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# Methionine 35 Oxidation Reduces Fibril Assembly of the Amyloid Aβ-(1–42) Peptide of Alzheimer's Disease*

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**The major component of amyloid plaques in Alzheimer's disease (AD) is Aβ, a small peptide that has high propensity to assemble as aggregated β-sheet structures. Using three well established techniques for studying amyloid structure, namely circular dichroism, thioflavin-T fluorescence, and atomic force microscopy, we demonstrate that oxidation of the Met-35 side chain to a methionine sulfoxide (Met-35$^{ox}$) significantly hinders the rate of fibril formation for the 42-residue Aβ-(1–42) at physiological pH. Met-35$^{ox}$ also alters the characteristic Aβ fibril morphology and prevents formation of the protofibril, which is a key intermediate in β-amyloidosis and the associated neurotoxicity. The implications of these results for the biological function and role of Aβ with oxidative stress in AD are discussed.**

Alzheimer's disease is characterized by the abundance of intraneuronal neurofibrillary tangles and the extracellular deposition of the Aβ peptide as amyloid plaques (1). Aβ is a normally secreted soluble peptide that under certain environmental conditions can produce β-sheet aggregates that eventually precipitate as amyloid fibrils. Numerous studies have established that the Aβ peptide becomes neurotoxic to cortical cell cultures when aggregated as β-sheet structures (2–4). Additional studies suggest that the longer, 42-residue Aβ-(1–42) is more pathogenic than the shorter, 40-residue Aβ-(1–40) because of its greater *in vitro* tendency to aggregate and precipitate as amyloid (5). Although the conversion of monomeric Aβ peptide building blocks into multimeric amyloid fibril β-sheet aggregates (β-aggregation) is now considered a seminal event in the progression of AD,[1] the molecular mechanisms associated with these processes remain unknown.

Evidence suggests that increased oxidative stress plays an important role in AD neurodegeneration (6–8). Many proteins and DNA undergo oxidation more readily in AD brains, and the exact role that Aβ plays in these processes is not clear (9). The binding between Aβ and metals such as copper and zinc accelerates β-aggregation and generates reactive oxygen species and $H_2O_2$, which in turn accelerates oxidative damage to other proteins (10, 11). In all of these processes, the Met-35 side chain of Aβ is thought to play some critical role because oxidized Met-35 (Met-35$^{ox}$) is found in variable amounts of postmortem AD plaques (12, 13) and, for many proteins, the methionine side chains are easily oxidized under physiological conditions (Met → Met$^{ox}$) (14, 15). Moreover, because oxidized methionine can be reconverted enzymatically back to its reduced state (14), it was proposed that, for some proteins, surface-exposed methionine residues may function as antioxidants and protect other amino acids from irreversible oxidative damage (16).

For Aβ, contradicting reports exist between the Met-35 oxidation state and its effects on β-aggregation, toxicity, fibril morphology, and biological roles. A few reports demonstrate that Met-35$^{ox}$ increases β-aggregation and toxicity (3, 17), whereas others showed the opposite effects (18–21). The reason for these discrepancies is not clear, although they could be the result of several factors such as differences with the handling of Aβ (the well known lot-to-lot variability) (3, 22, 23) and/or differences in the solution conditions (24), (*i.e.* pH, peptide concentrations, temperature, and the type of peptide employed (Aβ-(1–40) or Aβ-(1–42)). Because of the importance of the Met-35 side chain in oxidative stress and its possible role as an electron donor for the reduction of Aβ-bound $Cu^{II}$ to $Cu^{I}$ (15, 21), we undertook our own systematic analysis using the more pathogenic Aβ-(1–42) under native-like conditions (aqueous solution, neutral pH). Here, for the first time we show that oxidation of Met-35 to Met-35$^{ox}$ significantly impedes the rate of amyloid formation and alters the fibril morphology. These results provide support for the hypothesis that oxidative stress and Aβ amyloid formation are intimately related in AD pathology.

## EXPERIMENTAL PROCEDURES

*Materials*—Thioflavin-T (Th-T) was purchased from Sigma (81K3643), and the Aβ-(1–42) peptide was synthesized and purified as described (25). The primary amino acid sequence for the amyloid Aβ-(1–42) peptide is the following: $H_3N^+$-$D^1$AEFR$^5$HDSG-Y$^{10}$EVHHQ$^{15}$KLVFF$^{20}$AEDVG$^{25}$SNKGA$^{30}$IIGLM$^{35}$VGGVV$^{40}$IA$^{42}$-$COO^-$. Peptide identity was verified by NMR spectroscopy and mass spectrometry (MS).

*Preparation of Aβ-(1–42) Solutions*—Lyophilized Aβ-(1–42) peptide (1.8 mg, 40 μmol) was disaggregated first by thoroughly dissolving the peptide in dilute NaOH solution (1.9 ml, 10 mM) with sonication for 1 min (26). This disaggregation procedure, which removes potential interference from small aggregates ("seeds"), is vital for obtaining consistent results and overcomes many of the lot-to-lot discrepancies frequently encountered with Aβ peptides (23). The basic pH solution of Aβ-(1–42) (1.9 ml, 10 mM) was then combined directly with a potassium phosphate-buffered solution (6.1 ml, 10 mM, pH 7.3) to yield a final stock solution (8 ml) with a peptide concentration of 50 μM. The pH of the stock solution was checked and, if needed, carefully adjusted to pH 7.3 with dilute NaOH or trifluoroacetic acid solutions.

*Oxidation of Aβ-(1–42)*—Met-35 oxidation was done according to methods previously described for the Aβ-(1–40) peptide (19), which involved the addition of dilute $H_2O_2$ (5 μl, 7.5% by weight) to a phosphate-buffered solution of Aβ-(1–42) (1 ml, 50 μM). The reaction was

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[1] The abbreviations used are: AD, Alzheimer's disease; Met-35$^{ox}$, methionine sulfoxide; Th-T, thioflavin-T; MS, mass spectroscopy; AFM, atomic force microscopy.

done in 4 h, and a single product was obtained, as shown by matrix-assisted laser desorption ionization MS and two-dimensional NMR.[2] The product was freeze-dried to remove excess $H_2O_2$ and redissolved in water. In the MS, the molecular ion of the product had a molecular mass (4531 Da) that was 16 Da higher than the native Aβ-(1–42) (4515 Da). This established that Met sulfoxide was the only product (these results are also consistent with NMR spectral data; not shown). After 1 week, the MS analysis of the peptide solution containing the $H_2O_2$ gave identical results, demonstrating that other possible oxidations (such as forming a dityrosine adduct (7, 27)) did not take place. For the time-dependent Th-T and CD studies, a fresh 50 μM Aβ-(1–42) stock solution (7.5 ml) in phosphate buffer (10 mM) was partitioned into 5 equal fractions (1.5 ml) and incubated at 22 °C for specific intervals before initiating Met-35 oxidation (Met-35 → Met-35$^{ox}$). Met-35 → Met-35$^{ox}$ to fractions 2, 3, 4, and 5 was started by the addition of dilute $H_2O_2$ (8 μl, 7.5% by weight) at 0.08, 10, 22, and 72 h, respectively.

*Circular Dichroism Spectroscopy*—The CD spectra were obtained at 22 °C using a J-810 spectropolarimeter (Jasco) and a 1-mm path length cell (Hellma). For each sample, five accumulative readings were averaged and acquired with 0.2-nm resolution, a 2-s response time, and 50 nm/min scan speed. Spectra were obtained from 190 to 250 nm. Two methods were used to obtain the percent soluble β-sheet structure: 1) a comparison of the ratios between the β-sheet ellipticities at 195 and 217 nm, and 2) spectral deconvolution using the program CDANAL (28). Both methods gave comparable results, and both have been used previously for CD studies of the Aβ (29, 30). The percent soluble β-sheet determined by CDANAL is presented in Fig. 3*D*.

*Atomic Force Microscopy*—Images were generated on a Multimode AFM instrument equipped with a Nanoscan III controller (Digital Instruments) using an "E"-type scanner with an *xy* range of 12.5 μm. Imaging was done at 22 °C under the air-tapping mode with commercially available silicon NanoProbes (Digital Instruments). In general, the scanning parameters were as follows: 2 V, initial root-mean-square amplitude; set point at 80% of the free amplitude; 250–350 kHz, tapping frequency; 2 Hz, scan rate; and 2-μm size (512 × 512-pixel resolution). A three-dimensional rendering was used for processing, with the image tilted by an angle of 5° to allow better visualization of the periodicities. For sample preparation on the mica surface, we generally followed previous AFM methods employed with the Aβ peptides (31), which included diluting the Aβ-(1–42) aliquots (5–10 μl) with an equal volume of water (0.2 μm filtered) immediately before application to the mica surface as required to produce a monolayer. This dilution procedure required <30 s and did not affect the outcome of our study (31).

*Fluorescence Spectroscopy*—Fluorescence measurements were performed on a Cary Eclipse spectrophotometer (Varian) using excitation and emission slit widths of 5 nm. Fluorescence intensity was measured at excitation and emission wavelengths of 450 and 482 nm, respectively (32). At specific time intervals, aliquots (20 μl) were removed from the Aβ-(1–42) peptide solutions, thoroughly mixed with a Th-T solution (1 ml, 4 μM) containing potassium phosphate buffer (10 mM, pH 7.4), followed by immediate measurement of the fluorescence.

## RESULTS

*Secondary Structure Analysis of the Aβ-(1–42) and Aβ-(1–42)Met-35$^{ox}$ by CD*—Fig. 1 presents the CD spectra for Aβ-(1–42) and Aβ-(1–42)Met-35$^{ox}$ peptide solutions after 0.17 and 48 h of incubation or aging at 22 °C. Because the Aβ structure and aggregational properties are highly concentration-dependent, a 50 μM peptide concentration was selected to allow easy monitoring of conformational changes by CD (concentrations above or below this threshold undergo corresponding changes too quickly or slowly). To ensure that the two solutions were identical, they were prepared from an identical stock solution after which one of the solutions was oxidized immediately.

As shown in Fig. 1, after aging for 0.17 h both peptides adopted a predominantly random structure as indicated by the major negative bands at 198 nm. After 48 h of aging, the CD spectra of Aβ-(1–42) demonstrated that a conformational change occurred in which the 198 nm band disappeared, with the appearance of positive and negative bands at 195 and 214 nm, consistent with β-sheet structure. This random → β-sheet

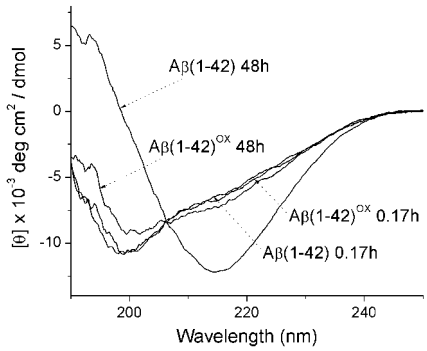


FIG. 1. **CD spectra of Aβ-(1–42) and Aβ-(1–42)Met-35$^{ox}$ solutions (50 μM, 22 °C, pH 7.3).** The two sets of spectra (obtained after 0.17 and 48 h of aging, respectively) demonstrate that Aβ-(1–42) shows a large conformational shift (random → β-sheet), whereas Aβ-(1–42)Met-35$^{ox}$ remains predominantly random.

conversion agrees with previous observations (24, 33, 34). By contrast, after 48 h Aβ-(1–42)Met-35$^{ox}$ remained predominantly random, with a modest reduction in the intensity of the 198 nm band. These results establish that Met-35$^{ox}$ hinders the random → β-sheet conversion associated with the β-aggregation process and Aβ neurotoxicity (2).

*Morphological Studies on Aβ-(1–42) and Aβ-(1–42)Met-35$^{ox}$*—To test whether Met-35$^{ox}$ affects Aβ fibril morphology, we used AFM, a well established technique for elucidating the fibril structures (31, 35–37). For an accurate comparison with the CD data, we used the same peptide solutions aged for 0.17 and 48 h (Fig. 1).

After aging for 0.17 h, the Aβ-(1–42) and Aβ-(1–42)Met-35$^{ox}$ solutions gave virtually identical AFM data, with essentially no detectable fibrils and only small aggregates. However, samples aged for 48 h gave a distinct sets of data, with the Aβ-(1–42) producing two well defined fibril types (Fig. 2*A*) and the Aβ-(1–42)Met-35$^{ox}$ producing no fibrils but only small globular structures (Fig. 2*B*). These globular structures had smaller dimensions and variable heights (0.7–3.3 nm, centered mostly at 1.4 ± 0.3 nm), appropriate to the assembly of small oligomers into larger conglomerates (51). For the Aβ-(1–42), the two types of fibrils were: large fibrils, brighter and sparsely dispersed (Fig. 2, *A* and *C–E*); and smaller, thin filaments. Many of the larger fibrils had average dimensions consistent with the Aβ protofibril, which is believed to be a neurotoxic intermediate formed during β-amyloidosis (39). These protofibrils had a 3.6 ± 0.6 nm average diameter and 21 ± 4 m periodicities, analogous to previously reported protofibril dimensions (3–4 nm average diameter, 20–22 nm periodicity) (31, 40). The second fibril type were thin filaments, also detected by Stine *et al.* (35), that had an 0.65 ± 0.07 average height with no clear periodicity. For the unmodified Aβ-(1–42), incubation times greater than 48 h lead to formation of longer protofibrils, as well as Type 1 and Type 2 mature fibrils (31, 35, 38, 40). Taken together, these data establish that Met-35$^{ox}$ markedly affects Aβ fibril morphology and inhibits production of both the thin filaments and the protofibril.

*Time-dependent Effects of Met-35$^{ox}$ on the Secondary Structure and β-Aggregation Rates of Aβ-(1–42)*—The CD results established that Met-35$^{ox}$ stabilizes the random structure and slows down production of the β-sheet structure found in fibrils (Fig. 1). To explore the possibility that Met$^{ox}$ not only slows down β-aggregation as an early event (random → β-sheet) but also at later stages (β-sheet (soluble) → β-sheet (amyloid fibril)), we undertook more detailed studies. These involved initiating Met-35 → Met-35$^{ox}$ at different time points during sample aging and monitoring the secondary structure and

[2] L. Hou, I. Kang, R. E. Marchant, and M. G. Zagorski, unpublished results.

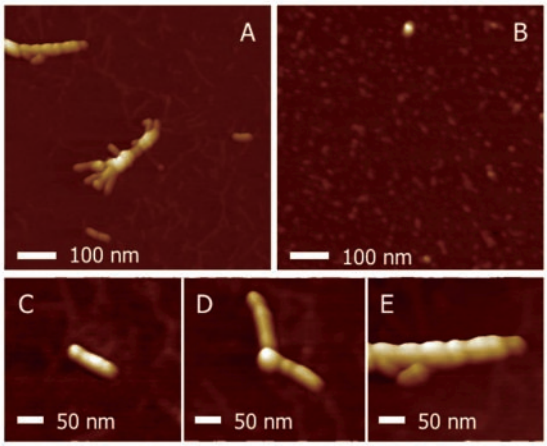


FIG. 2. **Representative AFM images of Aβ-(1–42) (*A*, *C*, *D*, *E*) and Aβ-(1–42)Met-35$^{ox}$ (*B*).** All images were taken from peptide solutions (50 μM) aged for 48 h (22 °C, pH 7.3). All images are presented with 10 nm in the Z-range. In the image of Aβ-(1–42) (*A*), protofibrils, aggregates of protofibrils, and mature fibers with a clear periodicity were observed. By contrast, Aβ-(1–42)Met-35$^{ox}$ (*B*) shows no fibrillar but only globular structures. The Aβ-(1–42) mature fiber had dimensions of 9.0 nm in height 294.9 nm in length with a periodicity of 38.3 ± 5.2 nm (*E*).

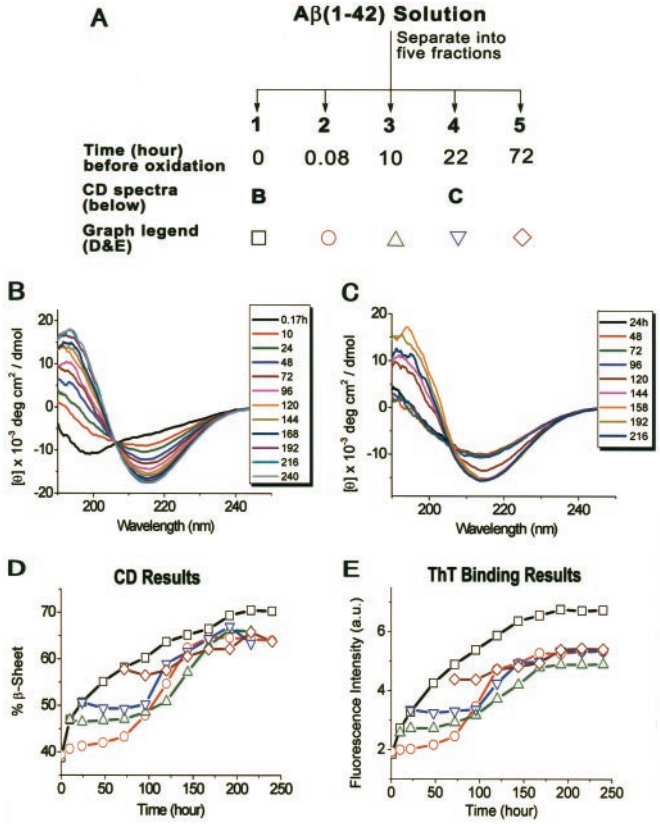


FIG. 3. **Time-dependent Effect of Met-35$^{ox}$ on the rate of random → β-sheet for Aβ-(1–42).** *A*, flow chart summarizing the partitioning of a single Aβ-(1–42) solution (50 μM, pH 7.3) into five equivalent fractions. For fractions 2, 3, 4, and 5, the time at which oxidation was commenced is also provided. Each fraction was monitored over time by CD and Th-T; representative CD spectra for fractions 1 and 4 are shown in *B* and *C*, respectively. The overlaid CD data for fraction 4 (*C*, oxidized at 22 h) does not include the 0.17- and 10-h traces, because these are the same as those for the unoxidized control (*B*). The graph legend symbols (*A*) summarize the percent β-sheet structure as determined from the CD data (*D*) and the Th-T fluorescence data (*E*). As shown, Met-35$^{ox}$ causes a significant lag phase and reduction in β-sheet production.

β-aggregation rates using CD and the Th-T binding assay (32).

Shown in Fig. 3*A* is an outline of the overall procedure. A freshly prepared Aβ-(1–42) solution was split into five equal fractions, and these solutions were then monitored by CD and Th-T for a period of up to 240 h. Fraction 1 represents the control, unoxidized sample, whereas fractions 2, 3, 4, and 5 were oxidized at 0.08, 10, 22, and 72 h of aging, respectively. Representative CD spectra for fractions 1 and 4 are presented in Fig. 3 (*B* and *C*) along with the estimated percent β-sheet structure and Th-T results for all five fractions (Fig. 3, *D* and *E*).

The increases in both the negative and positive ellipticities at 195 and 217 nm (Fig. 3*B*) demonstrate that the β-sheet content in unoxidized Aβ-(1–42) gradually increases over time, leveling off at 68–70% at 216–240 h of aging (Fig. 3*D*). In contrast, the Met-35$^{ox}$ samples show significantly reduced rates of β-sheet production. For example, with fraction 4 the β-sheet content (48–50%) remained nearly constant between 24 and 96 h followed by a rise at 120 h to 60% β-sheet (Fig. 3, *C* and *D*). Regardless of the time in which the Met-35 → Met-35$^{ox}$ was started, all samples showed similar lag phases (70–100 h), and all eventually went on to produce more β-sheet structures (Fig. 3*D*). The Th-T data shows comparable sigmoidal β-aggregation curves (Fig. 3*E*) in which the oxidized samples have reduced fluorescence because of their lower fibril content. On average, the final levels of β-sheet structure are 20% less with the oxidized samples, irrespective of the oxidation time. These data establish that Met-35$^{ox}$ hinders β-amyloidosis (random → β-sheet (soluble) → β-sheet (fibril)), regardless of the predominant solution conformation.

## DISCUSSION

It is now thought that therapeutic approaches for preventing the conformational conversions and fibrillation of Aβ could ameliorate the effects associated with oxidative stress and Aβ-induced neurotoxicity in Alzheimer's patients. Recent reports have demonstrated that soluble Aβ oligomers interfere with long-term potentiation (8), and another report shows that *in vitro* application of Aβ-derived diffusible ligands formed from synthetic Aβ-(1–42) causes neurological dysfunction in the hippocampus long before neurons degenerate (41). It is now evident that the research efforts directed at providing a molecular basis for the biological role of the Aβ and its relationship to oxidative stress, which is extensive and constitutes an early event in AD (42), will be a key factor in determining the most effective interventions.

The present biophysical data demonstrate that Met-35 to Met-35$^{ox}$ oxidation slows down β-aggregation and disrupts the characteristic fibril morphology. The decreased ability of Aβ-(1–42)Met-35$^{ox}$ toward β-aggregation could be the result of decreased hydrophobicity of the C-terminal tail, which leads to greater solubility and less hydrophobic induced aggregation (14, 43). A similar rationale was used to account for the decreased fibrillation of methionine-oxidized α-synuclein, a brain protein that is predominantly random but can produce the β-sheet aggregates found in Lewy bodies in Parkinson's disease (44). Similar lag phases for reduced fibrillation in Aβ-(1–42) (Fig. 3), irrespective of whether the major starting conformation is random or β-sheet, suggest that the increased polarity of Met-35$^{ox}$ leads to a nonproductive or alternative β-aggregation association that does not produce the characteristic amyloid-like fibrils.

A previous study (18) reported that Met-35$^{ox}$ inhibits β-aggregation of Aβ-(1–40), whereas another study (20), done in water/acetic acid solution, showed that the Aβ-(1–40)Met-35$^{ox}$ monomer did not aggregate beyond a dimer. Because the Aβ-(1–40)Met-35$^{ox}$ and Aβ-(1–42)Met-35$^{ox}$ monomeric structures

are predominantly random (45), these results demonstrate that Met-35$^{ox}$ prevents a random → β-sheet conversion, consistent with our present results with Aβ-(1–42). The discrepancies with previous reports, in which Aβ-(1–42)Met-35$^{ox}$ showed no inhibitory effect on β-aggregation or fibril formation (3, 21) or Met-35$^{ox}$ showed enhanced β-aggregation with Aβ-(1–40) (17), is probably because of the different solution conditions (such as pH and peptide concentration) and different peptide handling procedures (23). In the present work, we addressed these issues by thoroughly disaggregating the Aβ-(1–42) peptide (26) and partitioning this peptide solution into fractions, so that the only difference among the fractions is the Met-35 oxidation state. Our results were reproducible with the same or different Aβ-(1–42) peptide batches, regardless of whether synthetic and recombinant peptide was employed. Interestingly, our data also demonstrate that the inhibition is independent of the predominant solution conformation (random or β-sheet), suggesting that the slightly increased polarity of the Met-35$^{ox}$ side chain prevents intermolecular association of random monomers and/or β-sheet aggregates. This result may have implications for Aβ clearance, as the delay in β-aggregation may allow sufficient time for enzymes to degrade the monomer or dimers for excretion (46).

In addition, the present studies are the first to demonstrate a lack of a protofibril for Aβ-(1–42)Met-35$^{ox}$, which has been proposed to be a key intermediate in β-amyloidosis and neurotoxicity (31, 39, 40). This could explain why Met-35$^{ox}$ causes decreased cellular toxicity and lack of protein-induced oxidation (21). Although Aβ-(1–42)Met-35$^{ox}$ eventually forms β-sheet fibrils, the final levels of β-sheet structure are ~20% less than the reduced peptide (Fig. 3). These data suggest that the reduced and oxidized peptides may aggregate by alternative pathways, because the β-sheet fibril end points are different.

On the basis of the present results, we support the view that Aβ-(1–40) and Aβ-(1–42) peptides, which are both normal physiologic constituents, have a protective effect and under certain conditions function as antioxidants (47). Indeed, in normal individuals, low levels of Aβ are potent antioxidants and reduce the levels of oxidative stress, whereas in Alzheimer's patients, elevated Aβ production could be triggered by the increased levels of oxidative stress. Age-related events in the AD brain that either impair the ability of enzymes to promote Met-35$^{ox}$ → Met-35 (48) or impair the ability of Aβ to function as an antioxidant, may lead to neuronal cell demise by the accumulation of reactive oxygen species and reduced Aβ, which, according to the present results, is more prone to amyloid formation. We anticipate that additional studies aimed at establishing a more thorough connection between Aβ pro- and anti-oxidative properties and β-amyloidosis would facilitate the development of an appropriate therapeutic intervention for Alzheimer's patients.

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