several times to get concentrated Aβ solution.

4.3. MALDI-TOF

RPLC fractions were lyophilized and analyzed by MALDI-TOF (KRATOS, Manchester, UK).

Scanning was performed from mass/charge (m/z) 500-2000. At least 10 scans were summed before

transformation of the spectra to give the molecular mass of the uncharged peptides.

4.4. Congo red stain of amyloid fibrils.

HPLC fractions (I and II) were dissolved in mili-Q water at a concentration of 100µM were incubated for three days at 37 °C. These fibrils are treated with alkaline Congo red as described earlier (Westermark et al., 1999). Stained slides were observed with high intensity crossed polarized light in optical microscopy (EUROMAX, Holland). Amyloid stained with Congo red shows a bright golden yellow birefringence.

4.5. Circular Dichroism spectroscopy.

RP-HPLC fractions I and II were dissolved in milli-Q water and incubated for 24 hrs at 37 °C and the peptide solution was taken for Far UV CD measurement. The concentration of the peptide in the solution is determined by Fluroscamine florescence method. Circular dichroic spectra in the far UV region (190-240 nm) were obtained using Jasco J-715 Spectropolarimeter (Hachiaji, Tokyo, Japan).

4.6. Infra red spectroscopy.

RP-HPLC fractions I and II were dissolved in milli-Q water and incubated for 24 hrs at 37 °C and the peptide solution was taken for FT-IR measurement. The IR spectra were obtained using FTIR Avator-320 (USA) and analyzed for secondary structure.

4.7. Transmission electron microscopy.

RP-HPLC fractions II and I of brain extract containing Aβ41, Aβ43, Aβ54, and Aβ17-45 were prepared in milli-Q water, incubated at for 24 hrs (Naslund et al., 1994a and Nielsen et al., 1999). Amyloid fibrils on grids were covered by collodion stabilized Formvar film and then 5µL of freshly prepared 1.5 % uranyl acetate in water was added. After 2-3 min, excess fluid was removed with a filter paper and the grids were air-dried. The negatively stained specimens prepared in this way were examined and photographed in a JEOL-EM 100cx at 80 KV (USA).

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