

The Anti-amyloidogenic effect of natural product extracts on amyloid-like fibril formation of trypsin in aqueous organic solvents

Summary of Ph.D. Thesis

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Introduction

Amyloid aggregation is a hallmark of several central nervous system neurological disorders including neuro development diseases affecting brain and peripheral tissues. The formation of insoluble amyloid is associated with a number of fatal neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's diseases, or type II diabetes mellitus, transmissible spongiform encephalopathy, cerebellar ataxias and primary and secondary systemic amyloidosis. Amyloid formation is a generic form of a polypeptide conformation and most proteins have a potential to form amyloid-like structure under appropriate conditions. The core structure of the fibrils is stabilized by hydrogen bonding between atoms of the polypeptide backbone. Despite different proteins being responsible for each disease, all of them share similar features including beta-sheet-rich secondary structures and fibril-like protein aggregates.

Prevention is a more effective strategy than the treatment of chronic diseases. Functional foods that contain significant amounts of bioactive components play important roles in the prevention of chronic diseases. All proteins may form unbranched, long, amyloid fibrils with many beta-sheets *in vitro* under suitable conditions i.e., formation of amyloid might be a general property of the polypeptide backbone. The aggregation behavior of various peptides and proteins shows remarkable similarities. Unfolded or partially unfolded proteins associate with each other to form small, soluble aggregates that undergo further assembly into protofibrils or protofilaments and then into mature fibrils, which is associated with increasingly common and highly debilitating diseases. The amyloid diseases involve predominantly the aggregation of specific proteins, but fibrils can be formed by many other peptides and proteins too. Natural phenolic compounds, a wide panel of plant molecules, are one of the most actively investigated categories of potential amyloid inhibitors. One therapeutic approach is the screening or development of natural bioactive molecule

inhibitors of A β aggregation. Dietary natural products are a rich source of phytochemicals and active compounds, which have the potential for future drug development for AD. In the last decade, researchers have reported that certain fruits, vegetables, spices, drinks and beverages can potentially counteract AD pathogenesis. These natural products are effective at inhibiting oxidative stress, A β accumulation and toxicity, tau phosphorylation, and neuro-inflammation and exhibit antioxidant and anti-inflammatory properties. Researchers are currently searching for new, safer and more effective therapies that can target the pathophysiology of AD more precisely and inhibit the A β accumulation and toxicity.

Aims

Our primary interest here was to discover novel molecules possessing anti-neurodegenerative and anti-amyloidogenic properties through the screening of a diverse library of natural compounds. By considering these main objectives, our aims were:

- Collection, extractions of bioactive compounds and total phenolic analysis from 5 categories or groups of natural products.
- Modification of trypsin with phenylmethylsulfonyl fluoride (PMSF) for the formation of amyloid-like fibril in aqueous organic solvents.
- *In vitro* screening by turbidity, size exclusion chromatography, aggregation kinetics, CR binding, FTIR, CD and TEM on amyloid-like fibril formation of trypsin by natural product extracts in aqueous organic ethanol.
- Selection of suitable therapeutic agents for amyloid related neurodegenerative diseases.

Materials and methods

To answer the above questions, the following specific methods were used for the inhibitory activity of natural product extracts on amyloid-like fibril formation of trypsin in aqueous organic solvents like in 60% ethanol at pH 7.0:

- preparation of the extracts,
- assay of enzyme activity,
- modification of trypsin with PMSF
- turbidity measurements,
- determination of the total phenolic content,
- aggregation kinetics,
- CR binding,
- HPLC-MS,
- ECD measurements,
- size exclusion chromatography,
- FTIR spectroscopy analysis,
- Transmission electron microscopy,
- Statistical analysis.

Results and discussion

Recent studies on A β amyloid formation indicate that several natural small aromatic compounds and flavonoids interfere with aggregation pathways possibly by remodelling the amyloid intermediates through the different mechanisms or interactions. The effects of different inhibitory agents on PMS-trypsin aggregation can also be monitored *via* turbidity measurements. CR binding

assay was used to determine whether the aggregated species formed in the PMS-trypsin samples were fibril-like or not. CR binding properties of amyloid aggregates have been widely used to study anti-fibrillation activity of various inhibitors. Difference spectra demonstrated the spectral changes of CR upon binding to PMS-trypsin amyloid fibrils. Amyloid formation and morphology of aggregates were visualized by using transmission electron microscopy in the absence and presence of different inhibitory agents after 24 h incubation.

Inhibition of protein aggregation using herbal extracts

The sample without inhibitory agent shows maximum absorption value at 350 nm whereas the presence of different herbal extracts shows a marked decrease in it. The results from this comparative study indicated that peppermint extract displayed the greatest amyloid inhibiting function of the 7 samples tested. Based on our results, it was found that the anti-amyloidogenic effects of the herbal extracts could be related to their total phenolic content. CR differential spectra indicated that the presence of the peppermint extract decreased the amounts of amyloid fibrils in 60% ethanol at pH 7.0, and its inhibitory effect was dose-dependent. Our results showed that the peppermint extract had a preventive effect on aggregation of the PMS-trypsin, and it could effectively inhibit the *in vitro* fibrillation.

Inhibitory effect of the chili extract on the amyloid-like fibril formation

Turbidity measurement was used as an indication of the degree of aggregation in the presence of 27 commercially available spices. All examined agents showed varied effects. These experiments revealed that among the spices investigated chili was the best inhibitor. CR binding assay indicated that the presence of the chili extract decreased the amounts of amyloid-like fibrils and inhibited the fibril formation dose-dependently. The ability of chili extract to prevent aggregation was

visualised by TEM. TEM image of PMS-trypsin in absence of chili extract show fibrillar structure in 60% ethanol. However, in the sample in which chili extract was added to the PMS-trypsin, there was a significant lack of fibrils with only occasional scattered amorphous aggregates. The finding suggests that chili bioactive compounds may be potential therapeutic drug candidates.

Examination of the anti-amyloidogenic effect of the *P. ginseng* extract

Our results show that the presence of ginseng extract helps at maintaining the native structure of the protein. ECD experiments were made in two different sample positions. Significant change can be observed in the sample without the *P. ginseng* extract concerning the intensity and blue shift of spectral maxima for the sample closer to the detector. Adding *P. ginseng* extract to our sample decreased the light dispersion of the enzyme solution. This is due to the decrease of the rate of the protein aggregation or to the smaller size of the aggregates evolved. In the presence of *P. ginseng* extract, TEM image demonstrated that fibril formation was effectively inhibited.

Inhibition of amyloid-like fibril formation using the Eduscho coffee extract

The turbidity measurements were performed to detect the effect of different coffee extracts on PMS-trypsin aggregation. The greatest effect was exerted by the Eduscho coffee extract. CR binding assay can also be utilised to study the anti-fibrillation efficiency of small molecular inhibitors. CR binding assay indicated that the presence of the Eduscho coffee extracts decreased the amounts of amyloid fibrils in 60% ethanol at pH 7.0, and their inhibitory effects were dose-dependent. Gel filtration chromatography was used to separate the particles of different size. The presence of Eduscho coffee extract diluted increased the amount of the protein oligomers. We demonstrated that the inhibitory effect of the coffee extract on the fibril formation is due to its

capacity to stabilize the oligomeric form of the PMS-trypsin. The addition of the Eduscho coffee extract effectively prevented PMS-trypsin from undergoing helix-to-coil transition.

Inhibitory effect of the grapefruit seed extract on the amyloid-like fibril formation

The grapefruit seed extract was the best inhibitor among the food supplements investigated. The percentage of inhibition of amyloid formation did not change proportionally with the total phenolic content. The limonin concentration in the grapefruit seed extract was determined: it was 16.8 ng/ml. For the first time, our results indicated that limonin was an effective anti-amyloidogenic agent. The anti-aggregation effect of the grapefruit seed extract was dose dependent. TEM studies showed that there was no fibril formation in the presence of grapefruit seed extract diluted 500 times, indicating the inhibitory effect of grapefruit seed extract on the formation of amyloid-like fibrils by trypsin.

Summary of findings and conclusions

The present study can be concluded by the statement that natural product extracts may be efficient anti amyloidogenic agents, as they arrest PMS-trypsin fibrillation in a concentration dependent manner. We summarize the most important findings of our study as follows:

- It has been demonstrated that the peppermint extract could effectively inhibit PMS-trypsin amyloid fibril formation *in vitro*, and the process was concentration dependent of the peppermint extract. Our findings revealed that the anti-amyloidogenic activities of herb extracts might be related to their total phenolic contents. According to our experiments peppermint extract might serve as a valuable source of beneficial phenolic compounds for the prevention of protein aggregation.

- Our study demonstrated that the chili cayenne extract got the highest inhibitory effect on the formation of amyloid fibrils among all the spices investigated. The findings also suggest that the bioactive compounds in the chili extract may be a potential therapeutic drug candidate for amyloid related and neurodegenerative diseases including Alzheimer's disease. This study concludes that the possible nutritional importance of kitchen spices in the food system and the inhibitory effects of the chili extract clearly indicate that they could be used as a pharmacological agent to inhibit or retard amyloid fibril formation in Alzheimer's disease.
- The present study revealed that the *P. ginseng* extract is an efficient anti amyloidogenic agent against trypsin fibrillation in aqueous ethanol at pH 7.0 and the process is dependent on the concentration of the *P. ginseng* extract. Our results showed that the presence of the *P. ginseng* extract helped to maintain the native structure of the protein.
- According to our experiments, the degree of inhibition was found to change pro rata with the total concentrations of phenolic compounds of the coffee extracts. It can be concluded that the Eduscho coffee extract may be an efficient anti amyloidogenic agent, as it arrests PMS-trypsin fibrillation in a concentration dependent manner.
- We have successfully shown that the grapefruit seed extract inhibits the aggregation process of PMS-trypsin in 60% ethanol at pH 7.0. The inhibition of PMS-trypsin aggregation positively correlated with increased grapefruit seed extract concentration. Our results indicated, for the first time, that limonin effectively inhibited fibril formation.

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I convey my gratitude to all my friends for joining their hands during my work and encouraged me mentally during my difficult time.

Attestation of Authorship

*I, **Phanindra Babu Kasi** hereby declare that this submission is my own work and that to the best of my knowledge and belief, it contains no material previously published or written by author person.*

LIST OF PUBLICATIONS (MTMT number: 10053006)

Publications related to thesis

1. **Kasi PB**, Borics A, Molnár K, László L, Kotormán M. Eduscho Coffee Extract Effectively Inhibits the Formation of Amyloid-like Fibrils by Trypsin in Aqueous Ethanol. Nat Prod Commun 2018;13(12):1695–1698. Impact Factor: 0.809

2. **Kasi PB**, Borics A, Varga M, Endre G, Molnár K, László L, Kotormán M. Grapefruit Seed Extract Inhibits the Formation of Amyloid-like Fibrils by Trypsin in Aqueous Ethanol. *Nat Prod Commun* 2018;13(11):1437–1440. Impact Factor: 0.809
3. **Kasi PB**, Kotormán M, Borics A, Hervay BG, Molnár K, László L. The Inhibitory Effect of Panax Ginseng Extract on Amyloid-like Fibril Formation of Trypsin in Aqueous Ethanol. *Protein Pept Lett*. 2018;25(3):253–259.
doi: 10.2174/0929866525666171229231226. Impact Factor: 1.039


Publications not directly related to thesis

1. Kotormán M, **Kasi PB**, Halász L, Borics A. Inhibition of Amyloid-like Fibril Formation of Trypsin by Red Wines. *Protein Pept Lett*. 2017;24(5):466–470.
doi: 10.2174/0929866524666170214125847. Impact Factor: 1.039
2. Kotormán M, Kelemen Z, **Kasi PB**, Nemcsók J. Inhibition of the formation of amyloid-like fibrils using herbal extracts. *Acta Biol Hung*. 2018 Jun;69(2):125–134.
doi: 10.1556/018.69.2018.2.2. Impact Factor: 0.439
3. Kotormán M, Varga A, **Kasi PB**, Nemcsók J. Inhibition of the formation of amyloid-like fibrils with spices, especially cloves. *Acta Biol Hung*. 2018;69(4):385–394.
doi: 10.1556/018.69.2018.4.2. Impact Factor: 0.439

Oral/Poster presentations

1. **Kasi PB**, Kotormán M, Borics A, Hervay BG, Molnár K, László L. The Inhibitory Effect of Panax Ginseng Extract on Amyloid-like Fibril Formation of Trypsin in Aqueous Ethanol. Society for Experimental Biology and Medicine, Chengdu, China, October 19-21, 2018.

2. **Phanindra Babu Kasi**, Márta Kotormán, Attila Borics, Kinga Molnár and Lajos László.
The inhibitory effect of spice extracts on the formation of amyloid fibrils using trypsin in aqueous ethanol. 13th International Congress on Advances in Natural Medicines Nutraceuticals & Neurocognition & 14th International Conference on Clinical Nutrition, Rome, Italy, July 26-28, 2017.
3. **Kasi PB**, Borics A, Molnár K, László L, Kotormán M. Eduscho Coffee Extract Effectively Inhibits the Formation of Amyloid-like Fibrils by Trypsin in Aqueous Ethanol. Neurochemical Conference 2017 "Advances in molecular and epigenetic mechanisms in neurodegeneration and neuroinflammation: novel therapeutic approaches", Warsaw, Poland, October 19-20, 2017.

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Supervisor's/Coauthor's declaration

I, undersigned Márta Kotormán, the coauthor and supervisor of Phanindra Babu Kasi's Ph.D. work hereby certify that I am familiar with the Ph.D. thesis of the applicant Phanindra Babu Kasi entitled 'Anti-amyloidogenic effect of natural product extracts on amyloid-like fibril formation of trypsin in aqueous organic solvents'. I did not and will not use these results in getting academic research degree. There is no other Ph.D. student who can use these results in a doctoral process.

As the responsible author of the following publications, I declare that Phanindra Babu Kasi Ph.D. candidate has made a significant contribution to the creation of the following scientific publications and the results reported are not used in other Ph.D. dissertations.

Kasi PB, Borics A, Molnár K, László L, Kotormán M. Eduscho Coffee Extract Effectively Inhibits the Formation of Amyloid-like Fibrils by Trypsin in Aqueous Ethanol. *Nat Prod Commun* 2018;13(12):1695–1698.

Kasi PB, Borics A, Varga M, Endre G, Molnár K, László L, Kotormán M. Grapefruit Seed Extract Inhibits the Formation of Amyloid-like Fibrils by Trypsin in Aqueous Ethanol. *Nat Prod Commun* 2018;13(11):1437–1440.

Kasi PB, Kotormán M, Borics A, Hervay BG, Molnár K, László L. The Inhibitory Effect of Panax Ginseng Extract on Amyloid-like Fibril Formation of Trypsin in Aqueous Ethanol. *Protein Pept Lett.* 2018;25(3):253–259.

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