"In *Vitro* induction of amyloidosis and its counter measure"

A thesis submitted in partial fulfillment of the requirements for the degree of

Bachelor of Technology

in

Biotechnology

By

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Under the guidance of

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2015

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA.

CERTIFICATE

This is to certify that the thesis entitled **"IN *VITRO* INDUCTION OF AMYLOIDOSIS AND ITS COUNTER MEASURE "**submitted by **Mr. KALE KARUNAKAR** in partial fulfilment of the requirements for the award of bachelor of Technology in Biotechnology and Medical engineering with specialization in Biotechnology at the National Institute of Technology, Rourkela is an genuine work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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ACKNOWLEDGEMENT

I avail this opportunity to extend my sincere appreciation and gratitude to my guide Dr B.P Nayak, Biotechnology and Medical Engineering Department, for his invaluable academic and professional guidance, constant encouragement and kind help at different stages for the execution of this project. I want to thank Mr Vinay Kumar who guided me in my project work. I want to thank my friends Rashmi and Ranjan who always supported me and stood by me everywhere. Last but not the least ,I want to thank GOD and family for their blessings.

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Abstract:

Incorrect folding or mis-folding of proteins results in the formation of protein aggregates. Protein aggregates in general can be categorized into two types: amorphous aggregates, the ones with no long-range order, and amyloid fibrils, the ones with highly ordered structure. Aggregation of proteins leading to malfunction of organs leads to amyloidosis. About 17 different proteins have been found to form amyloid in vivo. Amyloid fibrils which are formed from those proteins share some common morphological features, but these proteins do not possess a conserved sequence or native structural motif. Recent studies show that amyloid formation is not only possible with disease-associated proteins, but also with proteins that are not associated with any known amyloid diseases under certain conditions. Non disease-related proteins can be induced in vitro to polymerize into amyloid fibrils under certain favorable conditions such as heating, agitation, low pH, pressure, and the presence of co-solvent. In the present study, an egg white lysozyme was used as protein for inducing amyloidosis through formation of amyloid fibrils. The amyloidosis was induced by applying extreme acidic environment (pH 2.0) by hydrochloric acid. Briefly, the lysozyme was isolated from the egg white by ethanol precipitation method. Results revealed that 40% ethanol concentration precipitation gave highest content of protein lysozyme. Lysozyme was treated hydrochloric acid (pH=2) along with salt. Amyloid fibril formation was verified by CD spectroscopy at far UV (200-260 nm). It revealed the presence of secondary beta sheet structure. Further, nicotinamide was used at serial concentrations of concentration of 22.5 mg and 45 mg to inhibit the amyloid formation. The outcome was also verified by CD spectroscopy. Nicotinamide successfully reduced the amyloid fibril formed in the egg white lysozyme. It was concluded that the inhibition of lysozyme amyloid formation by Nicotinamide is dose-dependent. Thus the current study shed light on a rational design of effective therapeutics for amyloidogenic diseases.

Keywords: Amyloidosis, amyloid fibril, hen lysozyme.

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Chapter-1

Introduction:

Introduction:

All the proteins present in the body are responsible for various functions and all them are biodegradable and recyclable. Amyloidosis is due to mis-functioning of these proteins ad and leads to formation of amyloid fibrils. Incorrect folding or mis-folding of proteins results in the formation of protein aggregates[1-3]. These amyloid fibrils deposit and accumulate in the body's tissues. If the amyloid fibrils are formed in the kidney, heart, liver, gastrointestinal tract or nerves then it causes those organs to function improperly. Thus the symptoms of amyloidosis are associated with the abnormal functioning of the organs involved. The symptoms of amyloidosis are mainly include unexplained weight loss, fatigue, shortness of breath, foamy urine, swelling in the ankles and legs well as numbness and tingling in the hands and feet. Treatments are designed either to dissolve the amyloid deposits or to interrupt their production. Left untreated, the disease can be life-threatening. Therefore, early and accurate diagnosis is the key to promoting positive outcomes

Amyloidosis effect several organs like heart, kidney, lungs, digestive tract and nervous system. There is no particular treatment available for amyloidosis reduction or inhibition is the main approach which is used[4]. Amyloid fibrillization is possible with the certain group of proteins associated with amyloidosis. Non disease-related proteins can be induced in vitro to polymerize into amyloid fibrils under certain favorable conditions such as heating, agitation, low pH, pressure, and the presence of co solvent[5]. The formation of fibrillary species in vitro from non-disease related proteins is the theme which is attracting renewed attention nowadays[6,7].

In the present study for formation of amyloid fibrils hen egg white lysozyme is used .It is used because it has some sequence similar with the human lysozyme. It is used for lysis of

cell walls of bacteria. By making some of the conditions which are unfavourable for the proteins they will denature leading to the mis-folding which in turn leads to the formation of the amyloid fibrils .By using hydrochloric acid (pH=2) these conditions are maintained for lysozyme leading to the formation of amyloid fibrils. Later detection is done by using CD spectroscopy .Inhibition of amyloid fibrils formed is the counter measure .It is done by using some of the drugs like Hsp 20,Nicotine, Benzoquinine, surfactants like SDS etc [8,9].can be used and results can be analyzed .In the present study nicotineamide is used for the counter measure of the amyloid fibrils which are formed .They are used in concentration dependent fashion and effects were analyzed according to it .

Objective:

- In Vitro induction of amyloidosis
- Counter measure for induced amyloid fibrils

Work plan:

Isolation of lysozyme from Hen egg.

⇓

Making conditions for formation of amyloid fibril

⇓

Testing for formation of amyloid fibrils by CD spectroscopy at far uv region (200- 260nm)

⇓

Making tests for reduction of amyloid fibrils by using nicotineamide which is counter measure.

Chapter -2

Literature Review:

2.1. Amyloidosis:

Amyloidosis is a condition where amyloid fibrils form in different tissues in the body .When this happens they normal function is being interrupted. It is the serious disease that leads to life threating organ failure. Many different proteins can lead to the formation of amyloid deposits, but only a few have been linked to significant health problems. The type of protein and where it collects determines the type of amyloidosis you have. Amyloid deposits may collect throughout your body or in just one area . The following are the different types of amyloidosis which are presently recognized[11]

The first type of amyloidosis is Primary (systemic AL)amyloidosis:

Primary (systemic AL) amyloidosis. This occurs without a known cause, but it has been seen in people with a blood cancer called multiple myeloma. This is the most common type of amyloidosis. "Systemic" means it affects the entire body. The most commonly affected body parts are the kidney, heart, liver, intestines, and certain nerves.

The second type is secondary (systemic AA) amyloidosis:

This is the result of another chronic inflammatory disease, such as lupus, rheumatoid arthritis tuberculosis, inflammatory bowel disease (Crohn's disease andulcerative colitis), and certain cancers. It most commonly affects the spleen, kidneys, liver, adrenal gland, and lymph nodes.

The third type of amyloidosis which is known is Dialysis-related amyloidosis (DRA):

It is most commonly seen in adults than in children .It seen in adults who have been on dialysis for more than 5 years. This form of amyloidosis is caused by deposits of beta-2 microglobulin that build up in the blood. Deposits can occur in many different tissues, but most commonly affects bones, joints, and tendons.

The fourth type found one is Familial, or hereditary, amyloidosis (AF):

It is hereditary type of amyloidosis . This is a rare form that is passed down through families. It is caused by an abnormal amyloid transthyretin (TTR) protein, which is made in the liver

Different proteins associate for different amyloidosis .The following is the list of different types of amyloidosis and their respective protein associated with it.

Table -1: Showing different types of amyloidosis and protein concerned.

Disease	Protein Featured	Official Abbreviation
Alzheimer's disease	Beta amyloid	A Beta
Diabetes mellitus type 2	IAPP Amylin	AIAPP
Parkinson's disease	Alpha- synuclein	none
Dialysis related amyloidosis	Beta 2 microglobulin	A beta2M
systemic AL amyloidosis	Immunoglobulin light chain	AL
Cerebral amyloid angiopathy	Beta amyloid	A Beta
Huntington's Disease	Huntingtin	none
Familial amyloid polyneuropathy	Transthyretin	ATTR
Rheumatoid arthritis	Serum amyloid A	AA
Aortic medial amyloid	Medin	AMed
Prolactinomas	Prolactin	APro

2.2. Lysozyme:

It is mostly found in saliva, tears and also found in the hen egg white. It is an enzyme consisting of 129 amino acids that lyses the cell walls of bacteria, has been comprehensively studied . It is a globular basic protein characterized by MW of approx. 14.4kDa. In a lysozyme molecule it contains four disulfide bridges (S-S), which can cause high thermal stability of the enzyme, together with six helix regions.

Approximately 40% of the hen egg white lysozyme is identical in sequence to the human lysozyme[12]. Recent studies have shown that, in addition to human lysozyme, several other variants of lysozymes are also capable of producing fibrillary species that exhibit characteristics of amyloid fibrils resulted from the group of proteins related to clinical amyloidosis. Generally in egg white, lysozyme accounts to 3.5% of the total egg white proteins .The following are the different types of proteins present in egg white.

- 54% Ovalbumin – Nutrition
- 12% Ovotransferrin - Binds iron
- 11% Ovomucoid - Blocks digestive enzymes[citation needed]
- 4% Ovoglobulin G2
- 4% Ovoglobulin G3
- 3.5% Ovomucin
- 3.4% Lysozyme - Kills bacteria
- 1.5% Ovoinhibitor
- 1% Ovoglycoprotein

- 0.8% Flavoprotein
- 0.5% Ovomacroglobulin
- 0.05% Avidin - Binds biotin
- 0.05% Cystatin

Lysozyme generally exhibits strong antibacterial activity against Gram-positive organisms. This phenomenon has found some practical application in the food processing industry, in medicine and also in the pharmaceutical industry.

The enzyme is also widely used as a preservative for meat, fish and their products, for milk and dairy products, as well as for fruit and vegetables. The pharmaceutical industry uses this enzyme mainly in the manufacture of adjuvant drugs for the antibiotics and analgesics in viral and also in bacterial infections, in the treatment of leukemia and neoplastic diseases.

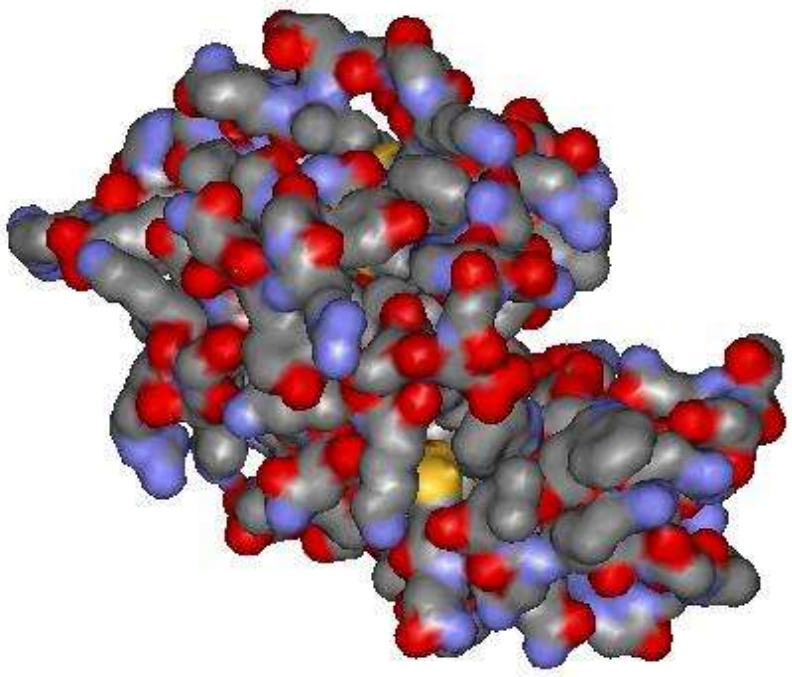


Figure-1: 3D Structure of lysozyme

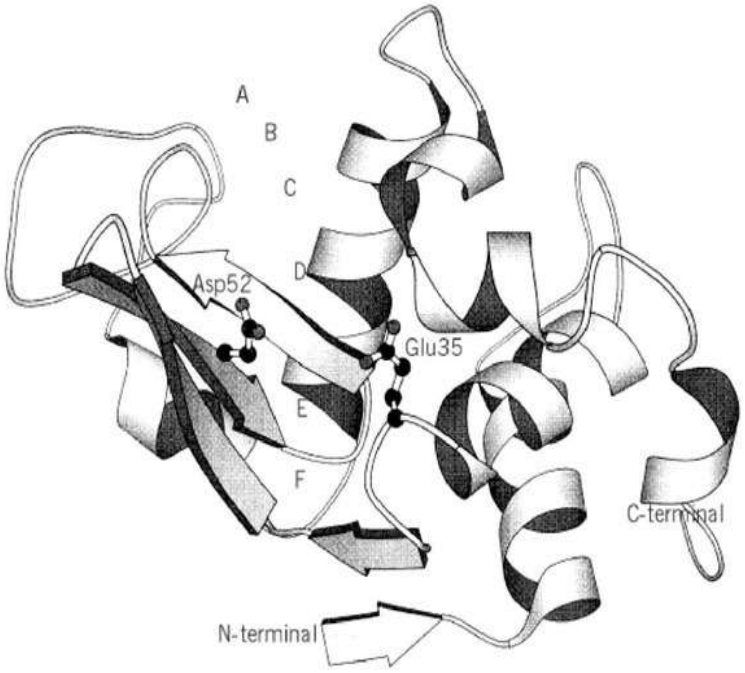


Figure-2: Lysozyme structure showing N-terminal and C-terminal.

2.3. Amyloid Fibrils:

Amyloid fibrils can be defined as fibrillar polypeptide aggregates with a cross-β structure**.** Amyloid is insoluble and is more dominately present with beta sheet structure. The amyloid fibrils are deposited extracellularly in the tissues and are thought to have a pathogenic effect. Well known examples of amyloid diseases include Alzheimer's disease, Diabetes type 2 and the spongiform encephalopathy's (e.g., Mad cow disease). fibrils are deposited extracellularly in the tissues and are thought to have a pathogenic effect[13]

Recently, a number of examples of functional amyloid have been identified including a constituent of melanosomes, curli and hydrophobins.

The following figure shows the formation of amyloid fibrils.

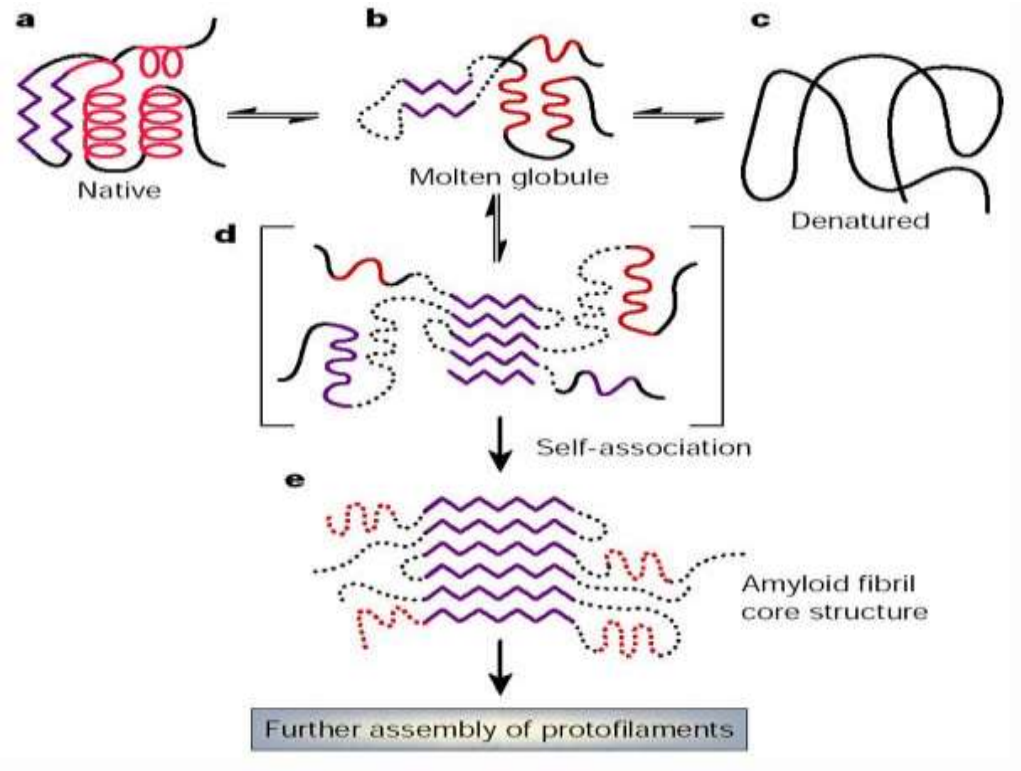


Figure-3: Amyloid fibril formation process: From native state to denatured and self-assembled amyloid fibrils.

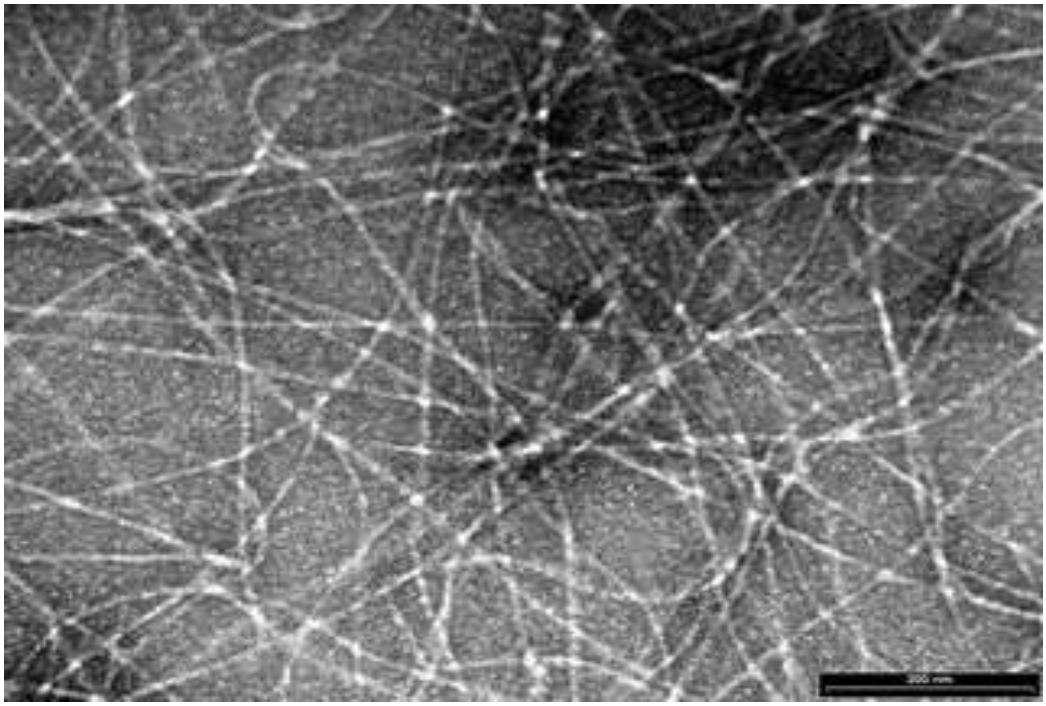


Figure-4: Showing amyloid fibril fiber like structure from microscopy.

2.4. Nicotinamide:

It a soluble crystal amide of nicotinic acid that is a component of the vitamin B complex and is present in most foods. Nicotinamide, also known as niacinamide and nicotinic amide, is the amide of nicotinic acid (vitamin B3 / niacin). It is a water-soluble vitamin and is part of the vitamin B group. Nicotinic acid, also known as niacin, is converted to nicotinamide in vivo, and, though the two are identical in their vitamin functions, nicotinamide does not have the same pharmacological and toxic effects of niacin, which occur incidental to niacin's conversion. Thus nicotinamide does not reduce cholesterol or cause flushing,[14] although nicotinamide may be toxic to the liver at doses exceeding 3 g/day for adults. In cells, niacin is incorporated into nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), although the pathways for nicotinic acid amide and nicotinic acid are very similar. NAD+ and NADP+ are coenzymes in a wide variety of enzymatic oxidation-reduction reactions. It's produced by the aqueous amino lysis of 3-cyanopyridine (nicotinonitrile) and subsequent crystallization.

Niacin and niacinamide are used to prevent niacin deficiency and to treat pellagra. Some clinicians prefer niacinamide for the treatment of pellagra because it lacks vasodilating effects. Pellagra may result from dietary deficiency, isoniazid therapy, or from decreased conversion of tryptophan to niacin in Hartnup disease or carcinoid tumors.[15]

Niacin and niacinamide are indicated for prevention and treatment of vitamin B3 deficiency states. Vitamin B3 deficiency may occur as a result of inadequate nutrition or intestinal malabsorption but does not occur in healthy individuals receiving an adequate balanced diet. Simple nutritional deficiency of individual B vitamins is rare since dietary inadequacy usually results in multiple deficiencies.

Nicotinamide occurs in trace amounts mainly in meat, fish, nuts, and mushrooms, as well as to a lesser extent in some vegetables

A safety study of niacinamide for the treatment of Alzheimer's disease is currently underway at the University of California, Irvine[16].

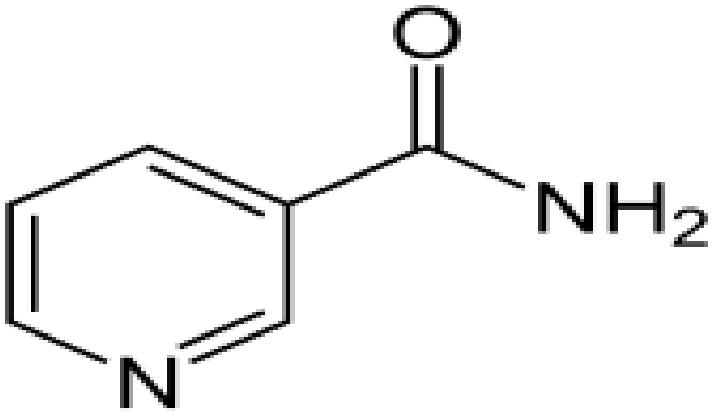


Figure-5: Structure of nicotineamide (niacinamide)

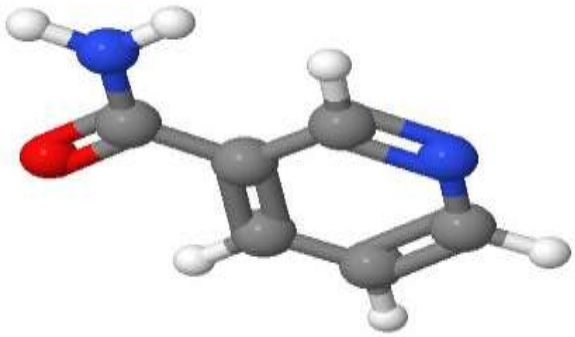


Figure-6: 3D Structure of nicotineamide.

2.5. CD Spectroscopy:

Circular dichroism (CD) spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light that arise due to structural asymmetry. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum which can contain both positive and negative signals [17].

CD can be used for:

- Determination if a protein is folded
- Characterization of secondary structure (α-helix, β-sheet)
- Detection of changes in structure upon mutagenesis
- Studying conformational stability of proteins:
    - pH stability
    - denaturant stability (urea, guadinium hydrochloride)
    - temperature
    - buffers
    - addition of stabilizers

- Detection of Changes in the conformation of a protein upon protein:protein interaction

Characterization of secondary structure is probably the most used CD spectroscopy application. Secondary structure can be identified in the "far-UV" spectral region (190-250 nm). The protein peptide bond is the chromophore, and it is possible to detect a signal if the protein is in a specific secondary structural conformation (α-helix, β-sheet). Alpha-helix, beta-sheet, and random coil structures each give rise to a characteristic shape and magnitude of CD spectrum. Like all spectroscopic techniques, the CD signal reflects an average of the entire molecular population.

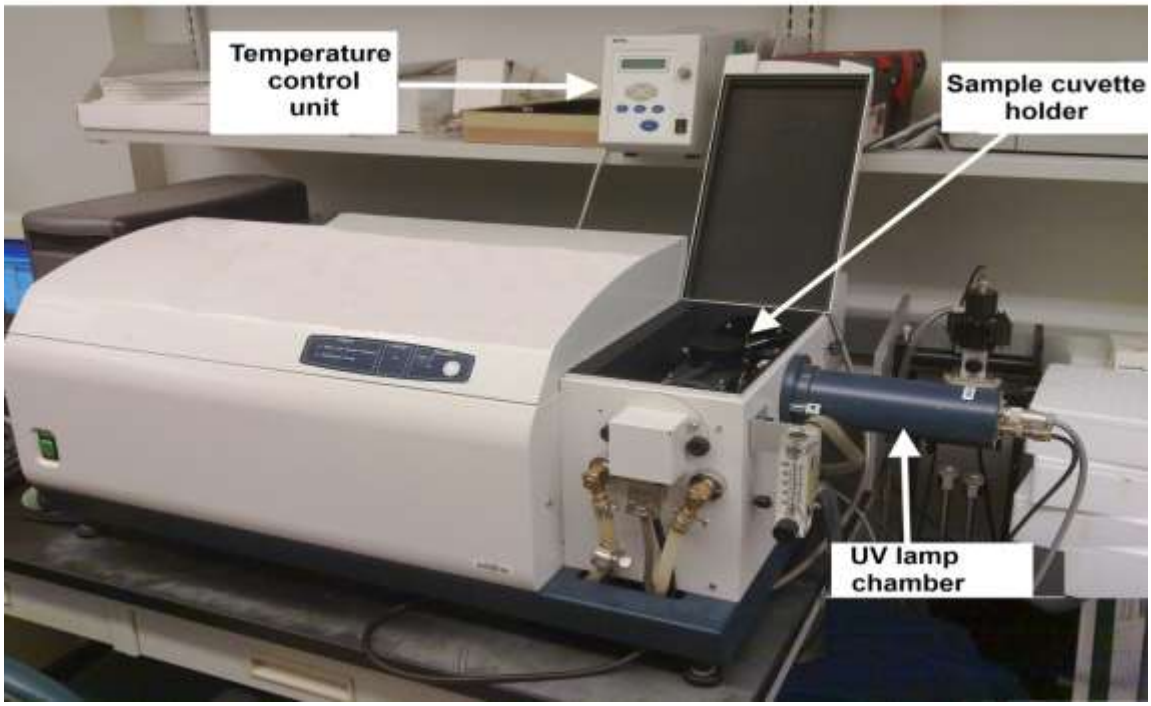


Figure-7: Jasco J-815 Circular Dichroism (CD) Spectropolarimeter:Showing the sample cuvette holder ,UV lamp source for focusing on the sample and also showing the temperature control unit.

Chapter-3

Materials and Methods:

3.1. Materials Required:

- Ethanol
- Nacl
- KCL
- 1 N acetic acid
- Hydrochloric acid
- Bradford Reagent
- Nicotine

Equipment's Required:

- UV Spectrophotometer
- CD Spectroscopy
- pH Meter

3.2 Methods:

3.2.1 Seperation of Egg white from total part:

Three or four hen eggs were purchased from market .Then separation of white part from yellow part is done. It is done by putting hole on egg shell and pouring in a beaker avoiding the yellow part carefully. Three or four eggs are taken and white part is seperated from the yellow part .The volume obtained by doing so is 50 ml.

3.2.2 Isolation of Hen Egg white lysozyme from white part:

Next Egg whites separated from the yellow ones were diluted to 3 fold by 0.05 M Nacl solution .100ml of 0.05 Nacl solution is added to 50 ml of egg white.0.05 Nacl is prepared in the following way by adding 2.922 grams of Nacl in 1 liter of distilled water.

Next pH of the mixture is kept 4.0 by adding few drops of 1 N acetic acid. Now the dilution is done by equal volumes of 20%, 30%, 40%(v/v) ethanol.

Next incubation is done for 7 or 8 hours at room temperature.

3.2.3 Centrifugation process:

Now the samples of each were taken 50 ml in falcon tubes for centrifugation process. Centrifugation is done at 7500 rpm for nearly 25 minutes .Then the three falcon tubes are taken out. Then precipitates are discarded from the three tubes .Now the supernatants containing the tubes contained required lysozyme. The further purification is done by Dialysis.

3.2.4 Dialysis process:

First gloves are weared. Then dialysis tube is cut according to the need of the sample. Three dialysis tubes were cut and then they are dipped in hot distilled water for expansion of the bags. Then they are rinsed several times for the removal of glyceral from the bags. Then one end of all the bags are tied with the help of thread and rubber. Then they are tested for their leakage. Then buffer solution is prepared by maintaining the pH 7.0 by mixing 8.85 gms of

disodium hydrogen phosphate and 3.4023 grams of potassium dihydrogen phosphate in 1 liter of distilled water. Then buffer solution is poured in the three beakers and supernatants are poured in the three bags and other ends are tied with the help of rubber or thread. Then checked for leakage. Then bags are dipped in beaker containing buffer solution .They are left for 24 hours. Then after dialysis tubes are cut for removal of the required lysozyme.

3.2.5 Lyophilization process:

Next the lysozyme sample taken from the bags were taken in three separate plates .They were sealed using the tape. Then they were subjected to lyophilization. It is done by using freeze drier with -44 to -47 ^{o}c and $50*10^{-3}$ and $100*10^{-3}$ bar vacuum.

Then the lyophilized samples are kept at -18^{o} c for different studies in the project process.

3.2.6 Estimation of lysozyme Protein content by using Bradford assay:

It is done for the three lysozyme samples obtained from three concentration ethanol used. The process is as follows.

At first 0.5 gm of each protein sample of three is dipped in 10 ml of distilled water in a test tube. Mixing is done with the help of the stirrer if necessary. Then 100 micro liter and 200 micro liter are taken from each test tube of 20%, 30 %, 40% named ones. Then it is made to 1 ml by adding required ml of distilled water to the test tubes .Now a total 6 tubes are made

.Now 100 micro liter is taken from each test tubes and placed in other test tubes. Then 1 ml of Brad ford reagent is added to each test tube followed by 2 ml of distilled water.

Then blank solution is made by adding 2.1 ml of distilled water followed by 1 ml of Bradford reagent in a test tube.

Now the absorbance is made at 595nm for the three concentrations of lysozyme samples.

From the standard curve the protein content is calculated for three concentrations of lysozyme.

3.2.7 Making conditions for the formation of amyloid fibrils in 40% concentrated ethanol separated lysozyme:

Since the 40% concentrated lysozyme has highest protein content, it is used for study. The conditions are done in the following way.

At first 0.08 g of lysozyme is dipped in the 48 ml of hydrochloric acid used along with the 0.18 gram of KCL and 5.2 gram of Nacl. Mixing is done by vortexing and then incubated in reciprocating shaker bath at 30 rpm under the condition of 55 degree. It is incubated for 24 hours during which the amyloid fibril formation taken place[18].

3.2.8 Checking amyloid fibril formation by CD Spectroscopy :

Circular dichroism (CD) spectra of lysozyme samples were recorded on a JASCO J-715 (150-S Type) spectrometer (Sunway Scientific Corporation) at 25 °C using a bandwidth of 2.0 nm with step interval of 0.1 nm and an averaging time of 2 s. It is done at far UV region ranging from 200-260 nm This region is mainly used for the detection of secondary

structure of protein generally. The path length of the cuvette is 1 mm. At first the buffer used is scanned for 2 seconds time .Three readings are taken and they are averaged generally.

Then sample is run for three readings and averaged. The main buffer scan readings are subtracted from the sample readings. Then a graph is plotted .It is between ellipticity (milli degree) verses wavelength. So, based on the graph the secondary structure analysis is done.

3.2.9 Counter measure :

This is the final process of the project. This is done with Nicotine drug. It is done in concentration depended fashion. It is used for reduction of the formed amyloid fibrils. At first the solution which is made for the formation of the amyloid fibrils is taken 15 ml in a flask Then Neurobion Forte tablet containing nicotine amide 22.5 mg is taken and made into powder and mixed with the 20 ml solution. It is left for 2 days .Similarly 45 mg Nicotine amide is taken and mixed with the 20 ml of lysozyme solution and left for 2 days. Later for checking the reduction of the amyloid fibrils it is subjected to CD Spectroscopy and results were analyzed .

Chapter -4

Results and Discussion:

4.1. Falcon tubes after centrifugation:

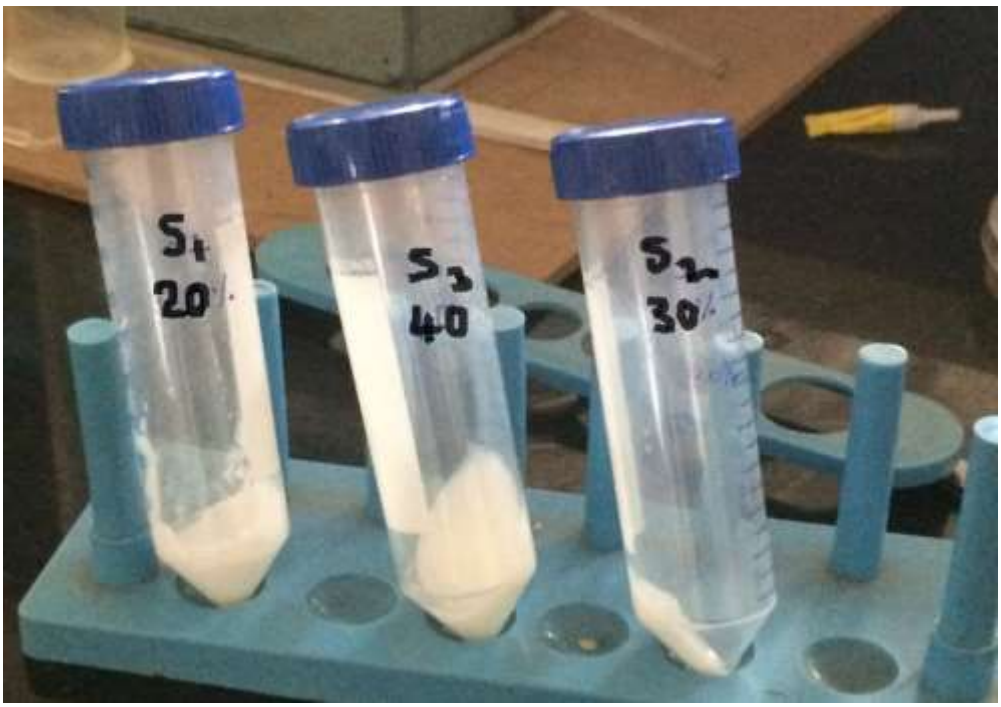


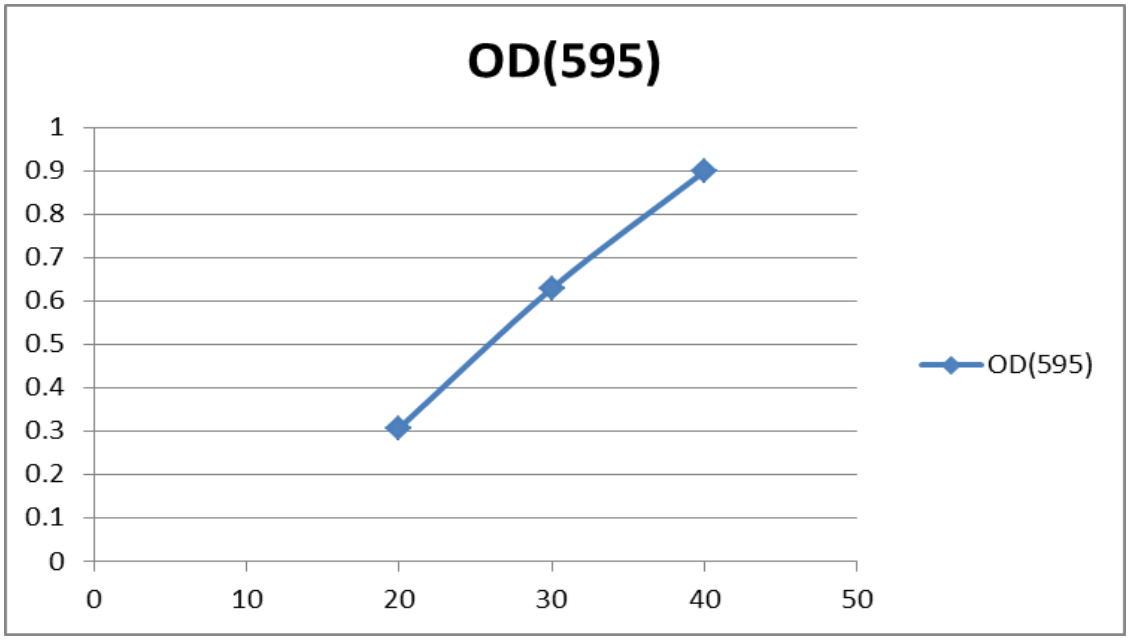
Figure-8: Falcon tubes after the centrifugation which are used for the isolation of the lysozyme represented by 20%, 30% and 40% ethanol concentration.

Centrifugation tubes which are marked as 20%, 30%, and 40%.These are the tubes in which supernanent containing lysozyme is separated. The solid part contains all the unwanted proteins .

4.2. Petridish containing lysozyme:

Figure-9: Petridish containing the lysozyme after the lyophilization process.

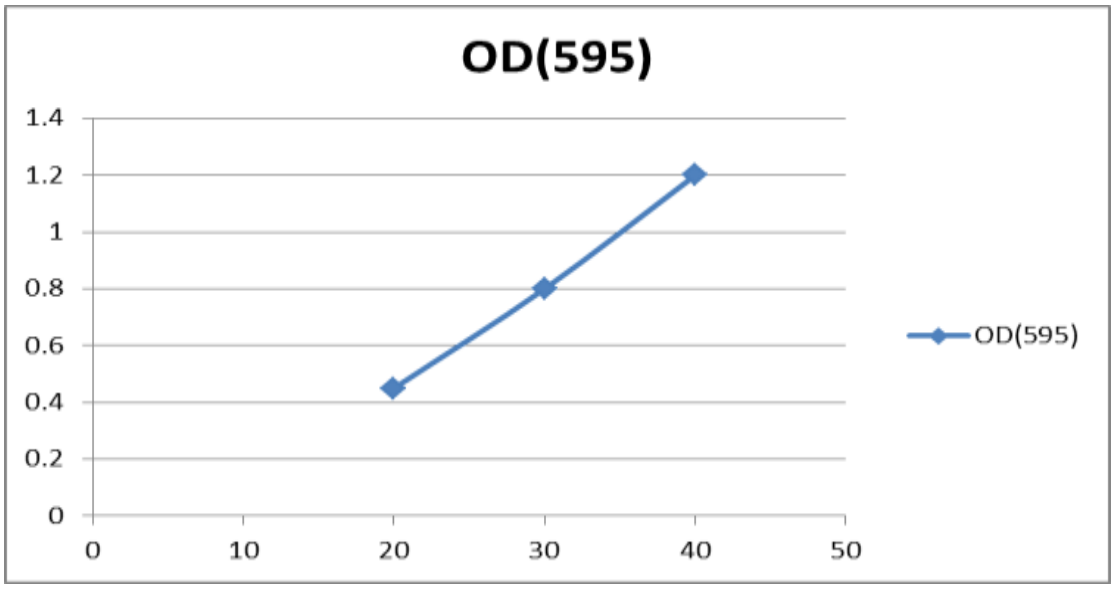
4.3. Protein estimation by Bradford assay.



Graph-1 showing the concentration taken on the x axis and O D taken on the Y axis .It is used for the estimation of protein content by using the molar absorption co efficient.

Table-2: Representing concentration of the solution taken and the protein content obtained for the graph-1 drawn above.

Concentration (100 µl)	Protein content(mg/ml)
20%	56
30%	78
40%	85



Graph-2: Showing the concentration (200 µl) taken on the x axis and the O D taken on the y axis got for the sample.

Table-3: Showing the concentration and the protein content achieved for the graph-2 shown above.

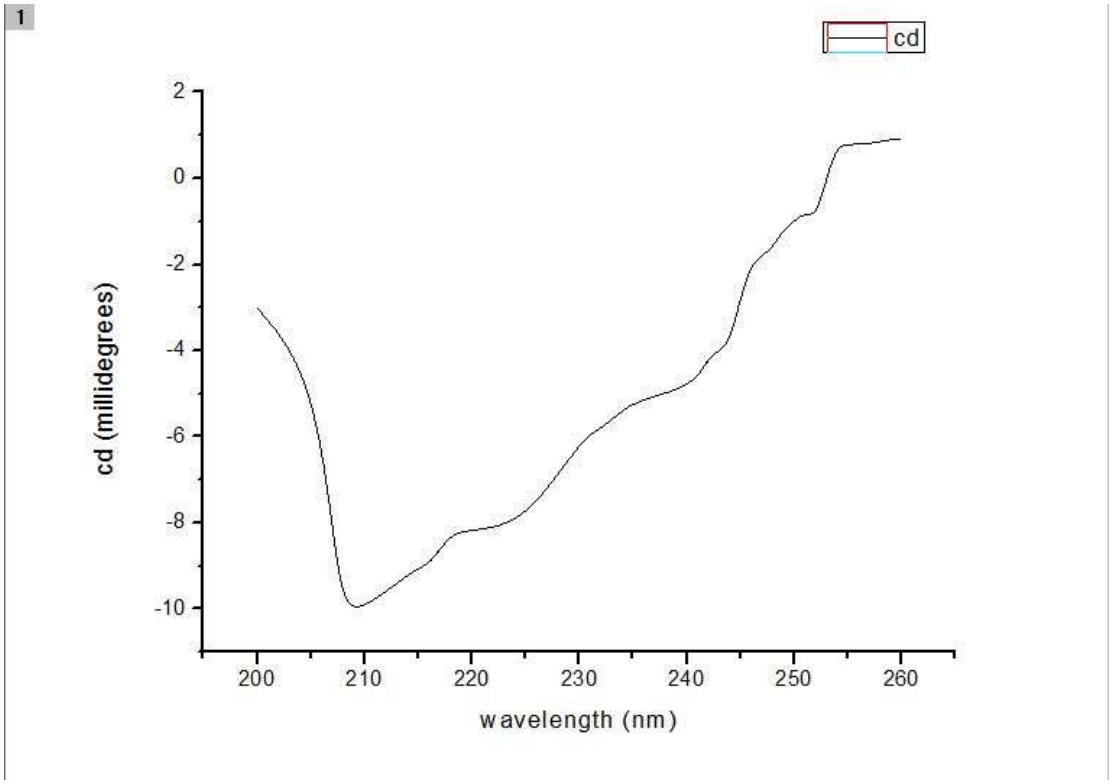
Concentration(200 µl)	Protein content (mg/ml)
20%	65
30%	72
40%	89

40 % ethanol concentration gave the highest protein content

4.4. Solutions containing lysozyme and buffer for formation of amyloid fibrils:

Figure-10: One beaker contains lysozyme along with buffer solution in which lysozyme was dipped. Right side beaker contains buffer solution which is used as a stream line solution during CD measurements

4.5. CD Spectroscopy graph at far UV (200-260)



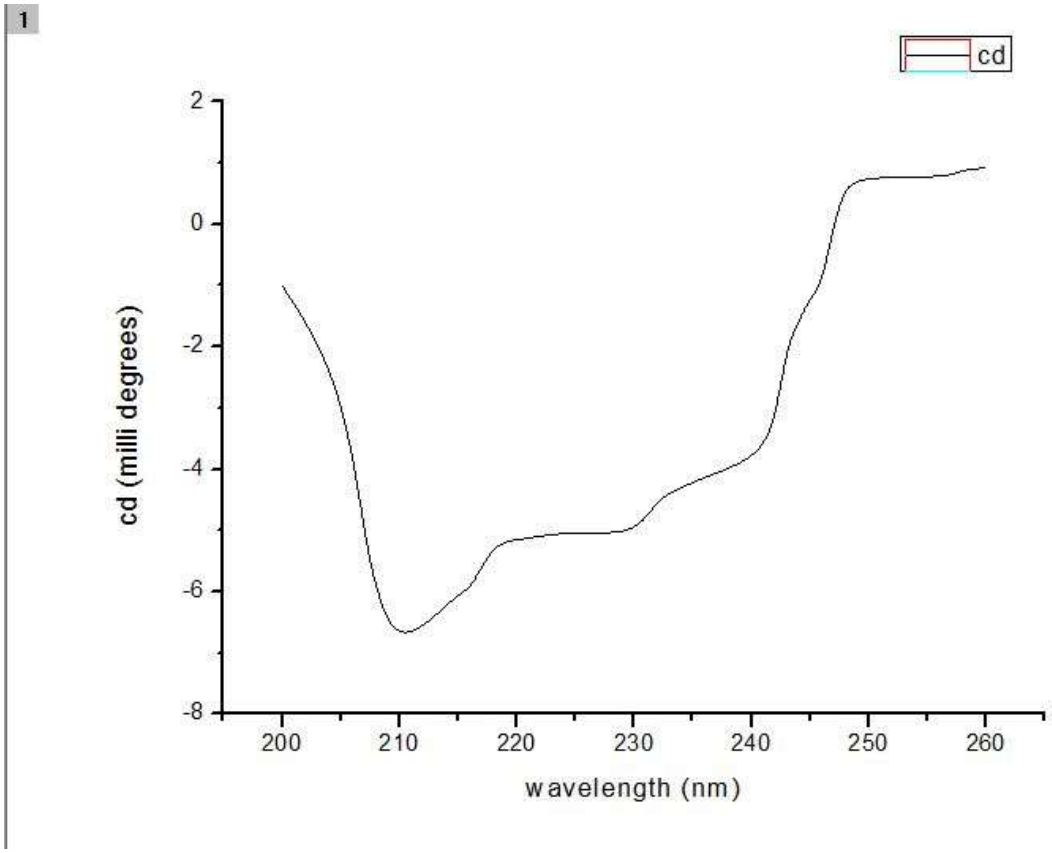
Description of the Graph: The CD spectroscopy was undertaken to follow the structural property, the secondary structure in particular, as aggregation/fibrillization proceed. The CD spectra obtained from HEWL were observed to exhibit a characteristic pattern of β-sheet-rich conformation, with an absorption minimum around 216 nm, clearly implying that the conformation of HEWL species was predominately *β*-structure..

4.6.**Solutions containing nicotineamide at different concentrations for inhibition of amyloid fibril formed**:

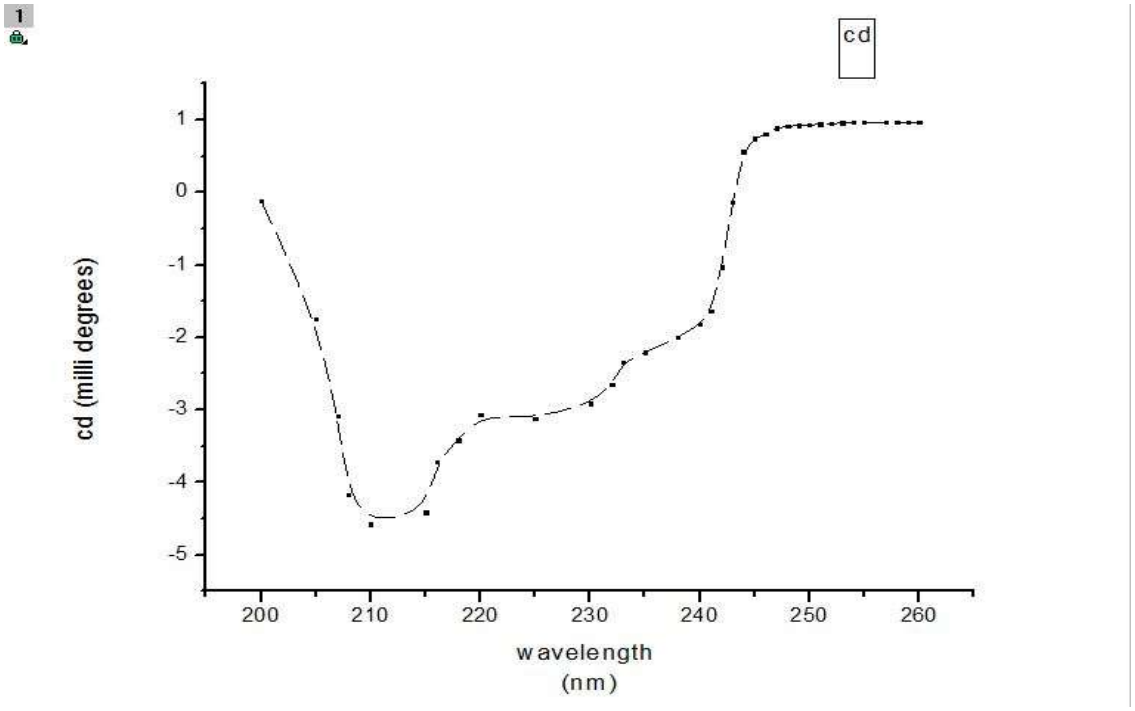
Figure-11: Samples containing nicotineamide 45 mg in the first beaker.The second beaker contains 22.5 mg of the nicotine amide mixed in the buffer solution which is used for the induction of the amyloid fibrils.These solutions are furthur tested or verified for inhibition of the amyloid fibrils which are formed earlier.

The first beaker contains lysozyme along with the inhibitor taken 45 mg and the second right beaker contain the lysozyme solution along with the 22.5 mg inhibiter.They were incubated for 2-3 days and they were checked for the amyloid inhibition from CD Spetroscopy.

4.7. CD spectroscopy for nicotine 22.5 mg treated lysozyme solution:



4.8 .CD Spectroscopy graph for 45 mg treated lysozyme solution



Description of the graphs: From the above two graphs ,HEWL conformer, a variation in the shape of the CD spectrum was detected upon the addition of inhibitor at 55 c. A closer look at the CD spectra obtained at 55 °C revealed that a slight difference. As shown in graphs that the negative peak in CD spectra at 218 nm disappeared when HEWLs incubated with 22.5 mg nicotineamide and 45 mg nicotineamide of the process. But more inhibiton is seen from the 45 mg nicotinamide treated solution .Clearly the graphs revealed that inhibition followed the concentration depended fashion.

Chapter-5
Conclusion:

Conclusion:

Hence Isolation of lysozyme is done by ethanol precipitation method and 40 % concentrated ethanol gave the highest lysozyme concentration. Next Induction of amyloidosis is successfully done in lysozyme by providing the low pH conditions by hydrochloric acid.. Later inhibition is done by using nicotine amide in concentration depended fashion Higher amount of nicotineamide gave the highest inhibition when compared to the lowest one. So, the inhibition followed the dose depended fashion. This experiment can be used to design better drugs for the treatments of amyloidosis.

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