Short communication

THE PROTECTIVE EFFECT OF CROCIN ON THE AMYLOID FIBRIL FORMATION OF Aβ$_{42}$ PEPTIDE *in vitro*

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Abstract: Aβ is the main constituent of the amyloid plaque found in the brains of patients with Alzheimer's disease. There are two common isoforms of Aβ: the more common form, Aβ$_{40}$, and the less common but more amyloidogenic form, Aβ$_{42}$. Crocin is a carotenoid from the stigma of the saffron flower and it has many medicinal properties, including antioxidant effects. In this study, we examined the potential of crocin as a drug candidate against Aβ$_{42}$ amyloid formation. The thioflavin T-binding assay and electron microscopy were used to examine the effects of crocin on the extension and disruption of Aβ$_{42}$ amyloids. To further investigate the relationship between crocin and Aβ$_{42}$ structure, we analyzed peptide conformation using the ANS-binding assay and circular dichroism (CD) spectroscopy. An increase in the thioflavin T fluorescence intensity upon incubation revealed amyloid formation in Aβ$_{42}$. It was found that crocin has the ability to prevent amyloid formation by decreasing the fluorescence intensity. Electron microscopy data also indicated that crocin decreased the amyloid fibril content of Aβ. The ANS-binding assay showed that crocin decreased the hydrophobic area in incubated Aβ$_{42}$. CD spectroscopy results also showed that the peptide undergoes a structural change to α-helical and β-turn. Our study shows that the anti-amyloidogenic effect of crocin may be exerted not only by the inhibition of Aβ amyloid formation but also by the

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disruption of amyloid aggregates. Therefore, crocin could be essential in the search for therapies inhibiting aggregation or disrupting aggregation.

Key words: Alzheimer's disease, Neurotic plaques, $A\beta_{42}$, Crocin, Amyloid, Neurofibrillary, Aggregation, Oligomerization, Protofibrils, Cytotoxic

INTRODUCTION

Alzheimer's disease (AD) is categorized as an advanced disorder involving brain nerve cell destruction. It is considered the most common kind of mental agitation or paranoia for which no valid diagnostic test has been found and is one of the major causes of death in the world [1-3]. The symptoms of this disease start with a loss of the capability to retain data, especially that of the short-term memory, and gradually move through loss of time awareness and the power of speech, to depression, and finally to death due to respiratory failure. Death occurs within 5 to 10 years of the onset of symptoms, but the disease actually begins about 20 years before symptoms appear [4].

The cause of the disease is the presence of two types of unusual structure called neurofibrillary tangles and aging plaques. Neurofibrillary tangles consist of microtubules associated with tau protein. These microtubule structures play important roles, such as participating in cell shape and structure, and producing cellular transport networks, which are important in moving micronutrients, neurotransmitters and organelles. Aggregation of $A\beta$ fibrils induces calcium entrance into the cell and activates a calcium-dependent kinase that hyperphosphorylates tau protein. Due to the high phosphorylation of tau protein, the tau and tubulin bonds are broken and microtubules become unstable. When the microtubules become unstable, they are deposited within the cells, giving rise to neurofibrillary tangles, which will ultimately lead to cell death [5-7].

Unlike intracellular neurofibrillary tangles, aging plaques can accumulate in extracellular positions. These plaques arise due to the accumulation of $A\beta$ peptides that consist of 40 or 42 amino acids ($A\beta_{40}$ and $A\beta_{42}$) and are formed by sequential cleavage of the amyloid precursor protein (APP), which is a membrane protein containing 695-770 amino acids [5, 8].

Cleavage of the APP occurs near the membrane through the action of a series of specific enzymes called secretases (types alpha, beta and gamma secretase). In AD, cleavage of APP by beta secretase results in the creation of a part that is again broken by gamma secretase and accumulates on the outside of neuronal cell bodies and creates $A\beta$ plaques. The plaques accumulate in vulnerable brain regions, such as the hippocampus and entorhinal cortex, which are sensitive to AD [9, 10].

High concentrations of soluble aggregates of $A\beta$ act as a poison and can lead to cell apoptosis. In addition, this state can induce oxidative stress that is regarded as an important factor in the onset of AD. This is due to an imbalance between free radicals and a shortage of their cleaner or a malfunction in the system that should

repair oxidized molecules. These stresses can cause injuries, such as the oxidation of membrane lipids and damage to DNA and cytoplasmic proteins [11, 12].

A common factor in the proposed mechanisms of $A\beta_{42}$ toxicity is amyloid oligomerization [13, 14]. The first phase of the oligomerization of $A\beta_{42}$ monomers includes the formation of pentamer/hexamer units that are called paranuclei. Paranuclei are the most basic and smallest structures that can be oligomerized into larger forms involving large oligomers, protofibrils and fibrils. Monomers, paranuclei and large oligomers are predominately unstructured with small amounts of β-sheet/β-turn and α-helix. During the formation of protofibrils, specific conformational changes occur causing the unknown structures of α-helix and β stranded structures to change into β-sheet/β-turn structures. A comparison of 34 physiologically relevant Aβ alloforms also revealed that an important feature controlling rapid oligomerization is the area of the C terminal. The side chain of residue 41 in $A\beta_{42}$ is important both for paranucleus formation and the accumulation and formation of large oligomers. The carboxyl groups of the C terminal of the side chain of residue 42 also affect paranucleus aggregation and oligomer formation [15].

Drugs that have been discovered with some potential to treat AD include acetyl cholinesterase enzyme inhibitors such as tacrine, donepezil, galantamine and rivastigmine, but they are very limited [16]. All these drugs have many potentially adverse side effects so attempting to discover new drugs with fewer side effects is an important area of research.

One of the materials that has been clinically evaluated for its effect on AD is crocin. Saffron is a good source of crocin. Saffron has three main chemical components: carotenoids with their bright red color, picrocrocin, and saffron. Crocin is a glycosylated carotenoid that is among the few water-soluble carotenoids with antioxidant properties. Thus, it is one of the chemicals that could be appropriate in the treatment of oxidative stress diseases such as AD [17, 18].

The purpose of this study was to evaluate the effectiveness, function and mechanism of crocin in preventing the $A\beta_{42}$ formation associated with early onset AD [19]. Spectroscopic studies of thioflavin proved that there was amyloid formation in the $A\beta_{42}$ peptide, but this was reduced in the presence of crocin. This finding was supported by the results of TEM imaging. ANS-binding experiments showed that the fluorescence intensity of $A\beta_{42}$ decreased in the presence of crocin, implying that the exposed hydrophobic region of $A\beta_{42}$ interacted with crocin. The data obtained from circular dichroism (CD) spectroscopy also proved that in both the fresh and incubated peptide, in the presence of crocin, the stability of the helical regions of the peptide increased. Results from this study provide helpful clues regarding the potential role of crocin in AD therapy and contribute to the development of an anti-amyloid therapeutic strategy.

MATERIALS AND METHODS

Chemicals and peptides

$A\beta_{42}$, DMSO, ThT, NaN_3, ANS and Na_2HPO_4 were all prepared by Sigma-Aldrich (St. Louis, USA). Crocin was extracted and purified from saffron as described by Bolhasani *et al.* [20].

Fibril formation of $A\beta_{42}$ as assessed with the thioflavin T-binding assay

Fibril formation of $A\beta_{42}$ (0.22 mM) was performed in the presence and absence of crocin (15.4 μM) [21] by incubation of $A\beta_{42}$ in Na_2HPO_4, 100 mM NaCl (pH 7.4) at 37ºC in an incubator (A-Q, Germany). The amyloid formation was detected by the increase in ThT fluorescence in a medium with 0.4 μM ThT in 50 mM Na_2HPO_4 and 0.05% (w/v) NaN_3 (pH 7.4). The excitation and emission wavelengths were respectively set to 440 and 490 nm with respective slit widths of 2.5 and 5 nm. The protective effect of crocin is calculated using the protection formula $((Ao - A) / Ao) \times 100$, where Ao and A represent the maximum fluorescence emissions respectively in the absence and presence of crocin. All of the experiments were independently repeated at least three times, and the results are shown as means ± SEM.

ANS-binding experiments of the $A\beta_{42}$ peptide

An ANS-binding assay of $A\beta_{42}$ (0.22 mM) in the presence and absence of crocin (15.4 μM) was done using a Varian spectrofluorometer at 37ºC for one hour. The experiment was done in a 10 mM phosphate buffer along with 150 mM NaCl at pH 7.4. The wavelengths of excitation and emission were respectively set to 446 and 400-600 nm with respective slit widths of 5 and 10 nm. The fluorescence emission intensity was measured in a 10-mm path length quartz cuvette in 350 μl samples titrated with 1 μl aliquots of a 10 mM ANS (1-anilino-8-naphthalene sulfonic acid) stock solution in a 50 mM phosphate buffer (pH 7.4).

Transmission electron microscopy (TEM) of $A\beta_{42}$ peptide

Formvar and carbon-coated nickel TEM grids (SPI Supplies, Westchester, USA) were prepared by the application of a 2 μl protein sample at a concentration of 0.22 mM. The grids were then washed three times with 10 μl Milli-Q water and negatively stained with 10 μl uranyl acetate (2% w/v; Agar Scientific, UK). The grids were dried with filter paper between each step. The samples were viewed under 25-64 K magnifications at an excitation voltage of 100 kV using a ZEISS LE9910 transmission electron microscope. The dimensions of the fibrils were estimated by observing the images at 100% zoom in Paintbrush (Microsoft Corporation, USA).

CD spectroscopy of $A\beta_{42}$ peptide

Far-UV CD (190-260) was obtained to explore changes in the secondary structure of $A\beta_{42}$ in the absence and presence of crocin. The experiments were done in a 10 mM phosphate buffer (pH 7.4). CD spectra were obtained with

a JASCO J-810 Spectropolarimeter (Jasco, Corporation, Japan) connected to a Desaga water bath. Spectra were recorded with a data interval of 1 nm, a response time of 4 seconds and a scan rate of 100 nm/min. Each spectrum was an average of 4 scans with a baseline scan subtracted.

RESULTS

Thioflavin T-binding assay

Thioflavin T, which has fluorescent properties, is a dye that is generally used for observing and quantifying amyloid assembly under both *in vivo* and *in vitro* conditions in such a way that the fluorescence intensity is increased by connection to amyloid fibrils [22].

In this study, an enhancement in the ThT fluorescence following the incubation of $A\beta_{42}$ peptide was observed, implying amyloid formation in the peptide (Fig. 1A).
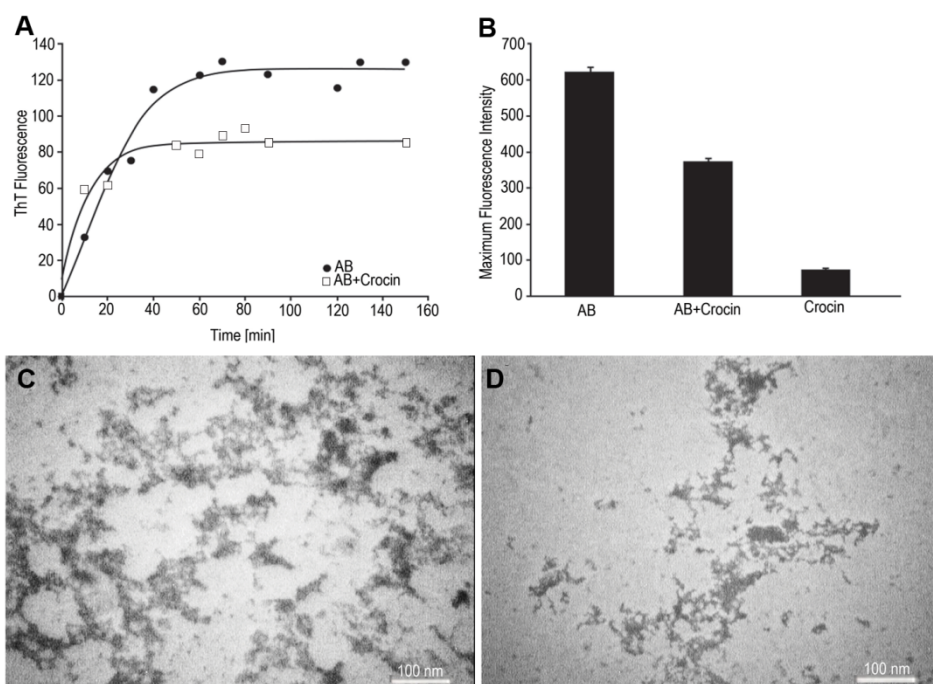

Fig. 1. Fluorescence data and electron micrographs of $A\beta_{42}$ incubated in the presence and absence of crocin. A – ThT fluorescence analysis of fibril formation after incubation with 0.22 mM $A\beta_{42}$ (●) alone and (□) in the presence of 15.4 μM crocin. B – ANS fluorescence data for 0.22 mM $A\beta_{42}$ and 15.4 μM crocin alone and in the presence of each other following excitation at 446 nm. Electron micrograph of 0.22 mM $A\beta_{42}$ incubated in the absence (C) and presence (D) of crocin. The scale bar represents 100 nm. Samples were incubated in 10 mM Na_2HPO_4 and 150 mM NaCl (pH 7.4) at 37ºC. ThT data were normalized to zero fluorescence intensity at the beginning of the experiment. Data shown are single measurements and are representative of three experiments.

However, in the presence of crocin, the fluorescence intensity was reduced, which means a reduction in the degree of amyloid assembly in the presence of crocin. This was also evident from the protective percentage: crocin provided 31% protection at the end point of 160 min.

ANS-binding study

ANS is a hydrophobic fluorescent molecular probe that has been used for examining the non-polar character of proteins and membranes [23-25]. ANS binding is used to study changes in exposed hydrophobicity in $A\beta_{42}$ upon incubation in the presence and absence of crocin. An increase in ANS fluorescence was observed in $A\beta_{42}$ until it maxed out and plateaued (data not shown). The ANS fluorescence maxima in the absence and presence of crocin $A\beta_{42}$ are shown in Fig. 1B. According to these data, $A\beta_{42}$ alone showed high ANS binding, which indicates hydrophobic protein surface exposure. In the presence of crocin, the ANS fluorescence intensity is reduced by 40% compared to the sum of the individual protein and crocin. The results show that hydrophobic surface exposure of $A\beta_{42}$ decreased in the presence of crocin, and this implies interaction between crocin and the protein and the formation of a complex between them.

Transmission electron microscopy (TEM)

TEM analysis was performed to obtain a morphological assessment of how crocin influenced $A\beta_{42}$ aggregation. Micrographs obtained for $A\beta_{42}$ incubated for 180 min displayed a large number of entangled fibril aggregates. In the presence of crocin it was apparent that the number of fibrils decreased (Fig. 1C and D).

CD spectroscopy study

Circular dichroism (CD) spectroscopy is widely seen as a valuable technique for testing the protein structure in solution [26]. Fig. 2A and B show the CD spectra of native and incubated $A\beta_{42}$ in the absence and presence of crocin. As shown in Fig. 2A, the spectrum obtained for native $A\beta_{42}$ is characteristic of an α-helix conformation, showing negative ellipticity at around the 220 nm region. The CD spectrum of incubated $A\beta_{42}$ is typical of a peptide containing significant portions of β-sheet structure, as evidenced by the curve with a characteristic minimum at 217 nm (Fig. 2B). The evaluation of the secondary structure of native $A\beta_{42}$ revealed 21.2% β-sheet contents, which is similar to the observations of other researchers [27]. Adding crocin to $A\beta_{42}$ induced a change in the features of the Aβ spectrum, raising the intensity of the 220 nm band and increasing the α-helix content to 28.3%. After the addition of crocin to the incubated peptide, the CD spectrum indicates a conformational transition from a largely disordered structure to one highly enriched in α-helix. This is indicative of a decrease in the contribution from the unstructured peptide that was rearranged to form the α-helix. The values obtained from this analysis are listed in Table 1.
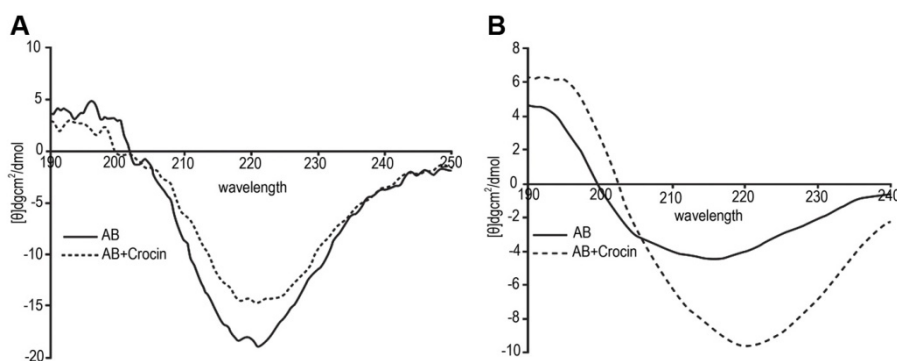
A

B

Fig. 2. Far UV CD spectra of the native (A) and incubated (B) $A\beta_{42}$ structure in the presence and absence of crocin. Spectra of $A\beta_{42}$ without crocin are shown as a solid line and with crocin as a dotted line. The protein concentration was 0.22 mM in 10 mM phosphate buffer (pH 7.0) at 37ºC in a JASCO J-810 spectropolarimeter with a 1-cm path length cell. The experiment was done in duplicate.

Table 1. Secondary structure prediction from far-CD spectra for native and incubated $A\beta_{42}$ in the absence and presence of crocin using the JASCO program.

Sample	β-Sheet [%]	α-Helix [%]	β-Turn [%]	Random coil [%]
$A\beta_{42}$ (native)	21.2	26.1	7.2	54.5
$A\beta_{42}$ + crocin (native)	14	28.3	9.0	48.7
$A\beta_{42}$ (incubated)	41.3	21	6.50	31.30
$A\beta_{42}$ + crocin (incubated)	4.8	31.2	26.20	37.0

DISCUSSION

Aβ fibrils are shown in the Alzheimer's Aβ peptide. In this sense, the alpha-helical structural changes to the beta sheet are of physiological importance and lead to the formation of amyloid structures [28-33]. Aβ peptides are composed of two isomers: $A\beta_{40}$ and $A\beta_{42}$, in which 10% of the total Aβ consists of $A\beta_{42}$ [30]. $A\beta_{42}$ is the most amyloidogenic form of the peptide because it is more hydrophobic than $A\beta_{40}$ [16, 35].
In this study, we used a thioflavin T-binding assay to provide some evidence about amyloid formation and interaction with crocin. Incubation of $A\beta_{42}$ at a temperature of 37ºC for 3 h induced the formation and accumulation of amyloid fibrils, as shown by the increases in the fluorescence intensity. This is in agreement with the results of other studies [37-39]. Crocin effectively prevented amyloid formation by incubated $A\beta_{42}$ by reducing the amount of amyloid fibril. This was proved by the reduction in thioflavin T fluorescence intensity. TEM examination of incubated $A\beta_{42}$ at 37ºC for 3 h also showed a great amount of amyloid oligomer [39, 40]. In addition, electron microscopy imaging revealed a decrease in the amount of amyloid fibrils in the presence of crocin (Fig. 2C and D).

The ANS-binding assay of incubated $A\beta_{42}$ showed very high fluorescence intensity due to the exposure of the hydrophobic region because of change in the peptide conformation [32, 41, 42]. However, adding crocin to $A\beta_{42}$ significantly decreased the fluorescence intensity, probably due to the interaction between crocin and $A\beta_{42}$.

Our CD results also showed that natively dissolved $A\beta_{42}$ develops a greater proportion of β-sheet structures upon incubation. Accordingly, as shown in Fig. 2A and B and Table 1, it is reasonable to conclude that after incubation with crocin, $A\beta_{42}$ develops a partial helical structure. The evaluation of the CD peak supports the idea that crocin causes $A\beta_{42}$ to form an alpha-helix structure that is a non-amyloidogenic conformer [25]. Possible reasons for this observation are provided below.

NMR structural investigation into the secondary structure of $A\beta_{42}$ has shown that the peptide contains two amyloid helical regions, including residues 8-25 and 28-38, which have been connected by a β turn [43, 44]. It has a hydrophilic N-terminal starting Asp and a hydrophobic C-terminal ending alanine in and it contains a central hydrophobic cluster (residues 17-21) that appears to be the main constituent of amyloid plaques as found in the brain of AD patients. In addition, residues 28-42 have been shown to have a high probability for β-sheet structure formation [43]. Presumably, incubation increases the interaction between hydrophobic areas and increases the β-sheet content (41.30%) which is important for amyloid formation [45]. These results are consistent with the aggregative ability and neurotoxicity. However, the β-sheet content in incubated $A\beta_{42}$ decreased to 4.8% in the presence of crocin. This is probably due to interaction between the crocin and $A\beta_{42}$ and stabilization of the protein structure. Since the content of β-sheet in incubated $A\beta_{42}$ in the presence of crocin is even less than that in fresh $A\beta_{42}$ (Table 1), we conclude that crocin interacts both with the aggregation-prone hydrophobic core (residues 17-21) and the C-terminal residue (residues 28-42). It is likely that crocin interacts with the C-terminal region and prevents its interaction with other monomers, forms paranuclei and subsequently amyloid fibrils. In addition, the binding of crocin to the aggregation-prone hydrophobic core stabilizes this region and prevents conformational change from an α-helix or random coil structure to a β-sheet, which is the key to forming fibrils and inducing cytotoxic effects [43].

Another explanation of the effects of crocin on amyloid may be related to its antioxidant properties. Many studies on antioxidants and their effect on amyloid fibril formation have been conducted. In studies on vitamin E and its isomers (forms of tocopherols), it was found that these vitamins may reduce the severity of amyloid fibril formation by reducing oxidative metabolites. Studies by Mishima et al. [46] also showed that the antioxidant power of α-tocotrienol is greater than that of α-tocopherol. The difference is in the greater number of double bonds that the former compound has (3 double bonds in the lipophilic tail). If the antioxidant activity has a direct relationship with the number of double bonds, then crocin, having several double bonds, will have a beneficial

antioxidant property [17]. Therefore, it can be concluded that crocin, like other antioxidants, will slow down the formation of amyloid fibrils in $A\beta_{42}$.

CONCLUSIONS

The inhibitory effects of crocin clearly indicate that it could be used as a pharmacological agent to inhibit or retard amyloid fibril formation in Alzheimer's disease. We have shown that crocin controls $A\beta_{42}$-mediated amyloid fibril formation *in vitro*, probably through the stabilization of the helical structure, which prevents fibril formation and dissolves previously formed aggregates. However, further studies, including molecular dynamic simulation, are needed into the hypothesis regarding the formation of a complex of crocin with protein to eliminate the free hydrophobic area or prevent interaction with the C-terminal region of amyloid fibrils.

The prevention of neuronal cell apoptosis has always been emphasized as a therapeutic strategy for the treatment of neuronal cell destruction. The main advantage for crocin as a drug for the treatment of AD appears to be that it has fewer side effects, it has economic benefits (it forms a greater percentage of saffron constituents and can be easily extracted) and it is water soluble. It is encouraging that crocin remains a candidate drug against AD. The experimental data described here may well indicate that the utilization of crocin can control amyloid fibril formation and contribute to the development of a therapeutic strategy against AD.

REFERENCES

1. Burns, A., Byrne, E.J. and Maurer, K. Alzheimer's disease. **Lancet** 360 (1998) 163-165.
2. Brookmeyer, R., Gray, S. and Kawas, C. Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset. **Am. J. Public Health** 88 (1998) 1337-1342.
3. Khalil, Z., Poliviou, H., Maynard, C.J., Beyreuther, K., Masters, C.L. and Li, Q.X. Mechanisms of peripheral microvascular dysfunction in transgenic mice overexpressing the Alzheimer's disease amyloid Abeta protein. **J. Alzheimer's Dis.** 4 (2002) 467-478.
4. Waldemar, G., Dubois, B., Emre, M., Georges, J., McKeith, I.G., Rossor, M., Scheltens, P., Tariska, P. and Winblad, B. Recommendations for the diagnosis and management of Alzheimer's disease and other disorders associated with dementia: EFNS guideline. **Eur. J. Neurol.** 14 (2007) e1-e26.
5. Veeranna, Kaji, T., Boland, B., Odrljin, T., Mohan, P., Basavarajappa, B.S., Peterhoff, C., Cataldo, A., Rudnicki, A., Amin, N., Li, B.S., Pant, H.C., Hungund, B.L., Arancio, O. and Nixon, R.A. Calpain mediates calcium-induced activation of the Erk1,2 MAPK pathway and

cytoskeletal phosphorylation in neurons: relevance to Alzheimer's disease. **Am. J. Pathol.** <u>165</u> (2004) 795-805.

6. Thomas, P. and Fenech, M. A review of genome mutation and Alzheimer's disease. **Mutagenesis** <u>22</u> (2007) 15-33.

7. Bajić, P.V., Su, B., Lee, H., Kudo, W., Siedlak, L.S., Živković, L., Spremo-Potparević, B., Djelic, N., Milicevic, Z., Singh, K.A., Fahmy, M.L., Wang, X., Smith, A.M. and Zhu, X. Mislocalization of CDK11/PITSLRE, a regulator of the G2/M phase of the cell cycle, in Alzheimer's disease. **Cell. Mol. Biol. Lett.** <u>16</u> (2011) 350-372.

8. Koo, E.H. The beta-amyloid precursor protein (APP) and Alzheimer's disease: does the tail wag the dog? **Traffic** <u>3</u> (2002) 763-770.

9. Wirths, O., Multhaup, G. and Bayer, T.A. A modified beta-amyloid hypothesis: intraneuronal accumulation of the beta-amyloid peptide—the first step of a fatal cascade. **J. Neurochem.** <u>91 </u>(2004) 513-520.

10. Howlett, D.R., Simmons, D.L., Dingwall, C. and Christie, G. In search of an enzyme: the beta-secretase of Alzheimer's disease is an aspartic proteinase. **Trends Neurosci.** <u>23</u> (2000) 565-570.

11. Yatin, S.M., Varadarajan, S., Link, C.D. and Butterfield, D.A. In vitro and in vivo oxidative stress associated with Alzheimer's amyloid beta-peptide (1-42). **Neurobiol. Aging** <u>20</u> (1999) 325-330.

12. Butterfield, D.A. Amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. **Free Radic. Res.** <u>36</u> (2002) 1307-1313.

13. Gandy, S., Simon, A.J., Steele, J.W., Lublin, A.L., Lah, J.J., Walker, L.C., Levey, A.I., Krafft, G.A., Levy, E.F., Checler, F., Glabe, C., Bilker, W., Abel, T., Schmeidler, J. and Ehrlich, M.E. Days to criterion as an indicator of toxicity associated with human Alzheimer amyloid-beta oligomers. **Ann. Neurol.** <u>68</u> (2012) 220-230.

14. Roher, A.E., Chaney, M.O., Kuo, Y.M., Webster, S.D., Stine, W.B., Haverkamp, L.J., Woods, A.S.C., Tuohy, J.M., Krafft, G.A., Bonnell, B.S. and Emmerling, M.R. Morphology and toxicity of Abeta-(1-42) dimer derived from neuritic and vascular amyloid deposits of Alzheimer's disease. **J. Biol. Chem.** <u>271</u> (1996) 20631-20635.

15. Kirkitadze, M.D. and Kowalska, A. Molecular mechanisms initiating amyloid beta-fibril formation in Alzheimer's disease. **Acta Biochim. Pol.** <u>52</u> (2005) 417-423.

16. Sallowaya, S., Mintzerb, J., Weinerc, M.F. and Cummings, J.L. Disease-modifying therapies in Alzheimer's disease. **Alzheimer's Dement.** <u>4</u> (2008) 65-79.

17. Bathaie, S.Z. and Mousavi, S.Z. New applications and mechanisms of action of saffron and its important ingredients. **Crit. Rev. Food. Sci. Nutr.** <u>50</u> (2010) 761-786.

18. Soeda, S., Ochiai T., Shimeno, H., Saito, H., Abe, K., Tanaka, H. and Shoyama, Y. Pharmacological activities of crocin in saffron. **J. Nat. Med.** 61 (2007) 102-111.

19. Yin, Y.I., Bassit, B., Zhu, L., Yang, X., Wang, C. and Li Y.M. γ-secretase substrate concentration modulates the Aβ42/Aβ40 ratio: Implications for Alzheimer's disease. **J. Biol. Chem.** 282 (2007) 23639-23644.

20. Bolhasani Sanjabi, A., Bathaie, S.Z., Moosavi-Movahedi, A.A. and Ghaffari, M. Separation and purification of some components of Iranian saffron. **Asia J. Chem**. 17 (2005) 725-729.

21. Pandreou, M.A., Kanakis, C.D., Polissiou, M.G., Efthimiopoulos, S., Cordopatis, P., Margarity, M. and Lamari, F.N. Inhibitory activity on amyloid-beta aggregation and antioxidant properties of crocus sativus stigmas extract and its crocin constituents. **J. Agric. Food Chem.** 54 (2006) 8762-8768.

22. Khurana, R., Coleman, C., Ionescu-Zanetti, C., Carter, S.A., Krishna, V., Grover, R.K., Roy, R. and Singh, S. Mechanism of thioflavin T binding to amyloid fibrils. **J. Struct. Biol.** 151 (2005) 229-238.

23. Kirk, W.R., Kurian, E. and Prendergast, F.G. Characterization of the sources of protein-ligand affinity: 1-sulfonato-8-(1')anilinonaphthalene binding to intestinal fatty acid binding protein. **Biophys. J.** 70 (1996) 69-83.

24. Matulis, D., Baumann, C.G., Bloomfield, V.A. and Lovrien, R.E. 1-anilino-8-naphthalene sulfonate as a protein conformational tightening agent. **Biopolymers** 49 (1999) 451-458.

25. Matulis, D. and Lovrien, R. 1-Anilino-8-naphthalene sulfonate anion-protein binding depends primarily on ion pair formation. **Biophys. J.** 74 (1998) 422-429.

26. Kelly, S.M., Jess, T.J. and Price, N.C. How to study proteins by circular dichroism. **Biochim. Biophys. Acta** 1751 (2005) 119-139.

27. Sureshbabu, N., Kirubagaran, R. and Jayakumar, R. Surfactant-induced conformational transition of amyloid β-peptide. **Eur. Biophys. J.** 38 (2009) 355-367.

28. Hasegawa, K., Ono, K., Yamada, M. and Naiki, H. Kinetic modeling and determination of reaction constants of Alzheimer's beta-amyloid fibril extension and dissociation using surface plasmon resonance. **Biochemistry** 41 (2002) 13489-13498.

29. Naiki, H. and Gejyo, F. Kinetic analysis of amyloid fibril formation. **Methods Enzymol.** 309 (1999) 305-318.

30. Sunde, M., Serpell, L.C., Bartlam, M., Fraser, P.E., Pepys, M.B. and Blake, C.C. Common core structure of amyloid fibrils by synchrotron X-ray diffraction. **J. Mol. Biol.** 273 (1997) 729-739.

31. Wetzel, R. Ideas of order for amyloid fibril structure. **Structure** 10 (2002) 1031-1036.

32. Dobson, C.M. Protein misfolding, evolution and disease. **Trends Biochem. Sci.** 24 (1999) 329-332.

33. Dobson, C.M. The structural basis of protein folding and its links with human disease. **Philos. Trans. R. Soc. Lond. B. Biol. Sci.** 356 (2001) 133-145.

34. Younkin, S.G. Evidence that Aβ42 is the real culprit in Alzheimer's disease. **Ann. Neurol.** 37 (1995) 287-288.

35. Kayed, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W. and Glabe, C.G. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. **Science** 300 (2003) 486-489.

36. Ban, T., Hamada, D., Hasegawa, K., Naiki, H. and Goto, Y. Direct observation of amyloid fibril growth monitored by thioflavin T fluorescence. **J. Biol. Chem.** 278 (2003) 16462-16465.

37. Bourhim, M., Kruzel, M., Srikrishnan, T. and Nicotera, T. Linear quantitation of Aβ aggregation using Thioflavin T: Reduction in fibril formation by colostrinin. **J. Neurosci. Methods** 160 (2007) 264-268.

38. Nybo, M., Svehag, S.E. and Holm Nielsen, E. An ultrastructural study of amyloid intermediates in A beta1-42 fibrillogenesis. **Scand. J. Immunol.** 49 (1999) 219-223.

39. Caesar, I., Jonson, M., Nilsson, K.P., Thor, S. and Hammarström, P. Curcumin promotes A-beta fibrillation and reduces neurotoxicity in transgenic drosophila. **PLoS One** 7 (2012) e31424.

40. Kanski, J., Aksenova, M. and Butterfield, D.A. The hydrophobic environment of Met35 of Alzheimer's Abeta(1-42) is important for the neurotoxic and oxidative properties of the peptide. **Neurotox. Res.** 4 (2002) 219-223.

41. Cardamone, M. and Puri, N.K. Spectrofluorimetric assessment of the surface hydrophobicity of proteins. **Biochem. J.** 282 (1993) 589-593.

42. Schein, C.H. Solubility as a function of protein structure and solvent components. **Nat. Biotech.** 8 (1990) 308-317.

43. Serpell, L.C. Alzheimer's amyloid fibrils: structure and assembly. **Biochim. Biophys. Acta** 1502 (2000) 16-30.

44. Crescenzi, O., Tomaselli, S., Guerrini, R., Salvadori, S., D'Ursi, A.M., Temussi, P.A. and Picone, D. Solution structure of the Alzheimer amyloid beta-peptide (1-42) in an apolar microenvironment. Similarity with a virus fusion domain. **Eur. J. Biochem.** 269 (2002) 5642-5648.

45. López De La Paz, M., Goldie, K., Zurdo, J., Lacroix, E., Dobson, C.M., Hoenger, A. and Serrano, L. De novo designed peptide-based amyloid fibrils. **Proc. Natl. Acad. Sci. USA** 99 (2002) 16052-15057.

46. Mishima, K., Tanaka, T., Pu, F., Egashira, N., Iwasaki, K., Hidaka, R., Matsunaga, K., Takata, J., Karube, Y. and Fujiwara, M. Vitamin E isoforms alpha-tocotrienol and gamma-tocopherol prevent cerebral infarction in mice. **Neurosci. Lett.** 337 (2003) 56-60.