EFFECT OF PLANT EXTRACTS ON AGGREGATION OF EGG PROEINS OVALBUMIN AND LYSOZYME

A dissertation project
submitted to Nirma University
in partial fulfillment of requirement
for the degree of

Master of Science

In

Biochemistry

Submitted by

Suresh Kumar
09MBC017

Manan Trivedi
09MBC019

Under the guidance of

Dr. Mili Das

INSTITUTE OF SCIENCE
NIRMA UNIVERSITY OF SCIENCE AND TECHNOLOGY
SARKHEJ-GHANDHINAGAR HIGHWAY
AHMEDABAD

THIS DISSERTATION PROJECT

IS DEDICATED TO OUR PARENTS

AND DR. MILI DAS

Acknowledgement

We take this opportunity to thank ***Prof. G. Naresh Kumar****, Director, Institute of Science, Nirma University, for his untiring help and Inspirational words. We thank him for all the facilities he provided us for the completion of our project.*

This work would not have been possible without the support and encouragement of our guide ***Dr. Mili Das*** *under whose supervision we chose our dissertation topic and began this thesis. Her valuable advice, guidance, extensive discussion about our work and constructive criticism was crucial and encouraging. No words can express our sincere and deep sense of reverence for her. We are extremely thankful to her for the scientific attitude she has installed in us which will definitely stand in our future endeavors and it was because of her that we were able to learn so much in this short period of time.*

We are thankful to all the faculty members, ***Dr. Shalini Rajkumar, Dr. Sriram Seshadri, Prof. Sarat Dalai, Dr. Amee Nair, Dr. Vijay Kothari, Dr. Sonal Bakshi*** *and* ***Dr. Nasreen Munshi*** *for their continuous encouragement and support. We thank* ***Mr. Hasit Trivedi, Mr. Sachin Prajapati*** *and* ***Mr. Bharat Anand*** *for extending their hands for help, for providing us with all kinds of possible help at anytime.*

We are grateful for the kind help provided by ***Prof. Sarita Gupta, MSU, Baroda*** *for providing us Sephadex G-75 matrix for our work.*

We would also like to acknowledge the genuine help from ***Dr. Preeti J. Mehta****, Institute of Pharmacy, Nirma University for allowing us to use UV - Visible Spectrophotometer in their laboratory.*

We enjoyed the opportunity of working with each other. We convey our special gratitude to all our group members and friends especially for their loving support during difficult times.

We owe thanks to our Parents, who indeed was a source of inspiration throughout our project and who taught us to stand against all odds of life and face every new challenge bravely whose love and affection strengthened us during our project work.

Last but not the least, we thank almighty for giving us the strength to work even in unfavorable conditions without losing faith in Him and our self.

Manan Trivedi *Suresh Kumar*

ABBREVIATIONS

AD	Alzheimer's disease
APS	ammonium per sulfate
BME	β-mercapto ethanol
BSA	bovine serum albumin
CD	circular dichroism
CM	Carboxy methyl
DEAE	Diethylaminoethyl
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ELISA	Enzyme Linked ImmunoSorbent Assay
ER	endoplasmic reticulum
GABA	γ-aminobutyric acid
G-CSF	granulocyte colony stimulating factor
GdmCl	guanidium hydrochloride
Hsp	heat shock protein
mAbs	monoclonal antibodies
MBP	mannose binding protein
NAG	N-acetyl D-glucosamine
NAM	N-acetyl muramic acid
NMR	nuclear magnetic resonance
OAN	native ovalbumin
OAR	Renatured ovalbumin
PAGE	polyacrylamide gel electrophoresis

PD	Parkinson’s disease
PEG	polyethylene glycol
SDS	Sodium Dodecyl Sulfate
TEMED	N,N,N,N-Tetramethylethylenediamine
TMAO	trimethylamine N-oxide

INDEX

1. INTRODUCTION AND REVIEW OF LITERATURE 1

1.1 Protein folding 2

1.2 Process of protein folding 3

1.3 Chaperones 3

1.3.1 Molecular chaperones 4

1.3.2 Chemical chaperones 4

1.4 Protein misfolding, aggregation and diseases 5

1.4.1 Aggregation of abnormal proteins 7

1.4.2 Mechanism of aggregation 8

1.4.3 Aggregation of therapeutic proteins 10

1.4.4 Protein aggregation in Crowded Environments 11

1.5 Egg proteins 12

1.5.1 Egg white 13

1.5.1.1 Ovalbumin 13

1.5.1.2 Lysozyme 16

1.6 Effect of various plant extracts on protein aggregation 18

2. OBJECTIVE 19

3. MATERIAL AND METHODS 21

3.1 Materials 22

3.2 Preparation of crude egg white from chicken egg 22

3.2.1 Purification of ovalbumin and lysozyme 22

3.2.1.1 Gel Filtration chromatography 22

3.3 SDS – PAGE of purified proteins 23

3.4 Denaturation and Renaturation 23

3.5 Preparation of plant extracts 24

4. RESULTS AND DISCUSSION 25

4.1 Protein purification by gel filtration chromatography 26

4.2 Aggregation Analysis of ovalbumin 27

4.3 Aggregation Analysis of lysozyme 28

4.4 Congo Red Binding assay for lysozyme 29

4.5 Aggregation of ovalbumin in the presence of plant extracts 29

4.6 Aggregation of lysozyme in the presence of plant extracts 32
4.7 Conclusion 38
5. SUMMARY 39
6. FUTURE PROSPECTIVE 41
7. APPENDIX 43
7.1 SDS-PAGE 44
7.2 Folin-Lowry method for quantitation of protein 46
7.3 Broad range protein molecular weight marker 47
7.4 phosphate buffer (0.1 M, pH 7.0) 48
7.5 phosphate buffer (20 mM, pH 6.2) 48
8. REFERENCES 50

LIST OF TABLES

1) **Table 1**: Cellular osmolytes 4

2) **Table 2**: Protein folding diseases and their sites 7

3) **Table 3**: Chemical composition of hen egg 13

4) **Table 4**: Major constituents of egg white and their properties 15

5) **Table 5**: Prevention of aggregation of lysozyme with different plant extracts 35

LIST OF FIGURES

1) **Figure 1.** Regulation of protein folding in ER 5
2) **Figure 2**. Schematic representation of the factors influencing protein folding and aggregation events in vivo 6
3) **Figure 3**. Mechanism of aggregates 9
4) **Figure 4**. The crowded state of the cytoplasm in eukaryotic cell 12
5) **Figure 5**. Structure of ovalbumin 14
6) **Figure 6.** Structure of lysozyme 17
7) **Figure 7.** Gel filtration chromatography of egg crude 26
8) **Figure 8.** SDS-PAGE of chromatography fractions 27
9) **Figure 9.** Aggregation analysis of denatured ovalbumin 27
10) **Figure 10.** Aggregation analysis of lysozyme in BME-phosphate buffer 28
11) **Figure 11.** Congo red binding assay 29
12) **Figure 12.** Aggregation analysis of ovalbumin in presence of amla extract 30
13) **Figure 13.** Aggregation analysis of ovalbumin in presence of cinnamon extract 31
14) **Figure 14.** Aggregation analysis of ovalbumin in presence of ginger 31
15) **Figure 15.** Aggregation analysis of lysozyme with amla extract 32
16) **Figure 16.** Aggregation analysis of lysozyme with ginger extract 33
17) **Figure 17.** Aggregation analysis of lysozyme with cinnamon extract 34
18) **Figure 18.** Aggregation analysis of lysozyme with amla extract in ELISA reade**r** 36
19) **Figure 19.** Aggregation analysis of lysozyme with amla extract in test tube 36
20) **Figure 20.** Aggregation analysis of lysozyme with amla in visible spectrophometer 37
21) **Figure 21.** Various bands of the broad range protein molecular weight marker 47

1. INTRODUCTION AND REVIEW OF LITERATURE:

The word **PROTEIN** comes from Greek language (prota) which means "of primary importance". This name was introduced by Jons Jakob Berzelius in 1838 for large organic compounds with almost equivalent empirical formulas. A protein is a group of complex organic macromolecules that contain carbon, hydrogen, oxygen, nitrogen, and usually sulfur and are composed of one or more chains of amino acids. Proteins are fundamental components of all living cells and include many substances, such as enzymes, hormones, and antibodies that are necessary for the proper functioning of an organism. They are essential in the diet for the growth and repair of tissue and can be obtained from foods such as meat, fish, eggs, milk, and legumes. Proteins are biochemical compounds consisting of one or more polypeptides typically folded into a globular or fibrous form in a biologically functional way.

The gene functions are manifested in the form of proteins. Proteins are the most abundant molecules in biology other than water. We contain perhaps 100,000 different types of protein and they stimulate or control virtually every chemical process on which our lives depend. Proteins are central to various biological processes. Proteins are synthesized on the ribosome and each protein molecule must fold into the specific conformational state that is encoded in its sequence in order to be able to carry out its biological function. This process happens in one of the most fascinating and challenging problems in structural biology, the process of "**protein folding**" (Dobson & Fersht, 1995).

The properties of the amino acid side chains and the peptide bond confer a high degree of conformational flexibility to the protein molecule, resulting in tremendous possible conformations from a single polypeptide chain (Cheolju & Myeong, 2005). The interior of a cell is an extraordinarily complex environment in which proteins and other macromolecules are present at a concentration of 300–400 mg/ml (Ellis & Minton, 2003).

1.1 Protein folding:

Protein folding is the process by which amino acids (the chemical building blocks of proteins) interact with themselves and form a stable three-

dimensional structure during production of the protein within the cell. Folding occurs very rapidly, probably within milliseconds after production of the string of amino acids, and results in 3-D conformations which usually are quite stable, which have specific biological functions *(*Anfinsen, 1973*)*. Importantly, folding not only allows the production of structures which can perform particular functions in the cellular milieu, but it also prevents inappropriate interactions between proteins, in that folding hides elements of the amino acid sequence which, if exposed, would react non-specifically with other proteins.

Protein folding has been studied in detail by both experimental and theoretical procedures. In vitro folding of some proteins has been investigated in detail by various optical techniques including NMR, circular dichroism and fluorescence spectroscopy (Kumeta H et al 2003).

1.2 Process of protein folding:

The folding pathway of a large polypeptide chain is very complicated. Many models have been proposed to describe protein folding. One model views folding as a ***hierarchical process*** where local secondary structures form first. Under this model, α helices and β sheets form first, with longer range interactions between helices and sheets forming super-secondary structures later. This process continues until the entire polypeptide folds. Another ***alternative model*** describes folding as a spontaneous collapse of the polypeptide into a compact state. This collapsed state is known as a **molten globule**.

1.3 Chaperones:

Chaperones bind to exposed hydrophobic surfaces of proteins, thereby sequestering unfolded polypeptide chains and preventing their aggregation. Chaperones mediate correct folding of other protein but do not themselves from a part of the final protein structure. Some chaperones are synthesized constitutively and affect normal intracellular protein trafficking, whereas others are induced by stress, such as heat (heat-shock proteins, e.g., hsp70, hsp90).

1.3.1 Molecular chaperones:

The term 'molecular chaperones' was originally used to describe the function of nucleoplasmin, a nuclear protein that ensures correct assembly of nucleosomes from DNA and histones. Molecular chaperones assist the folding of polypeptides as soon as they emerge from the ribosomal exit tunnel. The molecular chaperone proteins bind to the folding intermediates of polypeptides, which prevents protein aggregation (Jason et al, 2004).

Not only do chaperones ensure correct folding but also correct assembly of the units of an oligomeric protein and transport of a protein to its particular subcellular compartment (Chattopadhyay, 2001).

1.3.2 Chemical chaperones:

In certain disease conditions, some genes are mutated and form faulty protein. As the structural change is small, the proteins may not completely lose their biological activity but they are not properly processed and may be guided towards the degradation pathways. In the case of cystic fibrosis, the proteins get improperly folded due to certain mutations in gene. It is therefore retained in endoplasmic reticulum and not transported to the plasma membrane. At plasma membrane these proteins perform their biological activity.

Table.1. Cellular osmolytes (Dobson, 2004)

Amino acids & derivatives	**Carbohydrates**	**Methylamines**
Alanine	Arabitol	Betain
Glutamic acid	Glycerol	Glycerophosphocholine
Proline	Mannitol	Sarcosine
γ-aminobutyric acid	Mannose	Trimethylamine N-oxide
Taurine	Sorbitol	
Sucrose		
Trehalose		
Myo-inositol		

Folding defect is corrected by some low molecular weight substances like glycerol and trimethylamine N-oxide. These substances, which help to stabilize proteins in their natural conformation, are called 'chemical chaperones' (Welch & Brown, 1996). Some examples of chemical chaperones are organic solvents, trimethylamine N-oxide (TMAO), dimethyl sulfoxide (DMSO) and some cellular osmolytes as shown in **table 1**.

Chaperons bind to the misfolded or unfolded proteins and help them to escape from the cellular degradation pathway. In this way it plays an important role in minimization of aggregation.

1.4 Protein misfolding, aggregation and diseases:.

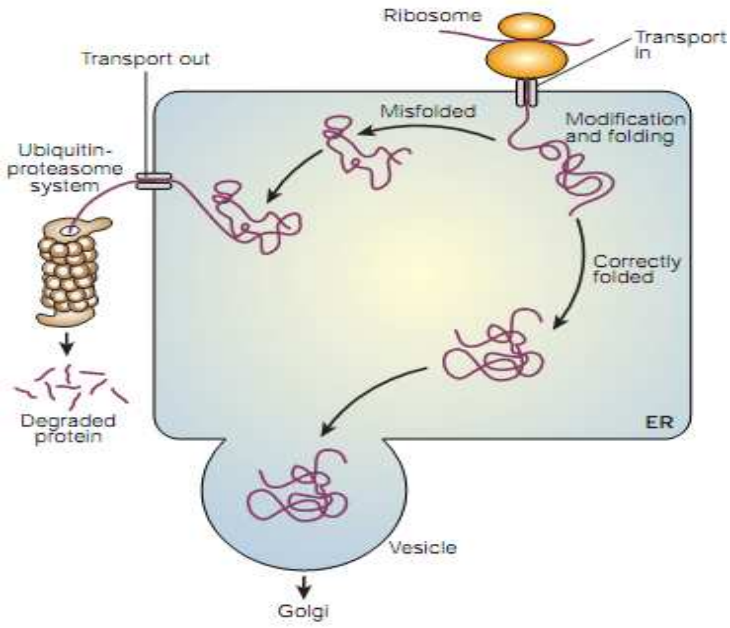

Figure 1. Regulation of protein folding in ER (Dobson 2003)

If the folding process is not successful, the damaged protein is degraded by the cellular protective machinery. One of the degradative processes involves ubiquitin which is added to the abnormal protein thereby marking it for degradation by the proteasome complex (in **Figure 1 & 2**). There are several mechanisms by which protein folding defects can

cause intracellular accumulations or result in disease which are listed in **table no. 2**.

Aggregation is considered to be a non-productive, off-pathway reaction that competes with correct folding reactions. Aggregation is a problem frequently encountered during the folding of polypeptide chains both in vivo and in vitro. Study of protein aggregation is important as aggregation is associated with several human diseases and poses a major problem in biotechnology industry. Diseases like Alzheimer's, Parkinson's etc. are caused due to unfolded or misfolded proteins which get accumulated in ER and trigger a number of cellular responses, called the unfolded protein response.

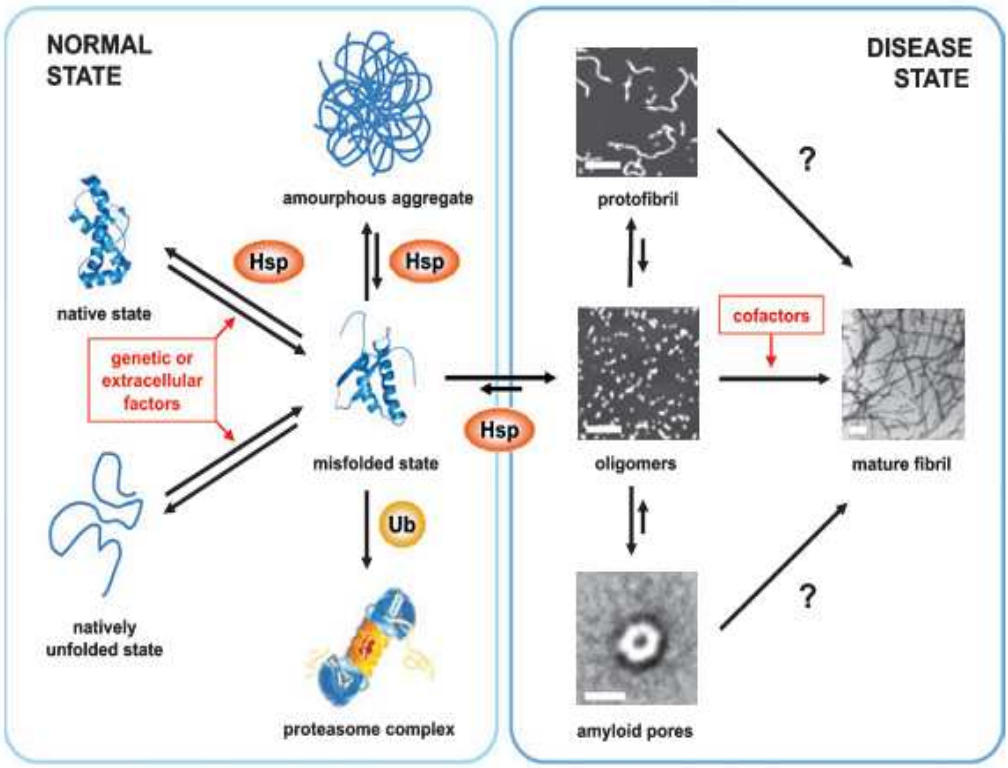

Figure 2. A schematic representation of the factors influencing protein folding and aggregation events in vivo (Jahn & Radford, 2005).

1.4.1 Aggregation of abnormal proteins:

Abnormal proteins may get deposited in tissue and interfere with normal cellular functions. These deposits can be intracellular, extracellular, or both. These aggregates cause the pathologic changes.

A wide range of human diseases (Dobson, 2004) is associated with protein folding process (in **table 2**). Mechanisms of protein aggregation are not yet fully understood, but several possible mechanisms have been proposed.

Table.2. Protein folding diseases

Disease	**Protein**	**Site of folding**
Hypercholesterolaemia	Low-density lipoprotein receptor	ER
Cystic fibrosis	Cystic fibrosis trans-membrane regulator	ER
Phenylketonuria	Phenylalanine hydroxylase	Cytosol
Huntington's disease	Huntingtin	Cytosol
Marfan syndrome	Fibrillin	ER
Osteogenesis imperfect	Procollagen	ER
Sickle cell anaemia	Haemoglobin	Cytosol
α-Antitrypsin deficiency	αl-Antitrypsin	ER
Tay–Sachs disease	β –Hexosaminidase	ER
Scurvy	Collagen	ER
Alzheimer's disease	Amyloid β-peptide/tau	ER
Parkinson's disease	α-Synuclein	Cytosol
Scrapie/Creutzfeldt–Jakob disease	Prion protein	ER
Familial amyloidoses	Transthyretin/Lysozyme	ER
Retinitis pigmentosa	Rhodopsin	ER
Cataracts	Crystallins	Cytosol
Cancer	p53	Cytosol

1.4.2 Mechanism of aggregation (in figure 3):

MECHANISM 1: REVERSIBLE ASSOCIATION OF THE NATIVE MONOMER

The tendency to reversibly associate (aggregate) is intrinsic to the native form of the protein. The surface of the native protein monomer is self-complementary so it will readily self-associate to form reversible small oligomers. There may be multiple "sticky" or complementary patches on the monomer surface. Those can then produce different types of interfaces, potentially producing multiple conformations for oligomers of the same stoichiometry and different patterns of oligomer growth. As the protein concentration rises, larger oligomers form over time and these large aggregates often become irreversible (Insulin being one example (Pekar et al., 1972)) Interleukin-1 receptor antagonist (rhIL-1RA) is an example of a product that undergoes reversible dimerization at high concentrations, followed by formation of irreversible dimers and trimers.

MECHANISM 2: AGGREGATION OF CONFORMATIONALLY-ALTERED MONOMER

Native monomer has a very low propensity to reversibly associate. However after it transiently undergoes a conformational change or partial unfolding, the resultant altered conformational form of the monomer associates strongly. The first step is a conformational change to a non-native state. Aggregation will be promoted by stresses such as heat or shear that may trigger the initial conformational change and will be inhibited by excipients or conditions that stabilize the native conformation. Two therapeutics where this mechanism has been reported are interferon (Kendrick et al., 1998) and G-CSF (Krishnan et al., 2002; Raso et al., 2005).

MECHANISM 3: AGGREGATION OF CHEMICALLY-MODIFIED PRODUCT

Protein conformation that precedes aggregation is caused by a difference in covalent structure. Usually this difference is caused by chemical degradation such as oxidation of methionine, deamidation, or proteolysis.

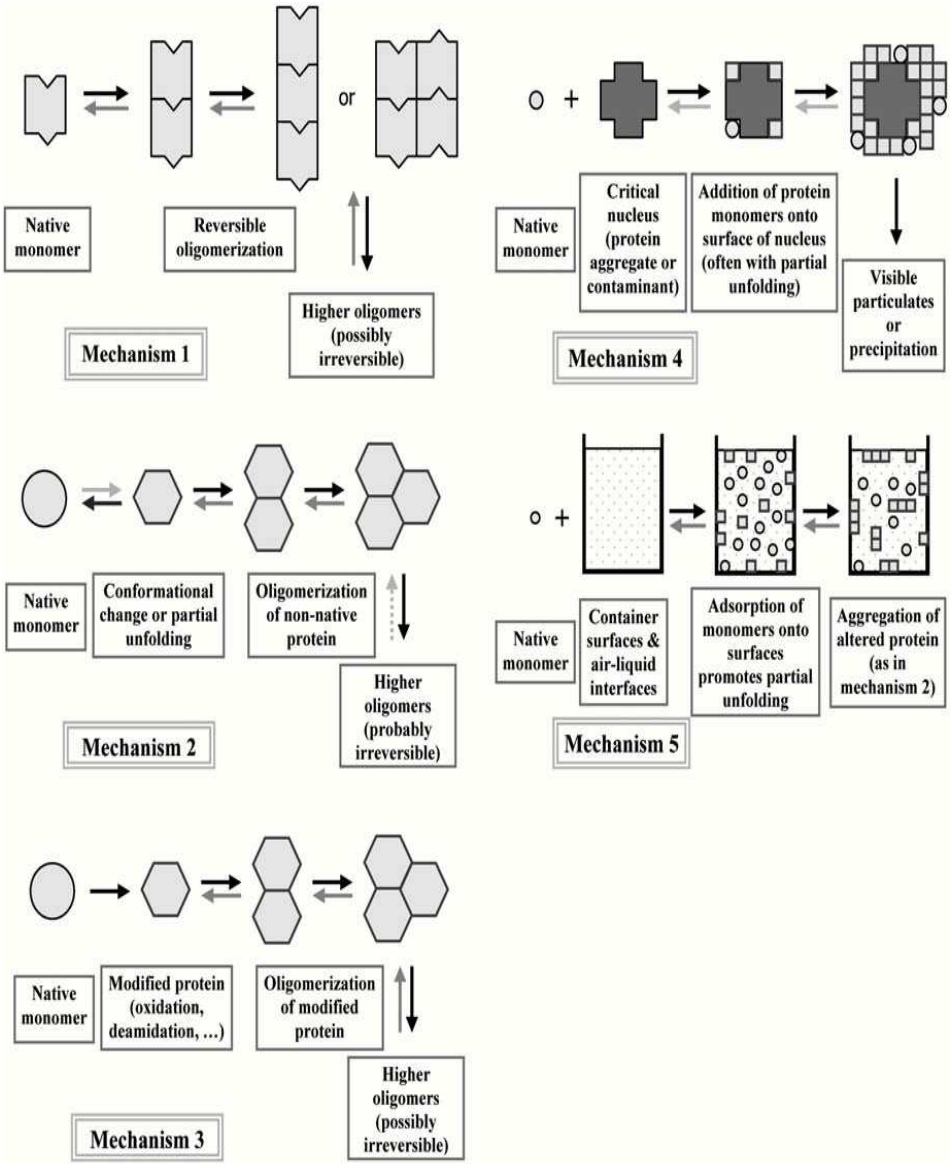

Figure 3. Mechanisms of aggregation (Philo & Arakawa, 2009).

Chemical changes create a new sticky patch on the surface, or change the electric charge in a way that reduces electrostatic repulsion between monomers. In many cases the chemically different species does not promote degradation, but remains as a normal variant within the product. For example, in glycoproteins there might be an unglycosylated or under-glycosylated fraction that is prone to aggregation. Improving the chemical stability will of the protein will reduce aggregation, and conversely attempts to improve the conformational stability of the monomer may not reduce aggregation. It is

also known that aggregates of chemically altered protein can be particularly immunogenic.

MECHANISM 4: NUCLEATION-CONTROLLED AGGREGATION

Nucleation-controlled aggregation is a common mechanism for formation of visible particulates or precipitates. In this mechanism the native monomer has a low propensity for formation of small and moderately-sized oligomers. An important feature of this mechanism is that the rate of formation of the large particles or precipitates usually exhibits a lag phase. That is, no particles or precipitates can be detected for a long period of time (perhaps months) but then rather suddenly the large species appear and accumulate. Two examples of contaminants reported to have served as seeds for aggregation are silica particles shed by product vials and steel particles shed by a piston pump used for filling vials.

MECHANISM 5

This aggregation process starts with binding of the native monomer to a surface. In the case of an air-liquid interface that binding would probably be driven by hydrophobic interactions, but for a container favorable electrostatic interactions might also be involved. After this initial reversible binding event the monomer undergoes a change in conformation (for example to increase the contact area with the surface). In a special case of this mechanism where the surface that induces aggregation is the surface of the critical nucleus. A second point about this mechanism is that during accelerated stability testing the tests that involve agitation or that try to induce shear forces by moving balls through the liquid will produce conformational stress and therefore may induce aggregation through Mechanism 2. It may also simultaneously produce significant exposure to surfaces; so it may be unclear which stress is actually inducing the aggregation.

1.4.3 Aggregation of therapeutic proteins:

Biotherapeutics are large macromolecules derived mostly from biological sources using recombinant DNA technologies. Such therapeutics include a diverse range of products including growth factors, cytokines,

hormones, receptors, enzymes, clotting factors, monoclonal antibodies (mAbs) and protein based vaccines.

Structurally altered proteins have a strong tendency to aggregate, often leading to precipitation. Irreversible aggregation is a major problem for long-term storage stability of therapeutic proteins and for their shipping and handling. Aggregates have been observed to form in therapeutic proteins during purification and storage, and the administration of proteins containing aggregates has been shown to stimulate immune responses, causing effects ranging from mild skin irritation to anaphylaxis (Wang W, 2005).

Some external factors like temperature, solution conditions and composition (pH, buffer type and concentration, ionic strength, protein concentration, metal ions, denaturing and reducing agents, impurities, organic solvents, containers/closures), sources of proteins, sample treatment, and analytical methodologies also affect the protein aggregation. Processing steps (fermentation/expression, unfolding/refolding), purification, freeze - thaw, shaking and shearing, pressurization, formulation/filling, drying, preparation of modified protein or delivery systems, etc., solid - state condition and composition also may cause aggregation.

1.4.4 Protein aggregation in Crowded Environments:

Biological macromolecules evolve and function within intracellular environments that are crowded with other macromolecules. Crowding results in surprisingly large quantitative effects on both the rates and the equilibria of interactions involving macromolecules, but such interactions are commonly studied outside the cell in uncrowded buffers (Ellis RJ, 2001). Living cells are highly crowded due to the high total concentration of macromolecules they contain: 20-30 volume % of total (Han and Herzfeld, 1993; Minton and Zimmerman, 1993; Record et al., 1998). In the cytoplasm of *Escherichia coli*, the total protein concentration has been estimated to be in the range of 200-300 g/L (Elowitz et al, 1999).

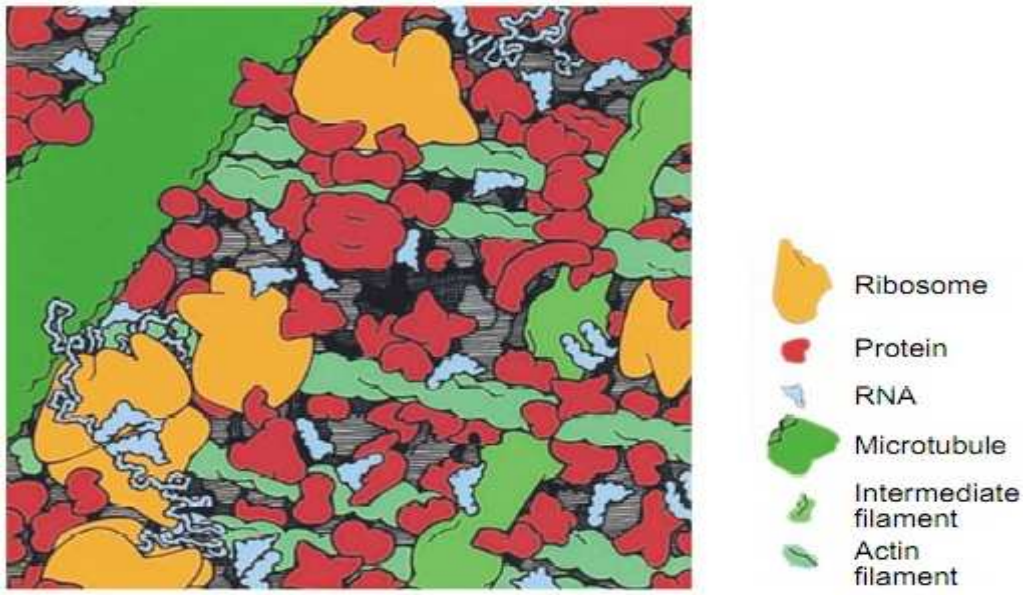

Figure.4. the crowded state of the cytoplasm in eukaryotic cell (Ellis RJ, 2001).

Exclusion volume theory also predicts that compact (native) states are stabilized under crowded conditions relative to less compact partially folded or unfolded states of proteins (Minton, 1981, 2000). In experimental studies, intracellular macromolecular crowding is typically mimicked by the addition of water-soluble polymers such as dextrans, Ficoll, PEG, and proteins such as hemoglobin and defatted BSA to concentrations ranging from50 to 300 g/L. When a complex of the denatured protein and SecB was preformed, SecB could completely prevent aggregation and promote folding of MBP and preMBP even in crowded solution (Kulothungan & Das et al, 2009).

1.5 Egg proteins:

Chicken eggs are probably one of the most multifunctional ingredients. Eggs are an excellent, inexpensive and low-calorie source of high quality protein and several important nutrients (Anon., 1989). One egg contains six grams of excellent quality protein and also contain folic acid, Vitamins A, B, D and E and iron (Davis & Reeves, 2002).

Chemical composition of hen egg:

It consists of 3 parts and their chemical composition is given in the **table 3:**

1) Egg yolk

2) Egg white

3) Egg shell and membrane

Table3. Chemical composition of hen egg (Craig & Reg, 2002)

	Water	**protein**	**Free sugar**	**Oligosa-ccharide**	**Lipid**	**mineral**	**Total**
Egg yolk	9.1	3.1	0.1	0.1	5.8	0.3	18.5
Egg white	28.5	3.5	0.1	0.2	0.0	0.2	32.9
Egg shell and membrane	0.1	0.4	-	-	-	5.9	6.4

1.5.1 Egg white

Protein (albumen) is the major component of egg white (9.7-10.6% w/v). The carbohydrates exist either in the free form or combined with protein. The amount of lipid in the egg white is negligible (0.01%).

Proteins of egg white:

The major constituents of egg white and some of their physicochemical and biologically or functionally important characteristics are summarised in Table 4.

1.5.1.1 Ovalbumin:

Ovalbumin, is the major component of egg white. It comprises half of the egg white proteins by weight and was one of the first proteins to be isolated in pure form (Hofmeister, 1889). Ovalbumin is a monomeric phosphoglycoprotein with a molecular weight of 44.5 kDa and an isoelectric point of 4.5. Its biological role in the egg remains largely unknown (Huntington & Stein, 2001). Ovalbumin contains four cysteine residues and

one cystine (Doi & Kitabatake, 1997). Ovalbumin is very similar in amino acid content to bovine serum albumin (BSA).

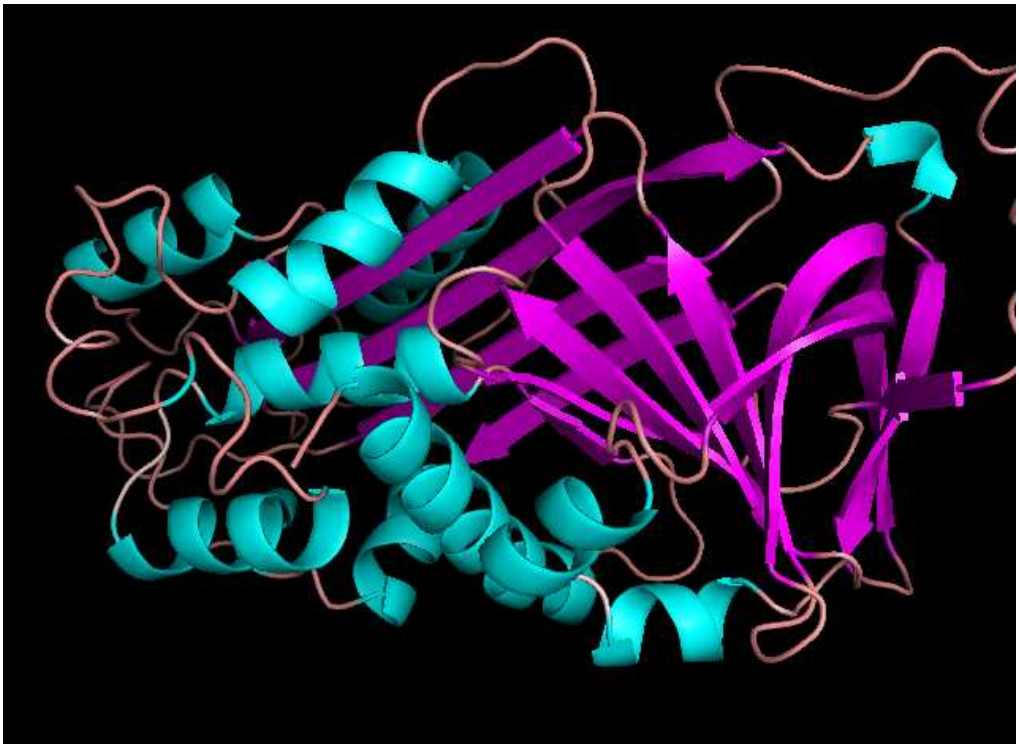

Figure 5. Structure of ovalbumin from PDB (uncleaved) PDB ID 1OVA.

Ovalbumin has become a key reference protein in biochemistry. As a carrier, stabiliser, blocking agent or standard, highly purified and crystalline ovalbumin has served the fundamentalists as well as the food industry. Ovalbumin is the only egg white protein which contains free sulfhydryl groups. The complete amino acid sequence of ovalbumin is 385 residues (Nisbet et al., 1981). The N-terminal amino acid is acetylated glycine and C-terminal amino acid is proline. Ovalbumin is converted into heat stable protein S-ovalbumin during the storage of eggs (Smith and Back, 1965). The denaturation temperature of native ovalbumin, S-ovalbumin and intermediated species were 84.5, 92.5 and 88.5ºC (Donovan and Mapes, 1976). New interest in the structure and function of ovalbumin was stimulated by the unexpected finding that this protein belongs to the serpin superfamily (Hunt and Dayhoff, 1980). The serpins include the major serine protease inhibitors of human

Table.4. Major constituents of egg white and their properties (Craig & Reg, 2002)

Proteins	**Amount (%)**	**M Wt (KDa)**	**pI**	**Characteristics**
Ovalbumin	54	45	4.5	
Ovatransferrin	12-13	77.7	6.0	Bind iron and other metal ions
Ovamucoid	11	28	4.1	Inhibits serine protinases
Lysozyme	3.4-3.5	14.3	10.7	Lysis of bacterial cell walls
Ovomucin	1.5-3.5	220-270000	4.5-5.0	Interacts with Lysozyme
G2 ovoglobulin	1.0	47	4.9-5.3	
G2 ovoglobulin	1.0	50	4.8	
Ovaflavoprot-ein	0.8	32	4.0	Binds riboflavin
Ovostatin	0.5	760-900	4.5-4.7	
Cystatin	0.05	12	5.1	Inhibits cysteine proteinases
Avidin	0.05	68.3	10.0	Binds biotin
Thiamine-binding protein	-	38	-	Binds thiamine
Glutamyl aminopeptidase	-	320	4.2	
Minor glycoprotein 1	-	52	5.7	

Minor glycoprotein 2	-	52	5.7	

plasma. Ovalbumin shows no protease inhibitory activity despite a 30% sequence homology with antitrypsin and other functional inhibitors of the serpin family.

The most widely used method for the purification of egg white ovalbumin is precipitation at a specific salt concentration, pH and temperature. Ammonium sulfate (Sorensen and Hoyrup, 1915) or sodium sulfate (Kekwick and Cannan, 1936) are salts commonly used for ovalbumin precipitation. Several procedures for ovalbumin purification have been developed either with cation exchangers like CM-cellulose (Rhodes et al. 1958), or with anion exchangers like DEAE-cellulose (Levison et al., 1992). Purity rates for ion-exchange chromatography are better than for other purification methods.

Folding of ovalbumin:

Ovalbumin can be completely renatured after chemical denaturation (using guanidine hydrochloride, urea etc). Ovalbumin renaturation will occur upon dilution of the denatured protein solution (Klausner et al, 1983). Renatured ovalbumin (OAR) has major differences from native protein (OAN) and the renatured protein can be separated from the native protein by affinity chromatography on Phenyl-Sepharose. Renatured ovalbumin (OAR) is checked by tryptophan fluorescence, u.v.-absorption and c.d. spectra (Klausner RD et al, 1983).

1.5.1.2 Lysozyme:

Lysozyme is a protein found in saliva, tears, hen egg white and mucus. It is a component of the egg white as listed in the above table. It is present in a minor amount in the egg white. Human lysozyme is commonly associated with innate immunity, which forms first line of defense against infections. It is also known as 1, 4 N-acetyl muramidase or N, O diacetyl muramidase . It is an anti-microbial enzyme. In egg white it comprises 3.4 % of total protein and has a molecular weight of 14.3 kDa. It is a single chain of 129 amino acid residues with $\alpha + \beta$ fold and 5 to 7 alpha-helices & 3 standard anti-parallel

beta sheets in it. Lysozyme catayzes hydrolysis of 1, 4 beta-linkages between N-acetyl muramic acid (NAM) and N-acetyl D-glucosamine (NAG) in peptidoglycan (Duane W, 2010).

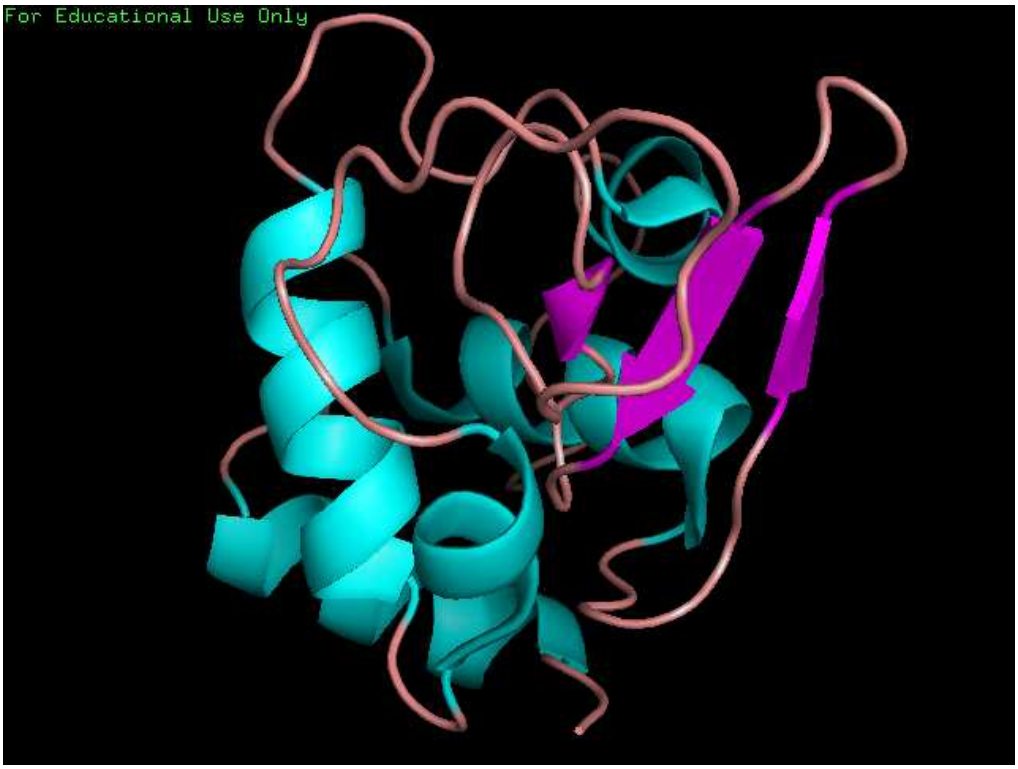

Figure 6. Structure of lysozyme from PDB (PDB ID - 1GXV).

It is used in cheese production to prevent late blowing, in human therapy for treatment of viral & bacterial infections and also in prophylaxis and therapy of leukopenia induced by antiblastic and ionizing radiation. It causes neutralization of acid substances released in the inflammatory process. Lysozyme decreases cell granulation leading to reduction of histamine release and subsequent anti-oedema effect (Ross Feldberg, 2005).

Lysozyme has an anti-microbial activity (Spark, 2004). It protects against bacterial infection by breaking down the carbohydrates in bacterial cell walls, thereby killing them. Hence it is a part of the innate immune response. In saliva it protects the oral cavity from pathogens. In times of increased inflammation, such as during inflammation of the salivary glands or after a tonsillectomy, there is an increase in lysozyme. This protein also works in

synergy with the bactericidal properties to inhibit viral replication and infection such as HIV. Lysozyme also interacts with and increases the function of histatin, a protein shown to be bactericidal and fungicidal. It has an important part in the process of cell lysis. Lysozyme, when reduced with β-mercapto ethanol (BME), forms aggregates (Puig, 1994).

1.6 Effect of various plant extracts on protein aggregation:

Some plants have different activities which remove and prevent the aggregation. Anti-amyloidogenic activity of garlic (*Allium sativum*) protecting neurons from Aβ (Alzheimer's β-amyloid fibrils) induced apoptosis has been reported (Gupta & Rao; 2007). An aqueous leaf extract of *Caesalpinia crista* prevents the formation of aggregates of oligomers from monomers (Ramesh & Rao; 2010). Turmeric (*Curcuma longa*) roots have polyphenolic compounds, curcuminoids, which give it a yellow color. The compound called Curcumin is a very important compound as it is known to have many medicinal properties. Curcumin has anti-oxidant properties and suppresses inflammatory responses of brain microgial cell (Begum et al, 2008; Cole et al, 2004; Miller et al, 2001). Protective effect of curcumin is also shown by reducing inflammation and oxidative damage in Alzheimer's diseases or by inhibiting protein misfolding and aggregation in Creutzfeld Jakob disease (Hafner-Bratkovic et al, 2008) and Parkinson's diseases (Ono et al, 2006).

Ginger, cinnamon, blueberries and turmeric have also been identified as in vitro blockers of β-amyloid aggregation (Guo et al, 2010). Amla is a rich source of tannins, similar to blueberries.

2. OBJECTIVE:

We wanted to examine certain plant extracts for their potential to prevent the aggregation of model proteins or disaggregate the already formed aggregates. Instead of working with expensive therapeutic proteins, we chose inexpensive model proteins for initial screening. If potential extracts are identified, then the same studies would be performed on actual therapeutic proteins or other proteins whose aggregation is known to pose several problems. We chose ovalbumin and lysozyme obtained from chicken egg white as our model proteins as they are abundant in nature, easily purified, and well known to form aggregates.

Plant extracts that we tested are of the fruit of Amla (*Emblica officinalis*), root of Ginger (*Zingiber officinale*) and bark of Cinnamon (*Cinnamomum zeylanicum*). We chose these plant extracts because they are all edible and normally included in or diet. Therefore they are expected to be non-toxic to the body and lead to minimum side effects. They are also known to have been used previously to treat certain diseases in Ayurvedic system of medicine, although without any scientific basis. Many of these diseases have been now shown to involve protein aggregation.

3. MATERIALS AND METHODS

3.1 Materials:

Chicken eggs, root of ginger, fruit of gooseberries (amla), bark of cinnamon, were bought from local market, Glycine, Acrylamide, Calcium Chloride, Citric Acid were purchased from MERCK, HEPES, Tris-Base, Poly Ethylene Glycol (PEG-20000), Guanidine hydrochloride ,beta-mercepto ethanol, Ammonium Persulphate were from SRL.

Bis-Acrylamide, Sodium Dodecyl Sulfate (SDS) were bought from HIMEDIA, Sephadex G-75 was from Sigma Aldrich., hydrochloric acid, ammonium sulphate, congo red, comassie brilliant blue were purchased from S.D. fine chem Ltd. Tri-sodium citrate bought from Qualigens Fine Chemicals, Sodium Chloride and was purchased from RANKEM, RFCL.

3.2 Preparation of crude egg white from chicken egg

A window was cut out in the shells of four washed and cleaned chicken eggs. The egg white was carefully separated from the yolk and poured into a beaker. It was then filtered through four thin layers of cheese cloth. An equal volume of saturated ammonium sulphate solution was added to the egg white to precipitate the proteins and placed on ice for half an hour. It was then centrifuged at 12000 rpm for 15 minutes. The supernatant was discarded while the precipitate was dissolved in 0.1M potassium phosphate buffer, pH 7.0.

3.2.1 Purification of ovalbumin and lysozyme:

Purification of ovalbumin was done by gel filtration chromatography.

3.2.1.1 Gel filtration chromatography:

Gel filtration separates molecules according to difference in size as they pass through a solid, porous medium packed in a column. For the separation of egg proteins, **Sephadex G-75 (**from Sigma Aldrich**)** was swollen in buffer and packed into a glass column (26 cm x 1.3 cm)**.** The fractionation range of Sephadex G-75 is 3 - 80 kDa. The molecular weights of ovalbumin

and lysozyme fall within this fractionation range. The mobile phase was 0.1M potassium phosphate buffer, pH 7.0. 300 µl of 27 mg/ml crude egg white extract was loaded into the column at a flow rate of 0.3 ml/min. 70 fractions of 0.5 ml each were collected and their absorbance at 280 nm was measured using a Shimadzu spectrophotometer. The presence of ovalbumin or lysozyme in the peak fractions were checked by SDS-polyacrylamide gel electrophoresis. The initial peak contained ovalbumin while lysozyme eluted at a later peak. Quantitation of the purified proteins was done by Folin-Lowry method.

3.3 SDS-PAGE of purified proteins:

SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) technique is used for the separation of proteins according to their molecular weight. The purity of the ovalbumin and lysozyme preparations were checked by 15% SDS-PAGE, using Amersham Biosciences SE 245 Dual Gel electrophoresis unit. Molecular weight of the purified proteins was checked using commercially available pure bovine serum albumin (BSA), lysozyme and protein molecular weight markers (from Bangalore Genei, Figure 21) as standards. The slab gels (1.0 mm thick) were run at constant current of 100 mA. Coomassie Brilliant Blue R250 (0.1% w/v in fixing solution) was used to stain protein bands. Destaining of the gel was done in acetic acid (7.5% v/v) and methanol (10% v/v) until the background was completely cleared and then the gel was photographed.

3.4 Denaturation and renaturation:

Ovalbumin was dissolved at a concentration of 9 mg/ml in 0.1M phosphate buffer, pH 7.0, containing 20 mM NaCl. Such solutions were found to remain stable for weeks. Denaturation of ovalbumin was done by incubating the protein with 6M guanidine hydrochloride (GdmCl) in phosphate buffer saline for three hours. Renaturation was then initiated by dilution with excess buffer. The buffer contained 30% PEG-20000 (polyethylene glycol) to mimic the in vivo crowded environment. Aggregation during refolding was monitored by visual inspection initially and then the kinetics of aggregate formation was monitored by light scattering at

365 nm in a Thermoscientific Spectronic 20D+ spectrophotometer or in an ELISA reader (Biorad) at 405nm.

Lysozyme formed aggregates upon reduction with beta-mercapto ethanol (BME). The purified protein was first dissolved in 20 mM potassium phosphate buffer, pH 6.2 at a concentration of 80 mg/ml. BME was added to a 12 mg/ml solution of lysozyme in buffer to a final concentration of 500 mM to reduce the disulfide bonds in the protein. This leads to formation of visible aggregates. The kinetics of aggregation as a function of time was monitored spectrophotometrically from light scattering.

Proteins can form two kinds of aggregates, amorphous aggregates and amyloid fibrils. Congo red is known to bind to only amyloid fibrils. To check which type of aggregates is formed by lysozyme under these conditions, Congo red binding assay was done. The lysozyme aggregates were taken and a solution of Congo red was added to it to a final concentration of 1M. This was then spread on a slide, air dried and observed under a light microscope at a magnification of 10x.

3.5 Preparation of plant extracts:

Various plant extracts were checked for their potential to prevent aggregation or disaggregate already formed aggregates of ovalbumin and lysozyme. We used the bark of Cinnamon and fruit of Amla, which are edible parts of the plants, for this purpose. 30 gm of dry amla was crushed in 200 ml of distilled water while dry cinnamon bark (30 gm) was crushed in 80 ml distilled water. The extracts were filtered through Whatman No. 1 filter paper. Filtrate obtained was 90 ml for amla and 40 ml for cinnamon. The filtrate was dried in a hot air oven at 50ºC for 5 hours. The dried extracted material was redissolved in phosphate buffer to a concentration of 250 mg/ml and 320 mg/ml for Cinnamon and Amla, respectively and used for further experiments.

Different concentrations of amla and cinnamon extracts were added to aggregation reactions before the initiation of aggregation. Formation of aggregates in the presence of these plant extracts was monitored by light scattering at 360 nm and 365 nm for lysozyme and ovalbumin, respectively.

4. RESULTS AND DISCUSSION

4.1 Protein purification by gel filtration chromatography:

Gel filtration chromatographic profile of crude egg white is shown in **Figure 7**. The first large peak starting from about 9.0 ml of elution contained ovalbumin while the lysozyme eluted at a much later peak centered at 20 ml.

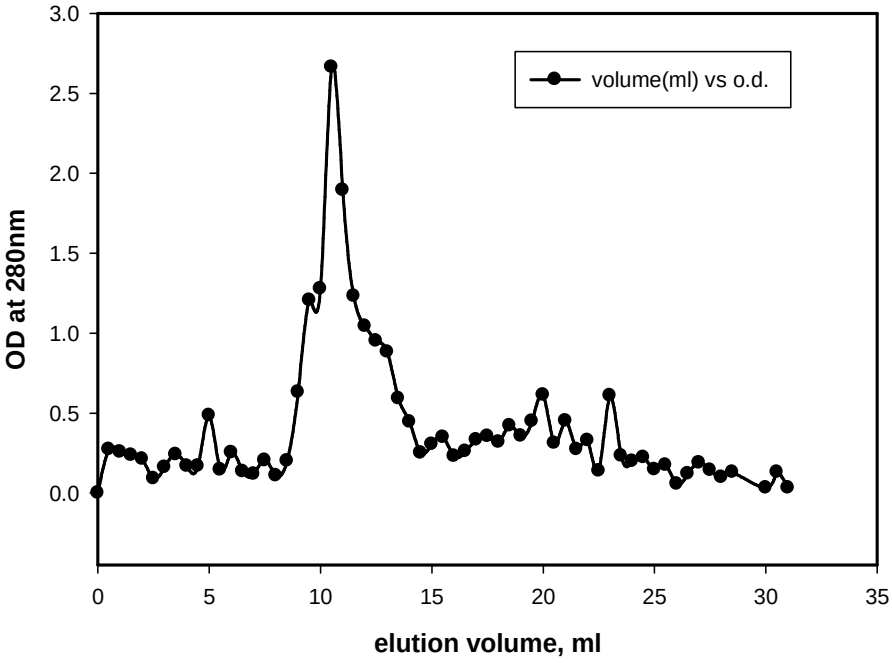

Figure 7. Gel filtration chromatography of egg crude.

The peak fractions were then analysed by SDS-PAGE shown in **Figure 8**. The bands of ovalbumin and lysozyme are clearly visible in the PAGE. Fractions containing pure ovalbumin or pure lysozyme were pooled and the pure protein was precipitated out by 100 % ammonium sulphate. They were placed on ice for half an hour followed by centrifugation at 12000 rpm for 15 minutes. The supernatant was discarded and the pellet was dissolved in the respective buffers to obtain concentrated solution of purified protein.

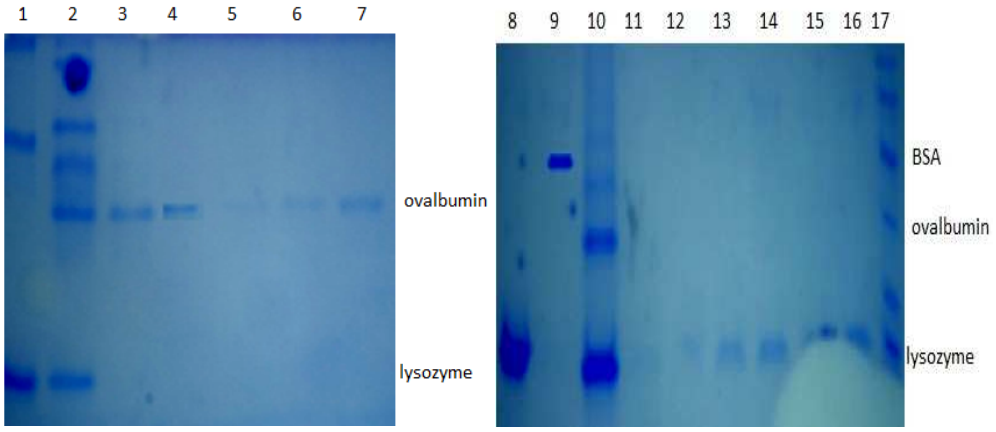

Figure 8. SDS-PAGE of chromatography fractions. Lane-1 is BSA and lysozyme marker; Lane-2 is crude egg white; Lane-3 to 7 is chromatography fractions of ovalbumin; Lane-8 is lysozyme marker; Lane-9 is BSA marker; Lane-11 to 16 is chromatography fractions of lysozyme and Lane-17 is protein molecular weight marker.

4.2 Aggregation analysis of ovalbumin:

(a) (b)

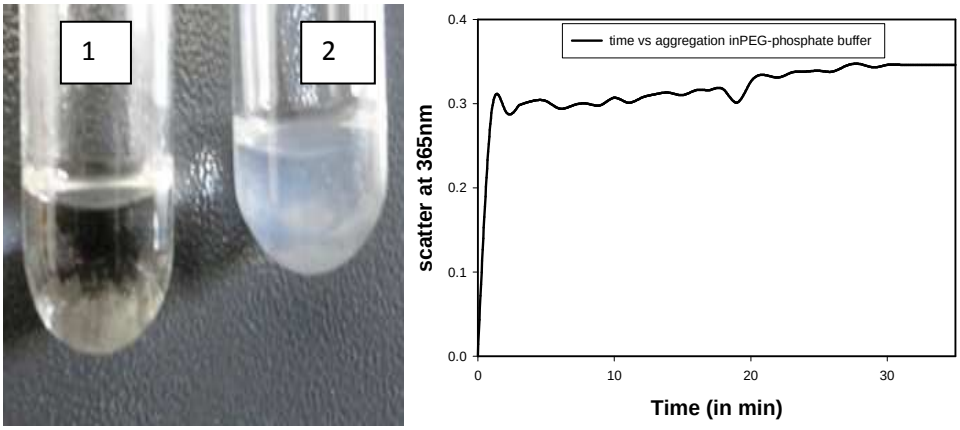

Figure 9. Aggregation of ovalbumin during renaturation in buffer containing 30% PEG 20,000. **(a)** Aggregation in test tube **(b)** Aggregation kinetics monitored spectrophotometrically by light scattering at 365 nm.

The aggregation occurring during refolding of denatured ovalbumin was monitored by light scattering at 365 nm. The amount of scatter increased with time, indicating formation of aggregates. Visibly also the solution turned very hazy (**Figure 9**).

In figure 9 (a) tube 1 is the clear buffer without any protein while tube 2 has hazy solution caused by aggregation of ovalbumin during renaturation. The aggregation kinetics shown in **figure 9 (b)** shows that the light scatter at 365 nm first increased with time and then reached a plateau in about 30 minutes, indicating that no further aggregation of the protein is occurring and all the protein has aggregated.

4.3 Aggregation analysis of lysozyme:

(a) **(b)**

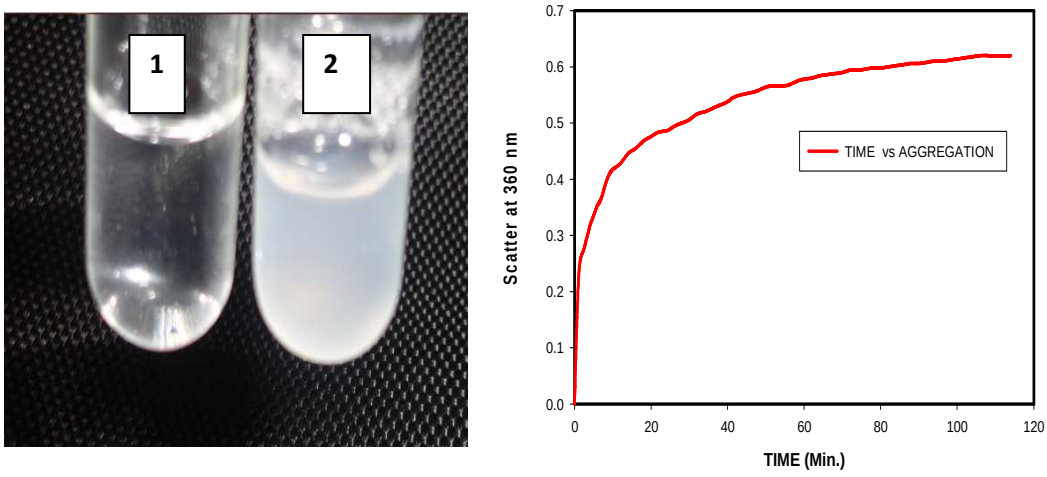

Figure 10. Aggregation of lysozyme during renaturation in phosphate buffer containing BME **(a)** in test tube, **(b)** in visible spectrophotometer at 360 nm.

Formation of visible aggregates of lysozyme upon reduction of its disulfide bonds by BME is shown in **figure 10(a)**. Tube 1 is the clear potassium phosphate buffer containing 500 mM beta-mercapto ethanol (BME). Tube 2 shows the hazy solution formed due to lysozyme aggregation after reduction with BME. The aggregation kinetics shown in **figure 10 (b)**

also shows an increase in light scattering initially which finally plateaus out after about 100 minutes, so aggregation forms rapidly and reach the point where it becomes constant.

4.4 Congo Red Binding assay for lysozyme (microscopic observation)

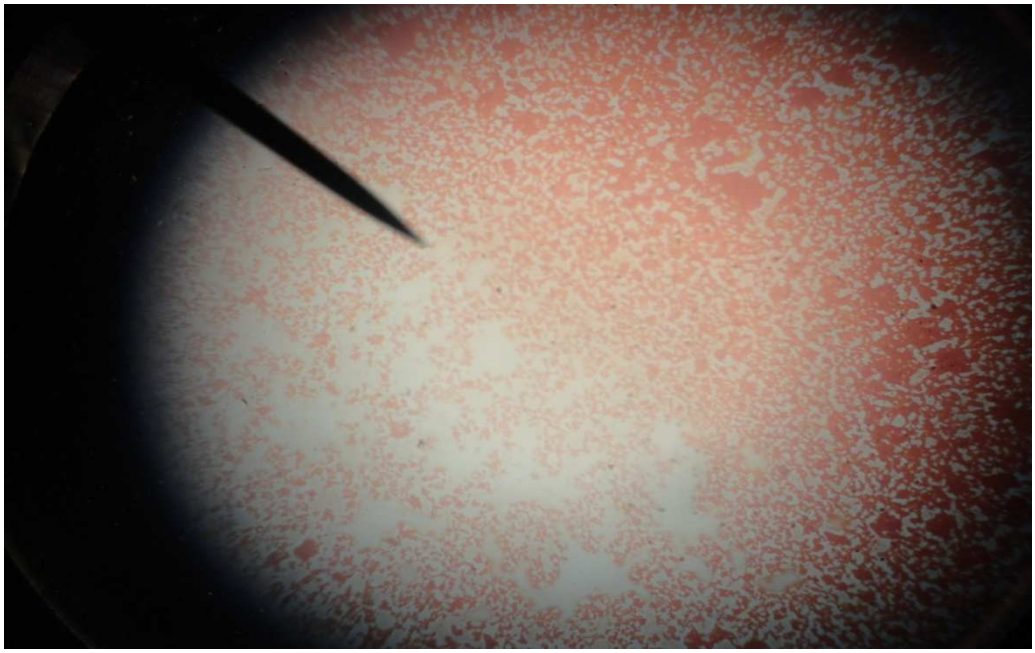

Figure 11. Congo red binding assay

The lysozyme aggregates were treated with Congo red and spread out on a microscopic slide and observed under a light microscope. **Figure 11** shows the slide. A network of red colored aggregates is visible indicating that the aggregates have bound the dye. This shows that the aggregates formed by reduced lysozyme are indeed amyloid fibrils.

4.5 Aggregation of ovalbumin in the presence of plant extracts:

Aggregation of ovalbumin was carried out in the presence of different concentrations of amla, ginger and cinnamon extracts to check whether any of these extracts could prevent the aggregation. Denatured protein was diluted into buffer containing varying concentrations of the extracts and the resulting

aggregation was monitored visually and by light scattering in a spectrophotometer. The results are given below.

Figure 12 (a) shows the extent of haziness due to protein aggregates in different concentrations of amla extract. Tube 1 has the clear buffer without any plant extract, while tubes 2 - 4 contain increasing concentrations of amla extract (12.5, 25 and 62.5 mg/ml, respectively). The haziness in the tubes decreased with increasing concentrations of the extract, indicating that high concentrations of amla extract could prevent formation of aggregates to a large extent.

(a) **(b)**

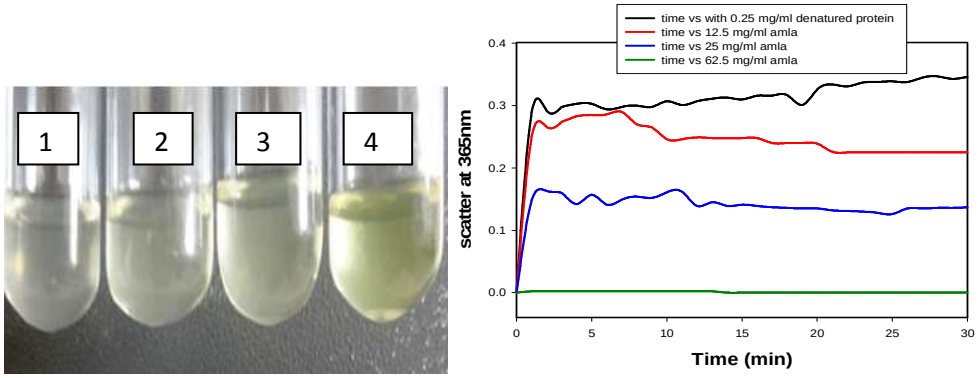

Figure 12. Aggregation analysis in presence of amla extract **(a)** in test tube, tube 1 is without plant extract and tubes 2 – 4 contain increasing concentration of extract (12.5, 25 and 62.5 mg/ml respectively) **(b)** in visible spectrophotometer showed the decrease in aggregation with increase concentration of extract.

Figure 12 (b) shows the ovalbumin aggregation kinetics in increasing concentrations of amla extract. Here also there is marked reduction in light scattering with increasing concentrations of the extract indicating decrease in aggregate formation. With 62.5 mg/ml extract, there was no light scattering indicating complete suppression of aggregation under these conditions. Thus amla extract was able to efficiently suppress aggregation of ovalbumin above certain concentrations.

(a) **(b)**

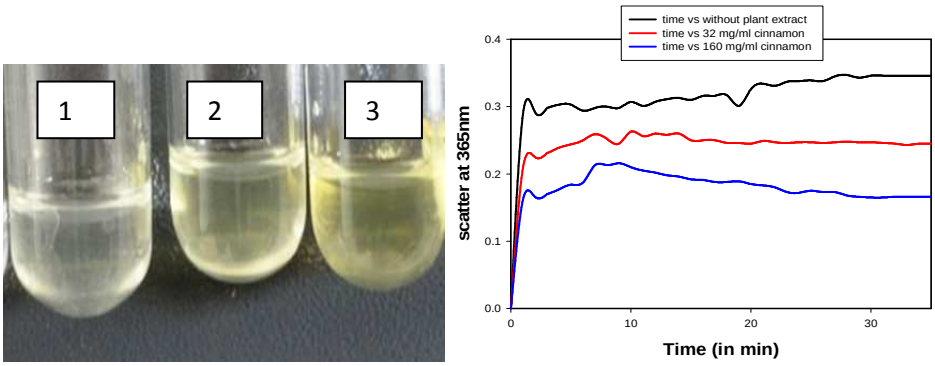

Figure 13. Aggregation analysis of ovalbumin in presence of cinnamon extract **(a)** in test tube, tube 1 is without plant extract and tube 2 & 3 contain increasing concentration of extract (32 and 160 mg/ml respectively) **(b)** In visible spectrophotometer, decrease in aggregation with respect to increasing cinnamon extract concentration.

In case of cinnamon, increasing concentrations of the extract decreased the haziness in the aggregation reaction tubes, but unlike amla extract, concentrations as high as 160 mg/ml in the cinnamon extract was unable to

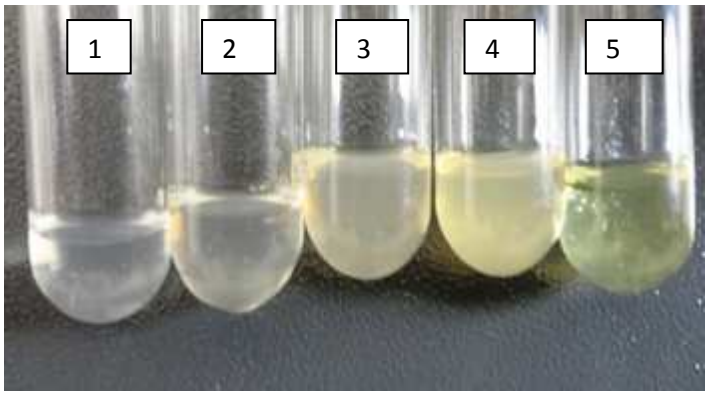

Figure 14. Aggregation analysis in presence of ginger. In test tubes haziness increased with the plant extract concentration. Tube 1 is without plant extract and tube 2 - 5 contain 12.5, 25, 37.5 and 125 mg/ml. At high concentration (125 mg/ml) of ginger extract indicate the prevention of aggregation.

completely prevent aggregation (**Figure 13 (a)).** The same result is shown in the aggregation kinetics monitored spactrophotometrically from light scattering (**Figure 13 (b)**).

In the case of ginger extract, lower concentrations were unable to suppress aggregation of ovalbumin. In fact, the amount of haziness in the tubes increased in comparison to the haziness in the tube containing aggregate in the absence of any extract as shown in **Figure 14**. Only very high concentration of the extract (125 mg/ml) was able to decrease the haziness totally, indicating total prevention of aggregation.

4.6 Aggregation analysis of lysozyme in presence of plant extracts:

Aggregation of lysozyme was carried out in the presence of different plant extracts like Amla, Ginger and Cinnamon to check which of these has effect on aggregation of lysozyme or which plant extract can successfully prevent the aggregation of lysozyme & disaggregate it.

1) WITH AMLA

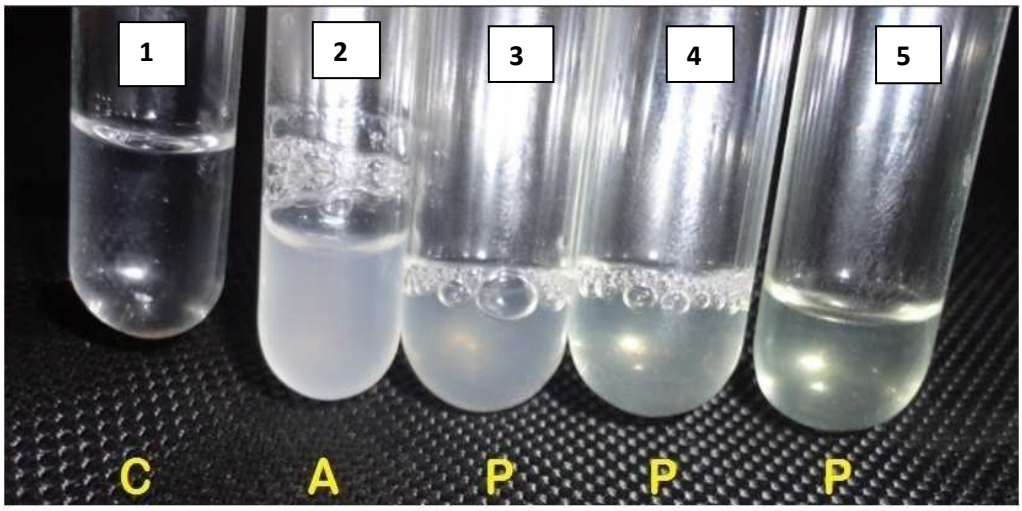

Figure 15. Aggregation analysis of lysozyme with amla extract. Tube 1 contain buffer with BME (500 mM), Tube 2 contain protein (12 mg/ml) in BME – buffer without any amla extract shows haziness. In Tube 3 – 5, haziness decreased with increasing concentration of amla extract (13, 26 &39 mg/ml, respectively)

Figure 15 shows the extent of haziness due to protein aggregates in different concentrations of amla extract. Tube 1 has the clear buffer without

any kind of aggregation. Tube 2 shows visible aggregates of lysozyme in the form of haziness because disulfide bond were reduced with BME, while tubes 3 to 5 contain increasing concentrations of amla extract (13, 26 and 39 mg/ml, respectively). The haziness in the tubes decreased with increasing concentrations of the extract, indicating that high concentrations of amla extract could prevent formation of aggregates to a large extent.

2) WITH GINGER

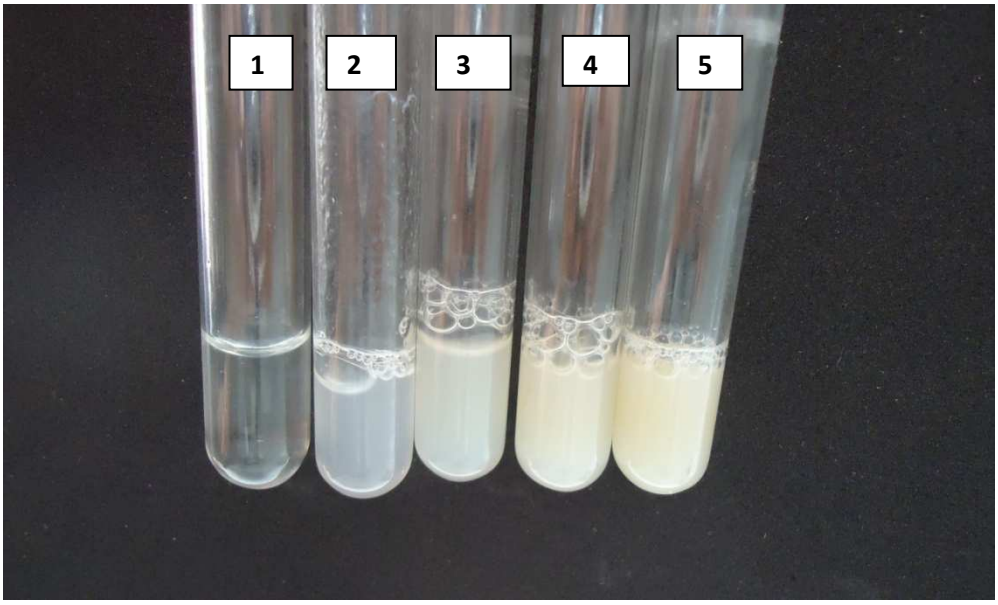

Figure 16. Aggregation analysis of lysozyme with ginger extract. Tube 1 contain buffer with BME (500 mM), Tube 2 contain protein (12 mg/ml) in BME – buffer without any ginger extract shows haziness. In Tube 3 – 5, haziness is not decreased with increasing concentration of ginger extract (13, 26 &39 mg/ml, respectively)

In **Figure 16**, tube 1 shows clear solution with buffer and BME, in tube 2 lysozyme at a concentration 12 mg/ml give the visible aggregates (haziness) and tube 3 to 5 contain increasing concentrations of ginger extract (13, 26 and 39 mg/ml, respectively). The haziness in the tubes increased with increasing concentrations of ginger extract. In fact, the amount of haziness in the tubes containing ginger extract increased in comparison to the haziness in

the tube containing aggregates, so ginger did not prevent the aggregation of lysozyme.

3) WITH CINNAMON

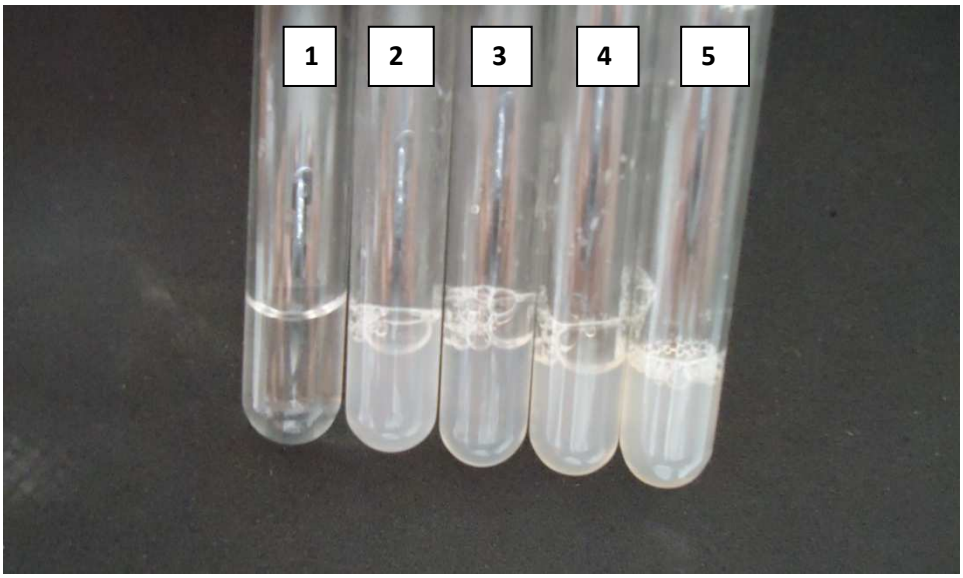

Figure 17. Aggregation analysis of lysozyme with cinnamon extract. Tube 1 contain buffer with BME (500 mM), Tube 2 contain protein (12 mg/ml) in BME – buffer without any cinnamon extract shows haziness. In Tube 3 – 5, haziness is not decreased with increasing concentration of cinnamon extract (13, 26 &39 mg/ml, respectively)

Here same experiment was performed with cinnamon extract Tube 1 in **Figure 17** shows clear solution with buffer and BME, in tube 2 lysozyme of concentration 12 mg/ml gave the visible aggregates (haziness) with buffer and BME and tube 3 to 5 contain aggregation in the presence of increasing concentrations of cinnamon extract (13, 26 and 39 mg/ml, respectively). The haziness in the tubes increased with increasing concentrations of cinnamon extract. In fact, the amount of haziness in the tubes containing cinnamon extract increased in comparison to the haziness in the tube containing aggregates so it gives the same result as the ginger extract in case of lysozyme.

So, both ginger and cinnamon were not able to prevent aggregation of lysozyme.

Table 5. Prevention of aggregation with different plant extract

PLAT EXTRACT	RESULT
Amla	Positive
Ginger	Negative
Cinnamon	Negative

From the above data, it was clear that only amla extract is able to prevent aggregation of lysozyme. Hence further experiments were carried out only with this extract.

Kinetics of lysozyme aggregation in the presence of amla extract was checked by two ways: one with ELISA reader and another with visible spectrophotometer.

Lysozyme aggregation analysis with amla extract in ELISA reader:

Figure 18 show the lysozyme aggregation kinetics in the absence and presence of different concentration of amla extract. Aggregation was prevented by 80 and 192 mg/ml of amla extract concentration. It is clearly seen that as the concentration of amla increased there was decrease in the aggregation of lysozyme hence amla extract at a concentration above 192 mg/ml may be useful to prevent aggregation.

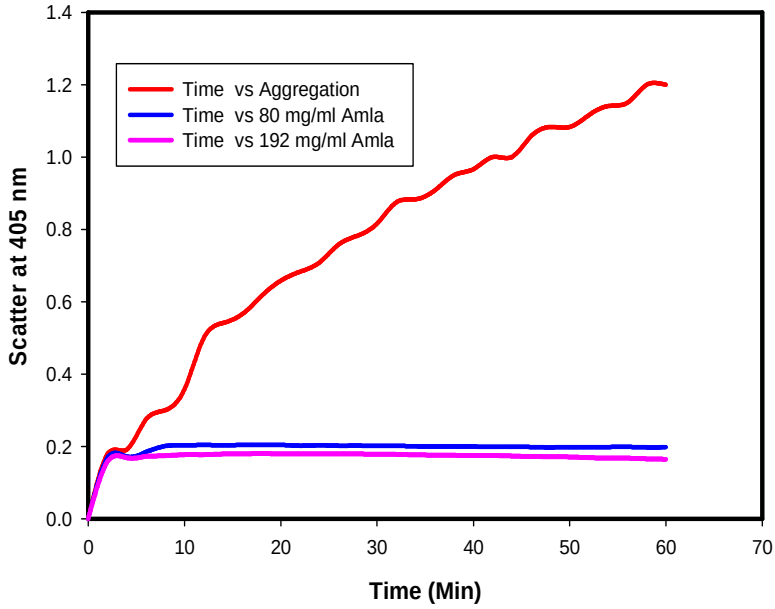

Figure 18. Aggregation kinetics of lysozyme with amla extract in ELISA reader. Red line shows the aggregation of lysozyme without any plant extract, blue and pink line shows prevention of aggregation with increasing concentration of amla extract (80 & 192 mg/ml, respectively).

Lysozyme aggregation analysis with amla extract in Visible Spectrophotometer.

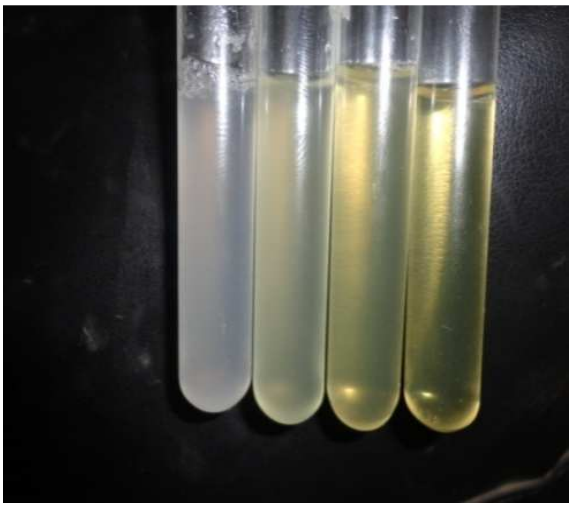

Figure 19. Aggregation analysis of lysozyme with amla extract in test tube. Tube 1 contain protein (12 mg/ml) with BME - buffer (BME - 500 mM) and Tube 2 - 4 shows the prevention of lysozyme aggregation with increasing concentration (40, 80 and 120 mg/ml respectively) of amla extract.

Figure 19 shows aggregation of reduced lysozyme carried out in the absence (Tube 1) and presence of 40, 80 and 120 mg/ml of amla extract (Tube 2, 3 and 4, respectively). With increasing extract concentration, the haziness in the tubes decreased, indicating less aggregation. Hence higher concentrations of amla extract was capable of preventing aggregation of reduced lysozyme.

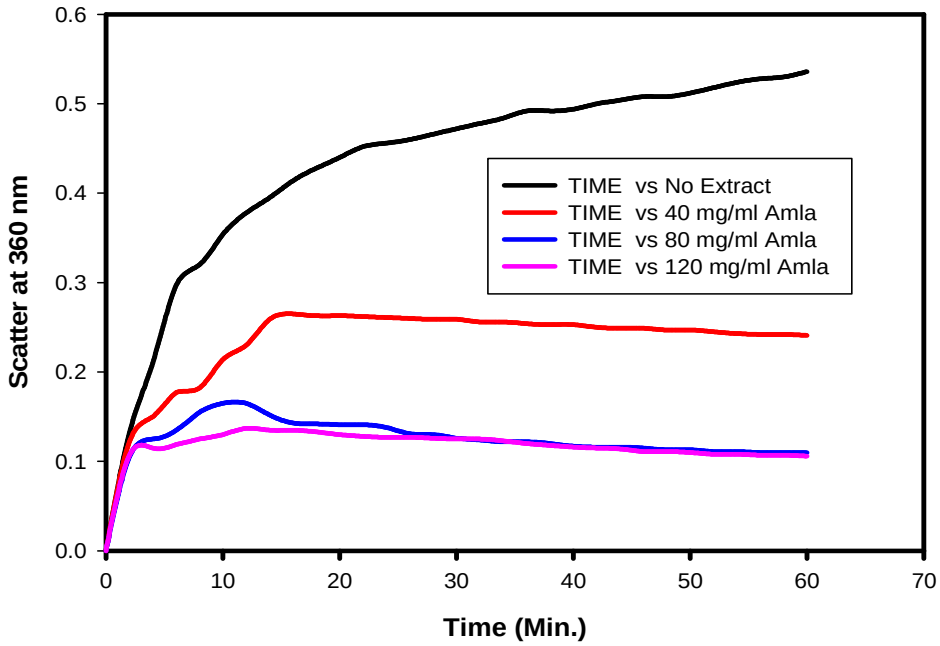

Figure 20. Aggregation kinetics of lysozyme with amla in visible spectrophotometer. Black line shows the aggregation of lysozyme without any plant extract. Red, blue and pink line shows prevention of aggregation with increasing concentration of amla extract (40, 80 & 120 mg/ml, respectively).

The kinetics of aggregation of reduced lysozyme monitored spectrophotometrically from light scatter at 360 nm is shown in **Figure 20.** In the absence of amla extract, the aggregation increases with time till it becomes almost constant after about 30 minutes. When aggregation was carried out in the presence of increasing concentration of amla extract, there was much reduced aggregation. Hence amla has the potential to prevent lysozyme aggregation at high aggregation

Conclusion:

From the above experiment we conclude that amla, cinnamon and higher concentrations of ginger extracts are able to prevent the aggregation of ovalbumin. Only amla extract is able to prevent the aggregation of lysozyme. None of these plant extracts promote disaggregation of ovalbumin and lysozyme aggregates. There may be certain bioactive compound or combination of bioactive compounds in these plant extracts which were preventing aggregation of egg protein. These extracts may solve the problem of aggregation of therapeutic proteins or diseases cause due to protein aggregation problem.

5. SUMMARY

Protein misfolding and aggregation can be a nuisance factor in many in vitro studies of protein or it can cause major economic and technical problems in biotechnology and pharmaceutical industries. Its effects are lethal in patients who suffer from a variety of diseases involving protein aggregation, such as Alzheimer's disease, Prion disease etc. Molecular chaperones guide the correct folding of protein in vivo. There are compounds called chemical chaperones, which help in the right folding of proteins in vitro. Plant extracts with medicinal importance, like ginger, amla and cinnamon etc. were checked for anti-aggregation activity on the purified model proteins, ovalbumin and lysozyme from chicken egg white. These proteins were purified from crude egg white using gel filteration chromatography. The aggregation of the ovalbumin and lysozyme was initiated by guanidine hydrochloride and β-mercapto ethanol (BME) respectively. Disaggregation or prevention of aggregation with plant extracts was monitored spectrophotometrically. None of the plant extracts show any results in disaggregating the aggregated model proteins. Ginger seems to be effective at very high concentration for preventing aggregation of ovalbumin, whereas amla and cinnamon have shown positive results with different concentrations for preventing aggregation of ovalbumin aggregates. In case of lysozyme only amla has shown positive result at low concentration for preventing aggregation.

6. FUTURE PROSPECTS

As we have identified the plant extracts which have the potential of preventing aggregation of the model chicken egg white proteins. The future plan for the project will be to identify the bioactive compound or the combination of the bioactive compounds which are responsible for their property of preventing aggregation and those compounds or combination of compounds will be tested for their action on aggregation of therapeutic proteins like insulin, which are responsible for many neurodegenerative diseases and major nuisance factor in bioprocessing and biotechnological and pharmaceutical industries. If these plant extracts are found to be effective in preventing aggregation of the therapeutic proteins then further it will be checked on cell cultures and thereafter on animal models.

7. APPENDIX

7.1 SDS-PAGE:

Regents:

1) 15 % resolving gel solution
   - Distilled water - 1100 µl
   - Tris-HCl (1.5 M, pH8.8) - 1300 µl
   - 30 %Polyacrylamide - 2500 µl
   - SDS - 50 µl
   - 25 % Ammoniumpersulfate- 50 µl
   - TEMED - 5 µl
2) 6% stacking gel solution:
   - Distilled water - 1150 µl
   - Tris-HCl (0.5 M, pH 6.8) - 330 µl
   - 30 %Polyacrylamide - 500 µl
   - SDS - 20 µl
   - Ammoniumpersulfate - 20 µl
   - TEMED - 5 µl
3) 2X sample buffer: 0.06 M Tris-Cl, pH 6.8, 2% SDS, 10% Glycerol, 0.025% Bromophenol blue
4) 5X Electrode buffer: 0.025 M Tris, 0.192 M glycine, 0.1% w/v SDS, pH 8.3
5) Polyacrylamide solution: 29.2 gm acrylamide and 0.2 gm N, N'–methylene-bisacrylamide add into distilled water and makeup volume 100 ml.
6) SDS solution (10 % w/v)

7) Ammoniumpersulfate solution (25 % w/v)

8) TEMED (N,N,N,N-Tetramethylethylenediamine)

9) Staining reagent: 40% Methanol, 10% Acetic Acid, 0.1% w/v Commasie Blue R-250.

10) Destaining reagent: Add 40ml methanol and 37.5 ml acetic acid into 400 ml distilled water and makeup 500 ml.

Procedure:

- Assemble the gel sandwich plates according to the instruction in manual.
- Prepare the resolving gel and poured between gel plates which covered the three fourth portion of plate.
- Layer about 0.5 mL isobutyl alcohol on the top of the resolving gel.
- Stay remains for half an hour till gel get polymerize.
- After half an hour make stacking gel and poured over the resolving gel and insert the comb for making wells.
- Stay remains this for half an hour and after that remove comb.
- Place plate on the Mini-PROTEAN cell.
- Add 1X electrode buffer to the lower buffer chamber and upper buffer chamber.
- Pipet 15 µl bromophenol blue (tracking dye) into each well allow visualization of the electrical front.
- Turn on electric supply for 15 minutes at 100v for run tracking dye.
- After 15 minutes add samples with 1X sample buffer in each well.
- Turn on electric supply. It will take approximately 1 hour.

- Turn off the power supply after complete running of sample & Remove plates and separate the gel between plates.
- Put gel into staining solution for half an hour.
- Stained gel put into destaining solution for 3 hours on gel rocker.
- Check the bands visibly after completely destain.

NOTE:

- Only check the pH of electrode buffer not changes pH with acid and base.
- Use gloves when handle polyacrylamide because it is highly neurotoxic.

7.2 Folin-Lowry method for quantitation of protein:

Reagents:

1. Complex forming reagent: Prepare immediately before use by mixing the following stock solutions in thr proportion of 100:1:1 (by vol), respectively

   Solution A: 2% (w/v) Na_2CO_3 in distilled water.

   Solution B: 1% (w/v) $CuSO_4.5H_20$

   Solution C: 2% (w/v) sodium potassium tartrate

2. 2N NaOH
3. Folin reagent: use at 1N concentration
4. Standards: use a stock solution of standard protein (BSA) containing 2 mg/ml protein in distilled water.

Procedure:

1. To 0.5ml of sample or standard, add 0.5ml of 2N NaOH. Hydrolyze at 100°C for 10min in a boiling water bath.
2. Cool the hydrolysates to room temperature add 5ml of freshly made complex forming reagent. Let the solution stand at room temperature for 10min.
3. Add 0.5ml of Folin reagent, using vortex mixer, and let the mixture stand at room temperature for 30-60min
4. Read the absorbance at 660nm.
5. Plot the standard curve as a function of initiation protein concentration and use it to determine the unknown protein concentration.

Stock solution(µl)	0	5	12.5	25	50	100	250	500
Water(µl)	500	495	488	475	450	400	250	0
Protein concentration(µg/ml)	0	20	50	100	200	400	1000	2000

7.3 Broad range protein molecular weight marker:

Protein molecular weight marker purchased from BANGALORE, GENIE. Marker consist following proteins:

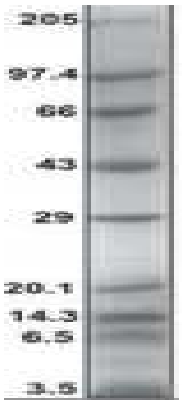

Figure 21. Various bands of the broad range protein molecular weight marker

.

Myosin, Rabbit Muscle	205,000
Phosphorylase b	97,400
Bovine Serum Albumin	66,000
Ovalbumin	43,000
Carbonic Anhydrase	29,000
Soyabean Trypsin Inhibitor	20,100
Lysozyme	14,300
Aprotinin	6,500
Insulin (α and β chains)	3,000

7.4 Pottassium Phosphate buffer (pH 7.0, 0.1 M) containing 20mM NaCl:

a) Monobasic phosphate (KH_2PO_4) (0.2M):

2.72 gm of monobasic phosphate in 100 ml distilled water.

b) Dibasic phosphate (K_2HPO_4) (0.2 M):

Take 3.48 gm of dibasic phosphate in 100 ml distilled water.

- Take 39 ml of monobasic solution (0.2M) and 61 ml of dibasic asolution (0.2 M) and mix the both solution.
- Add 20 mM NaCl (0.16 gm for 200 ml phosphate buffer) in this solution and make up the final volume 200 ml with distilled water.

7.5 Pottassium Phosphate buffer (pH 6.2, 20 mM):

c) Monobasic phosphate (KH_2PO_4) (20 mM):

0.272 gm of monobasic phosphate in 100 ml distilled water.

d) Dibasic phosphate (K_2HPO_4) (20 mM):

Take 0.348 gm of dibasic phosphate in 100 ml distilled water.

- Take 80.8 ml of monobasic solution (0.02M) and 19.2 ml of dibasic solution (0.02 M) and mix the both solution.

8. REFERENCES

Branden C, Tooze J. Introduction to protein structure, *2nd ed. New York: Garland Publishing* (1999).

Chattopadhyay MK. Chemical chaperones: *research news* (2001).

Cheolju Lee, and Myeong-Hee Yu. Protein folding and diseases. *JBMB*; **38**:275-280 (2005).

Claudio Soto. Protein misfolding and disease; protein refolding and therapy. *FEBS*; **498:**204-207 (2001).

Davis Craig and Reeves Reg. High value Opportunities from the chicken egg: A report for the Rural Industries Research and Development Corporation. *Publication No. **02**/094* (2002).

Dobson CM. Principles of protein folding, misfolding and aggregation: *Seminars in Cell & Developmental Biology*. **15**:3–16 (2004).

Dobson CM. Protein folding and misfolding. *Nature*, **426**:18-25 (2003).

Duane W.Sears. Overview of structure and function of lysozyme (2010)

Ellis RJ, Minton AP. Join the crowd. *Nature*; **425**:27–28 (2003).

Ellis RJ. Macromolecular crowding: an important but neglected aspect of the intracellular environment. *Curr Opin Struc Biol*, **11**: 114-119 (2001).

Ellis RJ. Macromolecular crowding: obvious but underappreciated. *Trends Biochem Sci*, **26**:597-604 (2001).

Elowitz MB, Surette MG, Wolf P, Stock JB, Leibler S. Protein Mobility in the Cytoplasm of Escherichia coli. *The Journal of Bacteriology*, **181**:197-203 (1999).

Gupta VB, Rao KSJ. Anti-amyloidogenic activity of S-allyl-l-cysteine and its activity to destabilize Alzheimer's [beta]-amyloid fibrils in vitro. *Neurosci Lett*, **429**: 75-80 (2007).

Hsuan-Liang Liu, Wen-Jang Chen, Shih-Nung Chou. Mechanisms of aggregation of alpha- and beta-amylases in aqueous dispersions. *Colloids and Surfaces B: Biointerfaces;* **28:**215 - 222 (2003).

Jahn, TR and Radford SE. The Yin and Yang of protein folding. *FEBS Journal*, **272**: 5962–5970 (2005).

Jason C. Young, Vishwas R. Agashe, Katja Siegers and F. Ulrich Hart. Pathways of chaperones mediated protein folding in the cytosol. *Nature*; **5**:781-791 (2004).

Jennifer A. Johnston, Cristina L. Ward, and Ron R. Kopito. Aggresomes: A Cellular Response to Misfolded Proteins. *The Journal of Cell Biology*; **143**:1883-1898 (1998).

John S. Philo and Tsutomu Arakawa. Mechanisms of Protein Aggregation. *Current Pharmaceutical Biotechnology*; **10**:348-351 (2009).

Klausner RD, Kemp C, Weinstein JN, Robert Blumenthal TR Renswoude JV. The folding of ovalbumin: Renuration invitro versus biosynthesis invitro. *Biochem. J.*; **212**:801-810 (1983).

Kulothungan SR, Das M, Johnson M, Ganesh C, Varadarajan R. Effect of Crowding Agents, Signal Peptide, and Chaperone SecB on the Folding and Aggregation of E. coli Maltose Binding Protein. *Langmuir;* **25**:6637-6648 (2009).

Mireille Weijers. Aggregate morphology and network properties of ovalbumin. *PhD thesis, Wageningen University, The Netherlands* (2005).

Puig A., Lyles MM, Noiva R, Gilbert HF. Anti chaperon behavior of BiP during the protein disulfide isomerase catalse refolding of denatured lysozyme. *Journal of Biological Chemistry*; **269**: 25889–25896 (1994).

Ramesh BN, Indi SS, Rao KSJ. Anti-amyloidogenic property of leaf aqueous extract of Caesalpinia crista. *Neurosci Lett*, **475**: 110-114 (2010).

Ross Feldberg. Structure and function of lysozyme. *Department of biology, Tufts university* (2005).

S.I. Park, M.A.Daeschel and Y. Zha. Functional property of anti microbial lysozyme – Chitosan composite Films. *Journal of food science*; **69:**M215-M221 (2004).

Thomasson W.A. Unraveling the Mystery of Protein Folding: A series of articles for general audiences. *FASEB* (1996).

Wang W. Protein aggregation and its inhibition in biopharmaceutics. *Int J Pharm*; **289 (1–2)**:1–30 (2005).

Weijers M., Peter A. Barneveld, Martin AC, Ronald WV. Heat-induced denaturation and aggregation of ovalbumin at neutral pH described by irreversible first-order kinetics. *Protein Science*; **12**:2693–2703 (2003).

Welch WJ, Brown CR. Influence of Molecular and Chemical Chaperones on Protein Folding. *Cell Stress Chaperon*; **1**:109-115 (1996).