A Report of the Research Work on

"Effect of Hydrogenated Vegetable Oil on Protein Fibrillation"

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Biochemistry and Molecular Biology Group
Department of Life Science
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In partial fulfillment of the requirements for the M.Sc. Life Science

By

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Candidate's Declaration

I hereby declare that the work which is being presented in this report entitled ***"Effect of Hydrogenated Vegetable Oil on Protein Fibrillation"*** in the partial fulfillment of the requirements for the M.Sc. thesis, submitted in the Department of Life Science, National Institute of Technology, Rourkela is an authentic record of my own work carried out under the esteemed guidance of Prof.(Dr.) Samir Kumar Patra, Epigenetics and Cancer Research Laboratory, National Institute of Technology, Rourkela.

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Date: Akash Tiwary

DEPARTMENT OF LIFESCIENCE

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Dr. SAMIR KUMAR PATRA
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Associate Professor and Head. Date: .....

CERTIFICATE

This is to certify that thesis entitled **"Effect *of Hydrogenated Vegetable Oil on Protein Fibrillation"*** which is being submitted by **Mr. Akash Tiwary, Roll No. 410LS2080** for award of degree of Master of Science from National Institute of Technology, Rourkela is a record of bonafide research work carried out by him under my supervision. The result embodied in this thesis is new and have not been submitted to any other university or institution for the award of any degree or diploma.

Dr. SAMIR K. PATRA

Associate Professor and Head,

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Akash Tiwary

Contents:

List of Figures

Figure

List of Figures

Page No.

List of Tables

1.0 Literature Review:

1.1 Proteins and its structures:

Proteins are naturally occurring polypeptide chains of hundred to several thousand amino acid residue which is generally folded into a globular and fibrous form. These macromolecules show great diversity in physical properties, ranging from water soluble enzymes to insoluble keratin of hair and horn. Similar to other macromolecules such as poly- sacccharide and nucleic acids, proteins are also essential parts of organism and participate in every process within cells.
It is possible to consider the structure of a protein on several levels as originally proposed by the Danish chemist, Kai Linderstrom-Lang:

1. ***Primary structure****:* the sequence of amino acids.
2. ***Secondary structure****:* the regular, repeating folding pattern (such as ? helix and ? pleated sheets) stabilized by hydrogen bond.
3. ***Super secondary structure****:* common repeating patterns of secondary structure that occur in many proteins. One common, recurring pattern is the ?-?-? motif.
4. ***Tertiary structures****:* It is the complete three dimensional structure of a polypeptide chain. Based on three dimensional structure protein is classified into two classes: fibrous and globular.
5. ***Quaternary structures****:* the interactions between different polypeptide chains to produce an oligomeric structure, stabilized by non covalent *bonds* only.

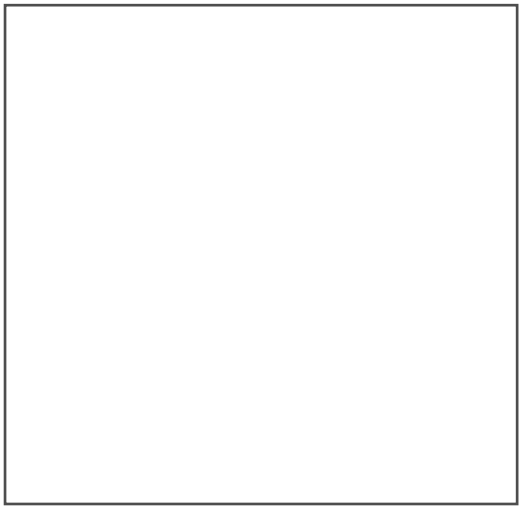


Fig 1: Hierarchy of protein structure

1.2 Pathways in Protein Aggregation and Fibrillation:

1.2.1 Protein Trafficking in the Secretory and Endocytic Pathways

The compartmentalization of eukaryotic cells has considerable functional advantages for the cell, but requires detail mechanisms to ensure that nascent proteins are correctly targeted to the appropriate compartment. This targeting occurs as the result of a series of decisions that commence at the time of synthesis of the nascent polypeptide. Proteins carry codes in their sequences that are read by targeting machinery at every stage of their voyage to their ultimate location. Proteins may be targeted to the cytosol, mitochondria, peroxisomes or chloroplasts. These proteins (if encoded in the nucleus) are synthesized on free ribosome. However, proteins destined for secretion, for the lumen of the ER, Golgi or lysosomes, or for the membrane of any of these organelles or the plasma membrane are synthesized on the membrane bound ribosome of the rough ER. They are then targeted to the appropriate cellular compartment. Protein Trafficking, Oligomerization and Folding are aided by chaperones and protein disulphide isomerase within the ER lumen. Aggregated and Fibrillated proteins are not permitted to pass further down the secretory pathway, but instead are degraded. Their continued association with chaperones prevents them from being packaged into transport vesicles destined for the Golgi. It has become clear recently that these proteins are degraded *in the cytosol* using the same ubiquitin-dependent machinery as cytosolic proteins. This means that polypeptides that are to be degraded have to be re-directed back through the translocation machinery. Thus, the translocation process can be reversed.

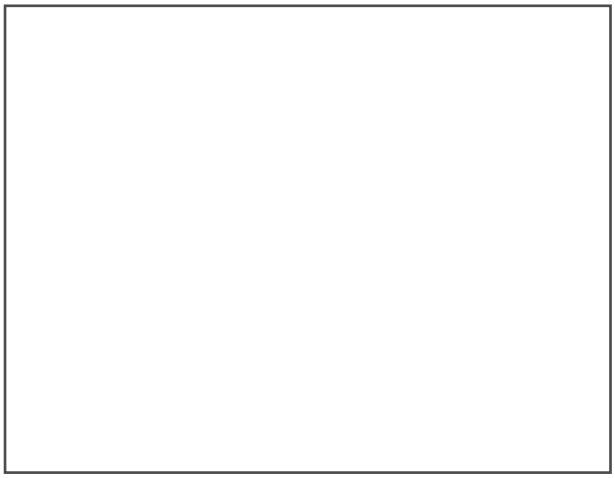


Fig 2: Regulation of Protein Folding in Endoplasmic Reticulum

1.2.2 Protein Aggregation and Fibrillation:

Under various adverse conditions, many proteins can aggregate to regular arrays of ?-sheet rich filaments or fibers of indefinite length, often coiled together in higher-order structures with high molecular weight assembly known as Amyloids which are insoluble protein aggregate sharing specific structural traits.[1] The process of formation of

Amyloids is known as amyloidogenesis which is responsible for various neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's diseases etc. The ability to aggregate and fibrillate is independent of the original native structure of the protein, whose amino acid sequence primarily appears to play a role in terms of filament arrangement, fibrillation kinetics and overall yield and stability of the fibrils. It typically occurs under conditions that stabilize partially folded or unfolded states.

Fig 3: Steps in Protein Aggregation and Fibrillation

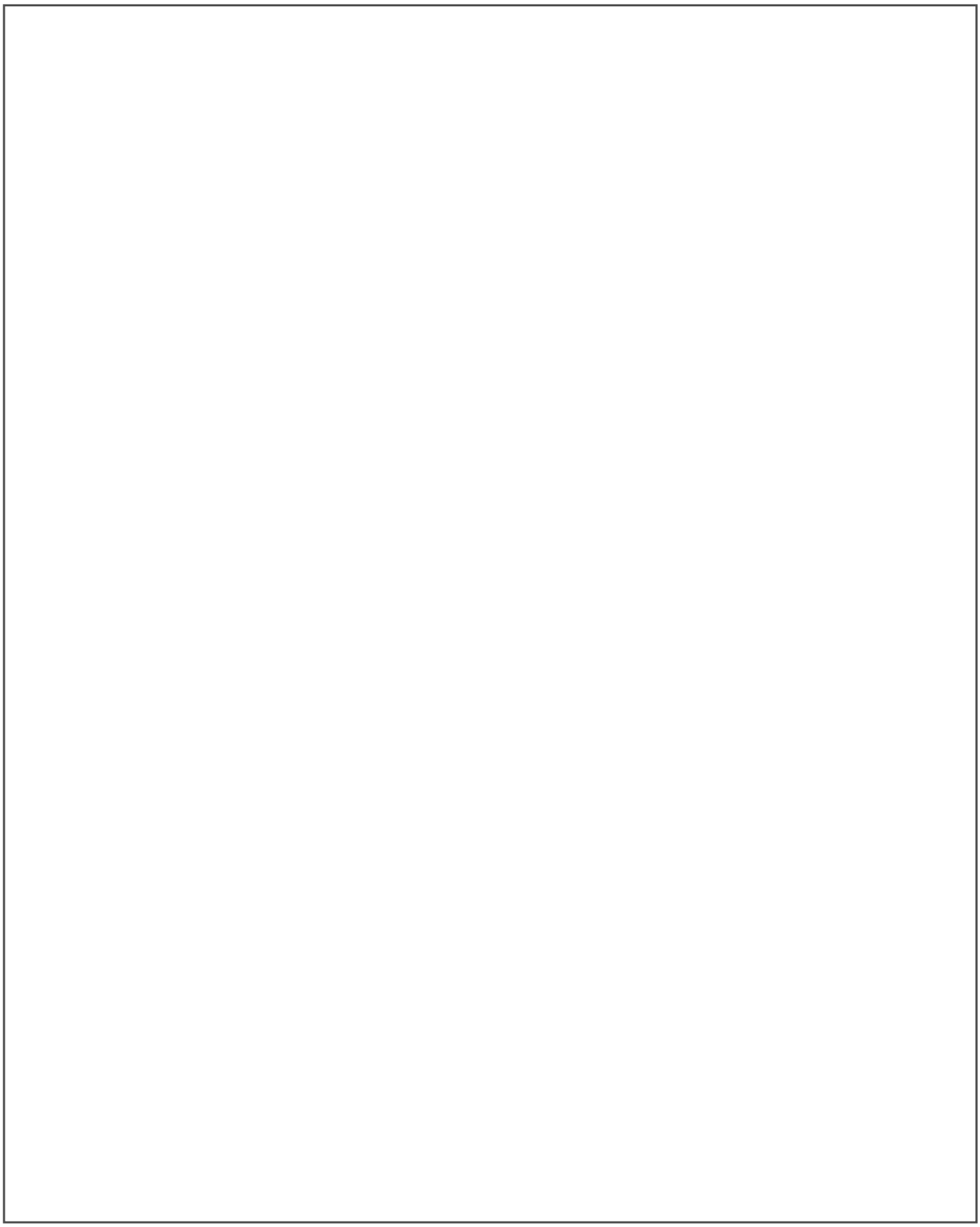


Fig 4: Neurodegenerative Diseases due to aggregation and fibrillation

Table 1: Characterization of Protein Aggregation:

Characterization	Description	Example
Productive Aggregation	These occur throughout the human body as well as other organisms controlling shape and mobility of the cells.	Glutamate Dehydrogenase and Actins
Unwanted Aggregation in Biology	This class includes ?-synuclein, amyloid ?, polyglutamine, and prions as common examples of proteins that aggregate and are suspected to play a key role in the neurodegenerative diseases such as Parkinson's, Alzheimer's, Huntington's, and prion's diseases, respectively	?-synuclein, amyloid ?, polyglutamine, and prions
Unwanted Aggregation in an Industrial settings	This class of aggregation usually produces amorphous aggregates and its control and understanding is important to the biotechnology industry for keeping proteins in a non-aggregated, bottleable, long-shelf-life form.	Insulin

1.3 Human and Bovine Serum Albumin: *Protein Models*

Human Serum Albumin (HSA) and Bovine Serum Albumin (BSA) are the most studied serum albumin proteins. There occurs almost 76% homology and a repeating pattern of disulphide which is conserved. The major difference between the two occurs with respect to the number and positioning of tryptophan residues in them. HSA has only one tryptophan rather than two present in BSA, located at position 214 which is equivalent to Trp-212 for BSA present buried in a hydrophobic pocket at sub domain IIA. BSA has one more additional tryptophan Trp-134, which is more exposed to solvent and found at sub domain IB. Thus BSA which is a homologous protein of HSA is selected as the protein model due to its medical importance, low cost, ready availability, and unusual ligand-binding properties.

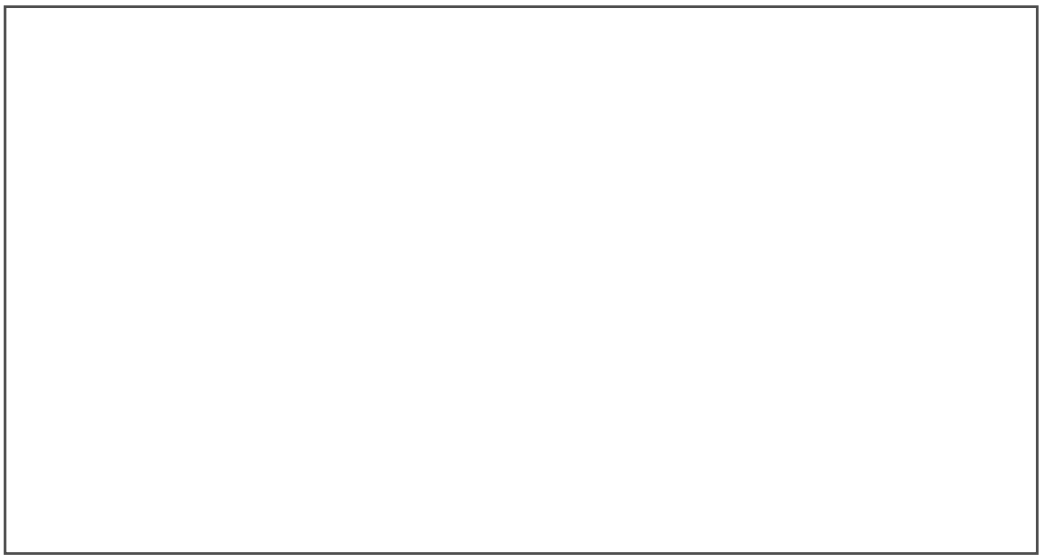


Fig 5 :Two side-on 3D graphic representation of a BSA model structure based on HSA X-ray crystal structure obtained from the Protein Data Bank (PDB ID:1UOR)

1.4 Serum Albumin: *structure, role and functions*

Serum albumin is a highly soluble multi-domain protein, without prosthetic groups that is very stable and available at high purity and low cost. It is highly soluble and of elliptical shape with a low intrinsic viscosity. Albumin is a very stable protein although more than 50 slight variants of the 585 amino acid sequences that comprises human albumin exists. Human serum albumin (HSA) structure has been revealed by high resolution X-ray image of the protein which is directed towards the determination of the tertiary structure of other mammalian albumins as they resemble closely to it. Thus serum albumin molecule can be described as a very flexible protein that changes shape with variations in environmental conditions and with binding of ligands. Despite this albumin has a resilient structure and regains shape easily owing to the disulphide bridges, which provides strength especially in physiological conditions. After their rupture the molecule can re-establish these bridges and regain its structure. Denaturation occurs only with dramatic and non-physiological changes in temperature, pH and the ionic or chemical environment. Albumin, the most abundant extra-cellular protein accounts for total 60 % of the total serum content in human. It is manufactured in the liver and is a single polypeptide with 585 amino acids and a molecular weight of 66.2 kDa. Serum albumin has one cysteine group (Cys-34) and low tryptophan content. The secondary structure consists of approximately 67 % of ?-helix as well as there are 9 loops and 17 disulphide bridges giving a heart shaped 3D structure confirmed by X-ray crystallography studies. The tertiary structure is composed of three domains I, II, and III, and each domain is constituted of two sub-domains A and B.

Serum albumin has been one of the most extensively studied and being the most abundant protein

in blood plasma with typical concentration of 50 g/L. Some of the albumins most commonly studied are human serum albumin (HSA), bovine serum albumin (BSA) etc.

Physiological Role:

1. Maintenance of the colloid osmotic pressure
2. Binding and transport, particularly of drugs
3. Free radical scavenging
4. Acid base balance
5. Pro and anti-coagulatory effects (inhibits platelets aggregation , enhances the inhibition of factor Xa by antithrombin III)
6. Effect on vascular permeability.

1.5 Hydrogenated Vegetable Oil:

Vegetable oils contain a mix of saturated, monounsaturated, and polyunsaturated fatty acids. The mono and polyunsaturated fatty acids have double bonds, all in the normal "cis" formation. These bonds can easily be broken down by oxygen. This produces compounds that make the oil rancid. Rancidity produces off-flavors in foods. To control this, food manufacturers use hydrogenated vegetable oils. These are not as likely to break down and will produce a product with a longer shelf life. Hydrogenation is a chemical process that adds hydrogen atoms to the available double bonds in the vegetable oil. As the degree of hydrogenation increases, the amount of saturated fats increases and mono and polyunsaturated fats decrease. Completely hydrogenated fat is solid at room temperature. Moderately hydrogenated fats are liquid at room temperature and contain more saturated fatty acids than the original oil. Hydrogenation will convert "cis" double bonds to "trans" double bonds, producing *trans* fatty acids. Hydrogenated vegetable oil that is solid at room temperature can contain 15–25 percent *trans* fatty acids. Partially hydrogenated oils are lower in *trans* fatty acids. Margarines are often mixtures of both hydrogenated fats and un-hydrogenated vegetable oils. Hydrogenated fats prevent rancidity. This gives products like crackers and other snacks a longer shelf life. *Trans* fatty acids can raise LDL cholesterol (the bad cholesterol) levels. At relatively high intakes they can also lower HDL (the good cholesterol) levels.

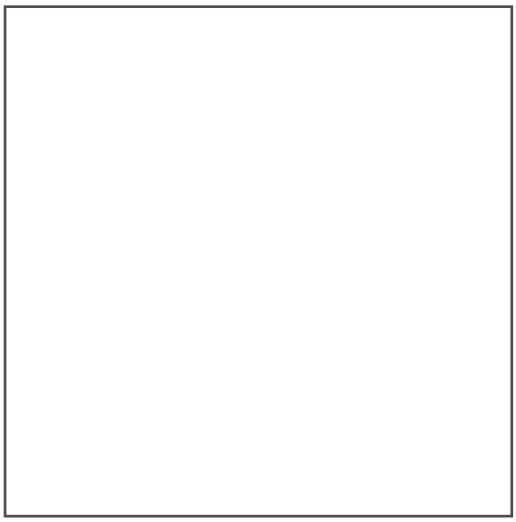


Fig 6: Hydrogenated Vegetable Oil

1.6 Spectroscopic and Morphologic Studies: *Techniques Used*

In this spectroscopic and morphologic study, the effect of Hydrogenated Vegetable Oil on Protein Fibrillation has been studied using spectroscopic methods including UV/Vis Absorption and Fourier Transformed Infrared (FT-IR) Spectroscopy and the morphologic study was done by Atomic Force Microscopy and Scanning Electron Microscopy. By spectroscopic studies the conformation changes of BSA and HSA has been studied.

2.0 Introduction:

Proteins may aggregate rapidly under the influence of a variety of factors, such as high temperature, agitation, or acidic pH [2]. Aggregation which is a high molecular weight assembly is a physical process that involves non covalent interaction of several protein molecules that are

believed to be at least partially unfolded. Similar to many other proteins, Bovine and Human serum albumin tends to adopt and maintain a three-dimensional structure in which its hydrophobic residues are buried by folding and assembly of individual molecules. It has been known for many years that heat treatment of various proteins, especially in acids, results in formation of protein fibrils The process of fibril formation has been found to involve nucleation, growth, and precipitation and to be dependent on pH (in the acid range) and the type of acid in which fibrils are formed. Intermolecular hydrophobic interactions were suggested to be the driving force of the fibrillation process and it was shown to be a nucleation-driven process with the partial denaturation and aggregation of intermediate states as the rate-limiting step. The morphology of HSA fibrils prepared in solution of Hexane and Hydrogenated Vegetable Oil has been studied by Atomic Force Microscopy and Scanning Electron Microscopy and the FT-IR studies of BSA and HSA were also done. HSA fibrils appeared as short, thick and un-branched fibrils with a marked degree of variations in the cross section when observed in AFM and SEM. However, little is known about the underlying molecular mechanisms of fibril formation, and identification of the interacting domains in the aggregates has been the subject.
Fourier transform infrared (FTIR) spectroscopy is also used in the study of protein secondary structure in the dissolved, aggregated, and solid state. FTIR spectroscopy is therefore a powerful tool for the study of conformational changes occurring during protein aggregation with formation of insoluble fibrils. With the introduction of methods for enhancing spectral resolution, it is now possible to obtain high quality spectra of proteins in different environments and under different conditions. In studies of thermally induced protein aggregation by FTIR spectroscopy, a common feature is the formation of a low-frequency band around 1632 cm−1, which is often accompanied by a weak band around 1639 cm−1. These bands are due to the formation of an intermolecular native ?-sheet structure in the protein aggregates.

3.0 Experimental Exploration:

3.1 Materials & Method:

3.1.1 Bovine Serum Albumin was purchased from HIMEDIA, INDIA and Human Serum Albumin was purchased from SIGMA ALDRICH, CHINA.

3.1.2 Hexane was purchased from NICE Chemicals, INDIA. Hexane used was petroleum fractions.

3.2 Preparation of Stock Solutions:

BSA and HSA stock solution (1mg/ml) was prepared in pH 7.4 phosphate buffer saline containing 0.1mol/l NaCl. The solid Hydrogenated Vegetable Oil was prepared by dissolving them in hexane. Finally the concentration of 1mg/ml of solution was prepared.

All other chemicals used were of analytical reagent grade and distilled water was used throughout.

3.3 Preparation of Working Solution:

1 ml of 1 mg/ml of protein sample was dissolved in 0.3ml of solution of Hexane and Dalda. The concentration of Hexane and Dalda was 1mg/ml. (Dalda is the solid form of Hydrogenated Vegetable Oil). The mixture was kept in water bath at 70^0 C till hexane gets evaporated.

3.4 Techniques Used:

3.4.1 **Spectroscopic Studies**: UV/Vis Absorption & FT-IR Spectroscopy

3.4.2 **Morphologic Studies**: Atomic Force & Scanning Electron Microscopy

4.0 Result:

The result was characterized by UV/Vis Spectrometer, FT-IR, AFM and SEM.

4.1 UV/Vis Spectrometer Result:

Investigation of the high temperature on ?-helix multi domain protein Bovine Serum Albumin was also done. It shows that at certain temperature BSA form thermal aggregates and this thermal aggregate have amyloid properties. They bind to the fibril specific dye Congo red shows elongated somewhat worm like structure. The graph shows that 100µg/l of BSA sample at 100^0C gives best result.

Fig 7: OD increases with temperature

[pic]

Fig 8: Aggregated protein at 95^0C
(A worm like structure will form)

4.2 Atomic Force Microscopy Result:

Atomic Force Microscopy was done in tapping mode and measurement was recorded with scanner Vecco Di Innova with cantilever frequency of 308 KHz. A cantilever will scan over the surface and upon contact a repulsive force will generate. Aliquots from protein sample incubated at 70^0C, were applied to a freshly cleaved mica surface which is fixed onto the metal plate. They are then desiccated overnight in Desiccators to transform liquid to solid form where they fix onto the mica. Excess protein solution on mica was rinsed off thoroughly under running MilliQ water.

Standard Protocol for experiment:

1. Cantilever Frequency: 308 KHz
2. Driving Force: 0.039 constant volts
3. Scan Rate: 0.05Hz
4. Sample/Line: 128
5. Scan Size: 10µM
6. Drive Amplitude: 0.47 V
7. Frequency: 308.05 KHz
8. Input gain: 4X

[pic][pic]
[pic][pic]

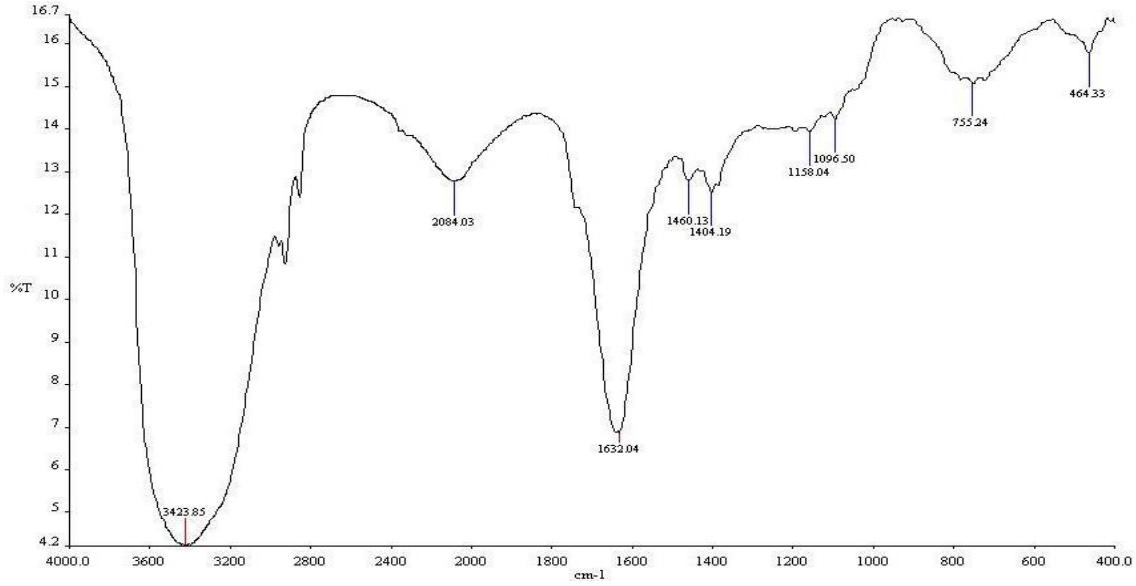
Fig 9: AFM picture of Human Serum Albumin

4.3 FT-IR Result:

To investigate which type of structural and conformational changes involved in the aggregation of BSA and HSA FT-IR experiment was done. FT-IR measurements were carried out at room temperature by Nuzol Method on Perkin Elmer Spectrometer equipped with a germanium attenuated total internal reflection (ATR) accessory. It is very difficult to interpret correlation between IR spectra secondary structural components. The sample prepared for FT-IR was in liquid form and was prepared in H_2O instead of D_2O. H_2O as a solvent is much more preferable than D_2O for studying protein structure. D_2O changes the protein properties somewhat in comparison with the native ones.

4.3.1 FT-IR sample of BSA

1 ml of 1mg/ml of BSA was taken for FT-IR.



Description:

Peak at 3423.85 shows strong IR absorption by H_2O with prominent peak. It is reported that 1633±2.0 shows ?-sheet formation when the sample was prepared in H_2O. The graph shows that ? sheet formation in the stock solution and this may happen due to longer storage of BSA at room temperature.

3.2 FT-IR sample of BSA+ Hydrogenated Vegetable Oil

1ml of 1mg/ml of BSA solution along with 0.3ml of 1mg/ml of Hydrogenated Vegetable Oil was mixed and heated at 70^0C till hexane gets evaporated. The FT-IR was done for the prepared sample.

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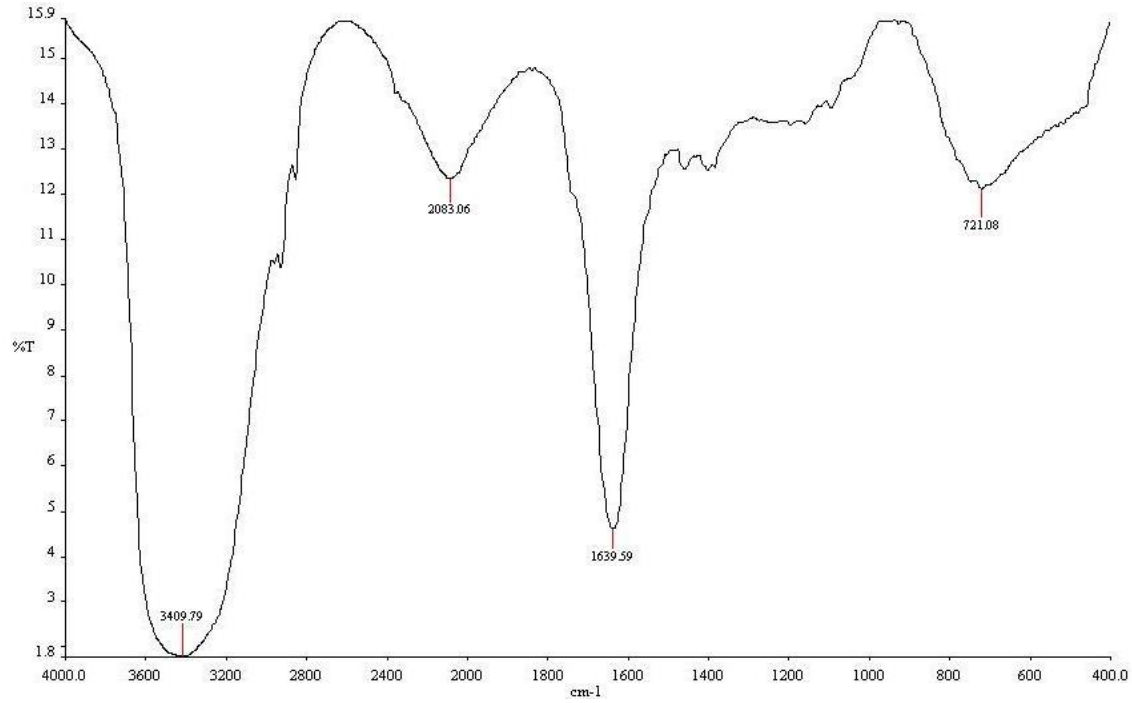

Description:

Peak at 3409.79 shows strong IR absorption by H_2O with prominent peak. Peak at 1639.59 confirm ?-sheet formation with 7cm of shift when compared with the stock solution IR band. The shift indicates that Hydrogenated Vegetable Oil increases the hydrophobic surface of the protein. The exposed hydrophobic core increases the intrinsic fluorescence capabilities.

4.3.3 FT-IR sample of HSA

1 ml of 1mg/ml of solution was prepared for FT-IR.

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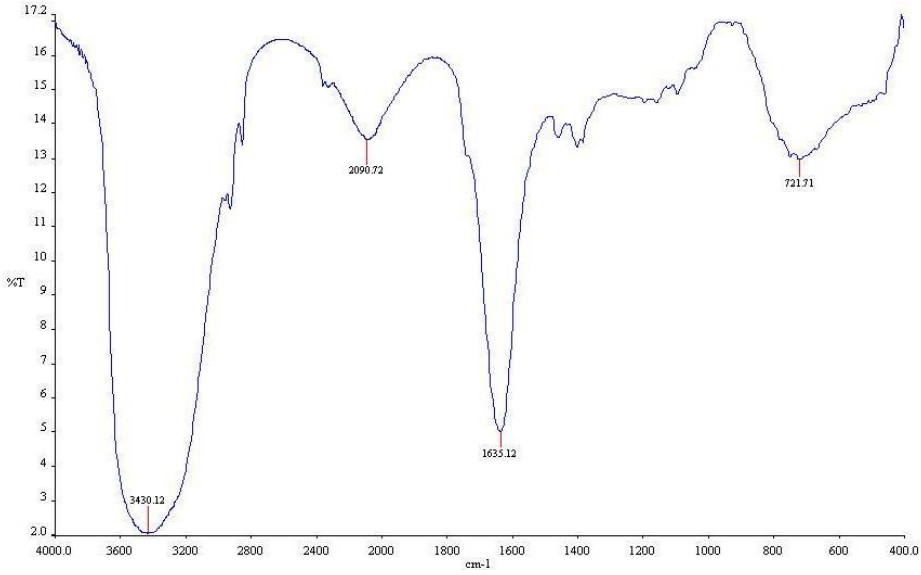
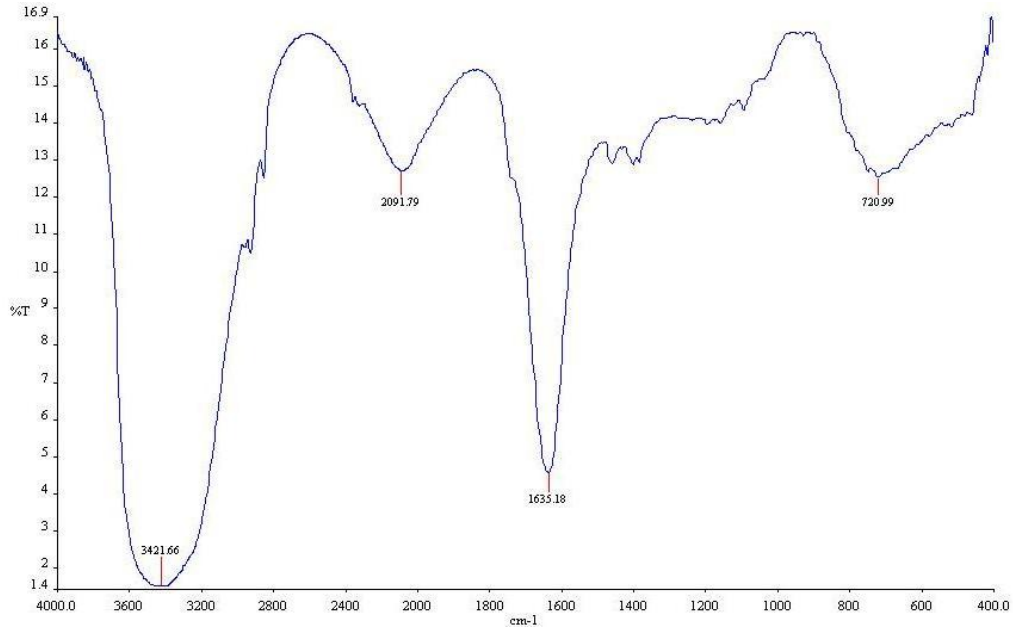


Description:
Peak at 3409.79 shows strong IR absorption by H_2O with prominent peak. Peak at 1635.12 confirms ?-sheet structure and this happen due to longer storage of HSA.

4.3.4 FT-IR sample of HSA and Hydrogenated Vegetable Oil

1ml of 1mg/ml of HSA solution along with 0.3ml of 1mg/ml of Hydrogenated Vegetable Oil was mixed and heated at 70^0C till hexane gets evaporated. The FT-IR was done for the prepared sample.

7



Description:

Peak at 3421.66 shows strong IR absorption by H_2O with prominent peak. Peak at 1635.18 confirms ?-sheet structure and this happen due to longer storage of HSA.

4.4 Scanning Electron Microscope Result:

SEM is an excellent tool for characterizing the homogeneity, structural and morphologic properties of fibrillated proteins. In Scanning Electron Microscope, the illumination system includes a field emission gun, which delivers a sub nanometer beam of 100-kV electrons onto a specimen. The image was focused at 2,500X. The image of the specimen is generated as the focused beam moves step by step over the specimen. SEM generally provides images of unstained sample.

The sample was applied on a clean glass plate and dried for half n hour. After sometime the

sample was coated with platinum and kept under vacuum for another 10 minutes. Then the sample was applied on SEM sample holder and was viewed under 2,500 KHz.

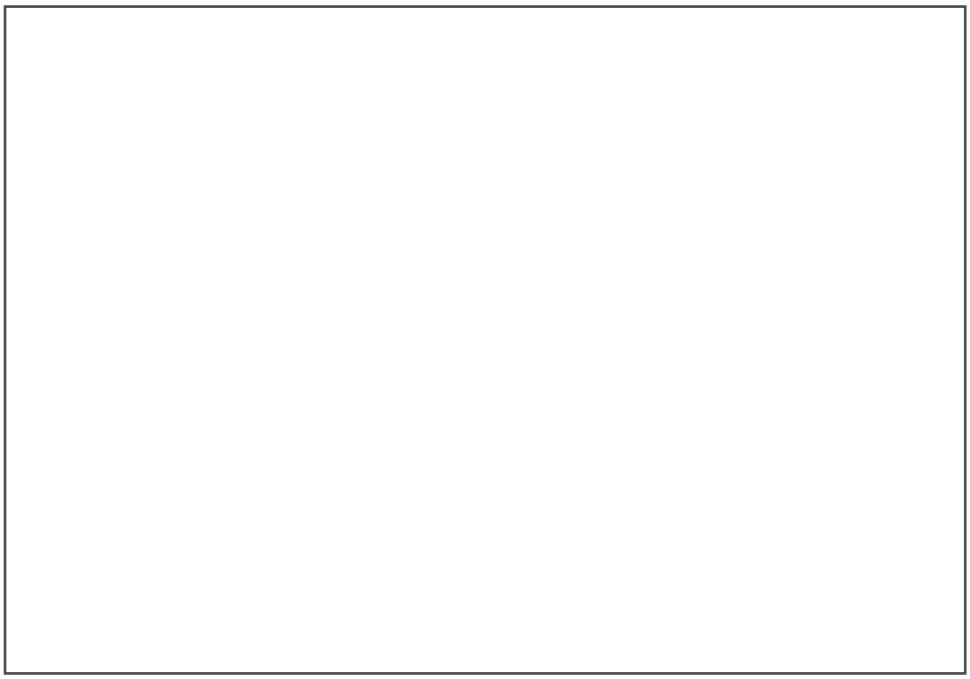


Fig 10: Scanning Electron Microscope view of Human Serum Albumin

5.0 Conclusion:

The molecular basis of fibrillation is still poorly understood. In recent years it has been noticed that various disease may occur due to fibrillation and its therapeutic aspects are emerging. Fibrillation is promoted by the factors that destabilize the classical self-association pathway from monomer to high order native assemblies, presumably by increasing the availability of the susceptible monomer. Fibrillation is also enhanced by partial unfolding of the monomer by a variety of perturbations, including urea, guanidine, co-solvents (such as ethanol), hydrophobic surfaces, stirring and high temperature (2, 3). Such interaction is also seen in BSA and HSA by using Hydrogenated Vegetable Oil. The interaction of Hydrogenated Vegetable Oil on BSA and

HSA in physiological buffer solution at pH 7.42 was studied by UV/Vis Absorption, FT-IR Spectroscopy, Atomic Force Microscopy & Scanning Electron Microscopy. The study indicated that both the protein model used was fibrillated when Hydrogenated Vegetable Oil is used. From UV/Vis Absorption Spectroscopy it can be said that with increase in temperature the OD (taken at 280nm) of the solution increase. Increase in temperature increase turbidity which confirms aggregation. From FT-IR band studies it can be said that native ? sheet conformation induced when Hydrogenated Vegetable Oil was used. Morphologic studies by Atomic Force Microscopy and Scanning Electron Microscope confirms morphologic changes in structure of protein sample used. Morphologic studies indicate that protein conformations changes and they get fibrillated with time.

6.0 References:

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