AN INVESTIGATION TO OBSERVE THE EFFECT OF DMSO AND GLYCEROL ON THE AGGREGATION OF LYSOZYME

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by

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NATIONAL INSTITUTE OF TECHNOLOGY
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CERTIFICATE

This is to certify that the thesis entitled **"An investigation to observe the effect of DMSO and Glycerol on the aggregation of Lysozyme"** by AMLAN KUMAR SAHOO (110BT0034) submitted to National Institute of Technology, Rourkela for the degree of Bachelor in Technology is a record of bonafide research work, has been carried out by him in the Department of Biotechnology and Medical Engineering under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other university/institute for the award of any degree or diploma.

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CONTENTS

ABBREVIATIONS

1. ThT – Thioflavin T
2. CR – Congo Red
3. DMSO – Di-methyl Sulphoxide
4. Gly – Glycerol
5. Lys – Lysozyme
6. AA – Amino Acid
7. HSP – Heat Shock Proteins
8. ATP – Adenosine Triphosphate
9. XRD – X-Ray Diffraction
10. H – Bond – Hydrogen Bond
11. UPP – Ubiquitin Proteasome Pathway
12. SDS – PAGE – Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis
13. FFF – Field Flow Fractionation
14. CD – Circular Dichroism
15. IR – Infrared
16. NMR – Nuclear Magnetic Resonance
17. HEWL – Hen Egg White Lysozyme
18. GdnHcl – Guanidine Hydrochloride

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Huntington's disease, Parkinson's disease, Alzheimer's disease, Prions Disease are few among many diseases caused due to the aggregation of misfolded proteins, which eventually leads to the formation of amyloids. Amyloids contain a large amount of β – sheets which make it highly stable in the body environment, thus making its lysis difficult. It is reported that the misfolded proteins form amorphous aggregates first which contain less number of β – sheets. These aggregates would further form amyloids as the number β – sheets increase. Inhibiting the formation of aggregates can be regarded as a therapeutic approach in the treatment of above mentioned diseases.

In the present investigation, two existing protocols for the formation of amorphous aggregates of lysozyme (Lys) were studied and a novel protocol for the same was proposed. This novel protocol showed high amounts of aggregate formation of lysozyme under laboratory conditions, as observed under Thioflavin T (ThT) assay. However, there was no formation of amyloids, as was observed under Congo Red assay. The effect of DMSO and Glycerol was investigated on the formation of Lysozyme aggregate. The results of these assays indicated that there was significant decrease in the amount of aggregation of lysozyme in solution. Thus, it was concluded that DMSO and Glycerol act as inhibitors for lysozyme aggregation.

CHAPTER 1

INTRODUCTION

1.1. Introduction:

There are three structural regimes of proteins, namely, primary structure, secondary structure, tertiary structure and quaternary structure of proteins. Primary structure of a protein refers to the sequence of amino acids that join together to form the polypeptide chain.

There are several regions in the primary structure which are folded in a particular way. These structures are called secondary structure. This structure is more regular and is stabilised by hydrogen bonds between the polypeptide chains. There are several secondary structures like α – helix, β – sheets etc. The protein is arranged in a coiled shape in α – helix structure. The "α" means if one looks down the spring, the coiling takes place in a clockwise manner. In the β – sheet structure, the sheets remain adjacent to each other by hydrogen bonding. The sheets lie anti – parallel to each other.

Tertiary Structure of protein is more complicated than the previous two structure. Here, the secondary structure folds itself into a three dimensional structure. This structure is stabilised by hydrogen bonding, van der waal's forces and sulphide bridges.

1.1.1. Protein Folding:

A protein exhibits a proper three dimensional conformation called the native structure. A protein is functional only in its native structure. A loss in the native structure of protein may result in the malfunctioning of the protein or its inactivation. A protein achieves this native structure via a series of guided pathways which help the peptide chains to fold in a desirable way (Lansbury 1999; Merlini and Bellotti 2003; Stefani and Dobson 2003; Sipe 2005). Any deviation from this pathway can result in misfolded proteins which may or may not be toxic.

Protein folding takes place under very vulnerable conditions, a change in which can cause a protein to misfold. This process is assisted by molecules called chaperones. Chaperones guide the protein folding process towards a pathway that causes the proteins to fold properly and attain its native state. Once a protein is misfolded, it could suffer three fates. It could be refolded back to the native state by special molecules called Heat Shock Proteins (HSP), or it could be lysed by the cell, or it could form aggregates by hydrophobic – hydrophobic interaction. These aggregates further form amyloids which are toxic and are the cause of several neurodegenerative disorders like

Parkinson's disease, Huntington's disease, Alzheimer's disease, Prions Disease, Fatal Filial Insomnia etc.

1.1.2. Protein Aggregation:

It is a biological phenomenon in which unfolded proteins aggregate either intra or extra cellularly. These aggregates often act toxically. The diseases that occur due to amyloids are called amyloidosis. After synthesis, a particular state/conformation is obtained which is called the native state. They are functional only in this state. This folding process is guided by the hydrophobic forces. However, proteins that are newly synthesized may not fold correctly or properly folded proteins can misfold spontaneously. In this case, if the cell doesn't assist the protein in refolding, or degrade the misfolded protein, the misfolded proteins form aggregates by interaction between the hydrophobic cores which become exposed to the surface of the protein when they misfold (Bennett 2005; Lee et al. 2001; Goedert and Spillantini 2006; Nguyen et al. 1995). There are several causes of protein misfolding. Firstly, mutations in individual proteins may encode proteins that are particularly prone to misfolding. Secondly, disruption of pathways to refold proteins (Ubiquitin Proteasome Pathway or UPP) or to degrade misfolded proteins.

A protein may undergo various aggregation pathways depending upon the environmental conditions. The aggregate process generally leads to soluble and/or insoluble aggregates which may precipitate.

1.1.3. Amyloids:

Amyloids, by definition, are insoluble fibrous protein aggregates sharing specific structural and functional properties. These structures of misfolded proteins alter their native configuration such that they erratically interact with one another and other components of the cell, thus forming fibrils that are insoluble. They are responsible for more than 20 human pathological and neurodegenerative disorders.

A more classical definition of amyloid is that it is an extra cellular proteinaceous deposit exhibiting β – sheet structure, which are generally identified by apple green birefringence when stained with Congo Red when observed under polarised light. A more accurate definition states that it includes any polypeptides which polymerises to from a cross β – sheet structure in – vivo or in – vitro. Formation of amyloids has been known to cause many disease like Alzheimer's disease, Parkinson's disease,

Huntington's disease, Fatal Familial Insomnia etc. Amyloid has several distinct characteristics and structural properties. It has an unbranched structure and ranges from 60 A^o – 120 A^o in diameter (Klunk et al. 1989). It possesses a particular cross X-Ray Diffraction (XRD) pattern. Amyloid fibrils are formed by proteins that have vastly different native topologies. Asparagine and Glutamine are commonly found in amyloids (Chiti et al. 1999; Fandrich et al. 2001). β – sheets assemble through the formation of H – bonds between adjacent segments of the polypeptide chains. They are either parallel or anti – parallel. The β – sheets stack parallel to the axis of the fibre with individual strands perpendicular axis.

1.1.4. Disaggregation of proteins:

Disaggregation of proteins is caused by a special class of molecules called the HSP (Heat Shock Proteins). All families and sub families of HSP have a common biochemical function – providing ATP to assist changes in protein folding and their assembly. HSP 100 plays a very crucial role in reversible refolding of proteins. It has further been classified into two more sub families - Class 1 HSP 100 (A, B, C, D sub types) and Class II HSP 100 (M, N, X, Y sub types).

Protein refolding can be achieved in two different ways. Firstly, the misfolded proteins can be refolded into native structure with the help of HSP molecules. Secondly, external molecules can be used to achieve the native structure of protein from its misfolded structure.

1.1.5. Induction factors causing protein aggregation:

Increase in temperature results in increased aggregation. Freezing and Thawing causes proteins to aggregate. Agitation Stress also is directly proportional to the formation of aggregates. Protein Concentration is also directly proportional to protein aggregation

1.1.6. **Methods used for analysis of protein aggregates:**

Table 1. Methods used for analysis of protein aggregates.

Method	Application
SDS - PAGE	Size estimation and to distinguish from reducible covalent from non – covalent aggregates.
Capillary Electrophoresis FFF	Size estimation and quantification (Soluble Aggregates)
Static Light Scattering	Size and shape estimation
Dynamic Light Scattering	Size distribution
Analytical Ultracentrifugation	Size, shape estimation and quantification
Coulter Counter	Size and member estimation
CD Fluorescence Spectroscopy IR Spectroscopy NMR Resonance	Structural Analysis

1.1.7. **Lumry – Eyring Two State Model:**

This is a model for protein aggregation. According to this model, the native protein undergoes first a reversible conformational change to an aggregation prone state, which subsequently assembles irreversibly to the aggregated state. The reversible conformational change is termed as ‘amorphous aggregate phase’ and the irreversible aggregated stage is termed as ‘amyloid’. Amorphous aggregates may or may not be toxic but presence of amyloids in the body is definitely harmful and fatal.

1.2. Objectives:

- To study the aggregation process of Lysozyme in vitro with an aim to develop a new protocol.
- To study the effect of DMSO and Glycerol on the aggregation of Lysozyme.

CHAPTER 2

LITERATURE REVIEW

2.1. Lysozyme:

Lysozyme is a single chain polypeptide of 129 AA cross-linked with four disulphide bridges. It hydrolyses β(1 – 4) linkages between N-acetylmuraminic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrin. The enzyme is often used for lysing bacterial cells by hydrolysing the peptidoglycan present in the cell walls.

This lysozyme preparation is purified from chicken egg white, crystallized three times, dialyzed, and supplied as a lyophilized powder. Protein content by UV absorbance is 90% with the remainder (~10%) being buffer salts such as sodium acetate and sodium chloride. The lysozyme thus prepared is called HEWL. Molecular weight of lysozyme is 14307 Da, making it easier to study due to its low molecular weight. Lysozyme functions optimally over a pH range of 6 – 9. The maximum activity of lysozyme is observed at a pH of 6.2. Its isoelectric point is 11.35.

Inhibitors:

Indole derivatives inhibit Lys aggregation, which bind to and disorient the active site, and imidazole, which forms a charge-transfer complex. It is also inhibited by surface-active agents such as sodium dodecyl sulphate, sodium dodecanate, and dodecyl alcohol. Other compounds having carbon chains of 12 or more carbons in length will also inhibit Lys. Its aggregation is also inhibited by DMSO and Glycerol as explained in this investigation.

Substrates:

The natural substrate for lysozyme is the peptidoglycan layer of bacterial cell walls. Low molecular mass substrates including murein degradation products as well as synthetic compounds have been used for various photometric, isotopic, and immunological lysozyme assays.

Compounds reported to be affecting Lys aggregation:

Table 2. Reagents affecting Lys aggregation.

Sl.No.	Compounds that inhibit aggregation of Lys	Compounds that accelerate aggregation of Lys
1	Acrinidine Derivatives	Glass
2	Curcumin	Steel
3	Kaempferol	Silicon
4	Surfactant Coplymers like Polyethylene Oxide – Polypropylene Oxide – Polyethylene Oxide (PEO – PPO – PEO)	Plastic
5	p - Aminophenol	Rubber
6	2 – Amino 4 - Chlorophenol	Oleic Acid

2.2. Study of molecules preventing Lysozyme aggregation:

2.2.1. Acrinidine Derivatives inhibit Lysozyme aggregation:

Formation of amyloids from their native structure has been recognized as a cause for over twenty human diseases, as well as Alzheimer's, Parkinson's and Huntington's diseases, sort II polygenic disease, prion-related transmissible spongiform encephalopathies, and hereditary disease (Taniguchi et al. 2005). The conversion of a selected protein or protein fragment from soluble native state into insoluble amyloid fibrils ends up in the formation of protein deposits during a style of organs and tissues with one predominant protein element that's characteristic of every disease. Varied proteins are known as forming amyloid in vivo.

Though the proteins take issue in their primary and tertiary structures, furthermore as their size and performance, the extremely ordered amyloid fibrils shaped from these proteins share common morphological and histochemical staining properties. The amyloid fibrils possess a typical cross-β structural motif, having b-strands familiarized perpendicular to the fibre axis, and that they bind by selection the aromatic dyes metal and ThT. as a result of the morphological similarities between several fibrils from completely different protein building blocks, it's been hypothesized that completely different proteins follow similar fibre formation pathways. However, the precise mechanism of the amyloid aggregation remains not clear. It's

typically accepted that protein aggregation has deadly consequence to completely different cell varieties suggesting its key role in cell impairment and death.

Recently, there's growing proof that soluble oligomers instead of mature amyloid fibrils is also the most deadly species in amyloid-related disorders. Though this idea was originally introduced within the investigation of the neurotoxicity of Ab oligomers and their role within the pathological process of Alzheimer's disease, this notion has currently been significantly expanded to incorporate many different proteins concerned in amyloid diseases. The explanation why early aggregates square measure additional deadly than mature amyloid fibrils isn't nevertheless clear. In isolated cells, such toxicity has been shown to result from enhanced membrane permeability with disruption of membrane integrity and formation of particle channels, aerophilic stress and freeing of cell physiological state by accumulation of animate thing amyloid. Additional recently, it's been found that amyloid chemical action isn't solely potential with disease-associated proteins, however conjointly with proteins that don't seem to be related to any known amyloid disease underneath bound conditions in vitro. Amyloid fibrils and pre-fibrillar assemblies shaped from non-disease connected proteins have similar morphological options and toxicity as those detected for disease-associated proteins. This has light-emitting diode to the suggestion that ability to make amyloid aggregates may be a generic property of peptide chains, which most or so all peptides and proteins have the potential to make such structures in vitro underneath applicable conditions. Therefore, the study of the amyloid aggregation of non-disease associated proteins will increase our understanding of potential inhibition of amyloid aggregation.

Hereditary general disease is related to one among the simplest known of all proteins-lysozyme. This disease results from single purpose mutations within the sequence giving rise to variant proteins that kind huge amyloid deposits within the liver and urinary organ of people tormented by this disease. Studies of those proteins have shown that amyloid formation of the variants is as a result of an inclination to favour part denaturated structures. The flexibility to make amyloid aggregates in vitro has been found for the only purpose mutants and wild-type human enzymes and conjointly for hen fixings lysozyme. Currently, there aren't any effective cures for amyloid diseases, however experiments from varied cell and animal models recommend that the reduction of amyloid aggregation is useful.

The anti-aggregating activity has been known for a spread of gear as well as the antibodies, artificial peptides, heat shock proteins, and chemical compounds. an excellent range of

numerous little molecule compounds are found to inhibit or scale back the aggregation of varied proteins, notably in relevance Ab deposition, aggregation of enzyme and transthyretin and also the formation of protease- resistant types of the particle protein. Recently, it's been detected that anthraquinones square measure ready to inhibit alphabetic character mixture formation in vitro and in cells. Similar impact was discovered conjointly for different low relative molecular mass compounds as phenothiazines, N-Phenylamine derivatives, polyphenols and porphyrins. Acridine based mostly compounds were known as potent inhibitors of protease-resistant types of the particle protein. Thus, little molecules may offer a basis for the event of tools for the treatment of amyloid pathology.

2.2.2. Amidated amino acids prevent heat induced aggregation of Lys:

Aggregation is an intrinsic development for peptide chain. The management of aggregation should be achieved inexpensively and simply for biotechnological and medical applications of valuable proteins. To scale back aggregation in vitro, numerous factors ought to be tested, such as pH, ionic strength, temperature, and protein concentration. A straightforward however effective approach to rising the aggregation drawback is that the addition of low quantity of potent substance to forestall protein aggregation. Many varieties of additives for reducing protein aggregation are developed.

Protein-denaturing reagents, usually guanidine and detergents, are used as an aggregation suppressor that weakens the hydrophobic building block interaction of proteins. However, these additives ambivalently decrease the soundness of proteins that typically accelerates aggregation. A compound synthesized through refolding in detergent followed by cycroamylose addition has been developed to operate as a man-made chaperone. Though non-denaturing reagents, like amino acids, are accustomed preserve protein answer, their use isn't adequate to unravel the issues of protein aggregation of these amino acids, essential amino acid (Arg) possesses a favourable property as an additive for the interference and dissolvation of aggregation; that's, it doesn't destabilize the native structure and has solely a minor impact on protein stability whereas it enhancing the solubility of aggregation-prone molecules throughout refolding.

Recently, we've rumoured that polyamines, specifically spermine and spermidine, forestall the heat-induced inactivation and aggregation of muramidase a lot of effectively than Arg. Polyamines slightly destabilize the native structure of muramidase however it markedly will increase the solubility of aggregation-prone molecules. The indispensable feature within the

structure of polyamines for his or her operate as an aggregation suppressor is that the presence of multiple amines. Essential amino acid ethylester (ArgEE) could be a lot of favourable additive for suppressing the heat-induced aggregation of muramidase than Arg. though Arg isn't effective at concentrations below one M, ArgEE is effective at concentrations one order of magnitude below that of Arg (Vieira et al. 2007; Guijarro et al. 1998). What is more, many organic compound derivatives equally forestall the heat-induced aggregation of muramidase as effective as ArgEE. Though organic compound alkylesters are promising candidate for preventing protein aggregation, these additives could also be hydrolysed to alcohols and amino acids in a solution. Therefore, organic compound alkylesters don't seem to be favourable for sensible applications that entail long-time storage.

2.2.3. Small molecules like p-Aminophenol and 2-Amino 4-chlorophenol act as inhibitors for Lys aggregation:

The conversion of soluble proteins or peptides into amyloid aggregates and their deposition in tissues are related to variety of necessary human pathologies, together with Alzheimer's and Parkinson's diseases, transmissible spongiform encephalopathies, type-2 polygenic disorder, and numerous general amyloidoses. Despite the dearth of sequence similarity between distinct disease-related amyloid-forming proteins and peptides, the method of aggregation looks similar all told cases, and therefore the ensuing materials share common tinctorial and morphological characteristics. Additionally, recent studies have shown that the power to create amyloid aggregates in vitro isn't associate degree exclusive property of proteins associate degreed peptides related to illness however rather looks to be an intrinsic characteristic of peptide chains.

Human muramidase variants are involved in chromosome hereditary general diseases. All told cases studied to this point, single aminoalkanoic acid mutations within the muramidase factor are shown to steer to the destabilization of the variants associate degreed to an accumulated propensity to create amyloid aggregates. However, beneath applicable in vitro conditions, wild-type human muramidase additionally forms amyloid aggregates that are nearly indistinguishable from those fashioned by the variants (Vernaglia et al. 2004). What is more, hen ingredient muramidase (HEWL), a protein that's not related to any illness, has recently been shown to bear amyloid aggregation in vitro at acidic pH and high temperatures. Those conditions destabilize the native structure of HEWL, pro the population of aggregation-prone

partly unpleated species. Muramidase is definitely obtained from completely different sources and its structure and folding mechanisms are extensively characterised.

These options build muramidase a stimulating model for the study of amyloid aggregation. Amyloid aggregates of varied proteins are deadly to completely different cell varieties, however the precise mechanisms of toxicity haven't been totally elucidated. It's clear, however, that protein aggregation into soluble oligomers, protofibrils, mature amyloid fibrils, or a mix of those may be a demand for toxicity. Thus, inhibition or reversion of amyloid aggregation could represent an attainable therapeutic strategy for the hindrance and treatment of amyloidosis.

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals Used:

1. Lysozyme
2. Monobasic Sodium Phosphate – Na_2HPO_4
3. Dibasic Sodium Phosphate - NaH_2PO_4
4. GdnHCl
5. ThT
6. CR
7. Distilled Water

3.2. Instruments Used:

1. UV/visible spectrophotometer for Congo Red (CR) assay.
2. Fluorescence spectrophotometer for Thioflavin T (ThT) assay.

3.3. Methodology:

3.3.1. Preparation of stock Lys solution:

1 mL of 100 μM Sodium Phosphate buffer was transferred to a test tube from the stock solution and was diluted to 2 mL with distilled water. 2 mg of Lys was weighed and was transferred to the test tube containing the buffer. Thus a 1 mg/mL concentrated Lys solution was prepared before every experiment for use in aggregation and further while performing ThT and CR assays.

3.3.2. Protocols used for Lys aggregation:

3.3.2.1. Protocol I:

The stock buffer was diluted to 20 mM and its pH was adjusted to 6.3±0.1. A 10 μM solution of lysozyme was prepared. It was transferred into a test tube along with 3M GdnHCl. The solution was transferred into a small beaker and was kept on a magnetic stirrer which was maintained at 60 rpm at 50 ^oC.

3.3.2.2. Protocol II:

A solution containing 1 mg/mL Lys and 50mM Sodium Phosphate Buffer was prepared and the pH was adjusted to 7.16. 200 μL aliquot of the stock solution was taken in a microtube and was then placed in a water bath. Temperature was increased from 25 ^oC to 90 ^oC at 1 ^oC/sec. It was stored at 25 ^oC for 20 min. It was then centrifuged at 15000 g for 20 min at 25 ^oC.

3.3.2.3. Protocol III (Novel Protocol):

A stock solution of 1 mg/mL Lys was prepared in 50 mM sodium phosphate buffer and the pH was adjusted to 7.16. 200 µL of the stock solution was taken in a test tube and the volume was adjusted to 1 mL using the buffer. It was then heated at 100 ^{o}C for 20 min in a water bath. It was incubated at 25 ^{o}C for 20 more min after heating.

3.3.3. Protocol used for studying the effect of DMSO on Lys aggregation:

Two test tubes were taken and were marked "BB" and "AB". 1 mL of 1 mg/mL Lys stock solution was taken in both test tubes. 100 µL of DMSO having volume fraction = 1 (pure DMSO) was added to the test tube marked "BB". Both the test tubes were closed using cotton plugs and aluminium foil. They were kept in the water bath for 20 min at 100 ^{o}C. After heating, both the test tubes were stored for 20 more min at room temperature to allow the DMSO to act properly on the aggregates formed in the test tube marked "BB" after boiling.

3.3.4. Protocol used for studying the effect of Glycerol on Lysozyme aggregation:

Two test tubes were taken and were marked "BB" and "AB". 1 mL of 1 mg/mL Lys stock solution was taken in both test tubes. 100 µL of Glycerol having volume fraction = 1 (pure Glycerol) was added to the test tube marked "BB". Both the test tubes were closed using cotton plugs and aluminium foil. They were kept in the water bath for 20 min at 100 ^{o}C. After heating, both the test tubes were stored for 20 more min at room temperature to allow the Glycerol to act properly on the aggregates formed in the test tube marked "BB" after boiling.

3.3.5. Thioflavin T (ThT) assay:

A 5 mM solution of ThT dye was prepared in sodium phosphate buffer of pH 7.16. This dye was stored in a dark and cold place for further use during ThT assay.

Four test tubes were taken and were marked "C" (Control Solution), "A" (Aggregated Solution), "BB_1" (Addition of reagent before boiling), and "AB_1" (Addition of reagent after boiling). The contents of the test tubes are portrayed in Table 3. All the test tubes were then left in the dark for 30 min before performing the assay because ThT dye is a light sensitive.

Table 3. Contents of the various test tubes in ThT Assay

Test Tube	Lysozyme	Reagent	ThT	Buffer	Total Vol.
C	Non – aggregated stock (100 µL)	-	15 µL	2885 µL	3 mL
A	Aggregated solution from test tube "AB" (100 µL)	-	15 µL	2885 µL	3 mL
AB_1	Solution from test tube "AB" (100 µL)	DMSO/Gly	15 µL	2785 µL	3 mL
BB_1	Solution from test tube "BB" (200 µL)	DMSO/Gly	15 µL	2785 µL	3 mL

3.3.6. Congo Red (CR) assay:

7mg/mL of CR solution was prepared in sodium phosphate buffer at pH = 7.16. This dye was stored in a dark and cold place for further use during CR assay.

Five test tubes were taken and were marked "CR" (Congo Red), "C" (Control Solution), "A" (Aggregated Solution), "BB_1" (Addition of reagent before boiling), and "AB_1" (Addition of reagent after boiling). The contents of the test tubes are portrayed in the following table. All the test tubes were then left in the dark for 30 min before performing the assay because CR dye is a light sensitive.

Table 4. Contents of the various test tubes in Congo Red (CR) Assay

Test Tube	Lysozyme	Reagent	CR	Buffer	Total Vol.
CR	-	-	15 µL	2985 µL	3 mL
C	Non – aggregated stock (100 µL)	-	15 µL	2885 µL	3 mL
A	Aggregated solution from test tube "AB" (100 µL)	-	15 µL	2885 µL	3 mL
AB_1	Solution from test tube "AB" (100 µL)	DMSO/Gly	15 µL	2785 µL	3 mL
BB_1	Solution from test tube "BB" (200 µL)	DMSO/Gly	15 µL	2785 µL	3 mL

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Protocol I for Lysozyme (Lys) aggregation:

Here, the protocol was used to test for Lys aggregation and was tested after 0h, 15h and 24 h for the amount of aggregation using the ThT assay. As expected, the buffer showed the least intensity. It was subtracted from the graphs of all the solutions to obtain accurate results without zero error. Although the aggregation in the sample was observed to gradually increase with escalating time, a major anomaly in the results were observed. The graph for the "Control" solution which contained 1mg/mL non-aggregated Lys solution in sodium phosphate buffer (pH=6.4) was observed to have suffered more aggregation that the samples which were prepared using this protocol and were tested after 0 h and 15 h, which is an anomaly considering the fact that the control solution contains "non-aggregated" Lys. This anomaly was attributed to the presence of GdnHCl (Guanidine Hydrochloride) in the solution which is thought to be the reason of decrease in aggregation in aggregated solutions tested after 0 h and 15 h, though the clear mechanism has not yet been understood. Another important factor that played a role in rendering this protocol ineffective is the difference in laboratory conditions.

4.1.1. Thioflavin T (ThT) Assay:

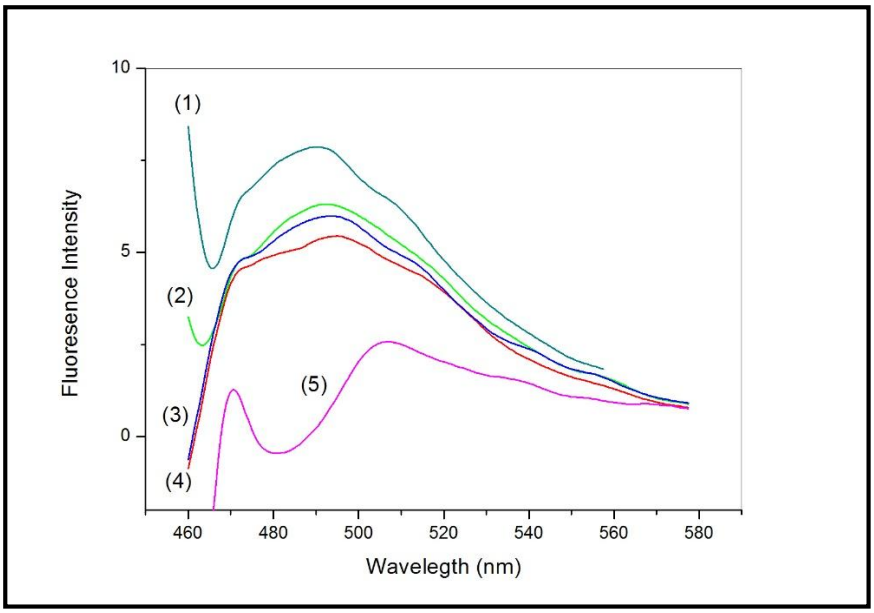

Figure 1. Graph of results of ThT assay performed on Lys aggregates formed by Protocol I. (1) Lysozyme aggregation observed after 24 h (2) Native lysozyme solution (3) Lysozyme aggregation observed after 15 h (4) Lysozyme aggregation observed after 0 h (5) Sodium Phosphate buffer.

4.2. Protocol II for Lys aggregation:

From the graph of ThT assay, we can observe that the aggregation is less in the test solution than in the control solution, which is absurd considering that control solution itself contain "non-aggregated" lysozyme. Moreover, no peaks were observed for control and test solutions in the CR assay, although a peak was observed for the CR solution. This indicated nothing about the formation of aggregates. As the results for both the assays in this protocol didn't predict desired results, this protocol was deemed inaccurate.

4.2.1. ThT Assay:

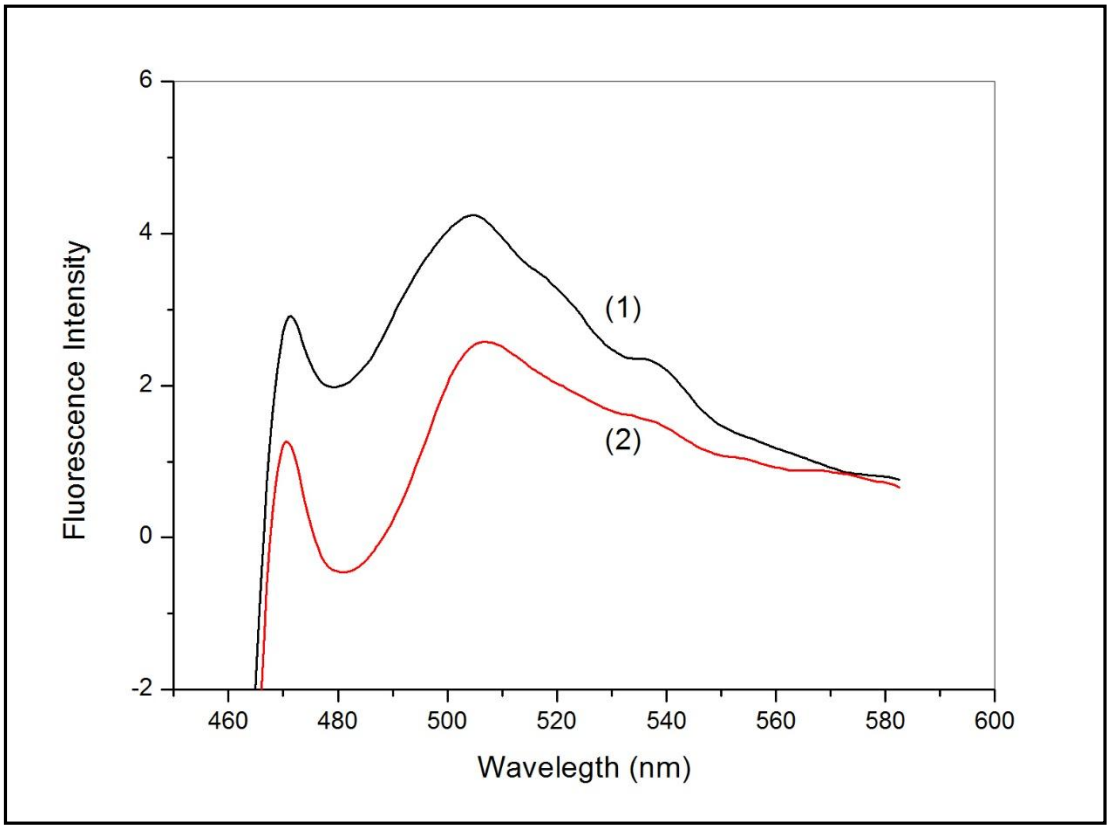

Figure 2. Graph of results of ThT assay performed on Lys aggregates formed by Protocol II. (1) Native lysozyme solution (2) Aggregated lysozyme solution.

4.2.2. Congo Red (CR) Assay:

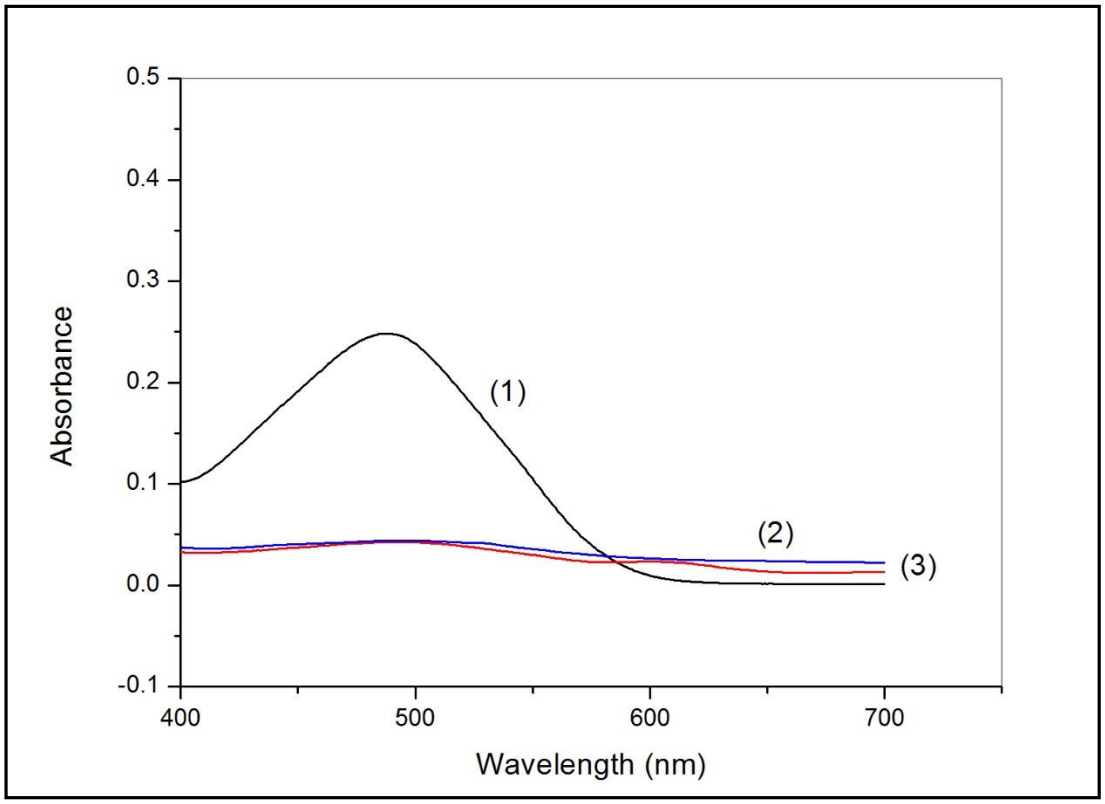

Figure 3. Graph of results of CR assay performed on Lys aggregates formed by Protocol II. (1) Pure Congo Red solution (2) Native Lysozyme solution (3) Aggregated Lysozyme solution.

4.3. Protocol III (Novel Protocol) for Lys aggregation:

As observed from the graph of ThT assay, there was a significant amount of aggregation in the test solution as compared to the control solution which is ideally termed as "zero aggregation state". This is based on a simple mechanism. Heating Lys at 100 ^{o}C for 20 min causes the protein to denature. This makes the hydrophobic cores of the protein to protrude out of the conformation which were on the inside in its native conformation. The hydrophobic – hydrophobic interactions causes the protein to aggregate.

There was very minimal difference in peaks and the wavelengths at which peaks were observed for control and test solution as observed from the graph of CR assay. This indicates that there was no amyloid formation in Lysozyme.

This protocol was dubbed as accurate and perfect for Lysozyme aggregation under the laboratory conditions.

ThT Assay:

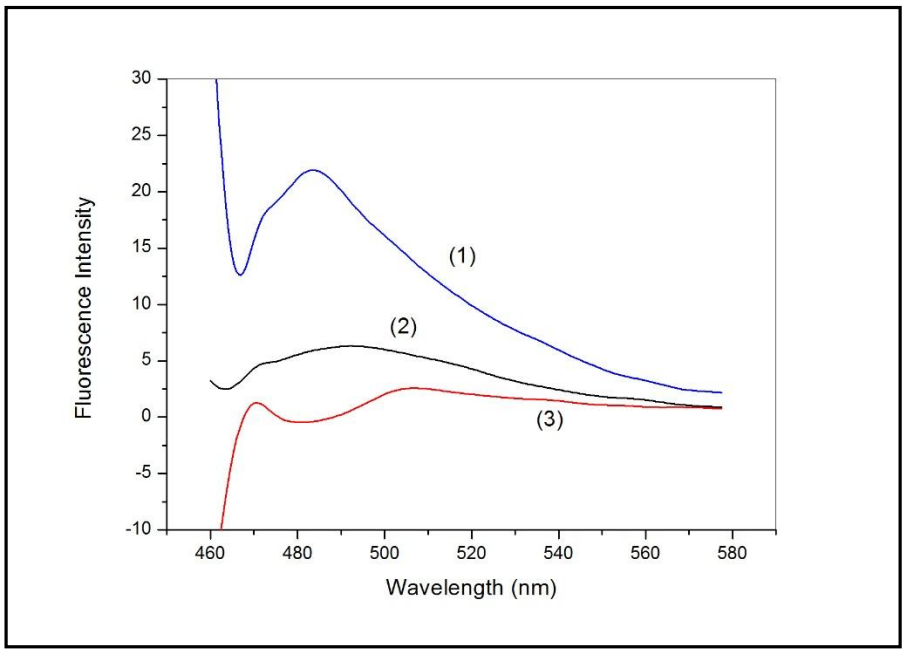

Figure 4. Graph of ThT assay performed on Lys aggregates formed by Protocol III. (1)Aggregated lysozyme solution (2) Native lysozyme solution (3) Sodium Phosphate buffer.

4.3.1. CR Assay:

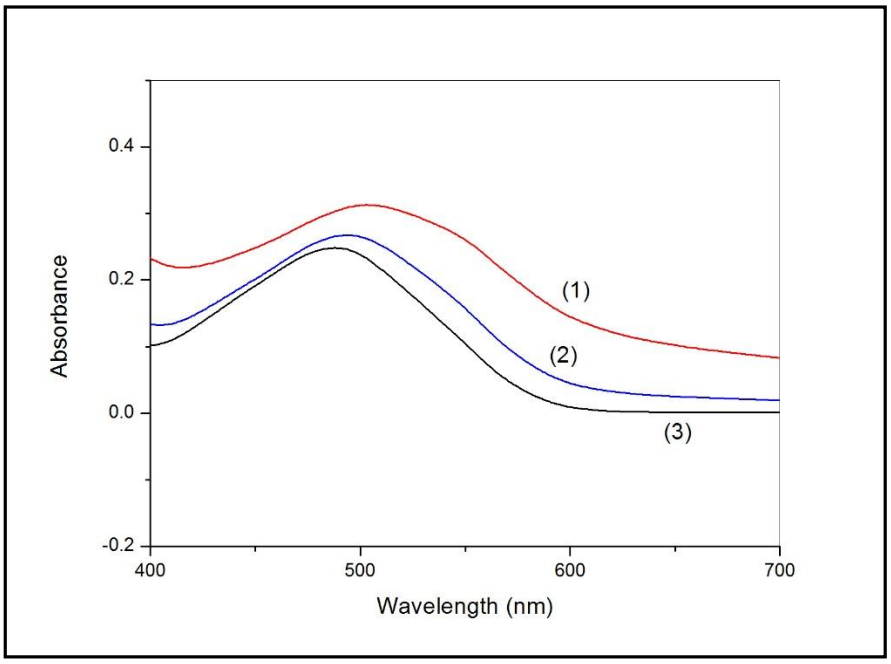

Figure 5. Graph of Congo Red assay performed on Lys aggregate formed by Protocol III. (1) Aggregated lysozyme solution (2) Native lysozyme solution (3) Pure Congo Red solution.

4.4. Effect of DMSO on Lys aggregation:

As observed from the ThT assay, DMSO significantly decreased the aggregation in Lys, both when added before and after boiling. This again follows a simple mechanism. At high concentrations of DMSO, the protein attempts to minimise the contact area between DMSO and amino acid residues for which DMSO is a bad solvent, so that it will rearrange in such a way so as to expose the units that were buried in native conformation and vice versa. In short, it causes the protein to flip it structure. Thus, flipping aggregates causes low levels of hydrophobic – hydrophobic interaction and thus reduces aggregation.

Again, no major peak shifts were observed in the CR assay indicating that there was no amyloid formation in the solution.

4.4.1. ThT Assay:

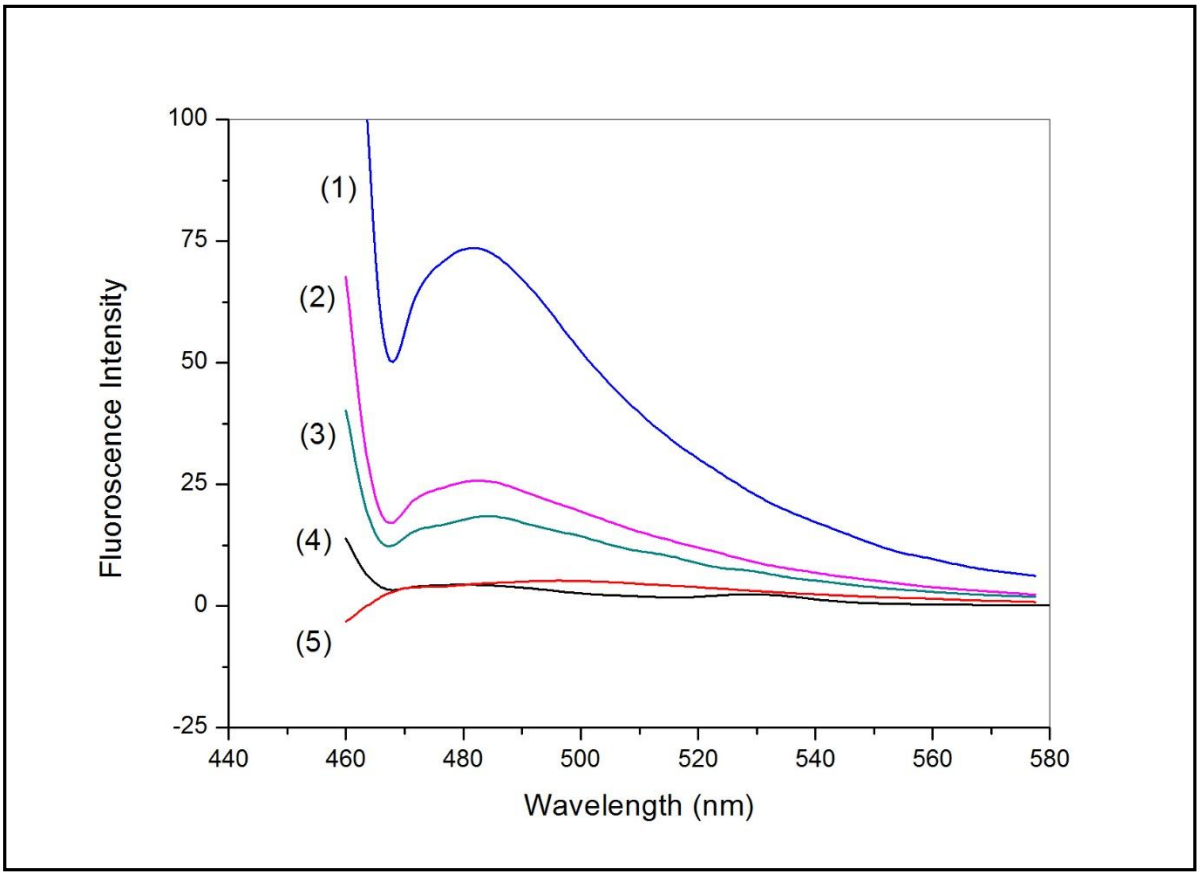

Figure 6. ThT Assay for effect of DMSO on Lys aggregation. (1) Aggregated lysozyme solution (2) DMSO was added to lysozyme solution before boiling (3) DMSO was added to aggregated solution after boiling (4) Sodium Phosphate buffer (5) Native lysozyme solution.

4.4.2. CR Assay:

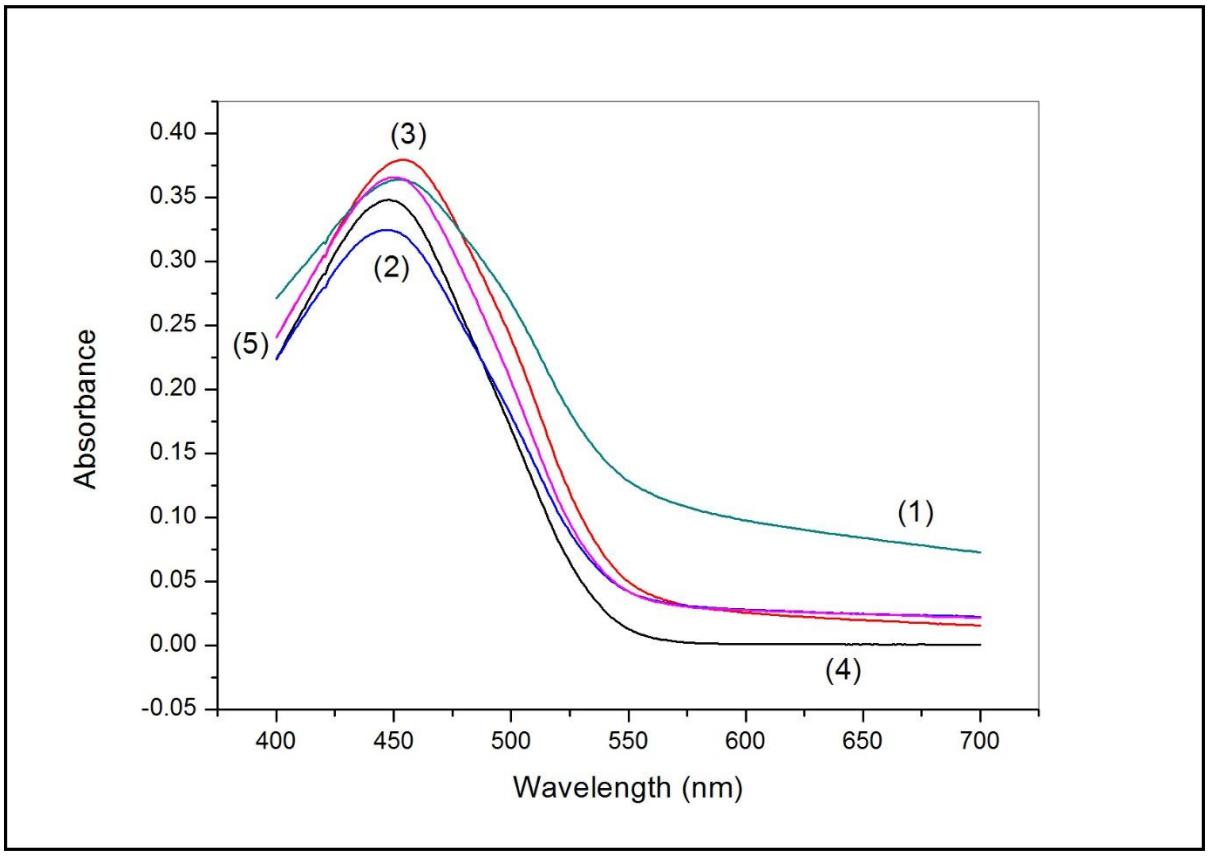

Figure 7. CR Assay for effect of DMSO on Lys aggregates. (1) DMSO was added to Lysozyme solution before boiling (2) Aggregated lysozyme solution (3) Native lysozyme solution (4) Pure Congo Red solution (5) DMSO was added to aggregated lysozyme solution after boiling.

4.5. Effect of Glycerol (Gly) on Lys aggregation:

As observed from the ThT assay, Gly significantly decreased the aggregation in Lys, both when added before and after boiling. This, again follows a simple mechanism. Poly co – solvents like glycerol increases the thermal stability of Lys. And proteins in general. This is explained by preferential hydration favouring the more compact native rather than the denatured state.

Again, no major peak shifts were observed in the CR assay indicating that there was no amyloid formation in the solution.

4.5.1. ThT Assay:

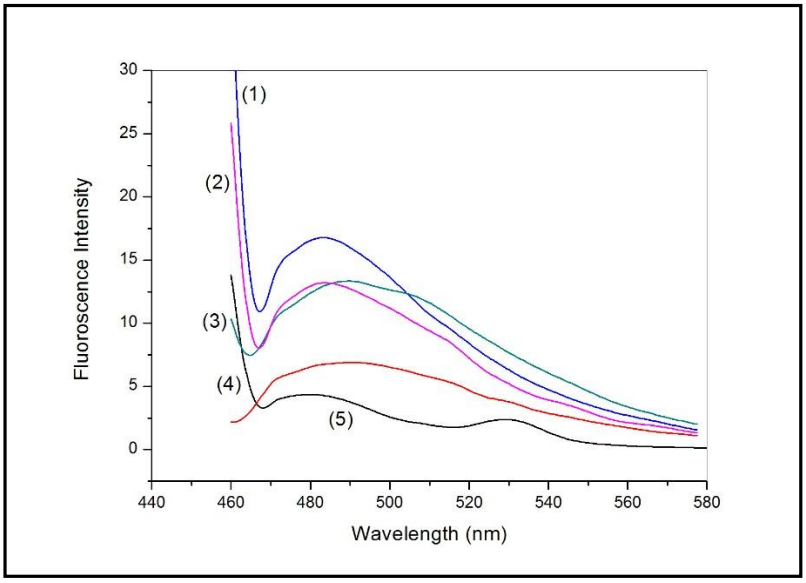

Figure 8. ThT Assay for effect of Glycerol on Lys aggregates. (1) Aggregated lysozyme solution (2) Glycerol was added to native lysozyme solution before boiling (3) Glycerol was added to lysozyme aggregate solution after boiling (4) Sodium Phosphate buffer (5) Native lysozyme solution.

4.5.2. CR Assay:

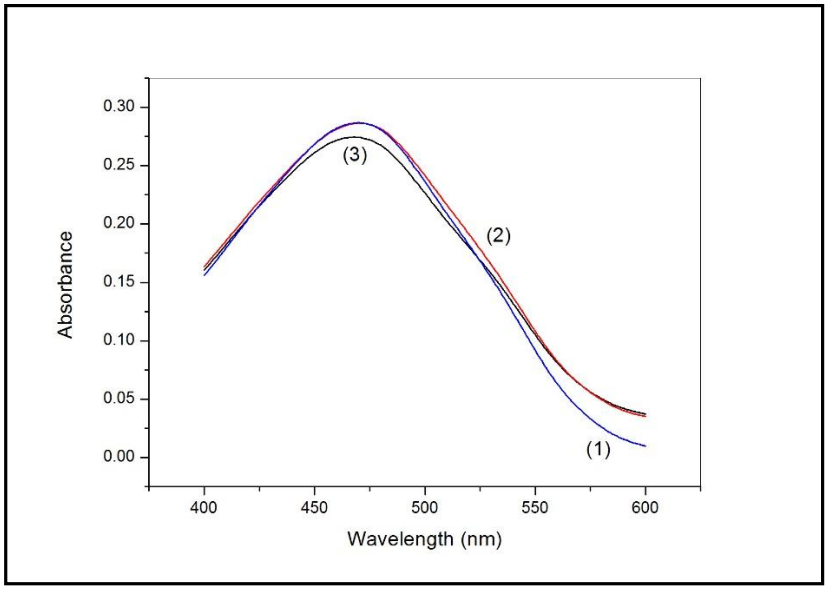

Figure 9. CR Assay for effect of Glycerol on lysozyme aggregates. (1) Glycerol was added to native lysozyme solution before boiling, and then the solution was heated (2) Glycerol was added to aggregated lysozyme solution after boiling (3) Native lysozyme solution.

CHAPTER 5

CONCLUSION

1.1. Conclusion:

The aggregates formed from the boiling of Lysozyme (Lys) at 100 ^{o}C for 20 min were quite significant. These aggregates are normally called 'amorphous' aggregates. Further increase in the number of β – sheets would result in the formation of amyloids which is the cause of several neurodegenerative disorders. An attempt was made to stop the amyloid formation pathway at the 'amorphous' aggregate stage and the effect of DMSO and Glycerol (Gly) were observed by performing ThT and CR spectroscopic assays.

DMSO and Gly were observed to be inhibitors of Lys aggregation. DMSO was found to prevent aggregation significantly, both when added before and after boiling. Gly also showed signs of inhibiting Lys aggregation, but was a weak one. Introduction of DMSO into the aggregated solution caused the proteins to flip their structure inside out due to preferential solubility of amino acid in DMSO. This reduced the hydrophobic – hydrophobic interactions amongst proteins and thus decreased aggregation. Gly prevented aggregation due to its preferential hydration favouring the more compact native structure rather than the denatured state. Although the mechanisms of action of both the molecules were different, they were found to be effective inhibitors of Lys aggregation.

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