DIVERSIFICATION AND MODULATION OF THE AGGREGATION PATHWAYS OF PROTEINS IN THE PRESENCE OF EXTERNAL ADDITIVES

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by

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CERTIFICATE

This is to certify that the thesis entitled, "***Diversification and modulation of the aggregation pathways of proteins in the presence of external additives***", being submitted by **Ms. Shivnetra Saha** to the **Indian Institute of Technology Delhi** for the award of the degree of **Doctor of Philosophy** in Chemistry is a record of bona fide research work carried out by her. Ms. Shivnetra Saha has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to my knowledge has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

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I owe this thesis to HIM

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ABSTRACT

The thesis titled **"Diversification and modulation of the aggregation pathways of proteins in the presence of external additives"** presents a study of the diverse behaviour of proteins under different environmental conditions. The key to controlling or manipulating the aggregation of proteins lies in the mechanistic understanding of this process, and one such route is to understand the kinetics and the thermodynamics of aggregation. The phenomenon has captured considerable attention, majorly because of its involvement in many neurodegenerative diseases, and also because of its undesirable, spontaneous occurrence during the processing and manufacturing of protein-based therapeutics. A brief description of the chapters is presented below.

Chapter 1 (Introduction) presents a brief idea about the necessity of studying the process of protein aggregation, the progresses till now in this field, various broad classes of mechanisms of aggregation and how the models developed/improved over the course of time. It also discusses about the intermediates in the aggregation pathway, and the various structural tools which are available for detecting the presence of various species in the aggregation pathway. **Chapter 2 (Materials and methodologies)** describes the proteins used in this study, their expression and purification protocol (as required), the procurement of the proteins and other chemicals used throughout the study, and the instruments used in the study. **Chapter 3 (Switch in the aggregation pathway of bovine serum albumin mediated by electrostatic interactions)** demonstrates that a strong denaturant, guanidinium hydrochloride (GdnHCl), can delay and alter the inherent aggregation pathway of bovine serum albumin (BSA) from downhill polymerization to a nucleated polymerization. It is hypothesized that such an alteration is closely connected to the conformational population of the protein, and ion-binding to such an ensemble. The study shows that the behavior in GdnHCl is not unique to it, but occurs in a certain class of cosolutes- those

which are charged and bind to BSA. **Chapter 4 (A paradox in the stability and aggregation of bovine serum albumin: uniqueness of a cosolute)** works as sequel to Chapter 3, wherein the aggregation has been carried out in the presence of urea, ethanol, and arginine. The three additives do not show a uniform behaviour towards unfolding and aggregation. Although partial unfolding is a prerequisite for the initiation of aggregation, this study shows that the phenomenon can be dominated by the presence of a particular cosolute too. **Chapter 5 (Modulations in the self-assembly of bovine serum albumin by enhanced depolymerisation and condensation induced upon stirring)** shows that stirring the solution of the BSA-CTAB system resulted into a reduced extent of aggregation, as compared to the unstirred solution. The apparent saturation phase phase which is attained faster when the solution is stirred, is a consequence of the condensation of aggregates predominating the aggregation pathway, beyond a certain aggregate concentration. The rapid approach to the condensation process ultimately leads to lesser incorporation of the monomers into the aggregates, thus reducing the aggregation. **Chapter 6 (Differential influence of additives on the various stages of insulin aggregation)** describes the effect of sugars and polyols on the aggregation of insulin which occurs by a typical nucleation-growth mechanism. Sugars and polyols affect the lag time and the rate constant of growth to different extents, suggesting that these additives affect both the pre- and post-nucleation processes. Sucrose and ethylene glycol effectively reduce the aggregation, whereas mannitol is not effective in suppressing the overall aggregation. However, it can delay the aggregation process. The varying behaviour of different additives suggests their interference in the different stages of aggregation, affecting the aggregation intermediates differently. In **Chapter 7 (Glycerol inhibits the primary pathways and transforms the secondary pathway of insulin aggregation)**, it is shown that glycerol could transform the secondary pathway of aggregation of insulin from fragmentation to

heterogeneous nucleation in a concentration dependent manner. Such a change in the secondary pathway was also accompanied by the formation of longer fibrils. The analysis of the kinetic traces suggested that the inhibitory effect was most significant on the primary pathways, although secondary nucleation and elongation were also inhibited. **Chapter 8 (Inverse effects of additives on the fibrillation and oligomerization of α-synuclein)** presents the aggregation of α-synuclein in copper sulphate and glycerol, in the presence and absence of dopamine. Both the additives increase the aggregation of this protein. However, it has been shown here that toxic oligomers which are usually promoted by dopamine, do not form in the presence of copper. Glycerol, however, promotes the formation of these toxic oligomers. We speculate that it is the binding of copper to the fibrils which stabilizes them to such an extent that the oligomers do not persist for long, and go all the way to form fibrils. Glycerol perhaps does not interact with the fibrils but facilitates the dopamine-oligomer interaction, leading to the stabilization of the oligomers. **Chapter 9 (Summary and future prospects)** presents the highlights of the study. It summarizes the diverse/similar behaviour a protein can exhibit under similar/different conditions, respectively. The pathway a protein takes, or the aggregates they form, can be manipulated by modifying the external conditions.

सार

"बाहरी योजक की उपस्थिति में प्रोटीन के एकत्रीकरण मार्गों के विविधीकरण और मॉडुलन" नामक थीसिस विभिन्न पर्यावरणीय परिस्थितियों में प्रोटीनों के विविध व्यवहार का एक अध्ययन प्रस्तुत करता है। प्रोटीन के एकत्रीकरण को नियंत्रित करने या जोड़ तोड़ने की कुंजी इस प्रक्रिया की यंत्रवत् समझ में निहित है, और एक ऐसा मार्ग है कि कैनेटीक्स और एकत्रीकरण के ऊष्मप्रवैगिकी को समझना है। इस घटना ने काफी ध्यान आकर्षित किया है, जो कि कई neurodegenerative बीमारियों में इसकी भागीदारी के कारण, और प्रोटीन आधारित चिकित्सा विज्ञान के प्रसंस्करण और निर्माण के दौरान इसकी अवांछनीय, सहज घटना के कारण भी। अध्यायों का एक संक्षिप्त विवरण नीचे प्रस्तुत किया गया है। अध्याय 1 (परिचय) प्रोटीन एकत्रीकरण की प्रक्रिया का अध्ययन करने की आवश्यकता के बारे में एक संक्षिप्त विचार प्रस्तुत करता है, इस क्षेत्र में अब तक की प्रगति, एकत्रीकरण के विभिन्न व्यापक कक्षाएं और कैसे समय के दौरान मॉडल विकसित / सुधारा। यह एकत्रीकरण मार्ग में मध्यवर्ती के बारे में भी चर्चा करता है, और विभिन्न संरचनात्मक उपकरण जो एकत्रीकरण मार्ग अध्याय 2 (सामग्री और पद्धतियों) में विभिन्न प्रजातियों की उपस्थिति का पता लगाने के लिए उपलब्ध हैं, इस अध्ययन में प्रयुक्त प्रोटीन का वर्णन करता है, उनकी अभिव्यक्ति और शुद्धि प्रोटोकॉल (आवश्यकतानुसार), पूरे अध्ययन में प्रयुक्त प्रोटीन और अन्य रसायनों की खरीद, और अध्ययन में इस्तेमाल किए गए उपकरण। अध्याय 3 (इलेक्ट्रोस्टैटिक इंटरैक्शन द्वारा मध्यस्थता वाले गोजाइन सीरम एल्बिन के एकत्रीकरण मार्ग में स्विच करें) दर्शाता है कि एक मजबूत डिस्टूटरेंट, ग्नाइडीनियम हाइड्रोक्लोराइड (जीडीएनएचसीएल), उतार-चढ़ाव पॉलिमराइजेशन से गोजातीय सीरम एल्बूमिन (बीएसए) के अंतर्निहित एकत्रीकरण मार्ग को एक न्यूक्यूबेटेड पोलीमराइजेशन ऐसा प्रतीत होता है कि इस तरह के परिवर्तन प्रोटीन की गठनात्मक आबादी से निकटता से जुड़ा हुआ है, और इस तरह के कलाकारों के लिए आयन-बाध्यकारी है। अध्ययन से पता चलता है कि जीडीएनएचसीएल में व्यवहार इसके लिए अनूठा नहीं है, लेकिन एक निश्चित श्रेणी के कॉस्सोलिट्स में होता है- जो कि चार्ज और बीएसए से जुड़े होते हैं अध्याय 4 (गोजातीय सीरम एल्बूमन की स्थिरता और एकत्रीकरण में एक विरोधाभास: एक सुसंस्कृतता की विशिष्टता) अध्याय 3 की अगली कड़ी के रूप में काम करता है, जिसमें एकत्रीकरण यूरिया, इथेनॉल और आर्गिनिन की उपस्थिति में किया गया है। तीन योजक उभरने और एकत्रीकरण के प्रति एक समान व्यवहार नहीं दिखाते हैं। यद्यपि आंशिक खुलासा एकत्रीकरण की शुरुआत के लिए एक शर्त है, इस अध्ययन से पता चलता है कि इस घटना पर एक विशेष संकाय की मौजूदगी भी हो सकती है। अध्याय 5 (उत्तेजित depolymerization और संयम पर प्रेरित condensation द्वारा बोवाइन सीरम Albumin के आत्म-विधानसभा में मॉड्यूल) पता चलता है कि बीएसए-सीटीएबी प्रणाली के समाधान को हल करने के कारण एकीकरण के एक सीमित हद तक परिणामस्वरूप, अनसुलझे समाधान की तुलना में। स्पष्ट संतृप्ति चरण चरण, जो समाधान प्राप्त हो जाता है, जब तेजी से प्राप्त होता है,

तो एक निश्चित सकल एकाग्रता से परे, एकत्रीकरण मार्ग को प्राथमिकता देने वाले समुच्चय का एक परिणाम है। संक्षेपण प्रक्रिया के लिए तेजी से दृष्टिकोण अंततः मोनोमर्स के समुच्चय को कम निगलना होता है, जिससे एकत्रीकरण कम हो जाता है। अध्याय 6 (इंसुलिन एकत्रीकरण के विभिन्न चरणों में additives के विभेदक प्रभाव) इंसुलिन के एकत्रीकरण पर शर्करा और पॉलीओल्स के प्रभाव का वर्णन करता है जो एक विशिष्ट न्यूक्लेयेशन-ग्रोथ मैकेनाइजेशन द्वारा होता है। शुगर्स और पॉलीओल्स समय के अंतराल को प्रभावित करते हैं और अलग-अलग विस्तार में वृद्धि की दर को प्रभावित करते हैं, यह सुझाव देते हैं कि ये योजक पूर्व और पोस्ट-न्यूक्लेयेशन प्रक्रियाओं को प्रभावित करते हैं। सुक्रोज और इथाइलीन ग्लाइकॉल एकत्रीकरण को प्रभावी ढंग से कम करते हैं, जबकि कुल एकाग्रता को दबाने में मनिटोल प्रभावी नहीं है। हालांकि, यह एकत्रीकरण प्रक्रिया को देरी कर सकता है। विभिन्न योजक के अलग-अलग व्यवहार एकत्रीकरण के विभिन्न चरणों में उनके हस्तक्षेप का सुझाव देते हैं, जो एकत्रीकरण मध्यवर्ती को अलग-अलग तरीके से प्रभावित करते हैं। अध्याय 7 में (ग्लिसरॉल प्राथमिक पथ को रोकता है और इंसुलिन एकत्रीकरण के माध्यमिक मार्ग को परिवर्तित कर देता है), यह दिखाया जाता है कि ग्लिसरॉल एकाग्रता निर्भर तरीके से विषुव न्यूक्लेशन को विखंडन से इंसुलिन के एकत्रीकरण के माध्यमिक मार्ग को बदल सकता है। माध्यमिक मार्ग में इस तरह के बदलाव में लंबे समय तक तंतुओं का गठन किया गया था। गतिज निशान के विश्लेषण ने सुझाव दिया कि निषेधात्मक प्रभाव प्राथमिक रास्ते पर सबसे महत्वपूर्ण था, हालांकि माध्यमिक न्यूक्लियेशन और बढ़ाव भी हिचकते थे। अध्याय 8 (α-synuclein के फाइब्रिलेशन और ऑलिगोमराइजेशन पर additives के प्रतिकूल प्रभाव) कॉपर सल्फेट और ग्लिसरॉल में α-synuclein के एकत्रीकरण को प्रस्तुत करता है, जिसमें डोपामाइन की मौजूदगी और अनुपस्थिति में। दोनों additives इस प्रोटीन के एकत्रीकरण में वृद्धि। हालांकि, यहां यह दिखाया गया है कि आमतौर पर डोपामाइन द्वारा विषैले ऑलिगोमरों को बढ़ावा दिया जाता है, तांबे की उपस्थिति में नहीं होता है। ग्लिसरॉल, हालांकि, इन विषैले oligomers के गठन को बढ़ावा देता है। हम अनुमान लगाते हैं कि तांबा की बाध्यता उन तंतुओं को बंधी होती है जो उन्हें ऐसी हद तक स्थिर करती है कि ओलिगोमर्स लंबे समय तक टिके नहीं करते हैं, और तंतुओं का निर्माण करने के लिए सभी तरह से जाते हैं ग्लिसरॉल शायद तंतुओं के साथ बातचीत नहीं करता है लेकिन डोपमाइन-ऑलिगोमर इंटरैक्शन की सुविधा प्रदान करता है, जिससे ओलिगोमर्स के स्थिरीकरण में वृद्धि होती है। अध्याय 9 (सारांश और भविष्य की संभावनाएं) अध्ययन के मुख्य आकर्षण प्रस्तुत करता है। यह विविध / समान व्यवहार को सारांशित करता है कि एक प्रोटीन क्रमशः समान / भिन्न परिस्थितियों में प्रदर्शित हो सकता है। एक प्रोटीन लेता मार्ग, या जो समुच्चय होता है, बाहरी स्थितियों को संशोधित करके इसका उपयोग किया जा सकता है

CONTENTS

Page No.

CHAPTER 7. GLYCEROL INHIBITS THE PRIMARY PATHWAYS AND MODIFIES THE SECONDARY PATHWAY OF INSULIN AGGREGATION

LIST OF FIGURES

LIST OF TABLES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
ANS	Anilino-1-naphthalenesulfonic acid
APS	Ammonium persulphate
BSA	Bovine serum albumin
CTAB	Cetyltrimethylammonium bromide
DCM	Dichloromethane
DCVJ	9-(2,2-Dicyanovinyl) julolidine
DLS	Dynamic light scattering
EPR	Electron paramagnetic resonance
FPLC	Fast protein liquid chromatography
GdnHCl	Guanidinium hydrochloride
HSA	Human serum albumin
IAPP	Islet amyloid polypeptide
ITC	Isothermal titration calorimetry
IFN-γ	Interferon gamma
LENP	Lumry Eyring nucleated polymerization
MPL	mass-per-length
PAGE	Polyacrylamide gel electrophoresis
RP-HPLC	Reverse phase high performance liquid chromatography
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
SOD1	Superoxide dismutase 1
ssNMR	Solid state nuclear magnetic resonance spectroscopy
STEM	Scanning tunneling electron microscope
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
TFE	Trifluoroethanol
ThT	Thioflavin T
TIRFM	Total internal reflection fluorescence microscopy
TTR	Transthyretin
UV	Ultraviolet