Inquiry: The University of Arkansas Undergraduate Research Journal

Volume 22 Article 5

2023

High Order Structures Formed by the Natural Aromatic Amino Acids

Gabriel Alexander Kupovics
University of Arkansas, Fayetteville

Zeina Alraawi
University of Arkansas, Fayetteville

Research Article

High Order Structures Formed by the Natural Aromatic Amino Acids

Gabriel Alexander Kupovics and **Zeina Alraawi**
***Faculty Advisor* – Dr. Thallapuranam Krishnaswamy Suresh Kumar**

J. William Fulbright College of Arts and Sciences, University of Arkansas, Chemistry and Biochemistry Department, Fayetteville, AR 72701

Abstract

Excessive concentrations of the natural aromatic amino acids phenylalanine and tyrosine are characteristic of the severe genetic abnormalities known as phenylketonuria (PKU) and tyrosinemia, respectively. Within this context, this feature article authenticates claims that tryptophan can form amyloid-like supramolecular structures *in vitro* and is the first to propose potential mechanisms of tryptophan self-assembly, including hydrophobic and electrostatic interactions. Thioflavin T (ThT) fluorescence kinetics and transmission electron microscopy (TEM) data suggest the formation of amyloid-like fibrillar structures by natural aromatic amino acids *in vitro*. Additionally, the propensity of amino acid aggregation increases in the presence of sodium dodecyl sulfate (SDS). The structures formed by these amino acids are likely nucleated via hydrophobic interactions and elongated by π-π interactions. Fluorescence kinetics reveal a higher propensity of phenylalanine to self-assemble at pH 2, while tyrosine and tryptophan assemble best at pH 7, suggesting the necessity of zwitterionic charges in self-assembly.

Profiles of the Authors

Gabriel Kupovics graduated from the University of Arkansas in 2020, where he obtained a degree in Biochemistry from the Fulbright College of Arts & Sciences. While at the University of Arkansas, he studied the mechanisms that underlie metabolic diseases, such as phenylketonuria and tyrosinemia. Gabriel is currently pursuing a degree in Osteopathic Medicine from the UNTHSC Texas College of Osteopathic Medicine, where he is exploring his interest in surgery.

Zeina Alraawi is a Ph.D. student in Cell and Molecular Biology at Fulbright College of Arts & Sciences, University of Arkansas. She is interested in researching biomolecule structures and how they function in the human body.

https://scholarworks.uark.edu/inquiry/vol22/iss1/5
DOI: 10.54119/inquiry.2023.22101

Dr. Thallapuranam is a professor of Chemistry & Biochemistry with significant interest in design of novel fibroblast growth factor (FGF)-based rational design of therapeutic principles for chronic wound care, anti-diabetes, anti-obesity, and osteoporosis. His research is supported by several Federal grants (NIH/DOE/NSF/USDA). He currently serves as the NIH sponsored AIMRC Bioenergetics core. He also currently serves as the Director of the Office of Undergraduate Research (in DRI). He has published about 10 US patents and 175 peer-reviewed research papers in high-impact Journals. He is the recipient of several teaching, research, and service awards including the Honors College Distinguished Faculty award, Honors College Faculty Gold medal, Collis Geren Outstanding Interdisciplinary Graduate Faculty award, elected as the member of the National Academy of Inventors, ARSC Master Teacher award. Golden Tusk Award, and the prestigious Nadine Baum Award for outstanding teaching. He is also the member of the University of Arkansas Teaching Academy. Dr. Thallapuranam also regularly serves on the grant proposal review panel of Federal agencies such as NIH, NSF, and DOE. He currently serves as the Associate Editor of the Frontiers of Molecular Biophysics journal and on the Editorial Board of several peer-reviewed journals including the prestigious Journal of Biological Chemistry.

Introduction

Amino acids are organic compounds that create the foundation of the essential biological macromolecules known as proteins. Proteins are synthesized by the sequential formation of peptide bonds between the amine (-NH^3) and carboxyl (-COOH) groups of a series of amino acids specific for that protein. Along with the amine and carboxyl functional groups, amino acids contain a unique side chain (-R), which determines their physical and chemical properties. In this project, the properties of the three canonical aromatic amino acids, phenylalanine, tyrosine, and tryptophan, were explored. Phenylketonuria (PKU) is an autosomal recessive inborn error resulting from the decreased activity of phenylalanine hydroxylase (PAH), the enzyme that is required to convert L-Phenylalanine to L-Tyrosine. Decreased activity of PAH interrupts phenylalanine metabolism allowing for accumulation of phenylalanine and its toxic catabolic by-products[1] in the plasma, cerebrospinal fluid and brain tissue[2]. This accumulation has been found to inhibit the transport of large neutral amino acids (LNAAs) into the brain[3,4]. Additionally, increased free phenylalanine in the brain is believed to result in the amyloid-like deposits which characterize PKU[2].

Tyrosinemia is another neurodegenerative autosomal recessive inherited metabolic disease characterized by an accumulation of tyrosine in body fluids and tissues. Tyrosinemia Types I, II and III are characterized by deficiencies of the critical tyrosine metabolism enzymes fumarylacetoacetate hydrolase (FAH), tyrosine aminotransferase (TAT) and 4-hydroxyphenylpyruvicdioxygenase, respectively[5]. The etiology of hypertryptophanemia was recently established to be a deficiency of tryptophan 2,3-dioxygenase, the metabolic enzyme responsible for the rate-limiting oxidation of L-Tryptophan to N-formyl-L-kynurenine in the kynurenine pathway. The disease is commonly associated with elevated levels of fasting plasma tryptophan[6]. Exploration of aromatic amino acid (AA) self-assembly processes has experienced increasing attention specifically within the context of human disease. Simulation of these processes has revealed the tendency of phenylalanine, tyrosine and tryptophan to form fibril-like structures[7].

Previous studies have suggested that the intermolecular interactions between single phenylalanine molecules are mediated through hydrophobic interactions, β-sheet generation, and π-π interactions[8,9]. Similarly, the formation of tyrosine and tryptophan aggregates has been proposed to be due to π-π stacking and hydrogen bonding interactions, producing cross-β-like structures[10,11]. The primary objective of this study is to determine the tendency aromatic (AAs) to form high order structures *in vitro*. In addition, to investigate the mechanism(s) of (AAS) self-assembly. In this context, the aggregation propensity of phenylalanine, tyrosine and tryptophan at millimolar concentrations were investigated utilizing ThT fluorescence experiments.

Methods

ThT fluorescence was observed at a wavelength of 485 nm for phenylalanine, tyrosine, and tryptophan over 30 days. Phenylalanine and tryptophan concentrations (mM): 1, 4, 6, 8, 10. Tyrosine concentrations (mM): 0.8, 1, 1.4, 1.6, 2. All extrinsic fluorescence spectroscopy kinetics were performed on a Hitachi F-2500 spectrophotometer at 25 ºC using a slit width of 2.5 nm and a quartz cuvette with a path length of 10 mm.

In addition, aromatic AA self-assembly morphology was investigated via transmission

https://scholarworks.uark.edu/inquiry/vol22/iss1/5
DOI: 10.54119/inquiry.2023.22101

electron microscopy (TEM). Formation of aromatic amino acids fibrils was achieved via 15-day incubation under physiological conditions (37 ºC, pH 7.4). The samples were centrifuged for approximately 40 minutes to isolate the self-assembled fibrils and washed three times with 150-µL aliquots of water distilled. The fibrils were ultimately suspended in 150 µL of distilled water. A 2-µL volume of each type of amino acid was added to a separate Formvar- and carbon-coated copper grid. Electron micrographs of aromatic amino acid aggregates were observed on a Hitachi H-7500 transmission electron microscope. Micrographs were obtained at a voltage range of 80-100 kV and magnifications ranging from 20000x to 100000x.

ThT fluorescence was also observed for phenylalanine, tyrosine and tryptophan in presence and absence sodium dodecyl sulfate (SDS). Additionally, ThT fluorescence of different concentrations of phenylalanine, tyrosine and tryptophan was observed at pH 2, 7, and 9 after 15 days of incubation.

Results

ThT is a small molecule that gives rise to fluorescence upon binding to the side chain channels along the long axis of amyloidogenic structures[11]. A significant rise in the relative fluorescence intensity of ThT at 485 nm (Fig. 1) suggests the amyloid-like aggregation of all three natural aromatic AAs at low millimolar concentrations. A lag time of approximately 10 days was experienced for all three AAs. Additionally, ThT signal saturation was achieved for phenylalanine and tyrosine after 30 days of incubation (Fig. 1a-b) whereas saturation for tryptophan samples was reached after 35 days (Fig. 1c).

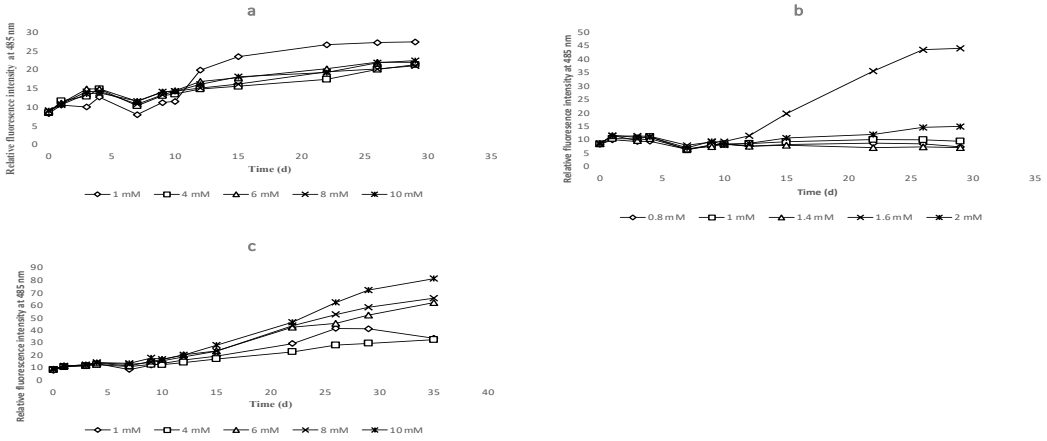

Figure 1
ThT fluorescence at various concentrations of phenylalanine (*Panel a*), tyrosine (*Panel b*) and tryptophan (*Panel c*) over 30 days. Phenylalanine and tryptophan concentrations (mM): 1 (*open diamonds*), 4 (*open squares*), 6 (*open triangles*), 8 (*crosses*), and 10 (*asterisks*). Tyrosine concentrations (mM): 0.8 (*open diamonds*), 1.0 (*open squares*), 1.4 (*open triangles*), 1.6 (*crosses*), and 2.0 (*asterisks*).

Aromatic AA self-assembly was further investigated with transmission electron

microscopy. Formation of aromatic AA fibrils was achieved via 15-day incubation under physiological conditions. The sample concentrations were based on those used in previous studies and their representative behavior of other millimolar concentrations studied[2,12,13]. The pre-formed fibrils were isolated and washed with buffer before transferring to a 400-mesh copper grid. Transmission electron microscopy (Fig. 2) reveals that the aromatic AA self-assemblies formed are fibrillar, exhibiting varying degrees of branching. Phenylalanine and tyrosine aggregates display minimal to no branching (Fig. 2a-d) whereas tryptophan supramolecular structures appear to be highly branched (Fig. 2e-f).

Additionally, the structures are discrete and well-organized, typical of aggregates formed by amyloidogenic proteins[14,15]. Previous studies suggest the possibility that π-π interactions play a role in amyloid fibril self-assembly processes due to the presence of aromatic residues[16]. We believe that the π-stacking hypothesis may be employed here as a potential mechanism of self-assembly and may contribute to the order and directionality of the process, specifically for phenylalanine.

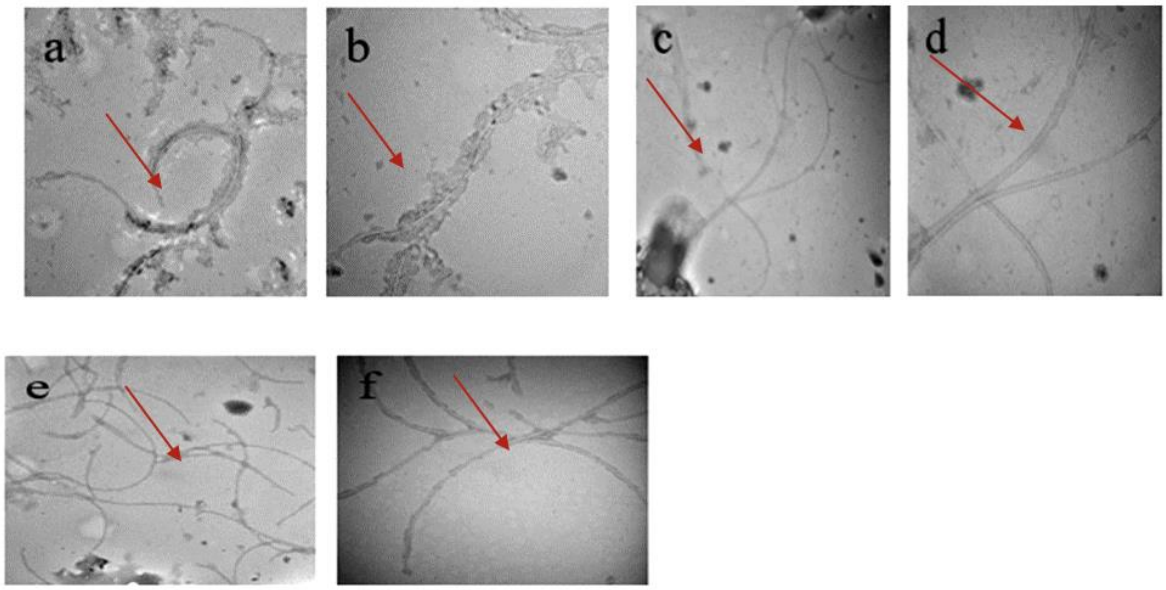

Figure 2
Transmission electron microscopy after 15 days of fibril formation. Phenylalanine (6 mM) is depicted at magnifications of 50000x (*Panel a*) and 100000x (*Panel b*). Tyrosine (1 mM) is shown at magnifications of 50000x (*Panel c*) and 100000x (*Panel d*). Tryptophan (6 mM) is depicted at magnifications of 50000x (*Panel e*) and 100000x (*Panel f*).

The buffer salt coating of phenylalanine fibrils observed in the micrographs support this hypothesis (Fig. 2a-b), as positively charged salts may stabilize the electron repulsion experienced between aromatic rings in the form of cation-π interactions. The lack of buffer salt coating of tyrosine and tryptophan fibrils prompts further exploration prior to addressing such an elongation mechanism. It is worth noting that the micrographs (Fig. 2b, 2d-e) depict small clusters of phenylalanine, tyrosine and tryptophan existing independent of their respective fibrils. These micelle-resembling clusters may serve as nucleation sites for self-assembly and suggest that

https://scholarworks.uark.edu/inquiry/vol22/iss1/5
DOI: 10.54119/inquiry.2023.22101

hydrophobic interactions play a significant role in nucleation of the fibrils. The results of this electron microscopy experiment confirm that the natural aromatic AA self-assemblies exhibit amyloid-like characteristics. Additionally, the results reveal that phenylalanine supramolecular structures may possess a π-stacking mechanism of assembly and that hydrophobic interactions could play a role in the nucleation of aromatic AA aggregation.

Sodium dodecyl sulfate (SDS) is an anionic surfactant which mimics the amphipathic character of the plasma membrane[17]. SDS-induced aromatic AA fibrils were incubated for 15 days at physiological temperature (37 °C) and pH 7.4. The results in Fig. 3 display a higher fluorescence intensity of ThT for samples incubated with SDS (compared to those without SDS) revealing a higher aggregation propensity in the presence of the detergent. The difference in fluorescence intensity was apparent at all monitored concentrations for tyrosine (Fig. 3b) while only at concentrations above 1 mM for phenylalanine (Fig. 3a) and tryptophan (Fig. 3c). The results of this experiment suggest that 0.2 mM SDS facilitates the formation of aromatic AA self-assembled fibrils. The SDS-induced fibrils were further investigated with transmission electron microscopy.

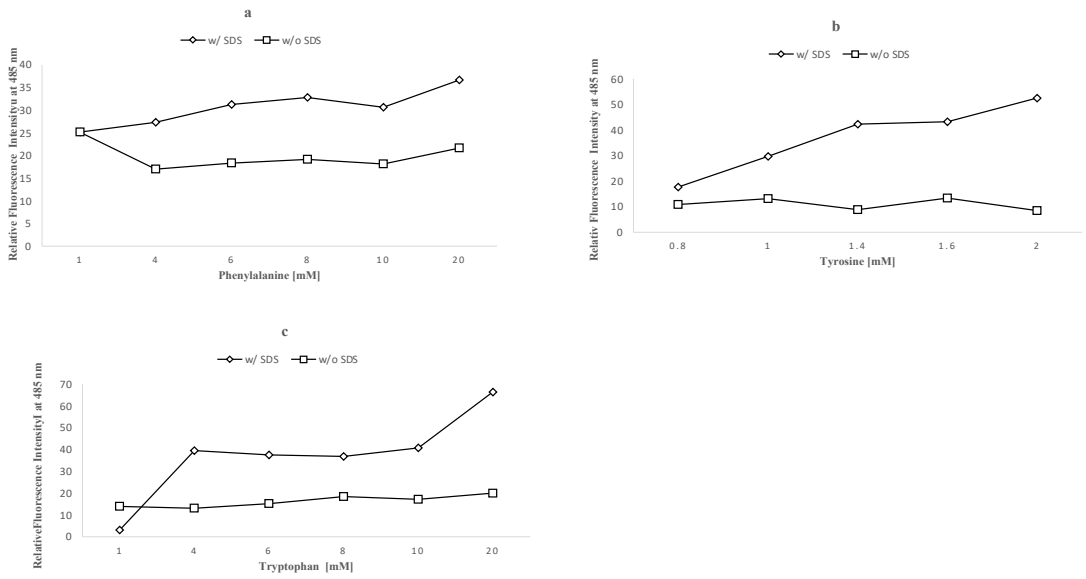

Figure 3
ThT fluorescence at varying concentrations of phenylalanine (*Panel a*), tyrosine (*Panel b*), and tryptophan (*Panel c*) in the presence (*open diamonds*) and absence (*open squares*) of 0.2 mM SDS after 15 days of incubation.

Samples of 6 mM phenylalanine, 1 mM tyrosine and 6 mM tryptophan were incubated with SDS under physiological conditions for 15 days. The aggregates formed were isolated and washed prior to microscopic examination. The micrographs (Fig. 4) depict well-ordered, fibrillar

structures, similar to the self-assemblies observed in the absence of SDS. Interestingly, SDS-induced phenylalanine self-assemblies (Fig. 4a-b) exhibit greater branching compared to those seen in the absence of SDS (Fig 2). The micrographs depict SDS micelles as dark structures (Fig. 4b, 4d, 4f) from which the fibrils project and elongate. All visible aromatic AA fibrils are depicted extending from micelles, which supports the hypothesis that SDS may play a facilitative role in the formation of these self-assemblies.

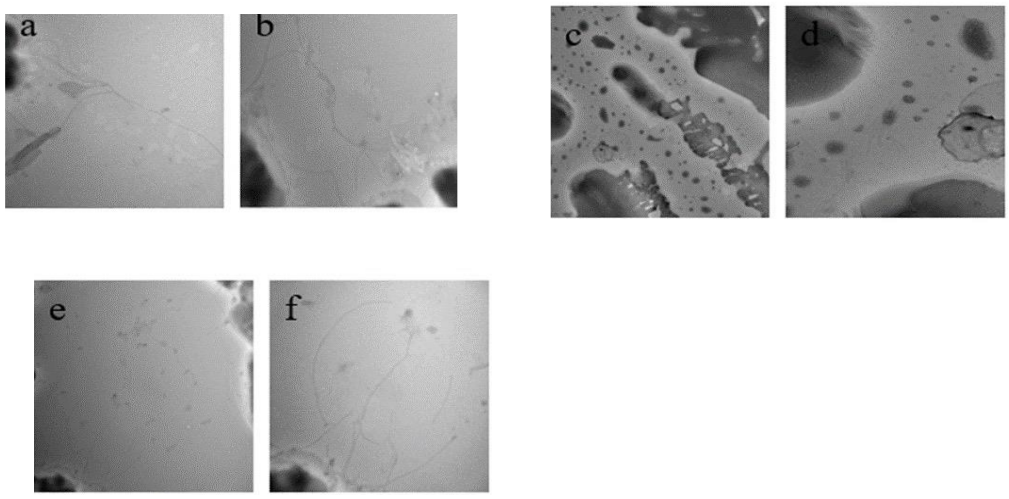

Figure 4
Transmission electron micrographs of SDS-induced aromatic AA fibrils. Phenylalanine (6 mM) is depicted at a magnification of 50000x (*Panel a* and *Panel b*). Tyrosine (1 mM) is shown at magnifications of 20000x (*Panel c*) and 100000x (*Panel d*). Tryptophan (6 mM) is depicted at magnifications of 25000x (*Panel e*) and 50000x (*Panel f*).

Additionally, the micelles appear to play a similar role to the AA clusters seen in Fig. 2b, 2d and 2e, further suggesting that hydrophobic interactions initiate aromatic AA aggregation processes. These results confirm that SDS can induce aromatic AA aggregation and proposes hydrophobic interactions as a potential self-assembly mechanism. The hypothesis that π-π interactions play a role in phenylalanine and tyrosine fibril formation suggests the importance of charge for aggregation of these AAs.

In this context, the effects of pH on aromatic AA aggregation were studied with ThT binding experiments. At low (acidic) pH, all groups of these amino acids are protonated and the net charge for each is +1. At neutral pH, the carboxyl group is ionized, giving the group a charge of -1. Therefore, the overall charge of each amino acid at neutral pH is 0. At high (basic) pH, the carboxyl and amine groups are deprotonated, and the net charge of each amino acid is -1. Aggregation propensities at pH 2, 7, and 9 were studied after 15 days of incubation at physiological temperature.

A higher fluorescence of ThT was observed at pH 2 for phenylalanine concentrations in the 1 to 10 mM range (Fig. 5a). This suggests that a +1 charge on phenylalanine is more favorable for its self-assembly. The plausible requirement of a positive charge on the AA supports the

https://scholarworks.uark.edu/inquiry/vol22/iss1/5
DOI: 10.54119/inquiry.2023.22101

hypothesis that π-stacking is the mechanism of fibril elongation. The π-electron repulsions may be stabilized by the positive charge on the amine group at low pH. Tyrosine and tryptophan exhibited a higher relative fluorescence intensity of ThT at a neutral pH (Fig. 5b, 5c), indicating that the zwitterionic form of the AAs is favorable for aggregation.

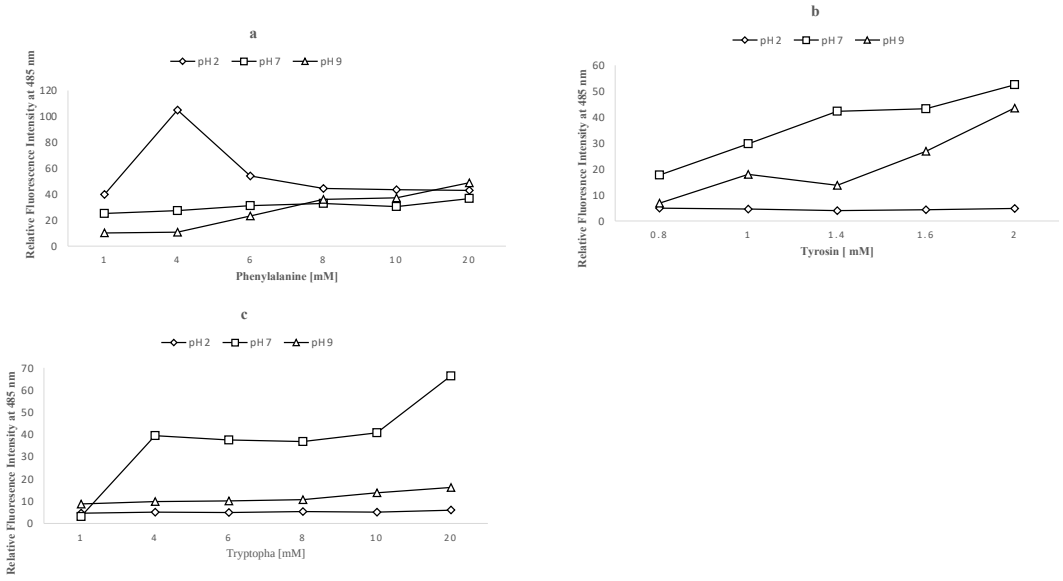

Figure 5
ThT fluorescence of varying concentrations of phenylalanine (*Panel a*), tyrosine (*Panel b*), and tryptophan (*Panel c*) at pH 2 (*open diamond*), 7 (*open square*), and 9 (*open triangle*) after 15 days of incubation.

Further, this finding suggests that electrostatic interactions may play a role in the formation of tyrosine and tryptophan high order structures. However, in contrast to tyrosine, basic pH appears to have an inhibiting effect on tryptophan aggregation, suggesting a deprotonated amine is unfavorable for its self-assembly. The results of this experiment support the π-stacking hypothesis of phenylalanine aggregation and reveal electrostatic interactions may play a significant role in the tyrosine and tryptophan self-assembly.

Conclusion

In summary, this study explores the etiologies of phenylketonuria, tyrosinemia and hypertryptophanemia and explores a possible mechanism of assembly for natural aromatic AA high order structures. Our findings authenticate reports of previous studies that phenylalanine and tyrosine form fibrillar structures *in vitro* at millimolar concentrations. The aromatic AA fibrils are discrete and well-organized, exhibiting amyloid-like characteristics. Positively charged salts from the phosphate buffer saline are depicted coating fibrils in phenylalanine electron micrographs. This suggests that π-electron repulsions are stabilized by the salts in cation-π interactions, supporting the π-stacking hypothesis of phenylalanine self-assembly. Micrographs of tyrosine and tryptophan depict small independent clusters of the AAs indicating the significant role of hydrophobic interactions in the initiation of these self-assembly processes. SDS-induced aggregation suggests that SDS serves as a nucleation site, further suggesting that hydrophobic

interactions may be responsible for the initiation of aromatic AA fibril formation. It is possible that the highly branched aggregates formed by phenylalanine in these membrane-like environments may contribute to the negative effects experienced by phenylketonuria patients.

Tyrosine and tryptophan exhibit higher propensities to aggregate at neutral pH suggesting the necessity of the zwitterionic AA form in the self-assembly process. In these contexts, it appears that hydrophobic interactions are responsible for nucleation of self-assembly processes in aromatic AAs, and π-π interactions may serve as the mechanism of phenylalanine fibril elongation whereas electrostatic interactions could be responsible for tyrosine and tryptophan aggregate extension.

Acknowledgment

This work is supported by the National Institute of Health (NIH) (R015569) and Department of Energy (DOE) (DE-02-01ER15161).

References

1. R. Williams, C. D. Mamotte, J. Burnett, *Clin. Biochem. Rev*., 2008, **29**, 31–41.
2. L. Adler-Abramovich, L. Vaks, O. Carny, D. Trudler, A. Magno, A. Caflisch, et al, *Nat. Chem. Biol.*, 2012, **8**, 701–706.
3. C. M. McKean, *Brain Res.*, 1972, **47**, 469–476.
4. C. M. McKean, et al, *J Neurochem*. 1968, **15**, 235-241.
5. C. R. Scott, *Am J Med Genet C Semin Med Genet*, 2006, **142**, 121-126.
6. P. Ferreira, I. Shin, I. Sosova, et al, *Mol Genet Metab*, 2017, **120**, 317-324.
7. S. Uyaver, H. W. Hernandez, M. G. Habiboglu, *Phys Chem Chem Phys*, 2018, **20**, 30525-30536.
8. T. D. Do, W. M. Kincannon, M. T. Bowers, *J. Am. Chem. Soc.*, 2015, **137**, 10080–1008.
9. E. Mossou, et al, *Acta Crystallogr C*, 2014, **70**, 326–331.
10. B. G. Anand, K. P. Prajapati, D.S. Shekhawat, K. Kar, *Biochem*, 2018, **57**, 5202-5209.
11. S.prabhijot,et al, Materials Science and Engineering, 2017, C72, 590-600.
12. C. Xue, T. Y. Lin, D. Chang, Z. Guo. *Open Science*. 2017, **4**, 1, 160696.
13. D. Banik, R. Dutta, P. Banerjee, et al, *J. Phys Chem*, 2016, **120**, 7662-7670.
14. D. Banik, S. Kundu, P. Banerjee, et al, *J. Phys Chem*, 2017, **121**, 1533-1543.
15. A. Sipe, A. Cohen, *J. Struct Biol*, 2000, **130**, 88-98.
16. J. Kelly, *Proc. Natl. Acad. Sci.*, 1998, **95**, 930-932.
17. E. Gazit, *FASEB J.*, 2002, **16**, 77-83.
18. B. Hammouda, *J Res Natl Inst Stand Technol*, 2013, **118**, 151-167.

https://scholarworks.uark.edu/inquiry/vol22/iss1/5
DOI: 10.54119/inquiry.2023.22101