TO INVESTIGATE THE ANTI-AMYLOIDOGENIC ACTIVITY OF DIFFERENT OXYGEN CONTAINING BIOLOGICALLY ACTIVE COMPOUNDS AGAINST THE FIBRILLATION OF BOVINE β-LACTOGLOBULIN

A Thesis submitted for the degree of

Doctor of Philosophy

by

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CERTIFICATE FROM THE SUPERVISOR

*This is to certify that the thesis entitled "**To investigate the anti-amyloidogenic activity of different oxygen containing biologically active compounds against the fibrillation of bovine β-lactoglobulin**" Submitted by **Sri Hasan Parvej**, who got his name registered on **20th November, 2015** for the award of **Ph. D. (Science) Degree** of **Jadavpur University**, is absolutely based upon his own work under the supervision of **Dr. Umesh Chandra Halder** and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.*

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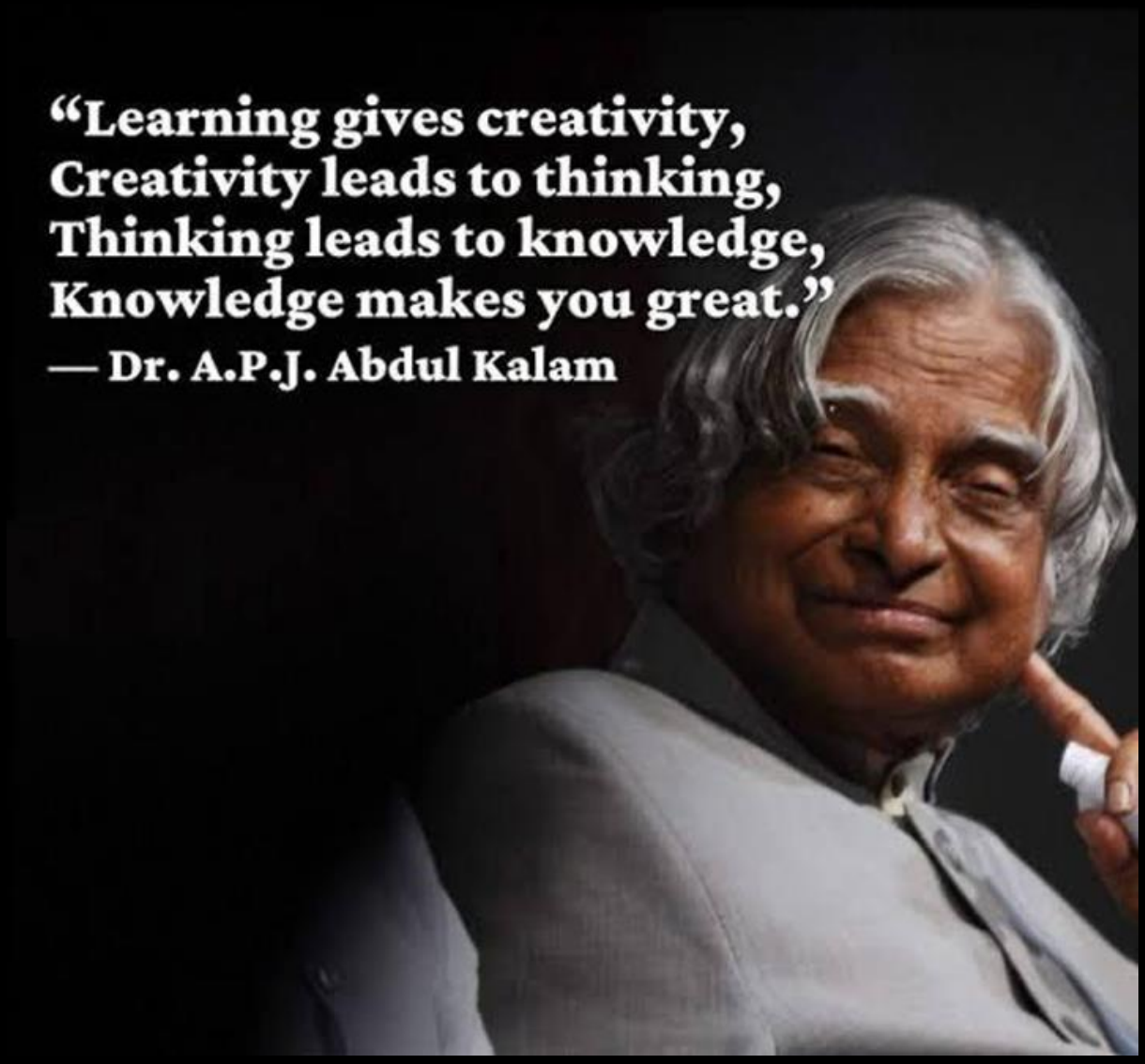
DECLARATION

I do hereby declare that the work entitled in this thesis **"To investigate the anti-amyloidogenic activity of different oxygen containing biologically active compounds against the fibrillation of bovine β-lactoglobulin"** which is being submitted for the degree of **Doctor of Philosophy (Science)** has been carried out by me under the supervision of **Prof. (Dr.) Umesh Ch. Halder** of Department of Chemistry, Jadavpur University, Kolkata, India. The thesis is neither in entirely nor in any part thereof has been presented anywhere earlier for any degree or award whatsoever.

Hasan Parvej

Date: **(Hasan Parvej)**

DEDICATED TO MY FAMILY AND TEACHERS


“Learning gives creativity,
Creativity leads to thinking,
Thinking leads to knowledge,
Knowledge makes you great.”
— Dr. A.P.J. Abdul Kalam

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Hasan Parvej

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List of Abbreviations:

α-lg – alpha lactoglobulin

α - alpha

ANS, 1-Anilino-8-naphthalenesulphonate

APS, Ammonium persulfate

AR, Analytical reagent

β, Beta

BBP, Bilin-binding protein

β -lg, Beta-lactoglobulin

BSA, Bovine serum albumin

CCK, Plasma cholecystokinin

CD, Circular dichroism

CR, Congo red

Cys, Cysteine

°C, Degree centigrade

DAC, Diacetylcurcumin

DMF, Dimethyl formamide

DTAC, Dodecyltrimethylammonium chloride

DTNB, 5,5'-Dithiobis (2-nitrobenzoic acid)

DOPA – dihydroxyphenylalanine

FA, Folic acid

FESEM, Field emission scanning electron microscope

FRET, Fluorescence resonance energy transfer

GABA – Gamma-aminobutyric acid

Gdn.HCl, Guanidine hydrochloride

Gdn.SCN, Guanidinum thiocyanate

Gln, Glutamine

Glu, Glutamic acid

HC, Hydroxychalcones

HFIP, Hexafluoro isopropanol

HIV-1, Human immunodeficiency virus 1

HSA, Human serum albumin

IOC, Isoxazole derivative of curcumin

Lys, Lysine

mg, Milligram

M, Molar

Met, Methionine

mM, Millimolar

µm Micromolar

mL, Milliliter

µl, Microlitre

MRE, Mean residue ellipticity

MUP, Major urinary protein

nm, Nanometer

ns, Nanosecond

OBP, Odorant-binding protein

PAGE, Polyacrylamide gel electrophoresis

PEA - Phenethylamine

PFOA, Perfluorooctanoate

PEG, Poly（ethylene glycol)

ps, Picosecond

PY, Pyrazole derivative of curcumin

RBP, Retinol-binding protein

SDD, Sodium dodecanoate

SDS, Sodium dodecyl sulfate

SEM, Scanning electron microscope

SIV, Simian immunodeficiency virus

SM – SAMLL MOLICULES

tBHP, tert-Butyl hydroperoxide

TEMED, N, N, N′, N′-Tetramethylenediamine

TEM, Transmission electron microscope

Th-T, Thioflavin T

TFE, 2, 2, 2-Trifluoroethanol

TMAO, Trimethylamine N-oxide

Trp, Tryptophan

Tyr, Tyrosine

UV, Ultraviolet

Table of Contents

PREFACE

Synthesized linear polypeptide chain of amino acids on ribosomes requires a highly ordered architecture to be active for the execution of its proper biological functions as each protein has unique 3D structure. Proteins are the most versatile and abundantly found biomolecule in the living cells. They can function either independently or in complex with other cellular components. Their functions include enzymatic catalysis, regulation of gene expression, signal transduction, immune response etc. Proper folding of protein into compact 3D structure minimizes the Gibb's free energy and maximizes hydrogen bond between polar groups. In vivo the protein folding is assisted by several folding accessories and chaperones.

Because of improper folding due to malfunctioning of any folding accessories or chaperones, many proteins and peptides have been found to form the stable self-assembly leading to insoluble aggregates termed as amyloid fibrils. Amyloid fibrils, generated due to protein aggregation, play an important role in a range of amyloid diseases, including Alzheimer's disease, dialysis-related amyloidosis, and type II diabetes mellitus etc. The structural hallmark of amyloid fibrils is the presence of high content of intermolecular β-sheet having the β-strands running perpendicular to the fibril axis. Thus preventing the formation of these soluble aggregates during the fibrillation procedure could serve as the key to the nonoccurrence of many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, type II diabetes and the spongiform encephalopathies etc. diseases. Many other proteins can also form amyloid-like fibrils in vitro under altered conditions of pH, ionic strength, and temperature. Insulin, lysozyme, bovine and serum albumins, beta lactoglobulins etc. are the examples of such proteins among the many others.

Several efforts have been devoted towards the discovery of novel anti-aggregating agents to combat the amyloid diseases. But the drugs developed for Alzheimer's disease were reported to be unsuccessful at the different phases of clinical trials. Small molecules are capable to cross the cell membranes and blood brain barrier and have been thought-out to be the potential drug candidates. They may include the small aromatic molecules like quinones, naturally occurring oxidants like curcumins and their derivatives, gallic acids, feluric acids, ascorbic acids, coumarin derivatives and the nanoparticles. They were reported as efficient inhibitors against the in vitro amyloid fibrils formation.

In my present research work, bovine beta-lactoglobulin (β-lg), a well-known globular whey protein (MW ~ 18.3 kDa), in ruminant milk, has been chosen as a model protein for studies of protein aggregation and amyloid formation. Bovine β-lg has been classified as an effective carrier-protein of oxidation-sensitive hydrophobic drugs, retinol, fatty acids and nutraceuticals and has a unique acidic pH resistivity and potential encapsulating property. At elevated temperature (above 60^0C), it undergoes a conformational change, with exposure of buried hydrophobic residues and the thiol (–SH) group (Cys-121). However, the β-lg is found to form amyloid fibrils when heated above 78^0C at physiological pH having all the characteristic features observed in many amyloid diseases. In order investigate the influence of small molecules on the thermal aggregation of beta-lactoglobulin (β-lg), I have employed several synthesized oxygen-nitrogen containing heterocycles like coumarin compounds, chalcones and the compounds related to neurotransmission like dopamine, phenylethyl amine and gamma aminobutyric acid.

My first work involves the synthesis of five different coumarin derivatives SM_0, SM_1, SM_2, SM_3 and SM_4 having substituents differing in their positions to the coumarin nucleus. Coumarin, a commonly used fluorescent dye having different biological applications. Substituents having electron donating and electron-withdrawing groups at different positions of the coumarin moiety can influence on its physical and chemical properties. Here I have investigated the effect of such substituents of coumarin on the structural and aggregation properties of a model protein, β-lg through a multi spectroscopic approach. It has been observed that coumarin methyl ester with an 8-hydroxyl group inhibits the thermal aggregation of β-lg. This compound binds at the hydrophobic site of β-lg and stabilize a particular protein conformation through the formation of hydrogen bonds and hydrophobic interactions. Hence a correctly designed compound can inhibit protein–protein interactions through the formation of protein–small molecule interactions. Other coumarin derivatives are also effective in the prevention of thermal aggregation of β-lg and the order of their inhibition is $SM_2 > SM_4 > SM_3 > SM_1 > SM_0$.

Second research work demonstrates the synthesis of the hydroxychalcones having different substituents. Chalcones are αβ-unsaturated ketone, which includes a variety of important biological compounds collectively known as chalcones or chalconoids. Chalcones and their derivatives demonstrate a wide range of biological activities including anti-inflammation.

These compounds were tested for the study of conformational change of β-lg leading to its amyloid fibrillation process. Depending upon electronic property of the substituent, chalcones

derivatives can interact differently with the protein and thus can affect their conformation change in different extents. Such binding of the compounds to the β-lg changes the protein conformation which exposes hydrophobic amino acids to the protein surface. It was also observed in the hydrophobic site specific ANS-assay. Exposure of hydrophobic amino acids to the protein surface, leads to the aggregation of the proteins. All the studies including Th-T binding assay, FT-IR, TEM and molecular docking studies show that the order of chalcone induced β-lg aggregation is HC1 > HC2 > HC3 > HC4.

My third research work address the potential application of the three therapeutic compounds dopamine (DOPA), phenylethyl amine (PEA) and gamma aminobutyric acid (GABA) related to neurotransmission, in the inhibition and depolymerization of amyloid fibrils formed by bovine β-lactoglobulin (β-lg) under thermal condition. Our investigation involves in vitro studies of aggregation mechanism using multi spectroscopic, microscopic, and computational approaches. Th T assay and TEM study clearly shows that DOPA inhibits completely the fibrillation of β-lg by preventing the structural change of native β-lg into the β-sheet rich fibrillar structure in a concentration dependent manner. Other two compounds PEA and GABA can also be used as anti-fibrillating agent to inhibit the aggregation process, but their effectiveness is less than DOPA. The effectiveness in the suppression of thermal aggregation of β-lg follows the order DOPA > PEA > GABA. Results of ANS fluorescence studies confirmed that DOPA is opposing the thermal exposure of hydrophobic patches and thus stabilizes the heat-treated β-lg structure. CD spectra confirm the transformation of β-sheet towards native-like structure in the presence of DOPA. Hydrogen bond formation and the hydrophobic interactions are the key forces involved in the stabilization of heat-exposed β-lg by DOPA. DOPA forms hydrogen bonds through its two –OH groups at C2 and C3 positions (as shown by molecular docking). ANS study for hydrophobic measurements shows PEA is less efficient to stabilize the thermally unfolded state of β-lg probably due to its inability to form the non-covalent bonds as it lacks two –OH groups in its structure. On the other hand, due to absence of both –OH groups and phenyl nucleus, GABA fails to stabilize the heat exposed β-lg structure leading to its aggregation.

Chapter:1

Review of literature on bovine beta lactoglobulin

1-1. Introduction

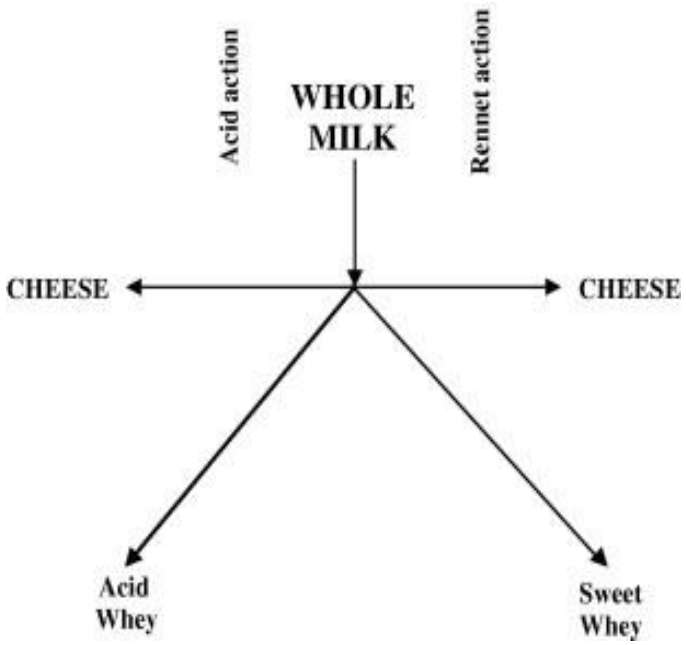
Protein are the large complex macromolecules, plays many vital and crucial role in biological System. In nature only 20 amino acids are available. Amino acids are joined together by peptide linkages leading to the formation of complex a protein molecule.[1] Most of the biological functions are catalysed by enzymes and these enzymes are also made of protein. Also, growth and developments of a biological system are depending on proteins and peptide hormones. Protein also helps to maintain proper pH in our body. Protein maintains the fluid balance of our body, also controls digestion, and helps in boosting the immune system. And all these functions of protein molecules depend on its physical interactions with other molecules. Hence the structure of protein plays crucial role on its biological function as each protein has unique three-dimensional structures and the function depends on the structure of the concerned protein [2]. Proteins are classified according to their functions and source of the respective protein.

1-1.1 Milk Proteins

To make a healthy and balanced diet, milk plays an important role. From the prehistoric period milk is consumed for its extensive nutritional value not only to the newly born babies but to the child, the elders and to the adult. Notable health benefits of milk are related to the proteins present in it, not only for the high nutrition value but also for their several biological functions. Therapeutic benefits such as antihypertensive properties, anticarcinogenic activities, immune system modulation, and other metabolic features of milk, are affiliated with its proteins. Milch animals, including, buffalo, goat, cow, and sheep are the main source of milk [3]. Bovine milk has about 13% total solids and about 9% solids without fat, making it an extremely liquid food (87% water). Presence of calcium, vitamin A, riboflavin, fortified form vitamin D, protein, vitamin B12, potassium, and phosphorus make the milk a nutrition reached supplement. Niacin precursor tryptophan is present in milk in sufficient amounts, making milk an excellent source of niacin equivalents. In addition, milk contains a variety of bioactive compounds with medicinal (nutraceutical) properties [4, 5]. In total, bovine milk contains approximately 3.5% protein by weight (36 g/L), which contributes almost 38% of its total solids and about 21% of its energy [6]. Furthermore, milk is one of the richest sources of biologically active peptides. Peptides derived from casein fractions and whey proteins, including casein phosphopeptides (CPPs), glycomacropeptide (GMP), antihypertensive peptides, opioid peptides, and lactoferrins take control of various physiological features, such as antibacterial, anti-hypertensive activities, immune stimulating activities, opioid-like features, and antiviral

impacts and also enhancement of calcium absorption [7-12] Scientific evidence shows that consumption of milk lowers the risk of cardiovascular diseases, hypertension, cancer, metabolic disorders, and some other diseases [13, 14].

The two major families of proteins: caseins (insoluble) and whey proteins (soluble) are the main constituent of milk. Approximately 80% of all protein consists of caseins (weight/weight). Casein is mainly composed of calcium phosphate-micelle complexes [15]. It is a heterogeneous family of 4 major components including alpha- (α_{s1}- and α_{s2}-casein), beta-casein, gamma- casein, and kappa-casein [16-19]. Isoelectric precipitation (the addition of acid, or the in-situ production of acid) or rennet-driven coagulation can readily recover these caseins from skim milk while releasing whey as a by-product (Scheme 1). [20].



Scheme 1: An overview of the acid-mediated and rennet-mediated processes involved in cheese making

1-1.2. Whey

Whey is the major part of milk. This greenish-yellow-coloured liquid, whey also referred to as cheese serum, is obtained by coagulating milk with acids or proteolytic enzymes following the separation of curd [21]. It is a liquid by-product of the milk contains many valuable constituents. Since its high biological oxygen demand and organic constituents levels make it

a major dairy waste, it has been considered a major waste for decades [22]. Previously it was not given that much of importance as it was separated as by-product. It is becoming more and more evident that liquid whey can be transformed into a wide range of valuable human food supplements as a result of the need to reduce waste [23]. In recent decades whey proteins (WP) are popular for their nutritional values and versatile functional properties and are widely utilized in the food industry. An estimate of the worldwide production of whey indicates that approximately 700,000 tonnes [24] of whey proteins are available annually as dietary food ingredients of which about 62% appears to be gainfully utilized [25].

There are two major types of whey, sweet (made by enzymatic coagulation commonly using chymosin) and acid (made by adding organic acids and mineral acids like tartaric, acetic, and fumaric acids). Sweet whey has a pH range between 5.8–6.6 and titratable acidity 0.10–0.2% LA, whereas acid whey has titratable acidity percentage > 0.40% LA, and pH > 5.0 [27].

Generally, fresh liquid whey produced as biproduct of cheese making process, is made of 94.2% water and 50% of the total solids of which 4.3% is lactose, 0.1% is fat, 0.5% is minerals and 0.8% is whey proteins [28]. Whey contains about 25% of total milk protein depending upon source, separation technique and on other environmental factors [29].

1-1.3 Whey Protein

Whey proteins are globular in structure. These molecules contain a high number of α-helix motifs, in which the acidic/basic and hydrophilic/hydrophobic amino acids are fairly distributed in a balanced way along their polypeptide chains. [24]. Whey proteins can induce excellent functional properties such as gelation, emulsification, and foaming [30]. Whey proteins exhibit several health-promoting effects, including anti-inflammatory, antioxidant, and antiviral activities [31]. In addition, whey protein contributes to the increase in lean mass and the enhancement of exercise endurance. Due to their higher leucine content, whey proteins have been proven to stimulate muscle protein synthesis more effectively than other proteins [32].

Table-1: Review of summarized work on whey proteins since last two decades

AIMS AND OBJECTIVE REVIEW	KEY NOTE	REFERANCES
Whey protein properties Use in dairy industry	Whey proteins, influence of high temperature on whey proteins, thermally induced complex between whey proteins and casein, Whey protein-based products, The application of whey proteins and whey protein products in acid fermented products. The application of the whey proteins and whey protein products in cheese making.	Jovanovi´c, Bara´c, and Ma´cej (2005). [33]
biological properties of whey proteins.	Whey protein system, Whey protein concentrates and whey protein isolates, β-Lactoglobulin, α-Lactalbumin, Bovine serum albumin, Immunoglobulins, Lactoferrin, Lactoperoxidase	Madureira et al. (2007) [34]
The physiological properties of bioactive peptides obtained from whey proteins, including the stabilities of such peptides during their gastrointestinal route.	Production of bioactive peptides; peptides with antihypertensive and antithrombotic activities; peptides with opioid and ileum-contracting activities; peptides with antimicrobial and immunomodulatory activities; peptides with nutrition system activities; other peptides with bioactivities; stability of bioactive peptides.	Madureira et al. (2010) [35]
Health benefits of whey proteins, especially research advances about their biological properties	Antimicrobial and antiviral activities, immune modulating activity, anticarcinogenic properties, physical performance, cardiovascular health, weight management, bone health, other health benefits.	Solak and Akin (2012) [36]

AIMS AND OBJECTIVE REVIEW	KEY NOTE	REFERANCES
The most recent advances on the controlled modifications of whey protein structures for specific functionalities	Native whey proteins, Structural characteristics of whey protein assemblies and related functionalities, Surface charge and apparent isoelectric pH values, Surface hydrophobicity.	Guyomarc'h et al. (2015) [37]
Whey protein, its fractions, and the therapeutic effect, and its application in the food processing and pharmaceutical field.	Whey proteins, Whey protein products, Major whey protein fractions, Minor whey protein fractions, Health properties of whey protein fractions such as anti-microbial and anti-viral, anti-oxidant, Immune modulating, and Wound healing.	Kassem (2015) [38]
Selected uses of whey and whey preparations in the food industry.	Meat and meat products, Products with reduced fat content, Bakery and confectionary products, Dairy products, other uses of whey in the food industry.	Krolczyk ´ et al. (2016) [39]
The review compares whey from different milk sources and puts whey proteins in the context of other known food antioxidants.	Do whey products show antioxidant activity in vitro? Can whey products boost intracellular antioxidant defences in vitro? Do whey products act as antioxidant protector in vivo?	Corrochano et al. (2018) [40]
Health benefits of the whey.	Commercial status, health benefits of the Whey that evolves from healthy aging, sarcopenia, bone health, physical performance, sports nutrition, weight management, infant nutrition, immunity, to gastrointestinal support.	Kadam et al. (2018) [41]

AIMS AND OBJECTIVE REVIEW	KEY NOTE	REFERANCES
How whey proteins can modulate cellular redox pathways and conversely how whey proteins can be oxidised during processing.	Whey proteins and their ability to modify redox pathways – studies in animal models and humans, Bioavailable antioxidant whey peptides, Redox modification of whey during processing, as well as oxidation, glycation, and racemisation of whey proteins.	Gilblin et al. (2019) [42]
This work reviewed basic principles of whey protein conjugation to carbohydrates, applicable in food products.	Whey proteins: practical limiting issues and potential solutions, Covalent conjugation via Maillard reaction, Preparation techniques	Doost et al. (2019) [43]
This review highlights the bioactive properties, functional characteristics, associated processing limitations, and applications of different whey protein fractions and derivatives in the field of food formulations, encapsulation, and packaging.	Whey protein derivatives: concentrates, isolates, and hydrolysates; biological properties of whey proteins associated with bioactive peptides; functional properties of whey proteins; current applications of whey proteins and its derivatives	Minj and Anand (2020) [44]

Main constituents of Whey proteins are α-lactalbumin and β-lactoglobulin with lactoferrin, immunoglobulins, glycomacropeptide and bovine serum albumin [45]. This soluble protein fractions of milk, whey proteins easily pass through the stomach and readyly release amino acids into the blood [46, 47].

Table-2: Protein profile of whey and primary structure with basic properties

Whey Protein Components	Concentrations (g/l)	Iso electric point (IP)	% of Whey protein	Molecular weight (KDa)	No of Amino Acid Residue	Benefits
β-lactoglobulin (β-lg)	3-4	5.2	50-55	18.4	162	Source of essential and branched chain amino acids, act as a carrier molecule, modulate lymphatic response, possess excellent gelling properties, stabilizing agent, anti-hypertensive and hypocholesterolemic activity
α-lactoglobulin (α-lg)	1.2	4.7-5.1	20-25	14.2	123	Source of essential and branched chain amino acids, anti-cancerous, anti-proliferative effects, positive effect on gastric mucosa, supplements for infant formulae, increase brain serotonin level
Immunoglobulins (Igs)	0.6-0.9	5.5-8.3	10-15	150-1000		Primary protein found in colostrum immune modulating benefits
Bovine serum albumin (BSA)	0.3-0.6	4.7-4.9	5-10	69	582	Source of EAAs, lipids synthesis, inhibits tumour growth, inhibit the growth of human breast cell line (MCF-7)
Lactoferrins	0.05	8.0	1-2	78	700	Antifungal, antioxidant, promotes growth of beneficial bacteria, naturally occurs in breast milk, saliva, tears, blood and mucus
Lactoperoxidase	0.006	9.6	0.5	89	612	Anti-oxidant, anti-viral, anti-bacterial, iron-binding glycoprotein
Glycomacropeptide	1.2-1.5	4.0-4.8	10-15	8.6	102-169	Source of branched chain amino acids, lacks aromatic amino acids, tyrosine, phenylalanine and tryptophan
Proteose-peptone	0.5		20-25	4-20	136	A mixture of proteins and peptides left in solution after heating/ acidification (pH-4.7)

1-2. The biological features of whey proteins

1-2.1. Antimicrobial and antiviral properties

Whey proteins has been more thoroughly reviewed for their antimicrobial function [48]. Helicobacter pylori-specific antibodies with high WPC produced by lactating cows prevents infections [49].

Proteins may chemically be modified by 3-hydroxyphthalic anhydride (3-HP), viz. β-Lg, α-Lg and BSA, were tested for their antiviral function against human herpes simplex virus type 1 (HSV-1), porcine respiratory corona virus and bovine parainfluenza virus type 3 shows only HSV-1 was sensitive to 3-HP-proteins. [50]. In all cases, chemically modified proteins showed antiviral activity against HSV-1 irrespective of whether they were tested before, during, or after infection.

1-2.2. Anticarcinogenic activities

As reported by Tsuda et al., whey proteins prevent breast and intestinal cancer in female rats when consumed. [51-53]. The whole whey protein system evidently protects against colon and mammary tumors that had been chemically induced in vivo [54, 55]. By increasing GSH concentration in relevant tissues, whey proteins result in anti-tumor effects in low-volume tumors, as they stimulate immunity through the GSH pathway, and they can also deplete tumor cells that contain a higher level of GSH than normal cells, making them more susceptible to chemotherapy [56, 57].

1-2.3. Immune system modulation

In vitro alloantigen-induced proliferation and lymphocyte mitogenesis, in mature murine lymphocytes solutions can be suppressed by whey proteins [58]. Addition of whey proteins from microfiltered-WPI to cell culture media stimulates in vitro proliferation of lymphocytes of murine spleen [59].

Following oral or parenteral administration of bovine LF, delayed-type hypersensitivity responses to a range of antigens, such as sheep red blood cells, ovalbumin, and Calmette-Guerin bacillus, were improved in mice via dose dependent manner [60]. The cysteine and glutamine residues in whey proteins are important for the coordinated T-cell response of macrophages and lymphocytes. This suggests that their ingestion may contribute to increase the level of free cysteine, and consequent production of GSH. In animals, GSH regulates

immunity and prevents cancer, improves immunity and liver function, and overcomes GSH deficiency in seropositives and Alzheimer's patients. Non-specific and specific immunity may be enhanced by whey proteins. In patients with cystic fibrosis, whey-based dietary supplementation increased lymphocyte GSH levels [61, 62]. Also proven effects are found on the patient of liver disfunction exhibiting chronic hepatitis B by this WPC long term consumption. Furthermore, whey proteins inhibit HIV-1 enzymes themselves, which is crucial in attempts to limit the virus' life cycle [63].

1-2.4. Nutritional benefits

A high level of protein breakdown can lead to muscle wasting, but amino acids, especially leucine, can minimize muscle wasting by controlling protein breakdown. In addition, whey protein contains sulphur-containing amino acids (methionine and cysteine), which are precursors of glutathione, an antioxidant, anticarcinogen, and immunostimulant. [64]. Whey proteins apparently facilitate attainment of favourable weight and composition found [65] to be more effective in satiation than caseins. Whey proteins may act as a helping agent towards bone formation and activation of osteoblasts (bone cells)– especially in what concerns proliferation and differentiation of osteoblastic MC3T3-E1 cells [66]. Whey Proteins also exhibit antioxidant activity. Whey products are less potent antioxidants than well-known plant antioxidants such as green tea, but they can be added to food at much higher concentrations. [67].

1-2.5. Therapeutic applications

In the aspect of physiological and biological properties, Caseins differs from whey proteins. Whey protein easily digested in intestine and separated in various EAAs and BCAAs. Whey proteins acts as an inhibitor for the occurrence and growth of chemically induced tumours and shows anticarcinogenic activity [68]. In According to Kent et al., in vitro studies demonstrated that whey protein isolates increased glutathione synthesis and protected human prostate cells from oxidative stress [69]. Whey proteins also act as an hypolipidemic, antioxidant, antitumor, antihypertensive, chelating agent, antibacterial, antiviral, and immunostimulatory agent. In recent cases several studies also show the activity of this whey proteins towards HIV virus, cardiovascular diseases, osteoporosis, and hepatitis B. Whey proteins helps to attainment of favourable body mass.

1-3. Beta lactoglobulin (β-lg)

Beta lactoglobulin is a very common protein widely available from milk of ruminants like sheep, buffalo, cow, goat etc. and in many other mammalian species. It is absent from the milk of rodents, lagomorphs, and humans. The bovine beta-lactoglobulin)β-lg(is one of the major whey protein constituents of cow's milk. Quantitively β-lg is the major product [55% (w/w)] of the whey protein part of the milk. β-lg was first discovered through salt fraction of cow's milk (Palmer 1934) [70]. During ultra sound study on milk and whey, a number of peaks are found in the sedimentation pattern (Pedersen 1936) [71]. Pedersen cognominated the peaks as α, β, γ etc and then identified β-peak as arising from the "Palmer's protein". First X-ray crystallography was obtained by Crowfoot (1938) [72]. Then β-lactoglobulin name was first given by Svedberg)1939([73]. Aschaffenburg and Drewry (1957(introduced the two genetic forms A and B. In case of ruminants, the β-lg exists as dimer, but in monogastric species, like pigs, dolphin, and horses, β-lg exists in a monomeric form of apparent molecular weight of M_r 18,300 Da [74]. It helps in transfer of passive immunity to the new-born, and in regulation of phosphorus metabolism at the mammary gland. β-lg is synthesised in the mammillary gland of the ruminants and then it incorporates with the milk. Ten genetic variants of bovine β-lg is already known. Two most abundant variants are labelled as β-lg A and β-lg B are differs only in two amino acid substitutions, Asp64Gly and Val118Ala, respectively [75]. The quaternary structure depends on the pH of the medium: mainly as a stable dimmer of molecular weight of 36,700 kDa, at pH between 7 - 5.2; as an octamer of molecular weight of 140,000 kDa, at pH

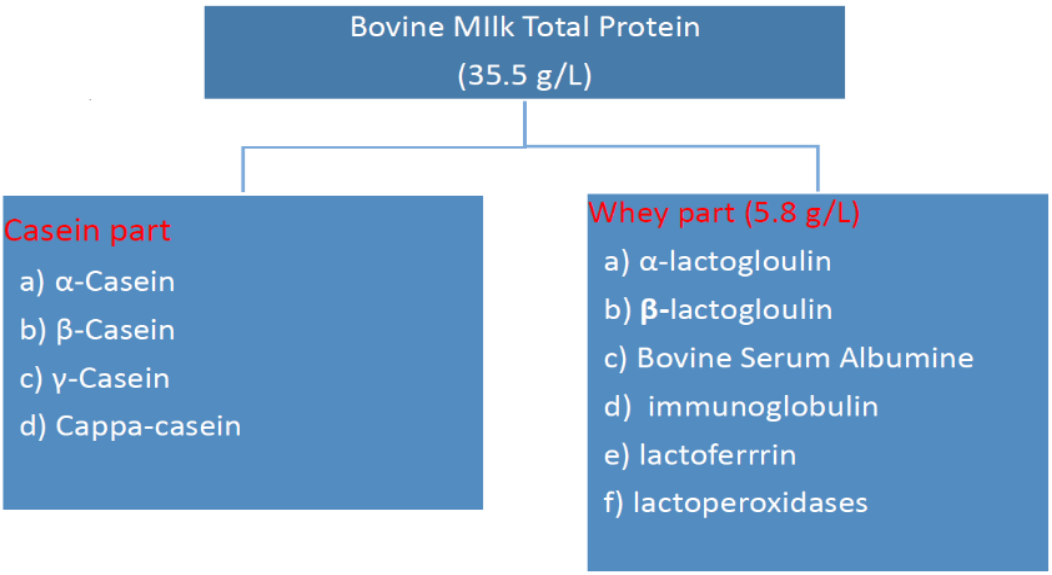


Fig. 1: Schematic Representation of protein content of bovine milk

values between 5.2 - 3.5; and as a monomer, with two-cysteine residues per monomer, at pH 3.0-0.2 and above 8.0 [76]. β-lg can binds with small hydrophobic ligands, such as fatty acids, retinol, triacylglycerols, protoporphyrin IX, alkanes, aliphatic ketones, aromatic compounds, cholesterol, vitamin D, palmitic acid and calcium (at pH 5.0) [77]. Considering its ability to bind hydrophobic molecules, it has been used to improve the encapsulation properties of liposomes and to sustainably deliver vitamin E to the body due to its ability to bind to hydrophobic molecules [78]. It shows several others therapeutic applications. β-lg is well studied protein for its biological function, availability, and separation technique. We choose this single whey protein, β-lg, as our model protein.

Table 3: Past Developments in isolation and purification of bovine β–lactoglobulin

Year	Isolation, Purification and X-ray Crystallographic Study	Reference
1934	Precipitation at pH 5.8 followed by Na2SO4 precipitation at 30ºC, dialysis and crystallization.	Palmer, 1934 [70]
1938	X-ray Crystallographic Study of "Palmer"s lactoglobulin"	Crowfoot et al., 1938 [72]
1941	X-ray work on β-lg crystal.	Crowfoot, 1941 [79]
1957	Na_2SO_4 precipitation at 40ºC followed by acidic precipitation at pH 2, dialysis and crystallization.	Aschaffenburg & Drewry, 1957 [74]
1959	X-ray diffraction study of derivatives of β-lactoglobulin	Green, 1959 [80]
1965	X-ray Crystallographic Study of β-lactoglobulin	Aschaffenburg et al., 1965 [81]
1967	(NH4)2SO4 precipitation at 20ºC, acidic precipitation at pH 2 followed by Na2SO4 precipitation at pH 6, dialysis and crystallization. Separation of β-lactoglobulin by trichloroacetic acid.	Fox et al., 1967 Armstrong et al., 1967 [82, 83]
1986	Crystal structure of orthorhombic lattice Y form.	Papiz et al., 1986 [84]
1987	Centrifugation, CaCl2 precipitation at pH 6.6, dialysis followed by anion exchange gel filtration and crystallization (lattice Z structure based upon lattice Y).	Monaco et al., 1987 [85]
1990	Gel filtration using Sephacryl S-200 and purification by diethylaminoethyl ion exchange chromatography	Yoshida, 1990. [86]
1991	Acidic precipitation at pH 4.6, Centrifugation, filtration followed by bio-affinity column chromatography.	Chiancone & Gattoni, 1991 [87]
1994	Large scale separation of β-lactoglobulin.	Mate & Krochta, 1994[88]
1996	X-ray Crystallographic Study of β-lactoglobulin.	Rocha et al., 1996 [89]
1997	Centrifugation, acidic precipitation at pH 4.6, filtration, dialysis, and gel filtration.	Felipe and Law, 1997 [90]

Year	Isolation, Purification and X-ray Crystallographic Study	Reference
	Acidic precipitation at pH 4.6, Centrifugation and N-retinyl-celite affinity chromatography. Triclinic lattice X at 1.8 Aº resolution and lattice Y at 2.1 Aº resolution. Isolated by combining a precipitation process and a diafiltration process.	Heddleson et al., 1997 [91] Brownlow et al., 1997 [92] Caessens et al., 1997 [93]
1998	Crystallographic presentation of external ligand-binding sites.	Sawyer et al., 1998 [94]
1999	Crystallographic three dimentional aspects of β-lactoglobulin and functional properties.	Sawyer et al., 1999 [95]
2001	Centrifugation, acidic precipitation at pH 4.4-4.5, base precipitation at pH 7.2, centrifugation followed by anion-exchange and gel filtration Chromatography	de Jongh et al., 2001[96]
2008	10-90% (NH4)2SO4 precipitation, cation-exchange chromatograph, dialysis and lyophilization.	Lozano et al., 2008[97]
2010	Gel filtration Chromatography at low pH using a Bio-Gel P10 column.	Naqvi et al., 2010 [98]
2012	One step method by anion exchange chromatography, high-performance liquid chromatography and mass spectroscopy.	Stojadinovic et al., 2012 [99]
2014	Combining ion exchange chromatography and gel filtration by adsorbed on DEAE-Sepharose FF packed column and Sephadex G-75 gel and SDS-PAGE.	Buyanbadrakh et al., 2014 [100]
2015	Gel filtration chromatography using Sephacryl S-200 at acidic pH 4.6	Aich et al., 2015 [101]

1-3.1 Molecular structure of bovine β-lactoglobulin

Due to its high abundance and relatively easy purification from easy source milk and to its propensity to form magnificent crystals, β-Lg was an early target of x-ray diffraction. In retrospect, this was a very ambitious project because β-lg was not the easiest protein to analyse partly because of the multiple crystal forms [102]. This study established that the monomer exhibits spherical shape with a block of electron density with a rod-like structure across one face. The secondary structure of β-lg was predicted to contain ~50% β-sheet ~15% α-helix, and 15–20% reverse turn [103]. Few residues present in the extended part of the native protein have a nascent propensity to form α-helical structures in the presence of trifluoroethanol or amphiphiles [104-106]. In 1986, the first medium resolution structure of β-lg was published [84]. β-lg has predominantly β-sheet structure (Fig. 3) with eight stranded anti-parallel (A-H) β-barrel, one small three turn α-helix on the outer surface and a β-strand 'I' immediately before

C-terminal end [84]. The crystal structures revealed till date, the typical lipocalin to be an eight stranded anti-parallel β-barrel oriented to form a hydrophobic calyx where hydrophobic ligands/ molecules are encapsulated [85. 92]. It is a globular protein, having 162 amino acid residues and a molecular mass of 18400 [107]. β-lg has a conserved three-dimensional structural domain (sequence motifs) and small hydrophobic molecules binding and

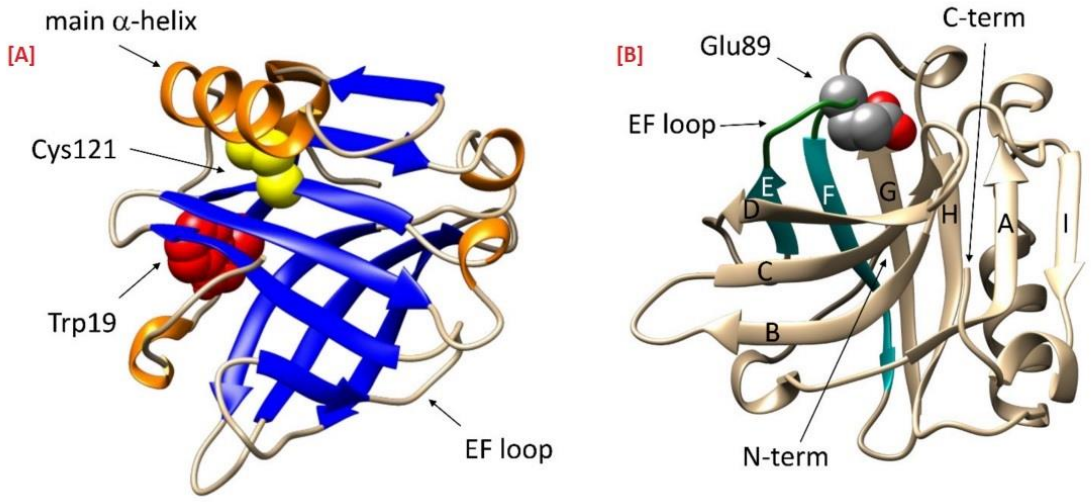


Fig. 2. β-lg native structure relevant to folding stability studies. In (A): Cys121 (yellow) lies underneath the main alpha-helix (orange); Trp19 (red) lies below the beta-barreled calyx (blue). In (B), protonation of Glu89 (in CPK colours) triggers closure of the "lid" formed by the EF loop (in dark green). Structures were generated using the free graphical software UCSF Chimera (version 1.14, University of California, San Francisco, CA, USA).

transportation property for which it is typified to the lipocalin family (Sawyer and Contopidis., 2000) [108]. β-lg has extensive sequence homology with the retinol binding protein and its function is thought to be the binding and transportation of retinol in mammals (Pervaiz et al., 1985; Perez et al., 1992) [109, 110]. β-lg exists as dimmer at physiological pH. Below pH 3.0 and above pH 7.0 it exists as a monomer. At pH 2.0, β-lg dissociates into its monomer form with a native conformation (McKenzie and Sawyer., 1967; Sakai et al., 2000) [111, 112]. β-lg is resistant to acid hydrolysis as well as pepsin digestion [113, 114, 115]. This pH dependent monomer ←→ dimmer transformation is strictly attributed to non-covalent and hydrophobic interactions [116]. The x-ray crystallographic presentations of β-lg shows that A—>D strands participate in formation of one side of the β-barrel and strand E—>H are involved in other side whereas strand 'I' is projected outward the calyx acting as dimmer interface by ionic strength/ salt dependent or through hydrogen bonding [117, 118].

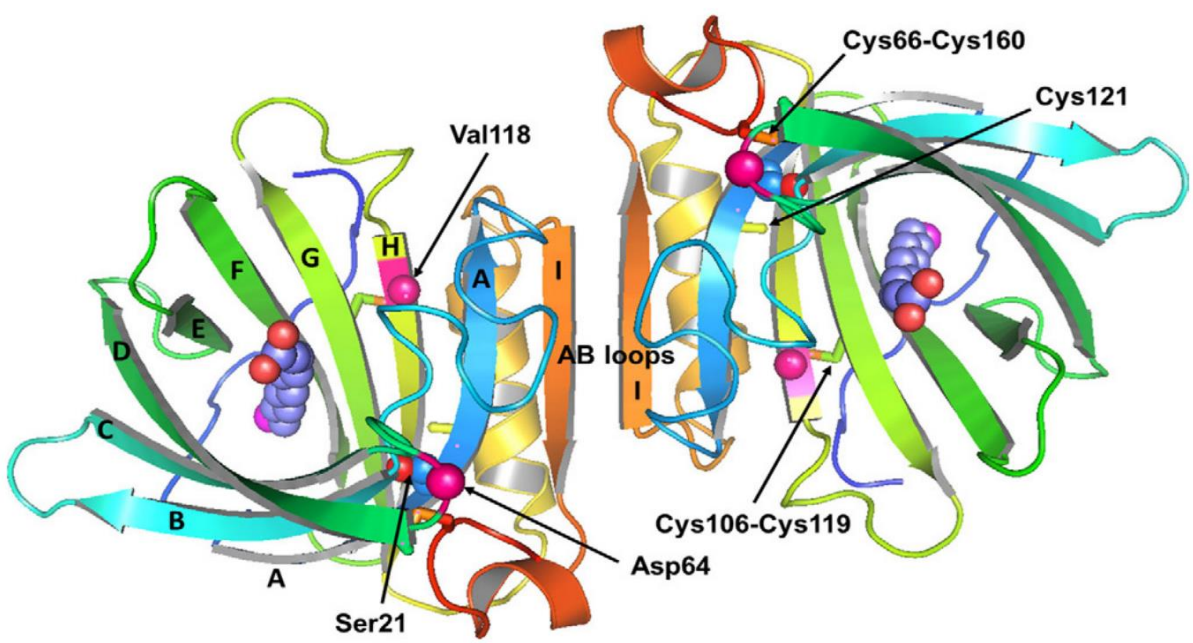


Fig. 3. Diagram of the dimeric structure of bovine β-lg A looking down the two-fold axis. The coordinates were taken from the structure of β-lg A in the trigonal Z lattice with 12-bromododecanoic acid bound (PDB code: 1bso). As shown in the diagram, the A to H strands form the barrel. I strand, together with part of the AB loop, forms the dimer interface at neutral pH. The locations of the differences between A and B variants are also shown. A rainbow-coloured structure begins with blue at the N-terminus and ends with red at the C-terminus. The 12-bromododecanoate anion and Ser21, which shows conformational flexibility, are shown as spheres. The figure was drawn using PyMOL (Delano, 2002).

In EF loop consisting of amino acid 85-90, the Glutamic acid (Glu89) plays a key role for the entry of the small hydrophobic molecule in the calyx of β-lg accompanied by the 'lid-motion' along with the Serine residue (Seri 16) via H-bond formation in pH dependent manner [117, 119, 120, 121]. β-lg has high helical propensity despite being a predominantly β-sheet protein. During its refolding from the fully unfolded state an intermediate with non-native α-helical structure accumulates because the local interactions between neighbouring amino acid residues favour the α -helical structure (Hamada et al., 1996) [122]. β-lg forms disulphide linked co-valent dimers or oligomers accompanied by the free thiol (Cysl21) group or disulphide exchange process during unfolding/ refolding path way under thermal stress [123. 124, 125, 126, 127]. Cys106 and Cys119 residues forms the disulphide bridge in the β-G and β-H strands, provides the α-helix remains packed against the exterior of the calyx. However, this disulphide bridge is not solvent-accessible and is shielded

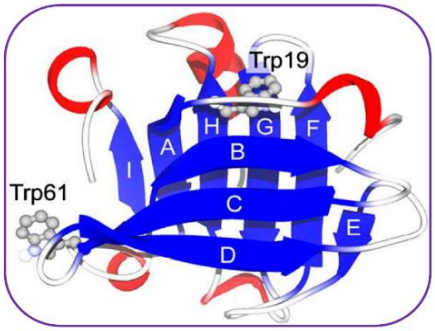


Figure 4. Tryptophan residues in β-lg

from the thiol of Cys121 by the side chains of Phe136, Ala139 and Leu140. Cys66-Cys160 makes the second disulphide bridge fastens the C-terminal loop to the exterior of the calyx [128]. Presence of Aromatic residues such as two tryptophan, four tyrosine and four phenylalanine residues make β-lg fluorescence active. These groups are also responsible for the near UV CD of β-lg spectra which helps in investigating site-specific conformational changes.

1-3.2 Secretion and amino acid sequence of β-lg

β-lg, as most of the milk proteins is produced in the secreting epithelial cells of mammary gland, under the control of prolactin hormone [129]. Messenger RNA coding β-lg is synthesized in the mammary gland, and it is translated into 180 amino acid long pre-β-lg [130]. Pre-β-lg contains a highly conserved signal peptide of 18 amino acids. The mature protein itself contains 162 amino acids and has a molecular weight of 18400D [107]. Milk is secreted from the bovine mammary gland upon fusion of lactose and milk protein-containing secretory vesicles with the apical plasma membrane. [131] The amino acid sequence of bovine **β-lg A** and **β-lg B**:)www.pdb.org, code IBEB(are as follows:

β-lg A:

1 LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR YVEELKPTP
51 EGDLEILLQK WENDECAQKK IIAEKTKIPA VFKIDALNEN VLVLDTDYK
101 KYLLFCMENS AEPEQSLVCQ CLVRTPEVDD EALEKFDKAL KALPMHIRLS
151 FNPTQLEFQC HI 162

β-lg B:

1 LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR YVEELKPTP
51 EGDLEILLQK WENGECAQKK IIAEKTKIPA VFKIDALNEN VLVLDTDYK
101 KYLLFCMENS AEPEQSLACQ CLVRTPEVDD EALEKFDKAL KALPMHIRLS
151 FNPTQLEFQC HI 162

Scheme 2: Amino Acid sequence of β-lg A and β-lg B. Change in amino acid sequences)position 64 and 118(in variants A and B has been highlighted in red and violet colour.

These two genetic variants only differ in two positions of the amino acid sequence. (Asp 64→Gly64 and Val 118→Ala 118) (Sawyer & Konotopidis, 2000) [107] and isoelectric point of β-lg A and β-lg B are 5.1 and 5.3 respectively. Both this variant contains two tryptophan residues (Trp 19 and Trp 61 residues). Trp19 residue remain hidden into the hydrophobic calyx of native β-lg, shows the major fluorescence property of β-lg. [Qin et al., 1998; Uhrinova et al., 2000] [117, 132] β-lg also have 4 tyrosine residues at positions 20, 41, 99, 102 in the amino acid sequence and have 15 lysine residues.

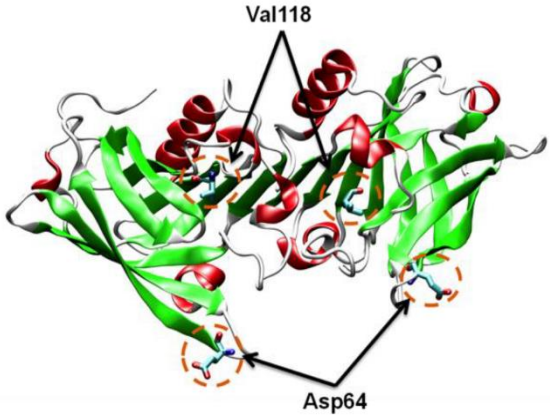


Fig. 5. Cartoon representation of the β-lg A dimer. The residues Asp64 and Val118 are represented within orange circles on both monomers in licoricey and coloured by atom type. These residues are substituted by Gly64 and Ala118 in β-lg B. [107]

However, the Asp64Gly substitution in these two genetic variant results in the alteration of CD loop conformations (Qin et al., 1999) [133]. The Val118Ala substitution causes no change to the structures, but the presence of bulky isopropyl substituent creates void space in the hydrophobic core of the B variant being less well packed, and may exhibit less thermal stability under some measurement conditions [133].

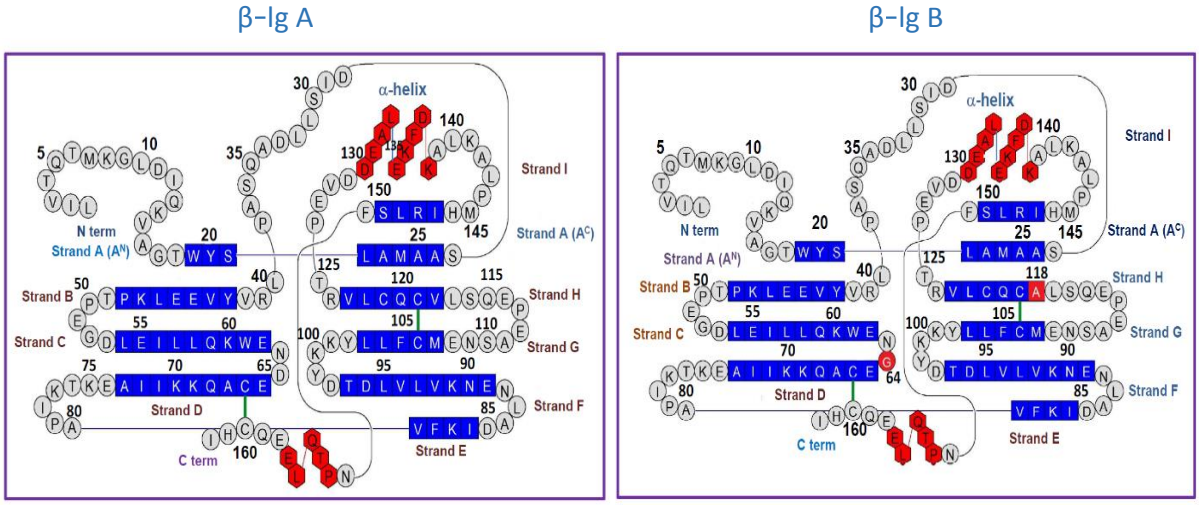


Fig. 6. Diagram illustrating the amino acid residues of the β-lg A and β-lg B sequence. Residues making up the α-helix, β-sheet, and loop are represented by hexagons in red, squares in blue, and circles in grey, respectively. Disulfide bonds are indicated by green lines. The substitutions Val118Ala (A→B) and Asp64Gly (A→B) are shown in red square and red circle respectively.

1-3.3 Structure of Bovine β -Lactoglobulin in Aqueous Solution

Using NMR spectroscopy protein structures in solution phase is developed [134]. The NMR technique is useful for monomeric proteins with molecular weights <∼25 kDa and usually requires recombinant singly (^{15}N) or doubly (^{15}N / ^{13}C) labelled material for protein molecules with molecular weights >∼8 kDa. Therefore, the NMR studies of bovine β-lg generally carried out at pH 2 to 3 and importantly at very low ionic strength, where the molecule is in monomer form. NMR study confirmed the presence of the eight-stranded β-barrel on wild-type-B variant [135]. Two groups from Tokyo and from Edinburgh and Palmerston North determined the full structure of β-lg by NMR techniques independently and near-simultaneously, this has provided very useful comparisons [136].

Kuwata et al. (1999) [137] and Uhrínová et al. (2000) [132] developed the β-lg structure in solution phase which indicates overall similarity to that established earlier structure by x-ray crystallography at pH 6.2.

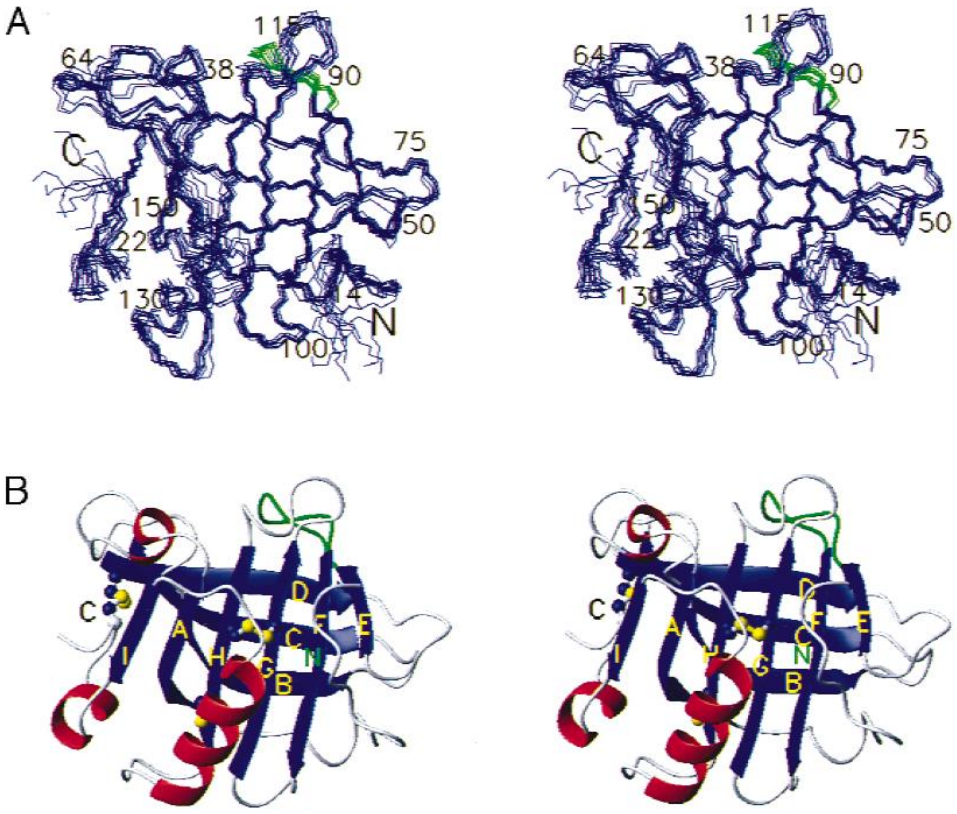


Fig. 7. (A) NMR and (B) X-ray structures of bovine β-lactoglobulin. An X-ray coordinate (3β-lg) and secondary structure locations are available from the Brookhaven PDB. A diagram illustrating the superimposition of the 17 NMR structures was created using MOLMOL (Koradi et al., 1996) [138]. Disulphide bonds (Cys66–Cys160 and Cys106–Cys119) and a free thiol group (Cys12) is shown in (B), helical regions are coloured red, and loop EF is a green colour.

It is quite evident that the protein's surface charge increased due to the decreased pH (which, in turn, caused the protein's pH to be lower) required to obtain usable NMR spectra from monomeric bovine β-lg. It is apparent from the X-ray structures that the side chains of the Glu89 'latch' are buried under the EF loop at pH 6.3, just as they are in the EF loop at pH 6.3. There is one major difference between the X-ray structure of the Z lattice structure at pH 6.2 and the structure of the three-turn a-helix at pH 6.2 [117], which is that the three-turn a-helix adopts a different position in relation to the β-barrel, perhaps as a result of the positive charge increased on the surface of the protein as a result of pH change [132]. As a result, the lack of a dimer interface at low pH also makes it possible to free up some restraints involved in the conformation of the AB loop, which acts as a constraint on the position of the helices when in dimers, but not in direct contact (see Fig. 7).

There was also a difference in the N- and C-termini; these differences may be explained because recombinant proteins with non-native N termini were synthesized for the NMR structure, while crystal packing effects may have affected the X-ray structure. Many of the residues from residues 152 to 162 in the C-terminus are either completely absent or poorly defined in electron density maps in some crystal forms.

1-3.3.1. The Monomer–Dimer Equilibrium of Bovine β-Lactoglobulin in Aqueous Solution

Over a long period of time, several techniques have been used to study the monomer-dimer equilibrium of bovine β-lg, such as analytical ultracentrifugation (AUC), light scattering, SAXS, and isothermal dilution calorimetry. Here Table-4 shows a comprehensive and critical tabulation of β-lg variants [139]. Although dimer dissociation is an essential step in the denaturation of bovine β-lg and its subsequent chemical processes, the rate constants associated with this phase have never been measured. In an essentially constant ionic strength medium of 100 mM NaCl, analytical centrifugation measurements confirmed earlier values for dimer dissociation equilibrium. Table 4 summarizes equilibrium constants and rate constants where possible. Equilibrium dissociation constants KD are 10 mM at pH 2.5 to 3.5 and 6.5 to 7.5. Towards the isoelectric point, KD decreases significantly and cannot be detected by the standard AUC method. This association rate constant is well outside the diffusion-controlled limit, and the dissociation rate constant is also quite slow. According to Mercadante et al. (2012) [139], this is due to the considerable restructuring of counter-ions during dimerization.

Table 4: Equilibrium[a] and Rate[b] Constants Calculated for β-lg A and β-lg B Dimer Dissociation over the pH Range 2.5 to 7.5 in 100 mM NaCl from Global Fits[c] of SE and SV Data

	$K_D^{2\text{-}1}$ (/μM)		k_{off} (/s^{-1})		k_{on} (calc.) (/M^{-1} s^{-1})	
	βLg A	βLg B	βLg A	βLg B	βLg A	βLg B
pH 2.5[d]	15 ± 3	8 ± 3	0.008 ± 0.005	0.009 ± 0.005	540	1,100
pH 3.5[d]	4.0 ± 1.5	1.4 ± 1.5	[0.007][f]	[0.041][f]	---[f]	---[f]
pH 6.5[e]	4.0 ± 1.5	2.5 ± 1.5	Fast (> 0.1)	Fast (> 0.1)	>25,000	>40,000
pH 7.5[e]	11 ± 3	9 ± 2	Fast (> 0.1)	Fast (> 0.1)	>9,200	>12,000

[a]Calculated from global fitting of sedimentation velocity (SV) and sedimentation equilibrium (SE) data.
[b]Calculated from global fitting of SV data (SE data contain no kinetic signal).
[c]Calculated error ranges represent the sensitivity of the values to changes in other fitting parameters.
[d]Citrate buffer.
[e]3-(N-morpholino)propanesulfonic acid (MOPS) buffer.
[f]Indicative value, as no error range could be determined; k_{on} not calculated.
Reproduced from Mercadante, Melton et al., 2012.

The counter-anions are restructured substantially at low pH, where the β-lg is strongly positively charged, and when pH is >7, where it is mildly negatively charged, the counter-cations are restructured and distance dependence is sharp for optimizing hydrophobic contacts. In isothermal dilution calorimetry at temperatures up to 35°C, the variant B of β-lg dissociates at a slower rate than variant A [139]. Dissociation constants measured by analytical ultracentrifugation (Table 4) are very similar to those determined by pH 7.0 phosphate buffer and 100 mM NaCl at 14.5(1) mM. Small extracellular proteins that possess similar properties, such as binding hydrophobic molecules and ligands to specific cell surface receptors, are termed lipocalins. There are 160-180 amino acids in lipocalins and they usually have a vastly preserved crystal structure (sequence motif) but a low degree of sequence similarity (Newcomer et al., 1984; Virtanen, 2001) [140, 141]. Normally "Kernel lipocalins" share three preserved sequence motifs (α-helix on the outer surface of β-barrel and amino acid sequence contains three structurally preserved regions together with one or more disulfide bridges) on the other hand "outlier lipocalins" have only one or two motifs [142, 143]. The presence of lipocalins can be found in a wide range of living organisms, including vertebrates, invertebrates, plants, and bacteria. Some lipocalins from different living sources are shown in Table 4.

1-4. The Lipocalins and β-lactoglobulin: Structure and Function

The lipocalin are the family of protein having ability to bind with the small, principally hydrophobic molecules steroids, bilins, retinoids, and lipids, specific cell surface receptors and form macromolecular complexes. The structural features of the lipocalin fold, a large cup-shaped cavity, within the β-barrel, and a loop scaffold at its entrance, are well adapted to the task of ligand binding: the amino acid composition of the pocket and loop scaffold, as well as its overall size and conformation, determining selectivity. This is an eight stranded antiparallel beta barrel with a repeated +1 topology enclosing an internal ligand binding site [144].

Table 5: list of Human Lipoclains [145]

Official Symbol	Official Full Name	Gene ID	Chromosome Location
AMBP	alpha-1-microglobulin/ bikunin precursor	259	9q32-q33
APOD	apolipoprotein D	347	3q29
APOM	apolipoprotein M	55937	6p21.33
C8G	complement component 8, gamma polypeptide	733	9q34.3
CRABP1	cellular retinoic acid binding protein 1	1381	15q24
CRABP2	cellular retinoic acid binding protein 2	1382	1q21.3
FABP1	fatty acid binding protein 1, liver	2168	2p11
FABP12	fatty acid binding protein 12	646486	8q21.13
FABP2	fatty acid binding protein 2, intestinal	2169	4q28-q31
FABP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	2170	1p33-p32
FABP4	fatty acid binding protein 4, adipocyte	2167	8q21
FABP5	fatty acid binding protein 5 (psoriasis-associated)	2171	8q21.13
FABP6	fatty acid binding protein 6, ileal	2172	5q33.3-q34
FABP7	fatty acid binding protein 7, brain	2173	6q22-q23
FABP9	fatty acid binding protein 9, testis	646480	8q21.13
LCN1	lipocalin 1	3933	9q34
LCN10	lipocalin 10	414332	9q34.3
LCN12	lipocalin 12	286256	9q34.3
LCN15	lipocalin 15	389812	9q34.3
LCN1P1	lipocalin 1 pseudogene 1	286310	9q34.2
LCN2	lipocalin 2	3934	9q34
LCN6	lipocalin 6	158062	9q34.3

Official Symbol	Official Full Name	Gene ID	Chromosome Location
LCN8	lipocalin 8	138307	9q34.3
LCN9	lipocalin 9	392399	9q34.3
LCNL1	lipocalin-like 1	401562	9q34.3
OBP2A	odorant binding protein 2A	29991	9q34
OBP2B	odorant binding protein 2B	29989	9q34
ORM1	orosomucoid 1	5004	9q32
ORM2	orosomucoid 2	5005	9q32
PAEP	progestagen-associated endometrial protein	5047	9q34
PMP2	peripheral myelin protein 2	5375	8q21.3-q22.1
PTGDS	prostaglandin D2 synthase 21kDa (brain)	5730	9q34.2-q34.3
RBP1	retinol binding protein 1, cellular	5947	3q23
RBP2	retinol binding protein 2, cellular	5948	3q23
RBP4	retinol binding protein 4, plasma	5950	10q23.33
RBP5	retinol binding protein 5, cellular	83758	12p13.31

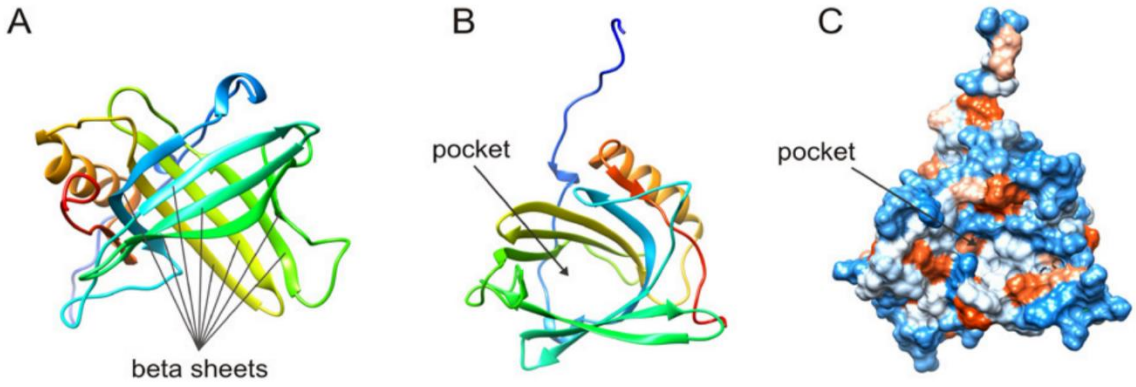


Fig. 8. Highly conserved lipocalin crystal structures consist of a single eight-stranded continuously hydrogen-bonded antiparallel β-barrel (A) delineating a calyx shape, which represents the internal ligand binding site (B). Hydrophobicity surface (C). Images were created from the RCSB PDB database (http://www.rcsb.org) (ID: 1NGL) using the UCSF Chimera package UFCS Chimera package that is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311) [146].

Apparently, there are lipocalin sequences from mammals (human origin) that are allergens (Table 8). Some of them are food allergens and some are respiratory allergens. Baby milk has been identified as a food allergen, but it is not clear why it is so allergenic. In 2004, Natale et. al. [166] reported that 45 percent of patients with allergic reactions to cow's milk were immunoglobin E antibodies specific to Ig E (L)-L (Ig E E E E).

Table 6: Lipocalin allergens [147]

Allergens	Animal	Remarks
Bla g 4	Cockroach	An indoor allergen in the warm humid climate.
Bos d 2	Cow	An occupational allergen in the farming environment.
Bos d 5	Cow	β-Lactoglobulin, a food allergen in cow's milk.
Can f 1	Dog	An indoor allergen sensitizing in homes, public places and in laboratory animal facilities.
Can f 2	Dog	Another dog allergen.
Cav p 1	Guinea pig	An indoor allergen sensitizing in homes and in laboratory animal facilities.
Cav p 2	Guinea pig	Another guinea pig allergen.
Equ c 1	Horse	An allergen related to occupational exposure and horse riding.
Equ c 2	Horse	Another horse allergen.
Fel d 4	Cat	A recently characterized feline allergen in the lipocalin family.
Mus m 1	Mouse	An indoor allergen sensitizing in homes and in laboratory animal facilities.
Ory c 1	Rabbit	An occupational allergen in laboratory animal facilities.
Ory c 2	Rabbit	Another rabbit allergen.
Rat n 1	Rat	An occupational allergen in laboratory animal facilities.
Tria p 1	Kissing bug	A salivary allergen from Triatoma protracta found in many regions of the Western Hemisphere.

Table 7: Mammalian Lipocalins from Human Origin [148]

List of Lipocalins	List of References
Tear Lipocalins（Tlc）	Redl, 2000; Breustedt et al., 2005 [149, 150]
Retinol Binding Protein（RBP）	Cowan et al., 1990; Zanotti & Berni, 2004 [151, 152]
α1－ microglobulin（α1m）	Akerstrom et al., 2000b; Larsson et al., 2004 [153, 154]
Glycodelin（GLy)	Koistinen et al., 1999, Halttunen et al., 2000 [155, 156]
Neutrophilic Lipocalin（NGAL）	Kjeldsen et al., 2000, Goetz et al., 2002 [157, 158]
Apolipoprotein D（ApoD）	Rassart et al., 2000 [159]
Complement component 8? （C8?）	Schreck et al., 2000, Ortlund et al., 2002 [160, 161]
Prostaglandin D Synthase（PGDS）	Urade & Hayasishi, 2000 [162]
Odorant Binding protein (OBP）	Tegoni et al., 2000, Briand et al., 2002 [163, 164]
A-1-acid glycoprotein（AGP）	Fournier et al., 2000 [165]

A small peptide (97-117) is bound to the first histocompatibility complex of [167, 168] and a large peptide bound to the second histocompatibility complex [169] contains the B-cell epitopes (amino acid residues 21-40, 41-60, 102-145, 148-168). Sakaguchi et al., 2002 identified two other peptide fragments: 30-47 and 142-162. As well, fragment 101-112 was also a predictable epitope region buried within the core of the β-lg molecule [170]. The most important and common type of allergy is lg-E assisted atopic allergy, which affects nearly 2.4% of babies in westernized countries [171]. As a result of this, β-lg, a lipocalin protein, is a significant food allergen.

1-5. The bioavailability of β-lactoglobulin

Bovine β-lg is the most important protein found in milk's whey. It is usually found in milk of animals such as cows, dolphins, baboons, pigs, sheep, horses (Conti et al., 1984) [172], goats, cats (Halliday et al., 1991) [173], buffaloes (Aich et al., 2014) [174], and bison among others. Human milk does not contain it (Sawyer & Kontopidis, 2000) [107]. Its average concentration in milk is 3 gL-1 [175]. Comparison of string sequences among lipocalins reveals that glycodelin, found in the human endometrium during early pregnancy, is most similar to β-lg (Koistinen et al., 1999; Halttunen et al., 2000) [176,156]. In 1972, Larson, reported Only glycodelin is established in human source. β-Lg is produced and secreted from the epithelial cells of the mammary gland under control of the prolactin hormone [129]. This type of transcript is synthesized in the mammary gland, after which it is translated into a later form of mRNA coding for a globin with 180 amino acids [130]. This is due to the existence of the signal peptide, which is a highly conserved peptide consisting of 18 amino acids, which precedes the 162 amino acids in the linear chains of β-lg. There are 162 amino acids in this linear peptide chain that are responsible for refolding to the native conformer for the linear peptide. Glycodelin has thus far not been revealed to have distinct functional aspects, but lipocalins are linked to β-lg [175].

1-6. Structural stability of bovine β-lactoglobulin

1-6.1. Impact of Heat-Processing on β-Lactoglobulin Structure

Bovine β-lactoglobulin is a globular protein that undergoes conformational changes based on pH or heat. The native noncovalently bound dimeric structure of lactoglobulin can be found to be the most thermodynamically stable structure in milk, and a small fraction of the protein can

be found in metastable partially unfolded monomeric states as well. This relationship, as a result of the dissociation of hydrogen bonds in the hydrogen bond network [177] may be shifted by heating and other types of processing and conditions [178-181]. A sedimentation velocity analysis revealed that β-lactoglobulin acquires monomer–dimer equilibrium at 100 mM NaCl and pH 2.5. Its initial conformation shift upon heating results from destabilization and partial unfolding of its globular structure, exposing histidine, tyrosine, and tryptophan residues to the solvent [181] with increased reactivity of buried thiol groups [182]. These limited and reversible conformational changes are followed by dissociation of intramolecular interactions and occupation of a partially unfolded, so-called molten globule state [183], reviewed in [184], which is also populated upon refolding of β-lactoglobulin [185], yielding exposure of the thiol group and the buried hydrophobic core of the protein [186]. Despite initial conformational switches occurring at temperatures as low as 40°C [187], fully denatured "β-lactoglobulin" can only be obtained at temperatures exceeding 130°C [188 – 190], suggesting a multistep process. The midpoint of transition between patterns of abrupt large-scale loss of spiral structural elements occurs around 65 °C [190], depending on environmental conditions.

It has been widely reported that partially denatured β-lactoglobulin proteins are prone to thiol/disulphide exchange reactions, which result in covalent aggregation, with non-covalent interactions playing a minor role [191- 199]. It has been observed under low salt, neutral pH conditions a quantitative kinetic model that describes the irreversible aggregation of β-lactoglobulin, as a function of thiol/disulphide exchange reactions at 60-75°C [199], upon heating at that temperature and then with increasing salt levels. As a result of radical polymerization reactions, involving initiation, propagation, and termination steps. These steps correspond to the exposure of a free sulfhydryl group, the exchange of a thiol for a disulphide, and the reaction of two reactive intermediates, respectively. In test tube conditions, studies like these have influenced our understanding of how β-lactoglobulin is denatured and aggregated. However, in many cases, these conditions are very different from the complex environment in milk, containing lipids, lactose, and other proteins. A recent study has demonstrated that β-lactoglobulin receptors are able to interact with other milk constituents, such as α-lactalbumin, caseins, and bovine serum albumin, as well as lactose and lipids, resulting in remarkably different behaviour of the β-lactoglobulin. The impacts of such interactions on β-lactoglobulin behaviour are discussed in bellow.

1-6.2. Effect of pH on bovine β-lactoglobulin:

Bovine β-lg undergoes a variety of local and global structural changes induced by pH. It exists as a dimer at neutral pH (pH 7) but dissociates into monomeric species with a pH of 2. At pH 0.92, most of the secondary structure of β-lg which has been reported by N. Taulier [200]. Between pH 2 and pH 1.5, the protein volume and compressibility increase, but decrease sharply below pH 1.5. According to N. Taulier, this transition is difficult to explain as all ionizable groups should have been protonated by pH 2 [200]. So, this transition may be due to the protein undergoes some electrostatically driven structural changes in response to an increased concentration of Cl- anions. The monomeric population at pH 2 attributed to the residues of the conformer of β-lg sited in the AB loops.

Specifically, near pH 3, the protein dimerizes with little alteration in structure [201]. Investigations into the monomer-dimer equilibrium has provided the information about the dimeric interface [202]. It should be noted that between pH 4 and pH 5, β-lg is reported to undergo the dimer-to-octamer transition as suggested by a variety of biophysical methods including optical rotation dispersion spectroscopy, ultracentrifugation, electrophoresis, light scattering, and NMR spectroscopy [203 - 205]. In particular, Timasheff et al. proposed that the N to Q transition facilitates octamer formation [206]. In contrary to these reports, no significant octamerization of the protein was found by N. Taulier around pH 4.5 [200]. McKenzie and Sawyer also reported that at pH 4.5, the dimer remains the persisting unit [207].

1-6.3. Tanford transition

The pH-induced transition of β-lg, which occurs between pH 6 and pH 8, was first described 50 years ago by Tanford and collaborators and commonly referred to as the Tanford transition [208]. EF loop displacement opens the interior of the calyx above pH 7.5, which is the main structural change of the protein [209]. Protonation of this residue is thought to trigger the Tanford transition. The Tanford transition occurs in conjunction with the expansion in the volume of the protein molecule with the opening of the EF loop, which is closed below pH 6.2 and opens above pH 7.1, and, therefore, the carboxyl group of Glu89, buried and exhibiting an anomalously high pKa value until now, becomes accessible to the solvent [210].

(A) pH 6.2 closed form. (B) pH 7.1 open form.

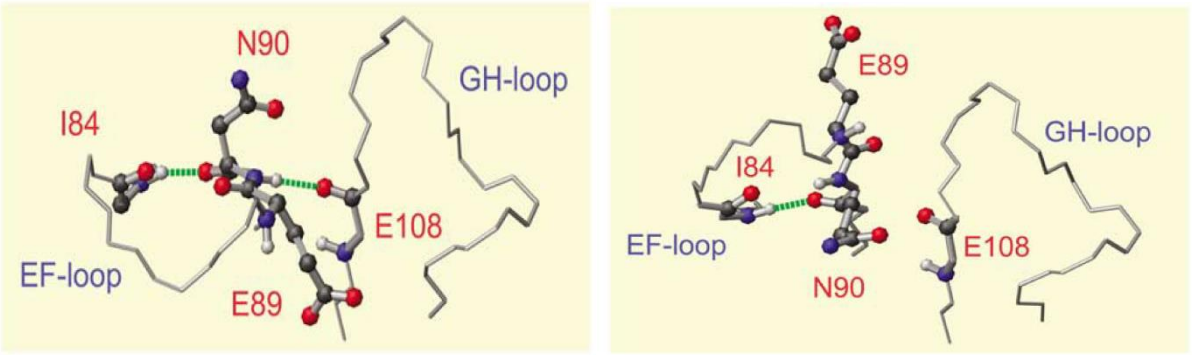


Fig. 9. Comparison of the closed and open forms of the β-lg crystal structure obtained at pH 6.2 and at pH 7.1, respectively. (A) and (B) Close-up of the EF-loop and GH-loop regions at (A) pH 6.2 and at (B) pH 7.1, respectively. Ile84, Glu89, Asn90, and Glu108 are shown as ball-and-stick. Broken green lines represent the backbone hydrogen bonds of Asn90 with Ile84 and Glu108. The hydrogen bond between Asn90 and Glu108 is not present in the open form. The sidechains are presented only for Glu89 and Asn90. The orientations of their side-chains are completely inverted between the two structures.

At low pH, loop EF serves as a door or lid to block access to the calyx. Tanford transition involves the alterations in secondary and in local microenvironment of aromatic residues of β-lg, with these alterations being rather local and causing no global change in protein conformation global change in protein conformation.

1-6.4. Base-induced transition

pH Dependent conformational change of EF loop is a characteristic feature to β-lactoglobulin. At pH 7.1 and 8.2, loop EF is folded back (open conformation) to reveal the interior of the calyx of β-lg. pH dependent property of β-lg was that an increase in pH (from 6 to 8) was accompanied by a decrease in sedimentation coefficients, and the increase in accessible surface area of β-lg. At high pH (>8.2), loop EF flips away from the calyx, which then becomes accessible for ligands. Except the conformation changes of exposed Glu89, no other charged residue undergoes a change from buried to exposed as a function of pH, although the main chain of Asp85 becomes partially buried at pH 8.2. Bin Y. Qin et al. suggested that in the closed, low-pH conformation, the hydrophobic side chain of Leu87 is partly buried and in contact with hydrophobic side chains of residues Leu39 and Lys60 [212]. In the high pH open

conformation, these side chains become more exposed and in the case of Leu87 completely exposed. The conformational change of loop EF is propagated into reorientations of the side chains for Met107, Ile84, and to a lesser extent Leu39. The environments of Tyr20, Tyr42 Tyr99 remain unchanged at 8.2. It is worth mentioning that the transition also results in

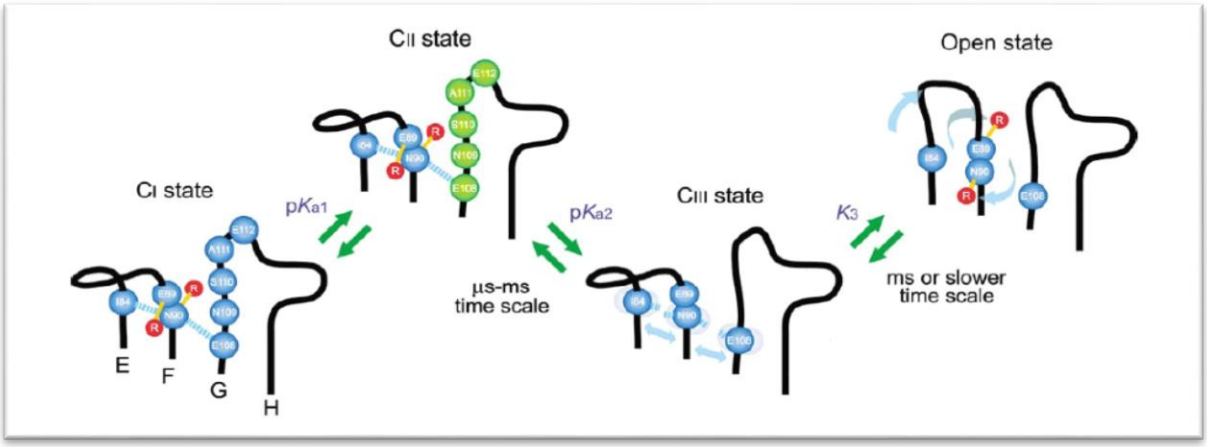


Fig. 10. A representation of the mechanism of conformational change in the EF-loop. The black lines with blue balls represent the backbone fold. The broken lines indicate hydrogen bonds. The red circles represent the directions of the side-chains of Glu89 and Asn90, which are omitted in CIII. At the CI–CII step, a certain conformational change occurs on the GH-loop with a pKa of 6.7. In the CII state, the backbones are fixed by hydrogen bonds at the hinge residues. In the CIII state, the hydrogen bonds of the hinge residues are cleaved. The timescale of the CII–CIII transition is of the order of micro- to milliseconds. In the open state, the collective conformational changes, and side-chain inversions of Glu89 and Asn90 take place cooperatively, leading to the EF-loop flip motion. The timescale of the CIII–Open transition is of the order of milliseconds or slower. The CII–Open transition is a pH-dependent EF-loop flip with an apparent pKa of 7.5. [211]

disruption of any remaining native-like dimeric structures into unfolded monomers at pH>9. Tyr42, which remains buried at the pH values of this study, can be identified as the tyrosine residue that remains buried and inaccessible to titration until the pH is above 12 [200]. It was demonstrated that 20% of β-sheets and 10% of α-helices are preserved at pH is above 12. This observation agrees with Casal et al. who have proposed that the protein retains some of its secondary structure even at highly alkaline pH [213]. Cys121 is buried at the interface between β-sheet and α-helix. Despite the detailed observations, the high-resolution structural data fail to explain some of the experimental evidence obtained in solution such as the pH-dependent reactivity of the free cysteine (Cys121) [214]. The recent work has been done the folding and self-assembly of β-lg from a reversible unfolded state at pH 10.5 in presence of methanol, 2-propanol, t-butanol and 2, 2, 2-trifluoroethanol (TFE) (Maity et al., 2016) [215].

1-6.5. Effect of Chemical Denaturants on Bovine b-Lactoglobulin

Sometimes Chemical denaturants are used to unfold proteins and to portray mechanisms and transition states of protein-folding processes. Alcohols, particularly 2,2,2-trifluoroethanol (TFE), Urea, 1,3- dimethylurea, 1,3-diethylurea, guanidinum thiocyanate (Gdn.SCN) and Guanidinium chloride (Gdn.HCl) are commonly used as denaturants. Theoretical calculations predict a significantly higher amount of a-helical secondary structure than is observed in native β-lg [216, 217]. This means that the native structure results from competition between the a-helix favoring local interactions and the β-sheet forming long-range interactions. Alcohols, such as TFE, can disrupt this equilibrium by weakening the hydrophobic interactions and strengthening the helical propensity [218]. The higher proportion of a-helical structures in the so-called TFE-state is found in the N-terminal half of the molecule [219]. As a result of magnetic relaxation dispersion measurements of the solvent nuclei, it has been shown that the structure is open, solvent-permeated (unlike the collapsed state of a molten globule), and that its formation is accompanied by a gradual swelling of the protein as the concentration of TFE increases [220]. High-protein and TFE concentration (8% v/w and 50% v/v, respectively) can lead to fibrillar aggregation and gel formation of bovine β-lg at both acid and neutral pH [221].

The ability of urea to induce protein unfolding is thought to be via a combination of hydrogen-bond formation with the protein backbone and a reduction in the magnitude of the hydrophobic effect [223]. Urea-promotes unfolding of bovine β-lg at acidic pH was first reported as a two-state process [223]. At pH 2.1 also urea induced unfolding as a two-state transition between folded protein and unfolded state via a cooperative unfolding of the β-barrel and the C-terminus of the major α-helix [224]. However, Dar et al. have provided evidence that urea also causes unfolding via an intermediate, albeit with structural properties between those of the native and unfolded states [225]. Addition of anionic amphiphiles, sodium dodecyl sulfate (SDS), or palmitate, causes b-Lg to resist urea-induced unfolding due to binding inside the calyx [226].

Often, guanidinium chloride is used instead of urea in protein stability studies. Typically, GdmCl is fully dissociated at neutral pH or acidic pH. At low concentrations of GdmCl (<~1 M), chloride ions screen electrostatic repulsion between positively charged protein groups [227]. Because GdmCl has extra electrostatic interactions in comparison with neutral urea, it is capable of stabilizing and destabilizing protein structure depending on its concentration [227]. D'Alfonso et al. have compared denaturation of bovine β-lg B with both GdmCl and urea between pH 2 and pH 8, as monitored by CD, UV differential absorption, and fluorescence

measurements [228]. Discrepancies between unfolding free energies obtained using the two denaturants could be reconciled if GdmCl denaturation was assumed to occur via an intermediate state. The secondary structure of this state is like that of the native protein, but with greater rigidity in the vicinity of the Trp residues consistent with the screening of electrostatic repulsion between charged residues [228]. The GdmCl-induced unfolding intermediate of bovine β-lg A at pH 2 has been reported to have increased α-helical structure [225]. Porcine β-lg has also been shown to unfold via an intermediate state on addition of GdmCl. The stability of the porcine protein was lower than that of its bovine counterpart, and the intermediate state was richer in a-helical structure. Most of the hydrophobic–hydrophobic interactions of the buried core of the native state are conserved between bovine and porcine β-lg. However, four pairwise interactions of the Phe105 side chain of bovine β-lg are lost on the change to Leu in the porcine protein. This indicates that the presence of the aromatic residue may play an important role in the increased stability of the bovine protein [229]. Hattori et al. reported unfolding/refolding studies on bovine β-lg with monoclonal antibodies as Probes and concluded that renatured β-lg molecules as a stable form unfolded in specific regions [230]. The two-step thermal denaturation process of β-lg was further investigated by J. A. Seo et al. in 20-100 °C temperature range by Raman Spectroscopy [231]. They reported that the first step corresponds to the dissociation of dimers associated with an increase of flexibility of the tertiary structure and in the second step; large conformational changes are detected in the secondary structure and described as a loss of α-helix structures and a concomitant formation of β-sheets.

1-6.6. Effect of Osmolytes:

Organic osmolytes—such as sugar and polyols—are known to play an important role in stress protection by stabilizing macromolecules. Osmolytes act as protein stabilizer in view of their impact on their thermodynamic stability toward thermal or chemical denaturation [232]. In addition to acting as Osmo protectants, natural osmolytes play an active role in the folding of proteins. Osmolytes such as glycine, N-methylglycine (sarcosine), N trimethylglycine (betaine), trimethylamine N-oxide (TMAO) and myo-inositol have been shown to double protein stability when compared to denaturants [233]. As protective osmolytes, TMAO, betaine, and sarcosine interact unfavorably with the unfolded state, providing relative stability to the folded state [231].

1-6.7. Effect of Pressure on Bovine β-Lactoglobulin

Pressure treatment as part of the processing regime has greater potential to produce dairy products with improved functional and organoleptic properties than those produced by thermal treatment alone [234]. β-lg is the most susceptible to pressure-induced change than other major whey proteins [235, 236]. Perhaps this is due to the relatively inefficient packing caused by the large solvent-exposed hydrophobic pocket and the lower number of disulfide bonds (two versus four in, for example, the similar-sized a-lactalbumin). It has been reported that bovine β-lg has a reduced molar volume at pressures as low as 10 MPa, possibly because the calyx has contracted [237]. According to several studies, β-lg becomes more susceptible to enzymatic cleavage when under pressure, possibly due to pressure-induced conformational changes. There is evidence that cysteine becomes free at 50 to 100 MPa [235, 238, 239]. β-lg's tertiary and quaternary structure is irreversibly altered by pressures greater than 300 MPa. When β-lg was exposed to pressures as high as 900 MPa at neutral pH, CD and fluorescence spectroscopy showed that pressure causes monomer formation with subsequent aggregation, but only small irreversible effects on its tertiary structure [240]. The results of tryptic hydrolysis suggest, however, that while exposure to pressures below 150 MPa does not permanently alter β-lg A's conformation, pressures above 300 MPa cause strands D and G to detach from the b-barrel and form disulfide-bonded oligomers [241]. When bovine β-lg is mixed with either α-lg or BSA at pH 6.6 under high pressure, intermolecular disulfide-bridged aggregates form only between β-lg and itself. No β-lg – α-lg or β-lg − BSA disulfide-bridged species are detected [236], in contrast to heat-treated mixtures where such species are observed [242].

In order to correlate the pressure-induced conformational changes with the protein's primary sequence, Belloque et al. have made NMR amide H-D exchange observations of β-lg A and B following exposure of solutions at neutral pH to pressures of up to 400 MPa [243]. A little H-D exchange was observed at 100 MPa, suggesting that conformational changes do not increase the exposure of most amide protons to the solvent compared to the native conformation at ambient pressure. There was an increase in conformational flexibility at 200 MPa and above based on a large increase in H-D substitution, however, the similarity between the spectra of control samples recorded in H_2O and D_2O before and after pressurization demonstrated that any conformational changes caused by pressure were largely reversible. The authors proposed that the A variant's structure was more sensitive to changes in pressure than that of the B variant

and that the F, G, and H strands of the protein's β-barrel were the most resistant to conformational change, the latter conclusion paralleling the effects of temperature [244, 245].

It has been found that even at 1GPa, the unfolded state has significant secondary structure [246]. Pressure and heat combined have shown that a change in temperature from 5 to 37 °C has a negligible effect on the susceptibility of β-lg to pressures up to 200 MPa [247]. Based on CD spectroscopic results, a molten globule with an a-helical structure was formed at 600 MPa/50°C [248] and 294 MPa/62°C [249] following the application of both moderate pressure and moderate temperature. Thus, when studying the effects of pressure on protein conformation and stability, it is important to consider the potential for temperature increases induced by rapid pressurization of the sample.

According to enzymatic proteolysis observations, β-lg is less susceptible to pressure-induced changes at acidic pH [250], but this may be confounded by pressure-induced changes in proteinases, thermolysin and pepsin. However, NMR measurements of monomeric β-lg at pH 2 while under pressure of up to 200 MPa have revealed that the two sheets unfold independently to produce two intermediate states of unfolded β-lg that are still characterized by significant secondary structure [251].

A three-step mechanism has been proposed for β-lg denaturation at neutral pH and ambient temperature, which broadly encompasses the above observations: A pressure of 50 MPa causes partial collapse of the calyx (with concomitant reduction in ligand-binding capacity) together with exposure of Cys121. When the pressure increases to 200 MPa cause further (partially reversible) disruption to the hydrophobic structure, together with a decrease in the molecular volume. Higher pressures cause irreversible aggregation reactions involving disulfide interchange reactions [252, 253].

1-6.8. Effect of surfactant

There is no doubt that surfactants denaturate native proteins. In addition to solubilizing insoluble proteins in water, they are also used to determine the molecular weight of proteins (SDS-polyacrylamide gel electrophoresis). Interactions between amphiphiles (surfactants/emulsifers) and β-lg have received much attention because of their diversity to induce conformational changes of protein. S. Maulik et al. investigated the binding of cetyltrimethyl ammonium bromide (CTAB) with G-lg.The binding occurs in two or three distinct stages, depending on the overall structure of the G-lg molecules [254]. The binding of

a perfluorinated surfactant perfluorooctanoate (PFOA), to β-lg has a denaturizing effect on the protein, decreases its thermal stability in aqueous solutions [255]. The neutron reflectivity of β-lg films adsorbed at the air–water interface in the presence nonionic surfactant hexaoxyethylene dodecylether (C12E6) in a concentration-dependent manner is measured by D. S. Horne et al. [256]. G. C. Kresheck et al. reported the temperature and pH dependence of the enthalpy of binding of sodium dodecyl sulfate (SDS) to the bovine β-lg Thermometric Titration Studies [257]. M. I. Viseu et al. research has shown that conformational changes as a result of presence of the cationic surfactant dodecyltrimethylammonium chloride (DTAC), is accompanied by partial unfolding of the β-lg at higher concentrations [102]. Unfolding kinetics shows that DTAC induces a β→α transition which constitutes an unfolding pathway that differs from that of the chemical denaturant guanidine hydrochloride [258]. The effect of model anionic surfactants such as sodium dodecyl sulfate (SDS) on the conformational changes of β-lactoglobulin are discussed in terms of the ionic charge arising for protein pH ranging from 2.0 to 5.8 at high temperature [259]. The anionic sodium n-alkyl sulfate, sodium dodecanoate (SDD), cationic n-alkyl trimethyl ammonium chloride, non-ionic n-alkyl maltopyranoside (alkyl length n differs from C8 to C14) surfactants interact with bovine β-lg and modulate the conformational propensity in a concentration dependant manner. This may be attributed to the length of the alkyl chain that greatly affects the strength of interactions between surfactants and protein [260]. Encapsulation of β-lg in sodium bis (2-ethylhexyl) sulfosuccinate reverse micelles leads to important conformational changes of the protein. The secondary structure of β-lg appears to evolve to a distorted -sheet, and this is probably related to the equilibrium between dimer and monomer.

1-6.9. Effect of co- solvents:

A cosolvent is a substance that makes immiscible substances mixable by adding it to the mixture. A cosolvent increases the solvent power of the primary substance in the mixture. Protein stability is enhanced by cosolvents such as trehalose, sucrose, glycerol, stachyose, and glucose. It is found that trehalose induces remarkable stability of β-lg against chemical denaturation by guanidinium chloride (Gdn.HCl) [261]. Cosolvents like sorbitol, glycerol and sucrose contains hydrogen-bonded when added to β-lg, the thermodynamic properties of proteins changes with extended thermal stability. Due to preferential hydration of the protein, which is exclusion of cosolvent molecules from the protein surface [262].

Maity et al. (2016) showed that the folding and self-assembly of β-lactoglobulin from a reversible unfolded state at pH 10.5 in the presence of methanol, 2-propanol, t-butanol and 2,2,2-trifluoroethanol (TFE) [215]. The extent of secondary and tertiary structure formation is in the order methanol < 2-propanol < t-butanol < TFE. Exposure of the hydrophobic core of the protein molecules in an apolar environment of TFE seems to promote intermolecular cluster formation. Methanol and TFE induce aggregation through the α-helical structure whereas isopropanol and t-butanol favour the formation of the β-structure leading to aggregation at higher concentrations.

1-6.10. The Effect of Chemical Modification:

A chemical modification is the process of chemically reacting a protein with chemical reagents. During this process, specific amino acid residue side chains are covalently grafted with modifying agents of interest. During chemical modification, the goal is to understand "relative reactivity of side chain groups, to quantify amino acids individually, and to develop affinity reagents, mechanism-based reagents for pharmaceutical use, cross-linking reagents, bioprostheses techniques, blocking reagents for peptide synthesis, and cleavage reagents [263]. According to Crane (1994), chemical modifications are extensively used in two applications: a) the identification of important groups involved in binding and catalytic sites and b) structural analysis [264]. In addition to primary structural analysis, the contribution of specific amino acid residues in the tertiary and secondary structural components has also been studied. Several chemical transformations, available for selective modification of protein, include the studies of protein-protein interaction, protein-ligand interactions, processing of bio-conjugates and protein micro-arrays [265]. Side chain residues of aspergine, cysteine, glutamine, histidine, lysine, tryptophan, tyrosine have been modified chemically in different proteins for several purposes. Since last few years, β-lg, a major whey protein abundant in cow's milk, has been exploited extensively in this regard. Modifications of β-lg not only serve the purpose for "structural biology" as a model protein but also have a direct significant approach in the "protein chemistry" field. Another point for the β-lg-derivatives is related to the developments in the design of therapeutics and toward the selective manipulation of bio-materials of precise uses. The different modifying agents for β-lg's modification have been discussed below briefly.

1-6.10.1. Phosphorylation:

Phosphorylation, with the help of phosphorus oxychloride or phosphorus pentoxide in phosphoric acid in alkaline pH to the amino nitrogen such as lysine or the hydroxyl oxygen,

like serine, of bovine β-lg has been reported previously. In addition, 31P NMR data indicate that phosphate may attach to lysine and histidine residues [266]. The level of phosphorylation greatly affected the emulsifying and gelling properties of β-lg [267]. It was then followed by phosphotylation under relatively mild conditions, and phosphotylation had the same impact on secondary structure and consequently solubility as in the previous press [268]. Various efforts have been made to further enhance the functional properties of milk proteins like β-lg for food industrial purposes.

1-6.10.2. The Maillard Reaction

When proteins are exposed to heat in the presence of carbonyl compounds, such as reducing sugars, the Maillard reaction occurs [269]. In this reaction a reducing sugar targets the "-amino group of lysine or the N-terminal group of a protein to form Amadori or Heyn's rearrangement products. As originally described by Hodge in 1953 [270], the advanced stages of the Maillard reaction involve degradation of the Amadori (1-amino-1-deoxy-2-ketose) and Heyn's products via a range of pathways, depending on the conditions under which the reaction takes place, involving Schiff bases, Strecker degradation, or fission products, ultimately giving rise to copolymers, brown nitrogenous polymers, melanoidins [271, 272].

1-6.10.3. Glycosylation:

Glycosylation technique is a well-accepted phenomenon in the field of chemical modification of β-lg. In this process, carbohydrates, such as glucose, maltose, lactose, gluconic acid etc., are covalently linked to β-lg and alter many physio-chemical properties of protein. The effects of glycosylation/ maltosylation greatly affect the properties like hydrophobicity, viscosity, and the fluorescence properties of modified protein [273]. The synthetic glycoproteins showed higher solubility and heat stability at isoelectric pH and lower ionic strength with increasing glycosylation [274]. Changes in β-lg glycation with lactose, both in powdered form and aqueous solution, affected the association behavior of multi-step denaturation and aggregation process of β-lg. The dimer interface of β-lg (AB loop, GH loop, P-strand "I", and helix part) that involved in non-covalently interaction was characterized by the means of immunochemical method after glycosylation [275, 276]. The properties like antigenicity and immunogenicity of β-lg were also reduced by glycosylation [277, 278, 279]. Besides all, recent work shows that the enzymatic activity of β-galacticidase was very likely to be affected when the lysine group of β-lg was modified by lactose and was concluded into the significance of lysine e-amino groups on its activating effect of the enzyme [280].

1-6.10.4. Free Thiol modification:

Cysteine is the most closable targeted residue for amino acid modification in the protein structure with chemical reagents. Free thiol of bovine β-lg at Cysl21 of β-H strand is completely buried under the C-terminal α-helix and have been targeted both in native and denaturing condition with several cysteine specific reagents such as Dithioerythritol (DTT); 5,5‟-dithiobis (2-nitrobenzoic acid) (DTNB) or 4,4‟-dithiopyridine; 2-mercaptoethanol (MCE); mercapto-propanoic acid and other fluorophore tagged maleimide derivatives for different structural elucidation purposes [281, 282, 283, 284, 285]. As the free thiol is very much susceptible to involve in disulfide linked oligomerization/aggregation pathways of heat treated β-lg, different thiol modifying agents have been employed to trace its specific role in the aggregation process as well as to insight the conformation and stability of the pristine protein [286].

1-6.10.5. Alkylation/Acylation:

Beyond conformational aspects of protein, research has been re-directed towards the functional aspects of protein after modification of side chain residues in a common platform by both the dedicated chemists and biologists. Recent progresses in structure activity relationship (SAR) of small proteins interacting with ligands/small organic molecules have shed a new light on the molecules of β-lg. Modification of β-lg by chemical means or by enzyme may induce conformational change and binding properties. Evidences show that N-methyllysyl-β-lg and N-ethyllysyl-β-lg, obtained by esterification or reductive alkylation, greatly influence the binding of several “terpenes” which are structurally related to retinol [287].

Adsorption behavior of lysine modified β-lg by acetylation and succinylation have been extensively studied in air-water interface as well as alumina-water interface [288, 289]. These results imply that the electrostatic forces manipulated after modification plays the key role in aggregation/dissociation properties of β-lg. The aggregation behavior was also studied in other protein system, Concanavalin A, after succinylation where the succinyl derivatives were quite reluctant to participate in aggregation process and fibril formation [290]. Results from other group of researchers showed that the succinylation of lysine residues exploited as an indirect proof of £-amino groups of some specific lysine residues are likely to be the binding sites of β-lg in the activation of β-galactosidase activity [280]. Nonetheless, enzymatic modification has also put the signature in the process of biophysical processes. Modification of lysines and glutamines of β-lg with transglutaminase from Streptoverticillium mobaraense (MTgase) can

be expoited for several physico-chemical processes such as surface tension, viscisity etc. [291, 292]. Earlier it was said that the importance of chemical modification is associated to the improvements in the design of therapeutic agents [293]. Recent reports suggests that modified β-lg, ty acetylation, succinylation and/or hydroxy pthalylation in the lysine residues, shows a great extent of anti-viral activities [294, 295], on the other hand, fine stranded, transparent gels were formed at pH away from iso-electric point and low ionic strength [295, 296, 297]. Increase in β-lg concentration increases the hardness of the gel. There is an apparent critical concentration of 2.5% (wt/V) which have been established by DLS, NMR and AFM studies [297].

1-6.10.6. Methionine modification:

The amino acid methionine (Met) is one of the most oxidation-prone amino acids and can be converted into methionine sulfoxide or sulfone derivatives by various mechanisms, such as hydrogen peroxide treatment, metal catalyzed reactions, or UV exposure [298]. Sanhita et. al. Investigated the effect of t-butyl hydroperoxide (tBHP)-induced oxidation on structure, compactness, and fibrillation propensity of β-lg at physiological pH. t-BHP-induced selective Met oxidation affects structural orientation of β-lg, reducing its resistance to thermal instability, as well as altering the internal polarity of the protein, resulting in the introduction of an internal strain [299].

1-6.10.7. Esterification

Proteins modified by esterification gain a more positive charge as the number of ionizable carboxyl groups becomes reduced. Moderate esterification of β-lg induces not only slight changes in its secondary structure, but also its tertiary structure. This leads to opening of the β-lg molecule to the cleavage of the peptide bond. Esterified β-lg is prone to hydrolysis by pepsin, because of 22 new sites of pepsin cleavage introduced by esterification [300]. Fourteen cleavage sites are pepsin-specific and their unveiling is due to the imposed tertiary structure changes. Eight of the new cleavage sites are esterified carboxylates recognized by pepsin [301]. M. I. Halpin suggested that the methyl-, ethyl-, and butyl-esters of β-lg showed enhanced surface activity, as well as hydrophobic probe binding activity, the most pronounced effect being that of methyl esters [302]. Due to positive charges situated on the protein molecules, enable them to interact with viral proteins or viral DNA, affecting viral replication, transcription or translation and, consequently, viral infectivity. Highly esterified β-lg at pH 7 showed DNA-binding capacities comparable to those exhibited by native basic proteins, such

as lysozymes and histones. Esterification of β-lg enhanced its antiviral activity against the avian influenza A virus (H5N1), the influenza virus A subtype H1N1 and the HSV-1 virus. Peptic hydrolyzates of esterified β-lg also displayed antiviral activity [303].

1-6.11. Modification by physical Methods

Physical parameters like UV-radiation, gamma rays, Ultrasound, pulsed electric field (PEF) and electrolysis, High hydrostatic pressure also promotes the alteration of structure and stability of β-lg.

UV radiation changes the molecular size distribution and disrupts the ordered structure of β-lg. This led to changes in the β-lg antigenicity and to altered activity of β- lg in the regulation of immunoglobulin production [304]. It was found that after 24 hours of UV-irradiation, 18.8% of the protein had been denatured and some of its aggregation had occurred, which led to some changes in the secondary structure of the protein. During the process of photo-oxidation, there has been a decrease in the number of total sulfhydryl groups, while a rise in the number of exposed sulfhydryl groups has been observed [305].

The changes in the secondary and tertiary structure of β-lg induced by γ-irradiation are similar to the alterations observed in β-lg that had been treated thermally under mild conditions [306] [270]. As a result of both changes, there is a reduction in the solubility of the protein as well as an increase in agglomeration. Application of γ-radiation up to a dose level of 10 kGy did not affect the molecular-weight distribution of β-lg, but reduced its solubility and increased its antigenicity [307].

Application of high-intensity PEF to β-lg resulted in the partial denaturation of the protein and its aggregation, including covalent cross-linking. PEF treatment increased the thermal stability of β-lg by 4 to 5 °C, as well as its gelation rate [308]. After the treatment by electrolysis, β-lg on the cathode showed markedly mitigated allergenic properties, attributed to the dislocation of the allergenic peptides from the protein surface [309].

The sonication process on β-lg forms had larger hydrophobic surfaces than native β-lg and, thus, more easily underwent cross-linking with phenol oxidase. Sonication had only a minor effect on the ability of β-lg to bind to IgE, both in vitro and in vivo [310]. Ultrasound mediated glycation in Maillard reaction exhibited radical scavenging ability and possessed a greater ferrous ion-chelating activity and better reducing power than the native protein [311].

In High hydrostatic pressure (HHP)-induced aggregation of β-lg, only dimers and trimers arose due to SH/S–S interaction [312]. The high-pressure denaturation of β-lg led to increased reactivity of the thiol group that was buried inside the native globule. This was a result of the exposure of the thiol group to the protein surface. The high hydrostatic pressure enhanced the enzymatic hydrolysis of β-lg by various enzymes [313].

1-6.12. Enzymatic Modifications:

The implementation of enzymes offers many advantages, including the ability to perform modifications under physiological conditions with great specificity and stereoselectivity, but without undesirable side reactions. Using transglutaminase (TG) Proteins can be cross-linked, which catalyzes an acyl group transfer between the ε-amino group of lysin residues and the γ-carboxamide group of glutaminyl residues in proteins, forming an iso-peptide bond. Treatment of β-lg in an excess of TG increase in the thermostability of the protein which may be attributed to partial unfolding of the protein molecule and subsequent re-arrangement of its conformation [314].

The application of laccase in the cross-linking of proteins is affected by the accessibility of their phenolic moieties, as proteins generally contain a small number of phenolic groups. In the presence of ferulic acid, laccase was able to form irreducible intermolecular cross-links in β-lg, as well as to induce oxidative modifications. The latter included: dityrosine formation, formation of fluorescent tryptophan oxidation products and formation of carbonyl derivatives of histidine, tryptophan and methionine, which resulted in the protein molecules exhibiting a higher surface tension [315].

Thalmann et al. reported that β-lg can be cross-linked by tyrosinase from Agaricus bisporus only in the presence of a low molecular weight phenolic compound, which probably acted as a bridging agent between the protein subunits [316]. Enzymatic hydrolysis of β-lg under HHP produces a higher yield of short bioactive peptides with potential antioxidant and anti-inflammatory effects.[313].

1-7. Biological functions of β-lg:

It binds retinol (vitamin A) in a hydrophobic pocket, protects it from oxidation, and transports it through the stomach to the small intestine where the retinol is transferred to a retinol-binding protein, which has a similar structure to β-Lg. It is not clear how retinol is transferred from the core of the fat globules, where it occurs in milk, to β-Lg and why some species lack this protein. β-Lg can bind many hydrophobic molecules, and hence its ability to bind retinol may be incidental. β-Lg is a member of the lipocalin family, all of which have binding properties [153]. It can binds with tri-glycarides and transportation of other small and long chain hydrophobic molecules [84, 317, 318, 95]. Two anti-bacterial fluoroquinonolones, Norfloxacin and Levofloxacin (Ebirini et al., 2006) [319], have been assessed for interaction with β-lg. A series of bio-active peptides have recently been found to bind to the peptide derived after proteolysis in vitro [320, 321, 322]. The hydrolyzed peptides are also believed to enhance passive immunity in infants. β-lg participates in removal of harmful organisms from neonatal after attaching to gut wall also [323]. β-lg acts a transporter of small, sparingly soluble molecules such as retinol or long chain fatty acids and vitamin D [62] but species distribution and variation in binding profile do not all fit with such a role. This has been supported by evidence that β-lg has been isolated with free fatty acids, mainly, palmitic acid and oleic acids, from fresh milk [63]. So β-lg shows a wide variety of functions, most of which involve some ligand-binding function.

1-8. Amyloid fibrils:

The term ‘amyloid’ was initially coined by Schleiden and then by Virchow in the mid-19th century to describe the iodine-stained deposits seen in liver at autopsy. At that time the deposits were thought to be carbohydrate in nature until the establishment of presence of high nitrogen content in it [324]. Nevertheless, the inaccurately descriptive name was retained for these highly proteinaceous deposits. Further tinctorial properties included the specific binding of amyloid to the dye Congo Red which produced an apple green birefringence when examined between cross polarizers in a light microscope [325]. The transmission electron monographs confirm the structure of the amyloid as fibrillar [326]. The term amyloid refers to fibrous, extracellular, proteinaceous deposits in tissues and organs. Structural studies have revealed that the fibrillar assemblies are composed predominantly of β-sheet structure in a characteristic cross-β conformation and these are inherently stable. Unlike other fibrous proteins it does not commonly have a structural, supportive or motility role but is associated with the pathology

seen in a range of diseases known as the amyloidosis [327]. Amyloid fibrils may be deposited in a variety of organs including brain, liver, heart, kidney, pancreas, nerve, and other tissues because of certain inherited and acquired disorders.

It was previously known as secondary amyloidosis. It occurs along with chronic infectious or inflammatory diseases, such as rheumatoid arthritis or inflammatory bowel disease. Hereditary (familial) amyloidosis is an inherited disorder that often affects the liver, nerves, heart, and kidneys. THE process of protein aggregation is a widely observed phenomenon in biology.

Table 8: Some amyloidosis and their respective precursors and amyloidogenic proteins [327].

Disease	Precursor protein	Amyloid protein
Alzheimer's disease	Amyloid precursor protein	Aβ peptides
Atrial amyloidosis	Atrial natriuretic factor (ANF)	Amyloid ANF
British familial dementia	Amyloid Bri Precursor Protein	ABri
Spongiform encephalopathies	Prion protein (PrPc)	PrPsc
Primary systemic amyloidosis	Immunoglobulin light and heavy chains	AL and AH
Senile systemic amyloidosis	Wild-type transthyretin	ATTR
Haemodialysis-related amyloidosis	β2-microglobulin	Aβ2M
Hereditary nonneuropathic systemic amyloidosis	Lysozyme	ALys
Type II diabetes	Pro-IAPP	IAPP or “amylin”
Injection-localized amyloidosis	Insulin	AIns
Secondary systemic amyloidosis	(Apo) serum amyloid A	Serum amyloid A
Hereditary cerebral amyloid angiopathy	Cystatin C	ACys
Finnish hereditary systemic amyloidosis	Gelsolin	AGel
Familial amyloid polyneuropathy I	Transthyretin variants	ATTR
Familial amyloid polyneuropathy II	Apolipoprotein A1	AApoA1
Ageing pituitary, prolactinomas	Prolactin	APro
Familial amyloidosis	Fibrinogen αA-chain	AFib

1-8.1. Amyloidogenesis: Assembly of Amyloidogenic Proteins:

In the evolution of mature fibrils, several metastable intermediates have been identified and isolated. [328 - 331] This includes very early species, such as dimers, trimmers, and tetramers (collectively termed oligomers) [332], diffusible ligands that are derived from Aβ, or ADDLs, and later bead-like structures up to 200 nm in length, called protofibrils. [333] As an alternative, fibrils can be formed by following an 'offset pathway' instead of producing fibrils by converting intermediates into amorphous deposits.

As proteins fold and misfold in vitro, several molecular mechanisms have been proposed to explain amyloid formation, such as the polar zipper [334] and domain swapping models [335, 336]. A number of molecular mechanisms have been proposed to explain amyloid formation. It has been proposed that amyloid formation is a generic property of all peptides since under denaturing conditions many normally globular, non-disease related proteins, have been shown to assemble to form fibrils [337, 338]. The propensity of a peptide to form amyloid is determined by several factors, including its hydrophobicity, sequence, and secondary structure. Thus, several algorithms have been developed to predict the propensity and rate at which different sequences will aggregate, and which mutations will increase or decrease aggregation rates [339-343]. In order to determine which regions of a polypeptide chain are involved in fibril formation or the formation of amyloid cores, these algorithms will prove useful.

1-8.2. The Structure of the Amyloid Fibril:

The fibrillar structures are thermodynamically very stable and are resistant to proteolytic degradation. Interestingly, although the proteins that form fibrils in various diseases are completely unrelated in structure and function, the final fibril structure is remarkably similar. The exact mechanism by which the diverse proteins aggregate to give rise to a common structure is still not clearly understood and is an active area of research.

Amyloid fibrils are insoluble and heterogeneous, so commonly used methods of structure determination are difficult. Therefore, most studies have involved X-ray fiber diffraction, electron microscopy [344] and more recently, solid state nuclear magnetic resonance (SSNMR) [345] and electro-paramagnetic resonance [346]. Electron and atomic force microscopy have given insights into the macromolecular structure of amyloid fibrils and have shown that fibrils are long, straight, and unbranched (Fig. 11) and are made up of individual subunits named “protofilaments” [347–349]. These may vary in number and are often observed to twist around

one another to form the mature fibril [347,349- 351]. Synthetic amyloid-like fibrils, may vary in morphology and this may depend upon the assembly conditions [347, 352].

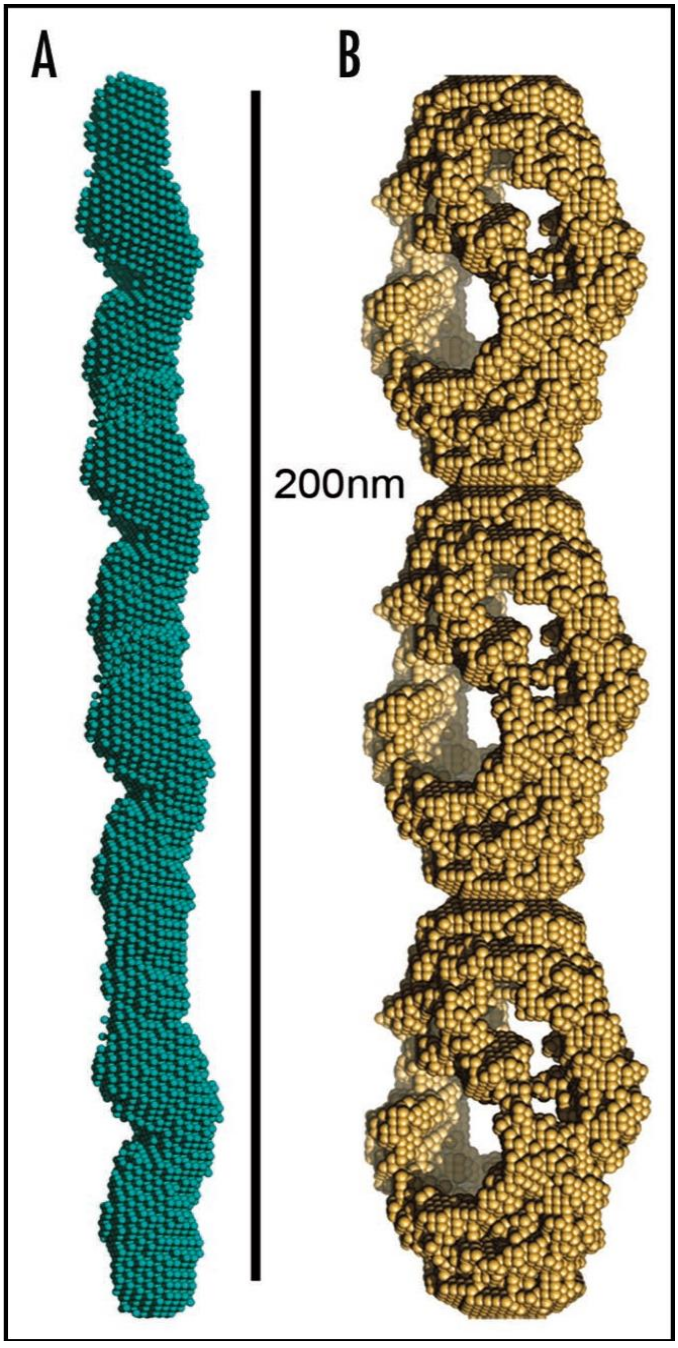


Fig. 11. Models of mature protein fibrils based on Small-Angle X-ray scattering solution data. (A) Human alpha-synuclein fibrils and (B) human insulin fibrils [353] The results suggest that insulin fibrils (B) are formed of three intertwining protofibrils, whereas a-synuclein fibril (A) consist of only one protofibril. Each protofibril is assumed to consist of two intertwining protofilaments. Four and three repeating units are shown for alpha-synuclein and insulin respectively.

The SAX study suggested that insulin fibrils are assembled from a helical fibrillation precursor composed of five to six insulin monomers. The mature amyloid fibril is composed of three individual filaments that wrap around one another [353] (Fig. 11). Small angle X-ray scattering from fibrils formed by alpha-synuclein revealed a contrasting structure in which a single filament appears to make up the mature fiber (Vestergaard, personal communication).

Cryo-electron microscopy studies revealed that synthetic amyloid fibrils formed by insulin [351], lysozyme [350] and Aβ (1–42) [352] are also composed of several protofilaments wound around one another. The numbers of protofilaments can differ from 2 to 6.[351]

The X-ray diffraction pattern given by amyloid fibrils is "cross-β," a diffraction fingerprint first identified for silk from the egg stalk of the lacewing, Chrysopa [354]. The pattern indicates that these fibrous molecules share a particular core structure consisting of β-sheet conformation in which the hydrogen bonding direction runs parallel to the fiber axis and the β-strands are perpendicular, much like the rungs of a ladder. The diffraction pattern consists of two major reflections at 4.7 Å and 10 Å found on orthogonal axes and arising from the hydrogen bonding distances between β-strands and side chain packing between the sheets respectively. [355]

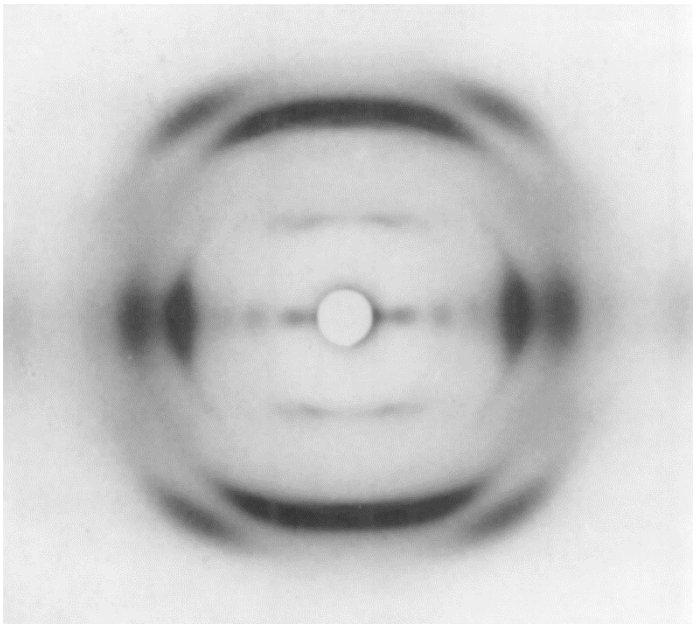


Fig. 12. X-ray fiber diffraction pattern from partially aligned amyloid fibrils showing the characteristic "cross-β" diffraction pattern [354].

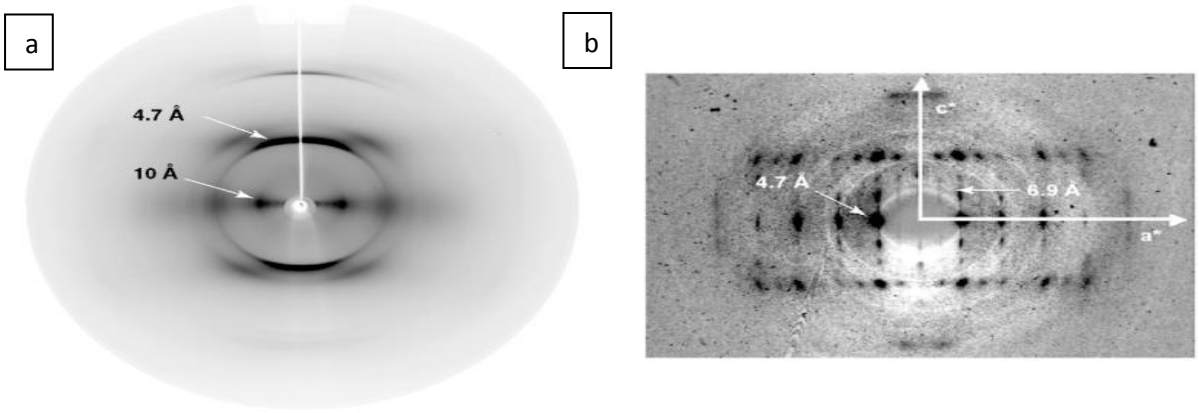


Fig. 13. Diffraction from fibrillar assembled proteins/peptides. (a) X-ray fibre diffraction showing cross-b signals characteristic of amyloid fibrils. This data was collected from IAPP amyloid fibrils. (b) Electron diffraction from fibrous crystals formed by assembly of a designed peptide. The diffraction shows signals corresponding to d = 4.7 A˚ (hydrogen bonding, inter strand) and d = 6.9 A˚ (repeat distance along the chain direction of a b-pleated strand). [355]

1-9. Aggregation of β-lactoglobulin:

In protein science and engineering, thermal aggregation plays a crucial role. It remains unclear how aggregates are formed, despite their biological importance. Accordingly, unfolding and aggregation are quite distinct processes, with the former consisting of successive unimolecular reactions, whereas the latter is bimolecular and considered a second-order reaction. Temperature, pH, protein concentration, and ionic strength affect both reactions differently [356-360]. Proteins such as α-lactoglobulin and β-lactalbumin in milk serum are sensitive to heating at 60-100 degrees Celsius. Denaturation, aggregation, and intramolecular reactions occur during heating. Dairy processing involves heating, which clearly results in a conformational change in β-lg. Many studies have shown that partial unfolding of polypeptide chains also leads to aggregation. Heuristic approaches are widely used in many studies on this subject. There is no clear physical picture on which to base predictions, and we still lack knowledge of the precise mechanisms of the processes. At room temperature and at physiological pH β-lg exists mainly as a dimer, in which the monomers are noncovalently linked, but it dissociates into monomers (molecular mass 18.3 kDa) at elevated temperatures. This dimer dissociation was shown to be a necessary step in the heat induced aggregation mechanism [361, 362]. Further heating (above 50°C) results in the protein undergoing a conformational change, exposing previously buried hydrophobic groups and thiol groups. This

state, however, can be referred to as a "molten globule state"[362] due to the retention of the nativelike backbone secondary structure. In the molten-globule state, hydrophobic interactions can cause protein molecules to aggregate. For the -lg heated at 65 °C with neutral pH and low ionic strength, Roefs and de Kruif proposed an analogous polymerization mechanism via thiol catalysts [363]. A progressive loss of β-sheet structure was observed with increasing temperature, while an abrupt loss of the helical conformation was detected near 65 ºC [364]. When the temperature is increased above 70 ºC, β-lg partially denatures and aggregates, leading to the formation of soluble aggregates if the protein concentration c is below the critical gelation concentration cgel or to the formation of a continuous network (also referred as gel) if c > cgel [260]. At temperatures higher than 80 ºC, noncovalent interactions become of increasing importance and the aggregation process is dominated by both interchange of disulphide bonds and hydrophobic interactions [365]. The kinetics of protein denaturation/aggregation is controlled by the heating conditions, temperature, and time [366] and by the chemical environment, protein concentration [356, 366], pH [357, 358], ionic strength [237, 359] calcium, and lactose concentrations [367].1H NMR kinetic experiments at 70ºC indicate that the folded form unfolds within several minutes and that subsequent aggregation and gel formation from the unfolded form involves a slow step (several hours) [368]. The thermally induced aggregation pathway of β-lg appears to proceed in a series of steps involving quaternary, tertiary, and secondary structural changes. In a report by Vetri et al., it was shown that conformational changes expose and/or cover specific residues and hydrophobic regions that play a fundamental role in the whole process and result in ‘aggregates’ with different structures, even at secondary and tertiary levels [214]. At 70°C and 90°C, protein conformations form nuclei through the addition of partially and/or fully unfolded monomers, resulting in the formation of spherical aggregates [369].

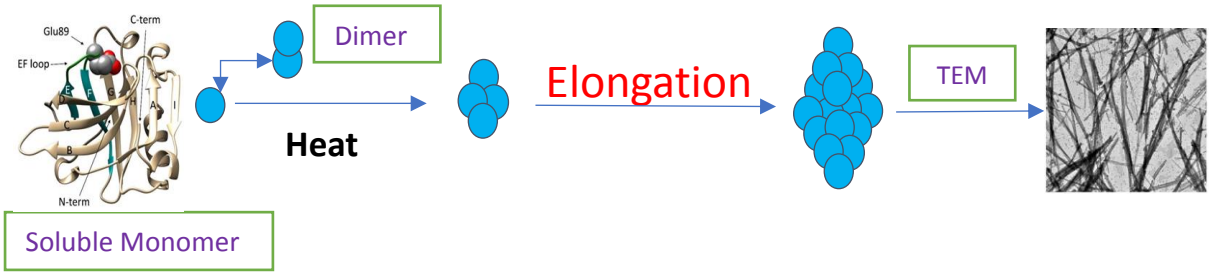


Fig. 14. Schematic representation: Aggregation occurs when complementary hydrophobic surfaces interact with one another, again through intermolecular interaction. The common intermediate in folding and aggregation can be seen.

In the case of pH close to the isoelectric pH (IEP), the interchange of disulphide bonds is less prone to occur, and the aggregation process is dominated by the electrostatic attractions and hydrophobic interactions [369, 370]. Below the IEP, at pH 2, the very low reactivity of the sulfhydric group inhibits formation of covalent bonds, the electrostatic repulsions are substantial, and aggregates based on noncovalent interactions (ionic, dipole, van der Waals, hydrophobic) are formed [371]. The denaturation and aggregation of β-lg follows by analogy with a radical-addition polymerization reaction at near-neutral pH and the free thiol group of β-lg plays the role of the radical [372]. Majhi et al. reported that maximum aggregation rates were observed in the pH range 4.3-4.8 [373]. Maith et al. reported the structural changes along

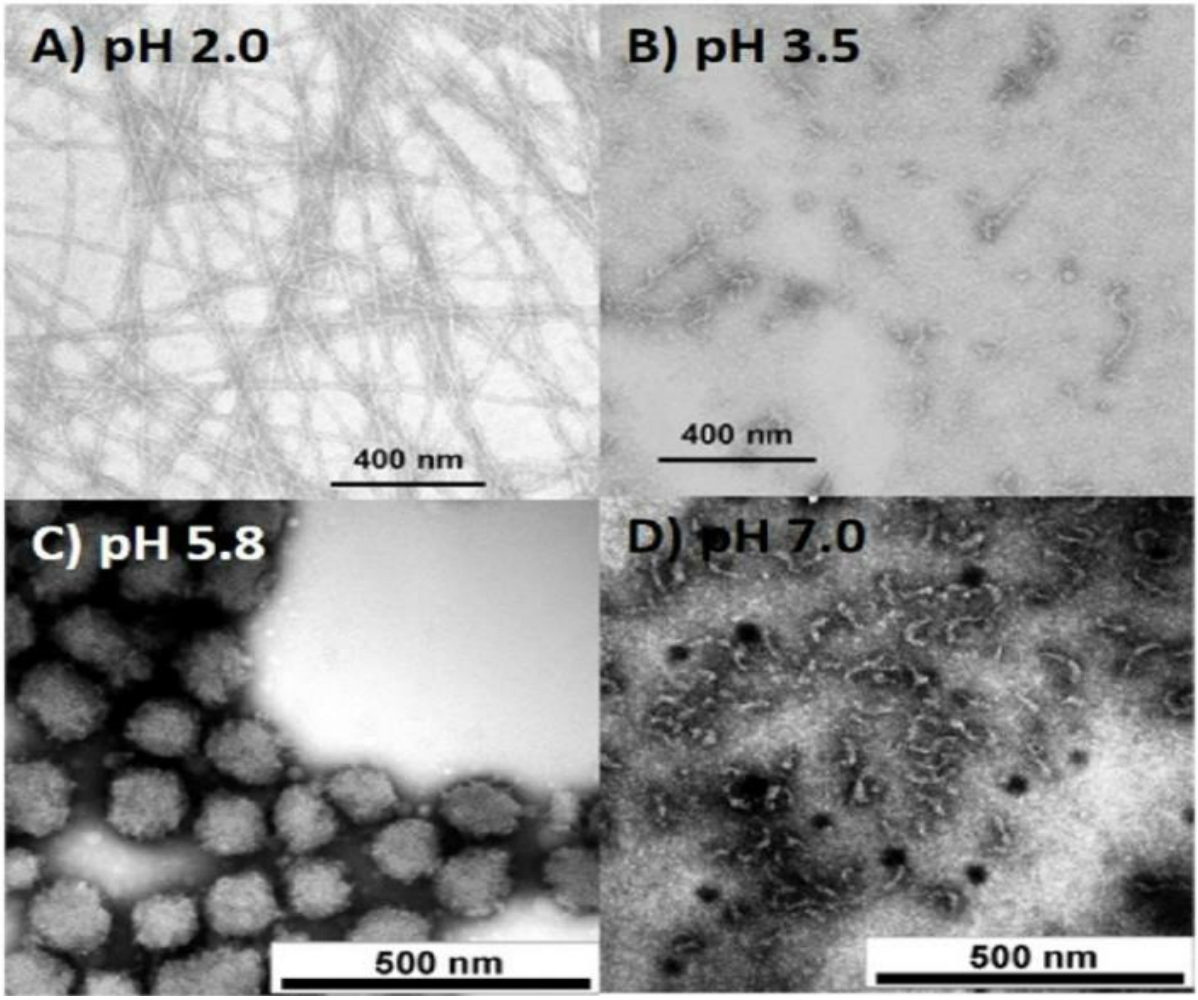


Fig. 15. TEM images of β-lg aggregates prepared at (A) pH 2.0 and (B) pH 3.5 (both 2.5% β-lg at 90 • C for 5 h, from Keppler et al. (2019) [374], and (C) pH 5.8 and (D) pH 7.0 (both 1.0% β-lg at 85 • C for 15 min, from Jung et al. (2008). [260]

the path of folding of alkaline β-lg in presence of three non- fluorinated alcohols MeOH, i-PrOH, t-BuOH and a fluorinated alcohol, 2,2,2-trifuoroethanol (TFE) [375]. As t-BuOH is less

polar and bulkier, it can induce secondary and tertiary structures at lower concentrations. By lowering the dielectric constants, TFE at much lower concentrations favoured intermolecular hydrogen bonding. At lower concentrations, all of them have increased α helicity and stabilized the secondary structure. In the presence of i-PrOH and t-BuOH, the formation of the β-sheet structure occurs concurrently with the loss of the α-helical structure. Both non-fluorinated and fluorinated alcohols accelerated the formation of non-native secondary structure, resulting in protein self-assembly. Interestingly, TFE has induced β-lg aggregation only through a-helical structure formation [375]. In addition, other proteins, such as bovine serum albumin and bovine serum fetuin, showed similar results [376, 377].

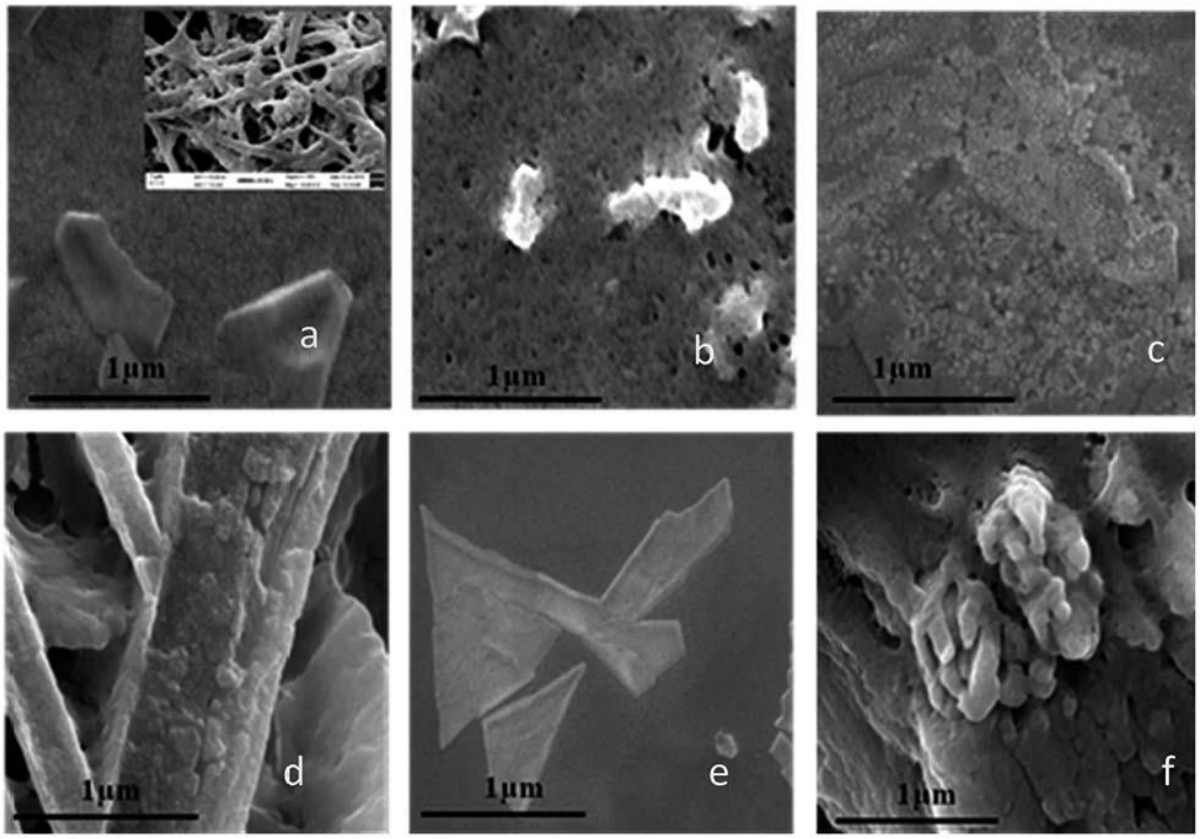


Fig. 16. FE-SEM images showing the formation of distinct self-assembled structure of β-lg at pH 10.5 and at: β-lg in absence of any alcohol (a) and native β-lg (inset), worm-like aggregates of 50–60 nm diameter in 80% (v/v) MeOH (b), smaller spherical nanoparticles in 70% (v/v) i- PrOH (c), long sheet-like aggregates in 60% (v/v) t-BuOH (d), flake-like and nanotube-like morphology with 15% (v/v) (e) and 30% (v/v) TFE (f) [375].

Nature of protein aggregates depend on the protein concentrations, temperature, pH and many other various conditions. Aggregates with widely different structures have been observed ranging from densely branched clusters to rigid rods [373]. Thermal incubation of β-lg aqueous dispersions at increasing pH from 2.0 to 5.8 to 7.0: rod-like aggregates, spherical aggregates,

and worm-like primary aggregates, respectively reported by Jung et al. [260]. Proteins at 70 °C and 90 °C form nuclei that grow via the addition of additional partially and/or fully unfolded monomers to give rise to the particles spherical aggregates [378]. It is possible to categorize amyloid (like) aggregates according to their size and flexibility. Generally, shorter and more flexible amyloid-like aggregates form when molecules are assembled relatively quickly and less precisely, for instance, due to a low repulsion force, high concentration, or a solvent's presence.[379] Factors that destabilize the native protein structure (such as high temperatures, zinc, solvent, hydrolysis, interfaces, oxidation) generally accelerate the aggregation, whereas stabilizing factors (such as glycerol) or hindering factors (such as phenolic compounds or sugars) slow it down.) [379].

1-9.1 Amyloid fibril formation by β-lactoglobulin

The association of partially unfolded or completely unfolded proteins or peptides results to the formation of specific aggregates called amyloid fibrils. Amyloid fibrils transform into an ordered and repetitive molecular architecture known as cross-β structure, in which β-strands from distinct protein/peptide chains associate to form an extended, intermolecular β-sheet with a parallel or antiparallel arrangement [378]. The kinetics of fibrillation process at neutral pH (pH 7.0) described by B. Ma involving the lag time for formation of stable nuclei (nucleation) and the apparent rate constant for the growth of fibrils (elongation) [380]. During heating at low pH, as cysteine residues are protonated, disulphide bonding between β-lg molecules does not occur to any significant extent [381]. Akkermans et al. reported with prolonged heating (20 h) at 85 ºC and pH 2, several chemical modifications occur to amino acid side chains, but no covalent crosslinking [382]. Besides crosslinking, another chemical reaction that is hydrolysis of peptide bonds can occur at low pH and high temperature [383]. J. Adamcik et al. revealed that heat-denatured β-lg fibrils at pH 2 have a multistranded helical shape with twisted ribbon-like structures supported by theoretical arguments and by the atomic force microscopy analysis [384]. It was suggested that strong repulsive electrostatic forces and low ionic strength are not sufficient alone to prevent amyloidal aggregation, and a strong energetic driving force must exist to promote aggregation at pH 2. Possible reasons for the attractive forces leading to aggregation might arise from the amphotheric nature of the protein fibrils, in which neutral residues occurring periodically along the contour length of the fibrils can promote strong attractive ‘hydrophobic’ interactions [385]. The formation of β-lg fibrillar aggregates upon thermal incubation in 3–5 M urea at 37 ºC and pH 7.0 for 10–30 days has been reported by

D.Hamada et al. [386]. They demonstrated that efficient fibril formation involves a balance between the requirement of a significant population of unfolded or partially unfolded molecules and the need to avoid conditions that strongly destabilize intermolecular interactions. The spherulite structures of amyloid fibrils of β-lg at pH 1.6 have been reported by K. R. Domike et al. [387]. Macromolecular crowding agents, Dextran 70 and polyethylene glycols (PEG), can effectively accelerate the fibril formation of β-lg at neutral pH [388]. Metal ions such as zinc and copper ions can effectively promote the heat-induced aggregation of β-lactoglobulin [389].

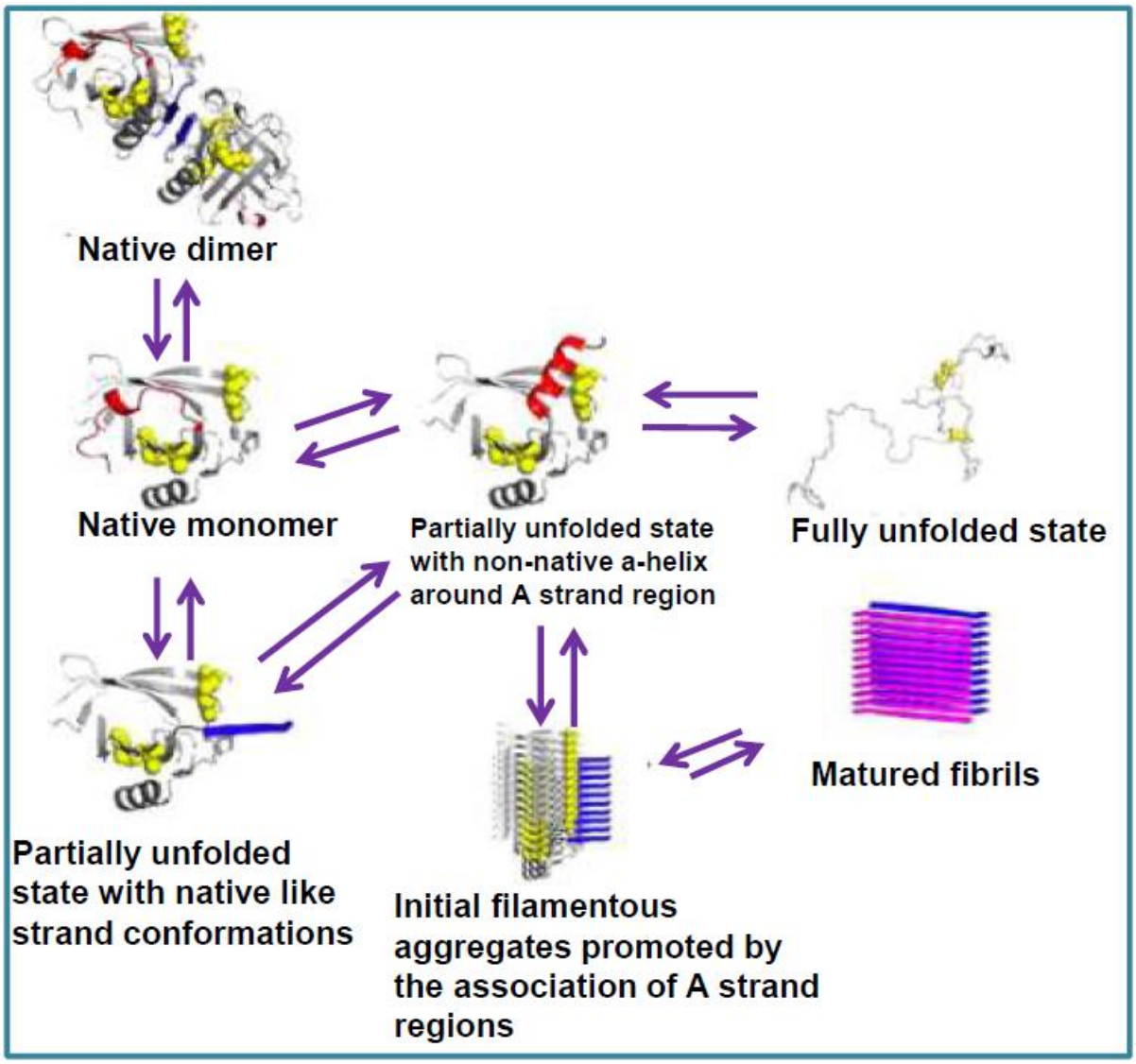


Fig. 17. Schematic view of a unified mechanism for amyloid formation by β-lactoglobulin.

1-10. Toxicity of Amyloid:

Amyloid deposition is a consequence of the amyloidosis; what is uncertain is whether it is a causative agent in its pathogenesis or a secondary event. The amyloid cascade hypothesis [390] proposed that altered metabolism of amyloid precursor protein (APP) initiates the pathogenesis of Alzheimer's disease (AD) leading to aggregation of Aβ and formation of neurotic plaques. These plaques would cause further pathological changes including the formation of neurofibrillary tangles and compromised synaptic connections ultimately resulting in neuronal cell loss and dementia. However, there was no correlation between the density of plaques and tangles and the severity of AD. Instead, the concentration of soluble Aβ appeared to correlate with cognitive impairment in other studies [391-394] This finding set the premise for studies suggesting that soluble nonfibrillar intermediates, such as oligomers (20 to >50 kDa globular aggregates, including ADDLs) and protofibrils (curvilinear structures 4–11 nm in diameter and ≤200 nm long) are the actual initiators of AD pathogenesis and that mature fibril formation represents the end point of the disease.

Usually, small organic compounds are screened and selected for their binding and inhibitory effects on amyloid precursors or the enzymes involved in their proteolytic processing. These potential anti-amyloid drugs are often highly toxic and have a profound effect on the immune system. Biological ligands such as monoclonal antibodies have been raised against amyloid and are more successful at plaque clearance and reduction in soluble Aβ levels in the CNS [394]. However, during phase II trials of an Aβ vaccine, 6% of subjects developed an inflammatory response (meningoencephalitis) which halted further clinical development. The search therefore for an effective and safe anti-amyloid drug continues. In the interim, several questions remain unanswered: (1) which intermediate species are responsible for the toxicity of amyloid; (2) what is its atomic structure and the exact mechanism of toxicity and (3) how can this toxicity be safely reversed.

1-11. Aims and Objectives of our research work:

The formation of amyloid fibrils due to protein misfolding primarily is the cause of several serious diseases, such as Alzheimer’s, Parkinson’s and Huntington’s diseases. It is found that the amyloid fibrils are composed of cross- β-structures and their morphological features are not related to behaviours of the specific proteins. Since the formation of amyloid fibrils generally can result in many neurodegenerative diseases, recent research has focused on the inhibition of fibril formation. It has been shown that the amyloid formation can be inhibited by various

naturally occurring and synthetic small molecular weight compounds, such as curcumin and curcuminoid compounds, feluric acid, coumarin derivatives, pyrroloquinoline quinine (PQQ), nitrophenols, epicatechin gallate, biocompatible nanogels, benzofurans, baicalein etc. compunds [395-397].

Scaffolds ranging from peptides and small molecules, both naturally occurring and synthetic, have been used to inhibit β-lg aggregation. The reported molecules target either the nucleation or the elongation step, thereby preventing insulin aggregation. A few molecules have been reported to disintegrate mature fibrils, while the modes of action of a few are still unknown.

Currently, there is no approved therapeutic agent directed towards the formation of fibrillar assemblies, which have been recently shown to have a key role in the cytotoxic nature of amyloidogenic proteins. Oneimportant approach in the development of therapeutic agents is the use of small molecules that specifically and efficiently inhibit the aggregation process. Small molecules, such as resveratrol, mitoxantrone, and pixantrone, derivatives of Congo Red, 1,4-naphthoquinon-2-yl-L-tryptophan (NQTrp), have been shown to inhibit the process of Aβ aggregation [398]. J. He reported that myricetin, a natural flavonolfound in many grapes, berries, fruits, vegetables, and herbs prevents fibrillogenesis of hen eggwhite lysozyme [399]. Several quinones, the oxidation products of polyphenols are found to inhibit insulin aggregation [400].

Present research proposal involves the synthesis and to study the interactions of the several chalcone compounds, different coumarin derivatives and oxygen containing neuro-transmitters like dopamine (DOPA), γ-amino butyric acid (GABA) and phenyl ethylamine (PEA) with the model protein β-lactoglobulin (β-lg) and efforts will also be given to investigate their effectiveness against the amyloid fibrillogenesis of the β-lg. The development of better β-lg aggregation inhibitors and insights into their mechanism of action will also enhance our knowledge of protein amyloidosis.

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Chapter:2

Review of literature on Coumarins

2-1. Introduction to coumarin compounds

Coumarins belong to a family of large and extensively researched compounds containing 2H-1- benzopyran-2-one core structure [1], which consists of fused benzene and α-pyrone rings mainly found in the families of Rutaceae and Umbelliferae, but also in fungi and bacteria [1-4]. Although more than 1300 natural coumarins have been identified to date [5]. A heterocyclic

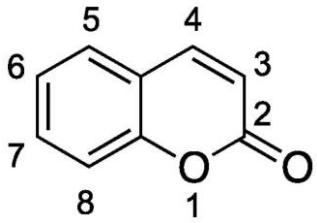


Fig.1. Structure and numbering scheme of coumarin

system such as this is also known as 1,2-benzopyrone, 2-oxo-1,2-benzopyran, 2H-chromen-2-one or o-hydroxycinnamic acid lactone [6]. In 1820 H. A. Vogel first isolated the simplest member of this family - coumarin from the tonka beans (Dipteryx odoranta Wild; Fabaceae family) called also Coumarou, a vernacular French name [7]. Later in 1868 the compound was first synthesised by W. M. Perkin [8]. Both natural and synthetic coumarins exhibit broad biological properties, including anticancer, antimicrobial, antiviral, anti-inflammatory, neuroprotective, and antioxidant properties. In this regard, coumarin skeletons can be considered as a promising scaffold for designing and synthesizing pharmaceutically active molecules [9 -16].

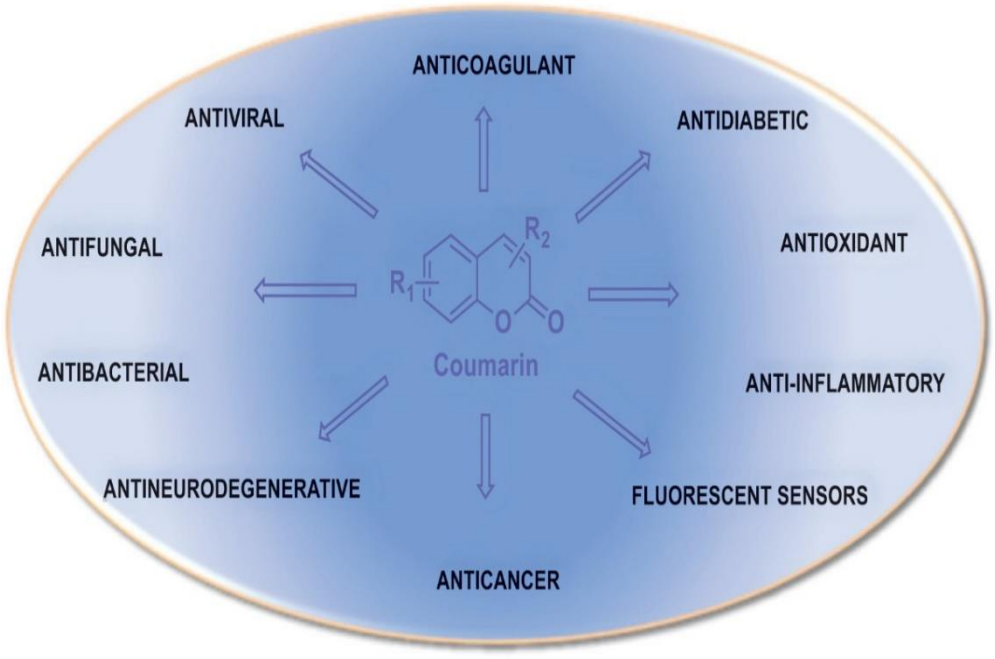


Fig.2. Schematic Representation of several biological functions of coumarins

2-1.1. Biosynthesis and Metabolism of Coumarins

Normally occurring coumarins are synthesized via the general biosynthetic pathway, leading to phenylpropanoids.[17] As a result of the shikimate biosynthetic pathway, phenylalanine is converted into trans-cinnamic acid, which can then be converted into the central metabolite-coumaryl-S-CoA by phenylalanine ammonia lyase (PAL). The transformation of this crucial intermediate into phenylpropanoids is shown in Fig. 3 in part [17]. In the biosynthesis of coumarin, the coumaroyl-S-CoA central metabolite is hydroxylated, the exocyclic double bond is isomerized trans > cis, and the final lactonization/cyclization is performed. A first and crucial step of the biosynthesis is the 6′ (ortho) hydroxylation catalysed by the 2-oxoglutarate-dependent dioxygenase F6′H1. Kai et al. proposed the radical mechanism of the isomerization and lactonization reactions in 2008[18].

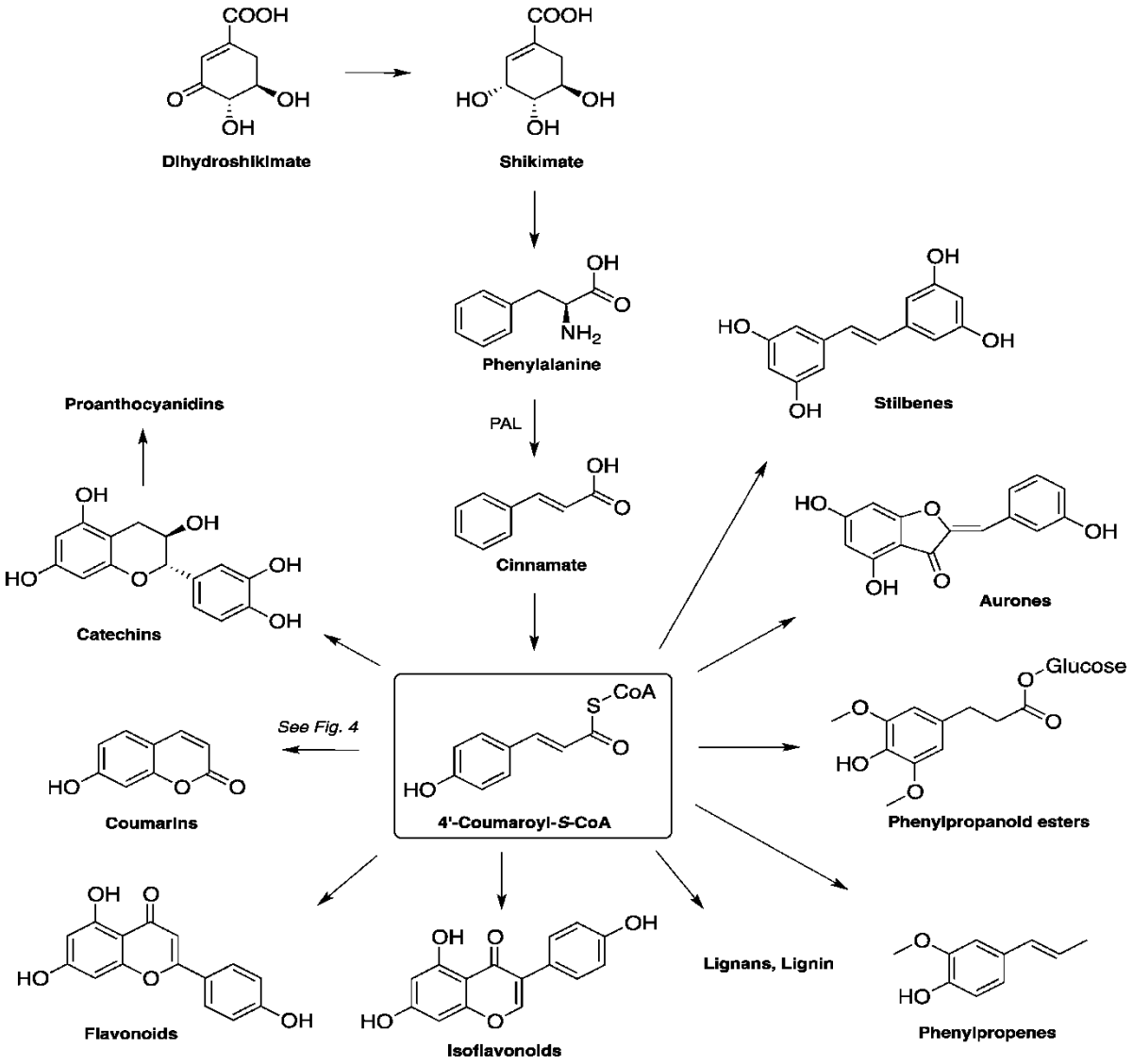


Fig.3. General biosynthetic pathway leading to phenylpropanoids [17]

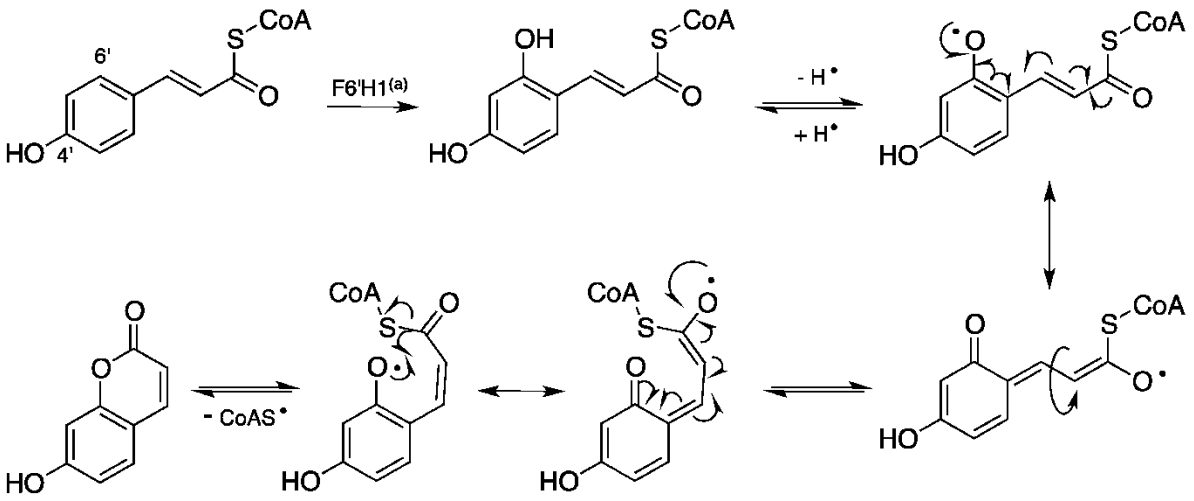


Fig.4. Proposed radical mechanism of 7-hydroxycoumarin biosynthesis from 4′-coumaroyl-S-CoA (adapted from Kai et al., 2008) [18].

There are two main pathways for the metabolism of coumarins that have been discovered so far: the 7-hydroxylation and the opening of the lactone ring with the loss of carbon dioxide (Fig. 4). This last reaction occurs in the metabolic pathway when coumarin 3,4-epoxide is formed as the intermediate in the first step. As a matter of fact, under aqueous conditions, this epoxide emits carbon dioxide and meta-hydroxyphenyl acetaldehyde (o-HPA) is produced, which can be further metabolized to produce appropriate acids (ortho-hydroxyphenyl acetic acid, o-HPAA) and alcohols (ortho-hydroxyphenyl ethanol, o-HPE). A nucleophilic attack by glutathione on the 3,4-epoxide can result in the formation of 4-HDHC-GSH (4-hydroxy-3,4-dihydrocoumarin-3-mercapturic acid), which can then be transformed into 3-hydroxycoumarin, which further converted into ortho-hydroxyphenyl lactic acid (o-HPLA), which eventually forms o-HPA and o-HPAA. As indicated in Fig. 5, other possible metabolites of coumarin are the 3,4-dihydrocoumarin (DHC) and hydroxylated coumarins at positions 4, 5, 6, and 8, which are formed at a much lower extent than at position 7. In Fig. 5, there are some pathways that have question marks indicated by arrows, which indicate reactions that are still under investigation, as well as reaction mechanisms.

Some cytochromes P450 (CYPs) play a pivotal role in the metabolic transformation of coumarin. CYPs are iron-containing enzymes (hemoproteins) that catalyse redox reactions between endo- and xenobiotics, such as drugs, food nutrients, and environmental pollutants [19, 20]. The CYP enzymes play a key role in the bioactivation, toxicity, and detoxification processes of xenobiotics [21]. Majority of drugs undergo oxidative biotransformation through

the CYP1 family, CYP2 family, and CYP3 family. An important drug-metabolizing enzyme in humans is CYP2A6 [22]. In human liver microsomes, CYP2A6 is the major enzyme involved in the metabolic transformation of coumarin to 7-hydroxycoumarin (Fig. 5) [23, 24]. CYP2A6 activity may be reduced due to large gene multiplicity and polymorphism, which may favour alternative metabolic pathways of coumarin, such as the one leading to 3-hydroxycoumarin under CYP3A4 catalysis. 3-hydroxycoumarin is suggested to promote the formation of the cytotoxic product o-HPA (Fig.5), which may be responsible or co-responsible for coumarin toxicity.

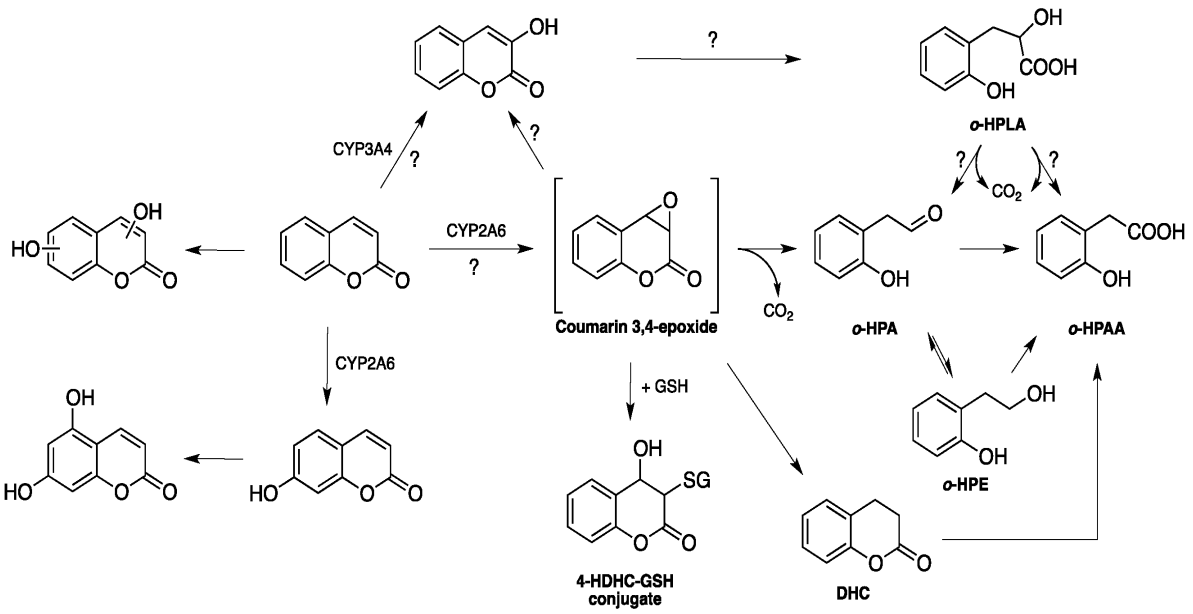


Fig. 5. Representative pathways of coumarin metabolism (adapted from Lake, 1999) [24].

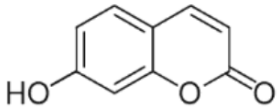
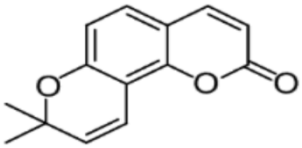
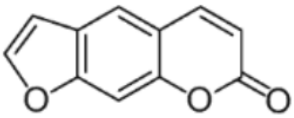
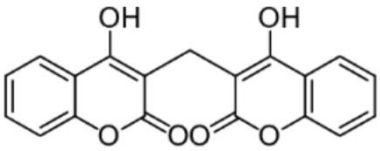
Millions of people around the world suffer from lymphedema, a chronic progressive and disabling disease. Coumarin is an effective pharmacological treatment for lymphedema. As already mentioned, this cheap and efficient drug has been banned in some countries due to possible hepatotoxic effects, unquestionably proven in rats and mice, but rarely in humans. Lymphedema patients with low active CYP2A6 would metabolize coumarin via the cytotoxic pathway leading to o-HPA, based on the previous studies. As a result of the preliminary identification (phenotyping) of these lymphedema patients, all other lymphedema patients with normally functioning CYP2A6 enzymes may use the inexpensive and effective coumarin with greater safety [25]. A kinetic study of the formation of 3,4-epoxide coumarin indicates that this hypothesis may be further supported [26].

2-1.2. Classification of coumarins

Coumarins are classified based on their chemical structure, occurrence, or synthesis, thus it creates several classes of coumarins. Here we mention some categorised coumarins on the basis of their structure [27]

a. Simple Coumarins
b. Pyranocoumarins
c. Furanocoumarins
d. Pyrone-substituted coumarin

Table 1: Classification of coumarins [27]

Classification	Examples	Features
Simple Coumarins	 Hydroxy Coumarin	Hydroxylated, alkylated, alkoxylated on coumarin
Pyranocoumarins	 Seselin	Six-membered pyrone ring attached to benzene ring
Furanocoumarins	 Psoralane	Five- membered furan ring attached to benzene ring
Pyrone-substituted coumarin	 Bishydroxy coumarin-Dicounmarol	Substitution on pyrone ring

2-2. Synthetic Strategies for the Preparation of Coumarins

A wide range of biologically active natural products, pharmaceuticals, agrochemicals, and polymeric [28] and optoelectronic [29] materials are characterized by the coumarin scaffold. Therefore, huge and continuous efforts have been made to develop new synthetic pathways and protocols for the facile cyclization of heterocyclic rings and their regioselective derivatization. In fact, more emphasis has been placed on developing more efficient and eco-friendly synthetic approaches for coumarin derivatives. In recent years, microwaves, and ultrasounds, as well as new catalysts and greener solvents (and even solvent-free reactions), have made it much easier to access coumarin derivatives. Among the numerous reactions proposed, those based on transition metal catalysts are the most exploited for high-yield syntheses of coumarins under generally mild experimental conditions [30, 31]. In a review of anticancer coumarins [32], various retrosynthetic approaches for preparing coumarin derivatives, including new methods leading to coumarins via the classical Pechmann condensation reaction, have been gathered and well-illustrated.

The widely used and classical methods to access coumarin derivatives e.g., the Pechmann [33 ,34] and Knoevenagel [35] reactions, are still the object of numerous investigations, mainly aimed at (a) using only recyclable and/or green catalysts and solvents; (b) devoloping better yields; (c) developing easy and straightforward work-up procedures. Solvent-free reactions and sonochemistry are also under investigation as alternative pathways. The newly published synthetic approaches for the synthesis of coumarins reported below are grouped according to their homogeneity and similarity.

2-2.1. Synthesis of Coumarins by the Pechmann Reaction

The most studied synthetic approaches for the synthesis of coumarinsis Pechmann condensation reaction. This Pechmann Reactionis still the under severalinvestigations. Some of the most recently proposed procedures are briefly reported herein.

2-2.1.1. $FeCl_3$-catalyzed synthesis of coumarin derivatives

Classical Pechmann reaction of activated phenols with β-ketoesters by using 10% mol of $FeCl_3 \cdot 6H_2O$ as catalyst provides Moderate to excellent yields [36].

2-2.1.2. Molybdate sulfuric acid-catalyzed synthesis of coumarins

Molybdate sulfuric acid has been used as a new and efficient catalyst in water–dioxane at 80 °C, and this Pechmann affording the expected coumarins in very good yields [37].

2-2.1.3. Synthesis of coumarins catalyzed by sawdust–SO_3H

The biodegradable and recyclable solid sawdust–SO_3H catalyst afforded coumarins via the Pechmann reaction at 110 °C, in high yields without solvants, within a short time (<1 h), and via easy work-up procedure [38].

2-2.1.4. Ionic liquid-catalyzed synthesis of coumarins

Ionic liquid catalysts, such as 1,3-disulfonic acid imidazolium hydrogen sulfate, can be effectively and re-used in solvent-free conditions for synthesis of coumarins. This process was carried out at 70 °C in less than 30 minutes without solvents and with high yields [39].

2-2.1.5. Ascorbic acid as promoter of the synthesis of coumarins.

Under solvent-free conditions at high temperature (180 °C) with a short duration of time (<30 min), l-Ascorbic acid (vitamin C) was shown to be an efficient and green promoter of high yielding coumarin and flavone synthesis. [40].

2-2.1.6. γ-Fe2O3@HAp-Ag NPs as catalyst in the synthesis of coumarins

It has been demonstrated that an easily prepared catalyst, namely Ag supported on hydroxyapatite-core-shell magnetic γ-Fe2O3 nanoparticles (γ-Fe2O3@HAp-AgNPs) efficiently catalyzed the Pechmann reaction. High yields of the desired coumarins were obtained with a magnetically recyclable catalyst under eco-friendly experimental conditions, and the procedure was easy to follow [41].

2-2.1.7. $SnCl_4$ grafted on silica as catalyst for the synthesis of coumarins

The heterogeneous catalyst promotes the coumarin formation under free solvent conditions at 120 °C in moderate to high yields [42].

2-2.1.8. Lewis acid grafted sulfonated carbon@titania composite as an efficient catalyst for the synthesis of coumarins.

Lewis acid C@TiO_2–SO_3–$SbCl_2$ catalyst, developed from carbon@titania composite showed excellent efficiency in the catalysis of the Pechmann reaction without solvents, at 60 °C [43].

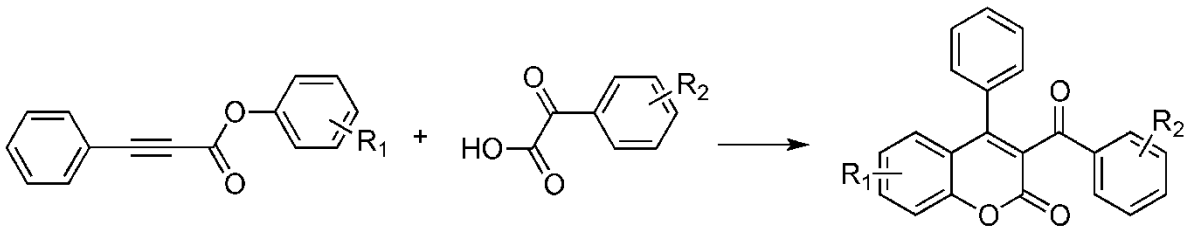
2-2.1.9. HFe(SO4)2.4H_2O-Chitosan Nano-Composite catalysed the Ultrasound-Accelerated Green Synthesis of Coumarins

HFe(SO4)2.4H2O-Ch NCs was applied as an efficient nano-catalyst in the green synthesis of coumarin derivatives through Pechmann condensation under solvent-free conditions at 100 °C, and also with ultrasonic-assisted at 70 °C. High yields of the products, short reaction times, and mild reaction conditions were perceived in both methods [44] .

2-2.2. Regioselective Synthesis of 3-Substituted Coumarins

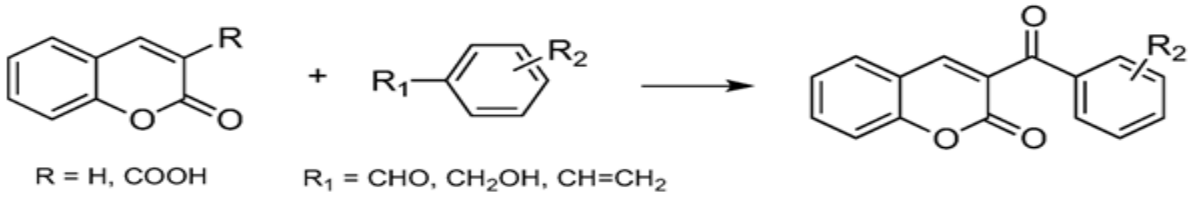
2-2.2.1. Synthesis of 3-aroylcoumarins 8 from the reaction of alkynoates with α-keto acids

Dipotassium peroxodisulfate, silver nitrate in water/acetonitrile in about 24 h, at 60 °C, in a sealed tube under an inert atmosphere undergo a convenient silver-mediated radical cyclization method for the synthesis in high yields of coumarin derivatives. [45]



Scheme 1: Synthesis of 3-aroylcoumarins from alkynoates

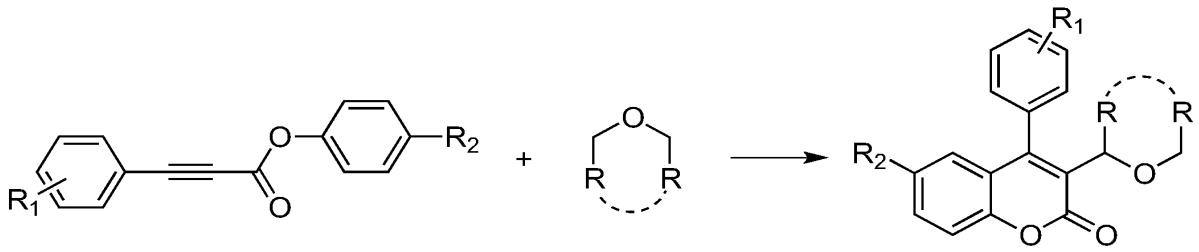
2-2.2.2. Synthesis of 3-aroyl coumarins 9 from the reaction of coumarins, or coumarin-3-carboxylic acids with benzaldehydes, benzyl alcohols, and styrenes



Scheme 2. Synthesis of 3-aroylcoumarins from coumarin or coumarin-3-carboxylic acid.

A metal-free, radical reaction has been performed with tert-butylhydroperoxide in water, chlorobenzene, for 20 h and at 100 °C, and in sealed tube [46]. Interestingly, the reaction takes place in good to high yields, also by using benzyl alcohols and styrenes as carbonyl surrogates.

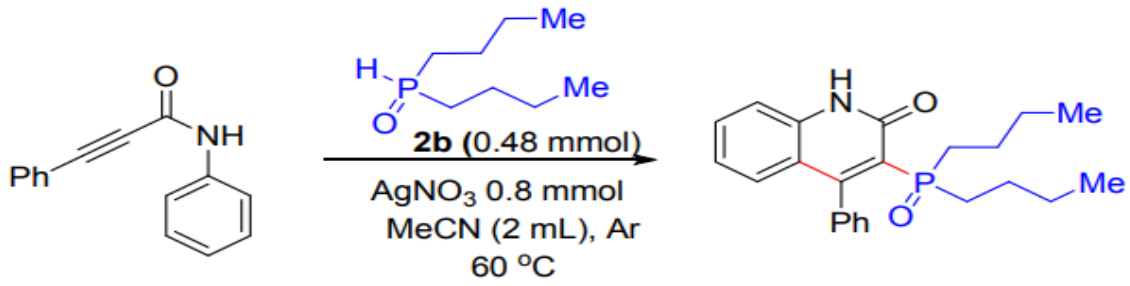
2-2.2.3. Synthesis of coumarins substituted at position 3 with alkoxy groups or saturated oxaheterocycles



Scheme 3. Synthesis of 3-substituted coumarins from alkynoates.

The reaction was performed in experimental conditions close to the ones reported in (A), i.e., with tert-butylhydroperoxide, tris(bipyridine) ruthenium (II) dichloride hexahydrate in decane, acetonitrile, at room temperature, under ultrasound irradiation and inert atmosphere [47].

2.2.2.4. Silver-mediated synthesis of 3-phosphorylated coumarins, quinolin-2(1H)-one and benzophosphole oxides

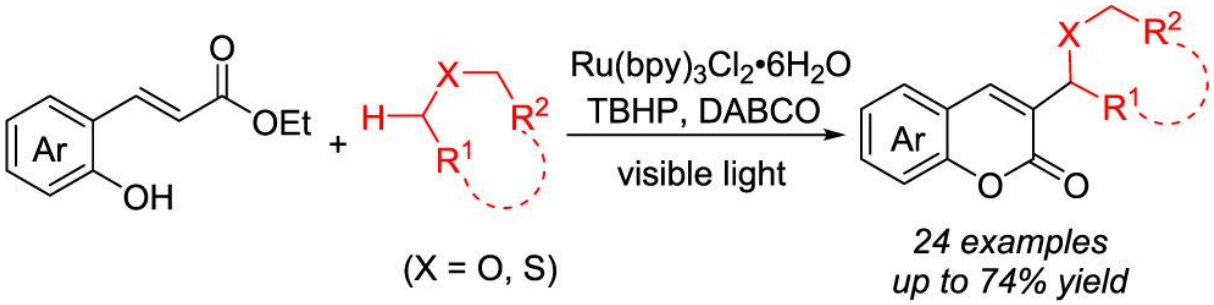


Scheme 4. The reaction of dibutylphosphine oxide 2b with propynoic amide 4a. [48]

The reaction with Aryl alkynoates,arylphosphine oxide, AgNO3 in a sealed tubeunder a nitrogen atmosphere, MeCN as solvent with a stir bar yields high desired products at 60°C for 24-48h. After reaction crude mixture was directly purified by flash columnchromatography on silica gel toget the desired products [48].

2-2.2.5. Visible-Light-Induced C(sp2)–C(sp3) Coupling Reaction for the Regioselective Synthesis of 3‑ Functionalized Coumarins

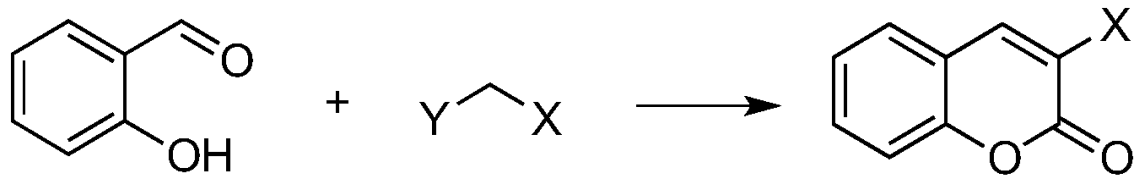
It was observed that with continuous stirring for 2- 8h with a magnetic stirring bar in a sealed tube salicylaldehyde (10.0 mmol),ethyl 2-(triphenylphosphoranylidene) acetate (12.0 mmol), and dryCH2Cl2 (40 mL) reacted together to provide enhanced yield of 3-Substituted Coumarins after purification bysilica gel column chromatography [49]



Scheme 5. Synthesis of 3-Substituted Coumarins via the C(sp2) − C(sp3) Coupling Strategies

2-2.2.6. Knoevenagel reaction:

2-2.2.6.A. Synthesis of 3-substituted coumarins 12 catalyzed by potassium phthalimide (KPhT)



Scheme 6. Synthesis of 3-substituted coumarins from salicylaldehyde.[50]

An expeditious, efficient, and green procedure for the KPhT catalyzed synthesis of 3-carboxy and 3-cyanocoumarins in high yields has been reported [50]. The reaction of salicylaldehydes with active methylene compounds (X–CH2–Y) was carried out under mild conditions in water at room temperature for 0.5–4 h.

2-2.2.6.B. Synthesis of 3-substituted coumarins catalyzed by $MgFe_2O_4$ nanocatalyst under ultrasound irradiation.

Knoevenagel condensation between various salicylaldehydes and 1,3-dicarbonyl compounds, by using $MgFe_2O_4$ nanoparticles as an efficient catalyst under solvent-free conditions and ultrasound irradiation, has been reported [51]. High yields, simple work-up procedure and short reaction times are further advantages of the proposed protocol.

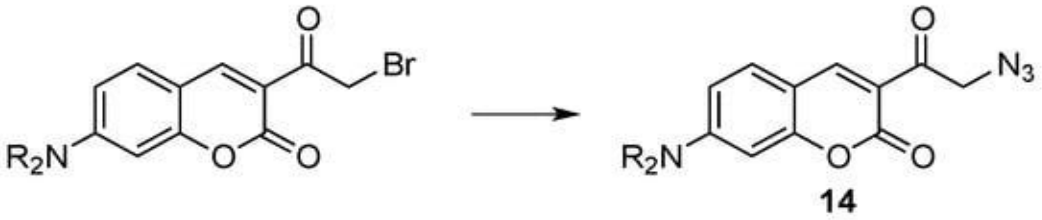
2-2.3. Miscellaneous Reactions for the Synthesis of Coumarins

2-2.3.1. Sonochemistry-based synthesis of coumarins.

Using active methylene compounds and 2-hydroxybenzaldehydes (Knoevenagel reaction) or resorcinol (Pechmann reaction), substituted coumarins were prepared via sonochemistry. In addition to good yields and short reaction times, this method is also easily adaptable to bulk production.[52]

2-2.3.2. Synthesis of azidocoumarins for click reactions.

It has been reported that azidocoumarins have been used as fluorescent reagents in the 1,3-dipolar click cycloaddition to synthesize substituted 1,2,3-triazoles [53, 54]. Evans and coll. Using the corresponding 3-acylcoumarins, they prepared 7-N-alkyl and N, N-dialkylamino coumarins 14 (Scheme 7) bearing an azidoacyl group at position 3.



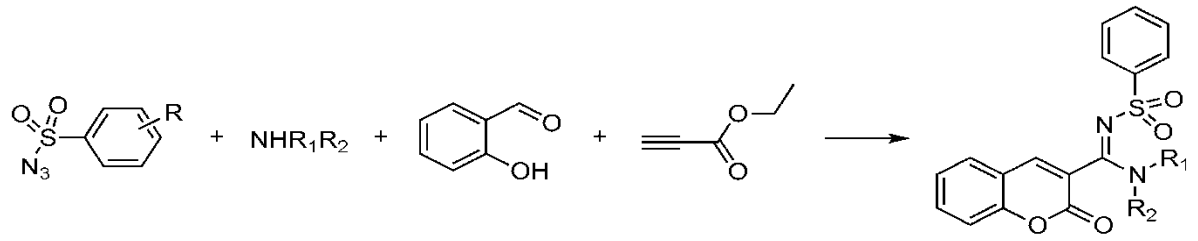
Scheme 7. Synthesis of 3-azidoacyl coumarins from 3-bromoacyl precursors.

2-2.3.3. Synthesis of coumarins by multicomponent reactions

2-2.3.3.A. Synthesis of 3-N-sulphonylamidine coumarins

The synthesis of title coumarins has been accomplished with moderate to high yields, by the coupling of salicylaldehydes, propiolates, sulfonyl azides, and secondary amines [55]. The four-component tandem reaction was carried out in 1,4-dioxane, in a sealed tube and inert

atmosphere, at 130 °C, under microwave irradiation, by using copper(I) iodide as catalyst. **(Scheme 8)**



Scheme 8. Four-component synthesis of 3-sulphonylamidine coumarins.

2-2.3.3.B. Multicomponent reaction for benzylpyrazolyl coumarin

It was observed that Potassium dihydrogen phosphate catalyzed the synthesis of benzylpyrazolyl coumarin and pyrano [2,3- c] pyrazole derivatives via cascade-One pot-multicomponent reaction. [56]

Although coumarins can be derived from various plant sources, or, as just noted, from a variety of chemical reactions, bulk production of diverse coumarin derivatives can be achieved by utilizing microorganisms such as bacteria (e.g., Escherichia coli) [57, 58] and fungi, namely Basidiomycetes and Ascomycetes [59].

2-3. Pharmacological Activities of Coumarins

As a result of their unique structure, coumarins are capable of an extensive range of pharmacological activities (e.g., simple coumarins, fused polycyclic coumarins, bis-coumarins). Among the most studied pharmacological activities, it is worth mentioning antitubercular [60], antibacterial [61,62], antifungal [63], antimutagenic [64], antioxidant [65], antiviral [66], anticoagulant activities[67] scavenging of reactive oxygen species (ROS) [68], anti-inflammatory [69], antithrombotic [70] anticancer [71], and cyclooxygenase [72], lipooxygenase [73], vasodilator [74], cholinesterase (ChE) and monoamine oxidase (MAO) inhibitory activities, CNS stimulant [75], and cytotoxic [76] effects.

A major reason for coumarin's numerous bio-pharmacological activities is its unique chemical structure and physicochemical properties, which allow easy binding to many protein targets. Planar, aromatic, and lipophilic, the 2H-chromen-2-one ring interacts with biological counterparts, mainly lipophilic binding sites, through strong hydrophobic interactions with aromatic amino acids, such as Phe, Tyr, and Trp, and more often through stacking interactions. Positively charged amino acids may bind coumarins through strong interactions with cations.

Further, coumarins have a lactone group that makes them capable of forming hydrogen bonds and dipole-dipole interactions, and sometimes of acylating proteins, as some enzyme covalent inhibitors do. It is likely that the compounds generated by the hydrolysis of lactone rings are responsible for the biological activity observed as a result of enzymes with esterase activity. As a result, coumarins act as pro-drugs, being bioactivated to release their active metabolites. It has been proposed that natural coumarins inhibit carbonic anhydrase, which has esterase activity as well [77]. As well as serine proteases like human leucocyte elastase (HLE) [78] and kallikrein [79], coumarins and 3,4-dihydrocoumarins may acylate their lactone rings. Accordingly, in both studies, suicide inhibitors were designed to attack an enzyme nucleophilic group at position 6 by placing a halomethyl group. In response to the attack of the catalytic serine on the lactone carbonyl, an electronic rearrangement on the intermediate acyl-enzyme takes place, which eliminates the halide and allows the synthesis of a highly conjugated exocyclic double bond, which undergoes an easy nucleophilic addition of a nucleophilic group, and is then trapped by two covalent bonds in a final inactivated form.

2-4. Infuence of coumarin compounds on protein aggregation

Coumarin derivatives such as enamide, enoate, methylprop (CDM) enamide show specific binding with Human Serum Albumin (HSA) [80]. These coumarins also act as a quencher to absorb fluorescence emitted by HSA at 360 nm. Coumarins bind extensively to human serum albumin with primary binding affinity constants of approximately 105 L/mole. The nature of coumarin-albumin binding is chiefly hydrophobic although hydrogen bonding and electrostatic interactions are also involved. It is shown that carrier protein, such as bovine serum albumin (BSA), can disintegrate the microcrystals of C6 to smaller fragments and trap them inside the hydrophobic domain of the folded protein [81]. It is also reported that daphnetin, one of coumarin derivatives, acts as a protein kinase inhibitor. It inhibit serine/threonine-specific protein kinases, including cAMP-dependent protein kinase (PKA) (IC(50) = 9.33 microM), tyrosine-specific protein kinase, EGF receptor (IC(50) = 7.67 microM), and protein kinase C (PKC) (IC(50) = 25.01 μM) in vitro [82] . Recently novel synthetic chalcone-coumarin hybrid has been employed to investigate Aβ aggregation reduction, antioxidation, and neuroprotection [83]

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Chapter:3

Coumarin derivatives inhibit the aggregation of β-lactoglobulin

3-1. Introduction

It is well known that soluble proteins can form insoluble amyloid fibrils. There are different types of amyloid fibrils that can form from proteins, and each one triggers different types of diseases [1]. There are different ways that proteins can form amyloid fibrils, which cause different types of diseases. Alzheimer's disease, Parkinson's disease, or mad cow disease are all neurodegenerative brain disorders caused by aggregation of Ab, synuclein, or prion proteins. Furthermore, it has been demonstrated that diabetes type 2 occurs when insulin amyloid fibrils form around it [1]. Since amyloid fibrils are insoluble in physiological conditions, they can deposit around the cell and tissues and cause a pathogenic effect [2]. Proteins that aggregate form very stable systems that facilitate the process. From a structural perspective, fibrillar assemblies generally have a cross-b conformation. It is interesting to note that fibrils can form regardless of the size, shape, and source of protein. Recently, hydrophobins, curli, and melanosomes have been identified as the expression of functional amyloidogenesis [1]. The development of a number of therapeutic strategies has been aimed at preventing the formation of amyloid fibrils. Some of the prevention techniques include site-specific glycosylation of proteins [3] and protein engineering [4]. As a result of being in a global free-energy minimum, most proteins are in a very stable conformational state in fibril form [5–7]. As a result, it is very hard to stop a protein from forming fibrils once it starts partial degradation. When the native conformation of a protein is stabilized, the partial degradation of the protein can be slowed, and this may lead to a reduction in fibrillation [8]. In the current therapeutic setting, this is the most effective strategy for preventing fibrils from forming. It is therefore vital to design new anti-fibrillating molecules.

A natural product found in many plants, coumarin is an aromatic heterocyclic lactone. There are thousands of compounds in the coumarin derivatives or coumarinoids family. The properties of these organisms are very interesting from the perspectives of photophysics [9], biology, and medicine [10]. In addition, to absorb the light at 280 nm, emission at 410 - 470 nm indicates that coumarin has fluorescence properties. As the substituents attached to the moiety are changed, this photophysical property is very much adjustable [11]. The use of these compounds as dyes is widespread as a result. In the past, some coumarinoids were developed for organic laser dyes that could be tuned from blue to green [11].

Among the whey proteins, there is β-lactoglobulin (β-LG), which has a wide range of nutritional and transport properties [12]. A barrel-like structure is formed by eight antiparallel β-sheets and occurs when exposed to heat. It is possible to isolate and purify the protein in high

yield using the procedure described by Aschaffenburg and Drewry, 1957 [13]. In this way, it is a very good model protein for protein aggregation studies. [14]

Warfarin, a derivative of 4-hydroxycoumarin, inhibits protein aggregation and is used to prevent blood clots [15]. These same compounds also have anticoagulant properties and are antagonists of vitamin K [16]. Several coumarinoids have been found to reduce biological effects associated with amyloid-β aggregation [17]. Some coumarinoids show inhibition of insulin fibrillation [18]. Coumarin hybrid molecules may also be designed to stop fibrillation in neurodegenerative disorders through multiple targeting mechanisms [18]. The fact that polyphenolic coumarinoids have anti-fibrillatory properties encourages the author to investigate them further. The purpose of this study was to synthesize and characterize polyphenolic coumarinoids and to determine their ability to inhibit fibrillation using the model protein β-lg.

3-2. Synthesis of coumarinoids

In this study, all the coumarin derivatives was synthesized except 4-hydroxycoumarins (**3**) which were collected from commercial source and used without further purification. Kayal et al., showed that polyhydroxy benzenes can be reacted with electron-deficient alkynes to produce polyhydroxycoumarinoids (**SM1–SM4**) with the help of 5 mol% CuO in refluxing toluene [19]. Here, this method was used for the synthesis of different polyhydroxycoumarinoids and it has been shown in the **Fig. 1**. All these compounds were well characterized and used in the protein aggregation experiments.

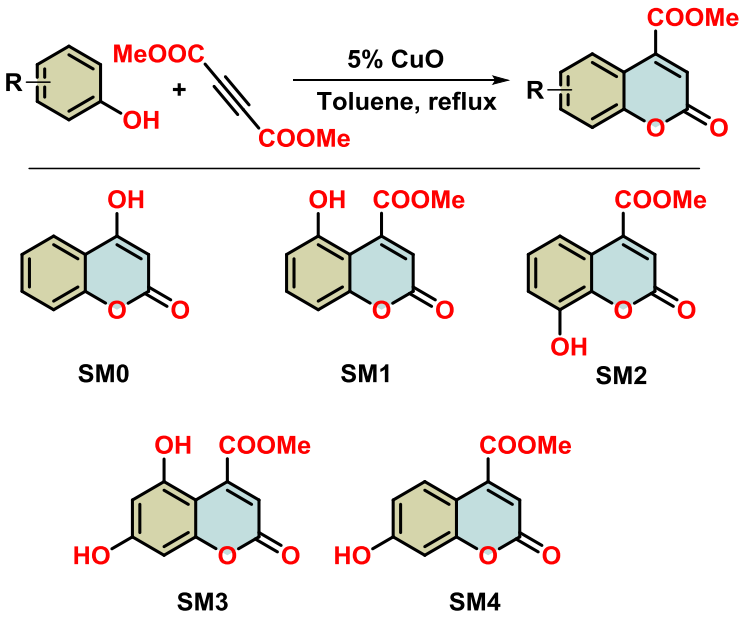

Fig.1: Synthesis of coumarin derivatives.

A particular feature of the compounds under investigation is the distribution of hydroxyl groups around the nucleus of coumarin [20]. At the C4 position, the hydroxyl group is present in compound **3**. The methyl carboxylate at C4 is attached to the OH at C5, and OH is shifted to the C4. C8 and C7 carbons are filled with OH groups in **SM2** and **SM4**, respectively. The positions C5 and C7 of **SM3** contain two hydroxyl groups. The molecular assembly of proteins is investigated here by examining the effects of both hydroxyl and methyl carboxylate groups.

3-3. UV-spectral characterization

It has been demonstrated that UV spectroscopy can be used to indicate the structural changes caused by interaction with small molecules [20]. In this study, we investigated the structural change of β-LG in the presence of coumarin derivatives. Due to the presence of Trp and Tyr moiety, a sharp characteristics peak is observed at λmax 280 nm for β-LG. The absorption spectrum of coumarin derivatives is distributed between 270 nm (for **3**, **SM1**, **SM2**, and **SM4**) and 350 nm (for **SM3**) as shown in Fig 2.

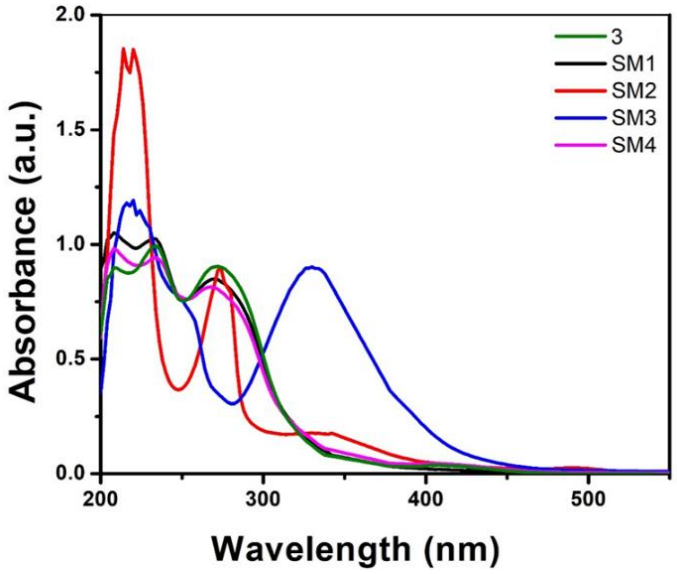

Fig. 2: UV-vis spectra of coumarin derivatives in the 10mM phosphate buffer (pH = 7.4)

The UV absorption intensity of native and heat-treated β-lg is lower with methyl 4-hydroxy-chromen-2-one (**3**) and methyl 8-hydroxy-2-oxo-2H-chromene-4-carboxylate (**SM2**) (Fig. 3). However, β-lg with methyl 5-hydroxy-2-oxo-2H-chromene-4-carboxylate (**SM1**), methyl 5,7-di-hydroxy-2-oxo-2H-chromene-4-carboxylate (**SM3**), and methyl 7-methoxy-2-oxo-2Hchromene-4-carboxylate (**SM4**) exhibit pronounced low-intensity bathochromic shifts compared to native.

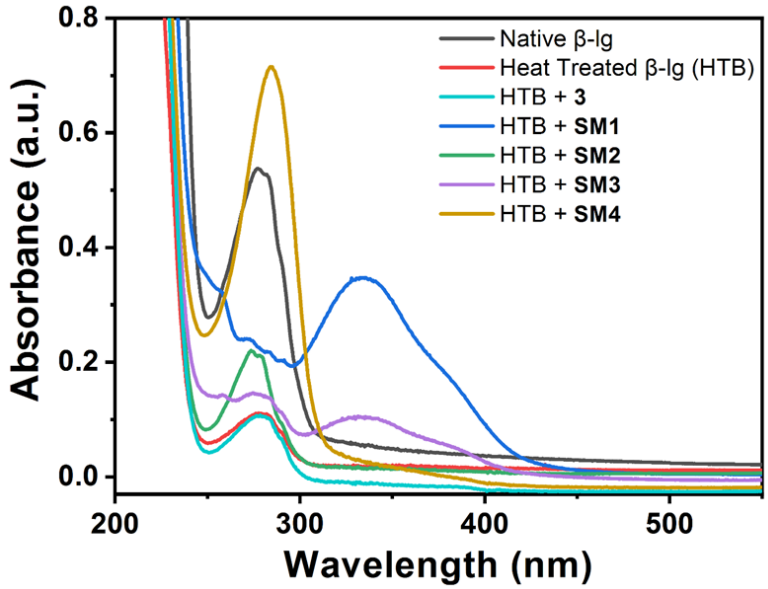

Fig. 3: Absorption Spectra of native β-lg and β-lg incubated at 78°C for 1 h in the absence and presence of different coumarinoid molecules.

It is possible to eliminate the contributions of the coumarin molecules in the aforesaid absorption spectra by adding them to the reference cell along with the sample cell (both the cell has the same concentration of coumarin derivatives). All the coumarin derivatives showed a single peak at 280 nm in this experiment (Fig. 4). Coumarin derivatives have stronger intensities in the spectra of proteins compared to native proteins and heat-treated proteins. Observations such as these indicate that the small molecule was likely to interact with the protein β-LG.

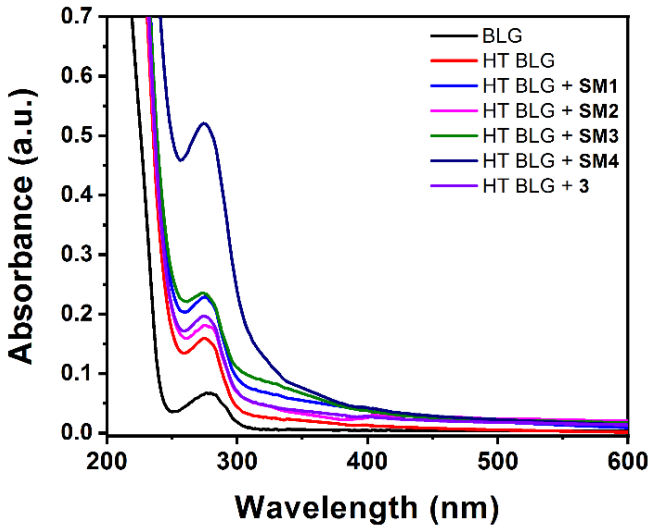

Fig. 4: UV-vis spectra native β-lg and protein under heating conditions in the presence and the absence of small molecules.

3-4. Intrinsic fluorescence

To monitor protein structure and how small molecules interact with proteins, fluorescence is an effective and sensitive technique. In most proteins, Trp and Tyr amino acid residues are responsible for the fluorescence caused by absorbing light of the corresponding wavelengths. β-LG only contains two Trp residues (Trp19 and Trp61) and four Tyr (Tyr20, Tyr42, Tyr102, and Tyr99) that contribute to its intrinsic fluorescence. Beta-lactoglobulin structural changes can alter the polarity of the fluorophore in its microenvironment due to the changes in its polarity. Aggregation of the synthetic ligand is associated with a decrease in solvent exposure, which is reflected in the emission spectra of fluorophores, primarily tryptophan. Here, aggregation of β-LG was investigated in the presence of coumarin derivatives.

A change in β-lg's intrinsic fluorescence can be attributed to its aggregation after heating at 78^{o}C for 1 hour (Fig. 5). In the case of **SM2** and **3**, the fluorescence intensities are slightly higher and lower with respect to heat-treated β-lg, respectively. When aggregation conditions are the same, **SM1** and **SM3** display greater fluorescence intensities than native β-lg and lower fluorescence intensities than heat-treated β-lg. In contrast, native β-lg is less intense when **SM4** is present.

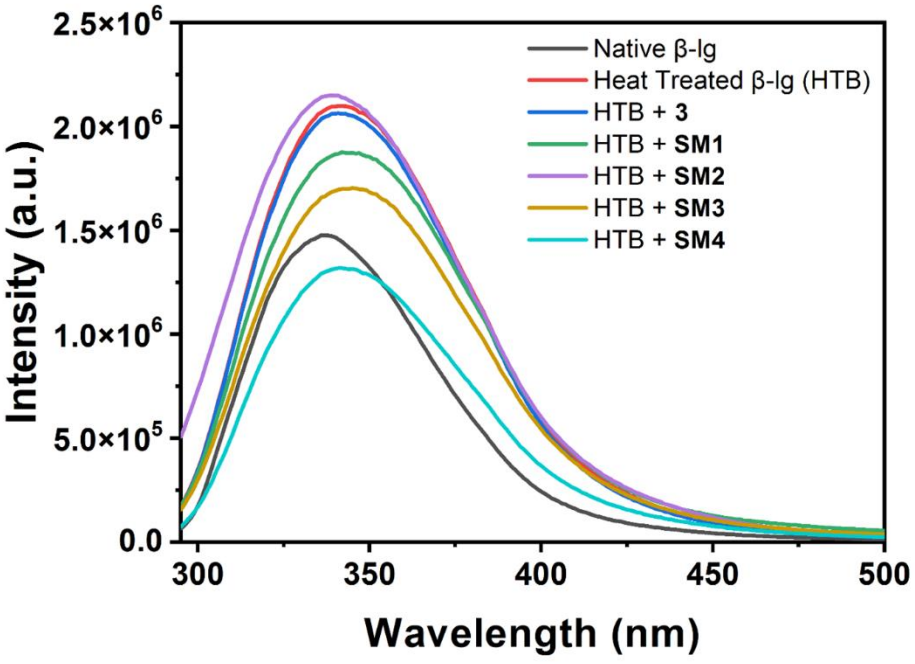

Fig. 5: Intrinsic fluorescence spectra of native β-lg and β-lg incubated at 78°C for 1 h in the absence and presence of different coumarin derivatives (protein: coumarin derivatives= 1: 1)

Therefore, coumarin derivatives interact with β-lg during its thermal exposure and can change its conformation. There is a possibility that compound **SM4** can interact with β-lg in such a way that Trp residue exposes more to solvent. Under the aforementioned conditions, it may explain the decrease in fluorescence of β-lg with **SM4**.

3-5. Aggregation of β-lg identification and quantification of the aggregates by Th-T assay

In response to an excitation of 480 nanometers at thioflavin T (Th-T), a benzothiazole dye, binds to the hydrophobic sites on fibrillar aggregates of proteins, increasing the fluorescence intensity at 480 nanometers [21]. During the heating process at 78^{o}C, native β-lg aggregates, can be measured through the intensity of the Th-T spectrum. The Th-T emission intensities of all other heat-treated β-lg samples with different coumarin derivatives are proportional to the number of aggregates that form in each protein solution. Because of the anti-fibrillating properties of coumarin derivatives, the Th-T will have a lower intensity due to lower aggregate formation. Since the two molecules (protein and **SM**s) have the same absorbance and emission peaks, determining their equivalent molar ratio through UV or fluorescence spectra can be very challenging. At a fixed [β-lg] in this case, we measured the intensity of the fluorescence of Th-T with coumarin derivatives with different molar ratios. We show this in Fig. 6.

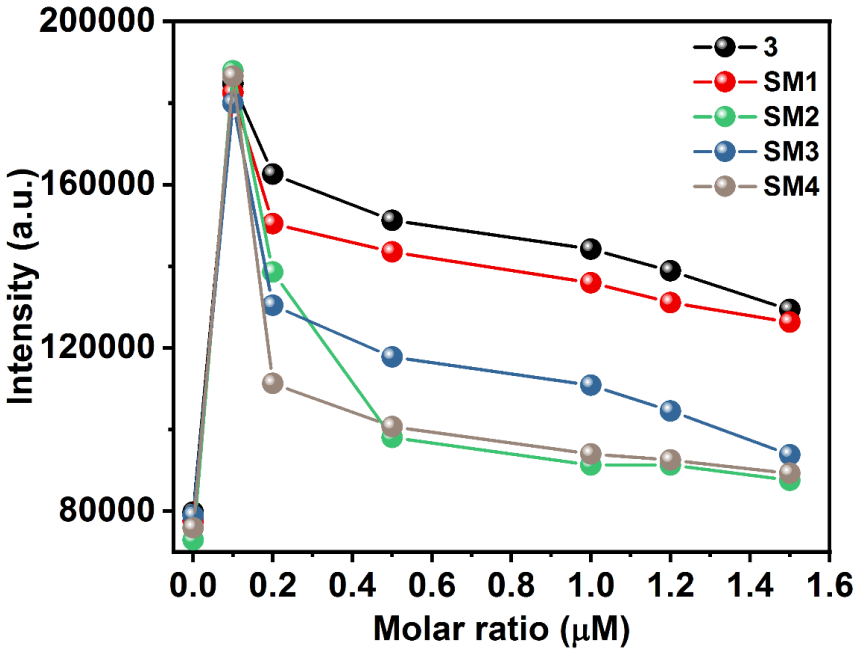

Fig. 6: Plot of fluorescence intensity of Th-T in the presence and absence of coumarin derivatives with different molar ratio at a fixed [β-lg].

Protein aggregation inhibition is most effective when **SM** compound ratios are 1:1 protein and protein. According to the Th-T assay, Figure 4 compares the aggregation patterns of native and heat-treated β-lg at pH 7.4 in the presence and absence of different coumarin derivatives (1:1). Figure 7 shows that heat-treated β-lg exhibits the most incredible Th-T intensity due to self-assembly formation. It appears that heat-treated β-lg with 3 shows slightly less fluorescence intensity than heat-treated β-lg alone under identical heating conditions. Nevertheless, when β-lg was incubated separately with **SM1**, **SM2**, **SM3**, and **SM4** there was a marked decrease in Th-T fluorescence due to the formation of smaller aggregated β-lg molecules. The synthesized coumarin derivatives can therefore inhibit the oligomerization of β-lg and act as effective anti-fibrillation agents. In this case, the inhibition of aggregation of coumarin derivatives follows the order of **SM2** > **SM4** > **SM3** > **SM1** > **3**.

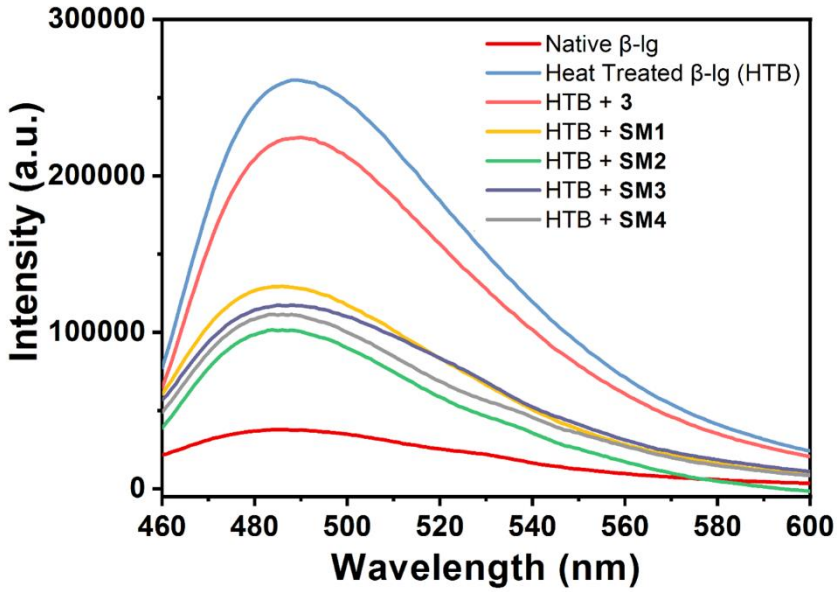

Fig. 7: Th-T(λex = 440 nm and λem = 485 nm) assay of native β- lg and heat-treated β-lg(incubated at 78°C for 1 h) in the absence and presence of different coumarin compounds (1:1) at pH = 7.4, excitation wavelength was set at 440 nm and emission wavelength was 480 nm.

3-6. ANS-fluorescence study to monitor the hydrophobicity changes

A fluorescent probe called 1-anilinonaphthalene-8-sulfonate (ANS) can bind to the surface of protein aggregates by forming an electrostatic and hydrophobic interaction at the hydrophobic site [22]. The hydrophobic interactions involved in aggregation were monitored by fluorescence experiments conducted with ANS.

The protein, β-lg, contains two potential binding sites for small hydrophobic molecules. The first site is the inner side of the beta-barrel and the second site is at the channel between the barrel and the alpha helix. After binding with the hydrophobic site of the protein, the dye emits fluorescence around 480 nm with greater intensity.[23]

In heat-exposed β-lg (78^{o}C, 1 h), ANS fluorescence intensity was enhanced at 480 nm. The increase in fluorescence intensity during aggregation may be explained by ANS having more access to hydrophobic patches in heat-treated β-lg (Fig. 8). Since the ANS intensity is lower in the β-lg sample than in the heat-treated sample, the aggregation process is slowed down. In addition, the aggregate surfaces are less hydrophobic than the fluids. Therefore, β-lg is likely to undergo conformational changes when thermally exposed.

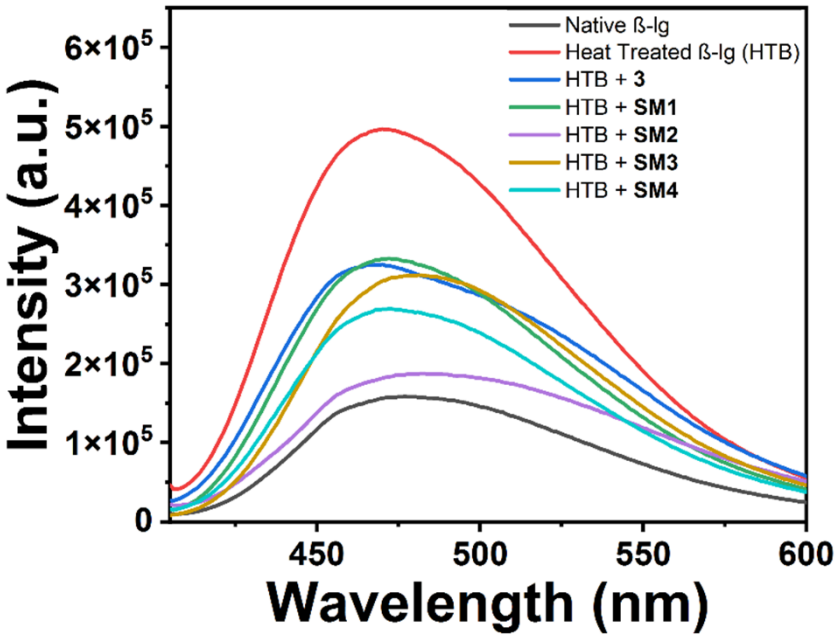

Fig. 8: ANS- fluorescence emission spectra of native β-lg and β-lg incubated separately at 78°C for 1 h in the absence and presence of different coumarin derivatives (1: 1) at pH = 7.4. β-lg concentrations throughout all emission experiments were kept at 0.25mg mL^{-1}. Results were the mean of three different experiments

Incubation with **SM2** at 78^{o}C yielded significant results for β-lg. According to the findings, the ANS fluorescence intensity decreased significantly, and it was much closer to the native β-lg fluorescence intensity, implying that the hydrophobic loops of β-lg were less exposed, demonstrating that ANS had less affinity for β-lg, and **SM2** effectively inhibited the thermal aggregation of β-lg by decreasing protein–protein interactions.

Despite very close spectral correspondence between **SM3, SM1 and 3**, ANS fluorescence intensities have been reduced by almost 55% when incubated with β-lg with **SM3, SM1 and 3**. The presence of 3, 1 and 3 resulted in less hydrophobic loop opening, thus decreasing protein–protein interactions, whereas ANS binding and hydrophobicity were greater than 2. It can thus be concluded that the fibrillation of β-lg occurs when hydrophobic sites of the protein are exposed to the solvent and that binding coumarin derivatives to these hydrophobic sites stabilizes them such that they prevent the protein from undergoing fibrillation. Therefore, coumarin derivatives are capable of stabilizing protein conformation to a greater extent with lower ANS intensities. It is found that the order of stabilization is **SM2** > **SM4** > **SM3** > **SM1** > **3**. In light of the above data, this result supports the Th-T results.

3-7. Rayleigh light scattering (RLS) study

RLS measurements can also be used to investigate protein aggregation. As colloidal particles in the medium scatter light, they increase the scattering of light. A higher protein aggregate can therefore scatter light more efficiently than an aller aggregate. A B-lg solution was incubated at 78°C for 1 h without and with coumarin derivatives, and RLS data were collected. According to Figure 6, heat-treated β-lg is observed to scatter the most in the absence of coumarin derivatives. A protein's structure is lost upon thermal denaturation, resulting in maximum aggregate formation. As coumarin derivatives were added to β-lg, RLS intensities decreased, indicating aller aggregates were formed. In this regard, coumarin derivatives contribute to maintaining the structural integrity of proteins. The minimum RLS intensity value obtained for 2 (closer to that of native β-lg) was found to be superior to that of other coumarin derivatives during thermal exposure (Fig. 9). During thermal incubation, RLS intensities decreased, confirming fewer aggregates formed. In our present study, **SM2** > **SM4** > **SM3** > **SM1** > **3** were found to be the most effective in inhibiting β-lg thermal aggregation by coumarin derivatives.

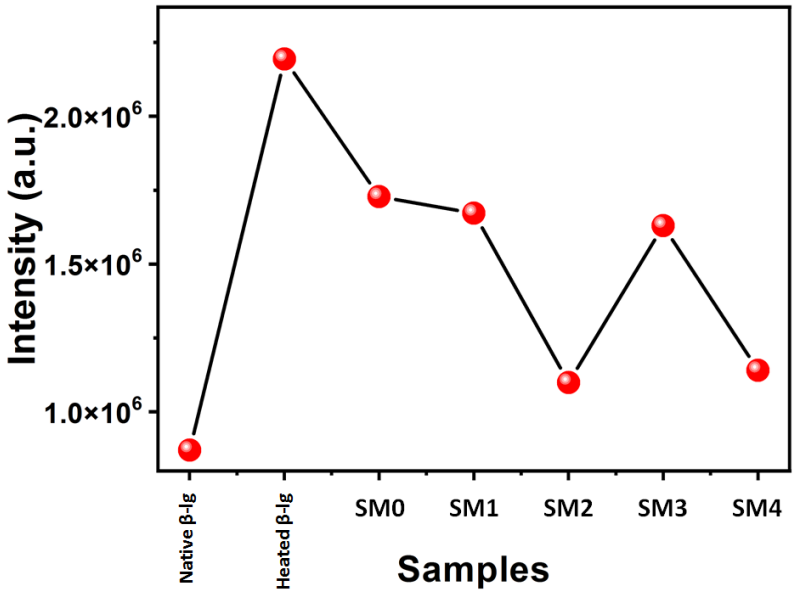

Fig. 9: Rayleigh light scattering data of β-lg at native form and β-lg incubated at 78°C for 1 h in the absence and presence of different coumarin derivatives (1: 1) at pH = 7.4.

3-8. Dynamic light scattering (DLS) measurements

We were able to identify and measure the hydrodynamic size of heat-induced oligomers of β-lg by using dynamic light scattering (DLS). Various coumarin derivatives as well as native β-lg and its heat-treated form are shown in Figure 7 along with their size distribution profiles. Different sized protein aggregates formed after heating (78ºC, 1h) β-lg with or without coumarin compounds. Native β-lg had a hydrodynamic radius between 12 and 50 nm, and its size was increased to 1250 to 3200 nm after incubation at 78°C for an hour, indicating large β-lg aggregates with more significant light scattering effects. There was a decrease in the scattering intensity of the β-lg solution in the presence of different coumarin derivatives. When 3 was present and in the range 1500-2750 nm, protein aggregate sizes decreased. It is known that incubation with **SM2** produces a minimum hydrodynamic radius. As coumarin molecules were incubated with aller diameter aggregates, the size of the aggregates decreased minimally between 100 and 450 nm. As coumarin derivatives are present in protein aggregates, hydrodynamic radii are observed to follow the following order: **SM3** > **SM1** > **3** > **SM4** > **SM2**. Therefore, all of these coumarin compounds have potent anti-fibrillatory properties, with **SM2** being the strongest inhibitor (Fig. 10). **SM4** is the second strongest inhibitor, with **3** being the third strongest inhibitor (Fig. 10). Several β-lg aggregates were also imaged with AFM to confirm this result.

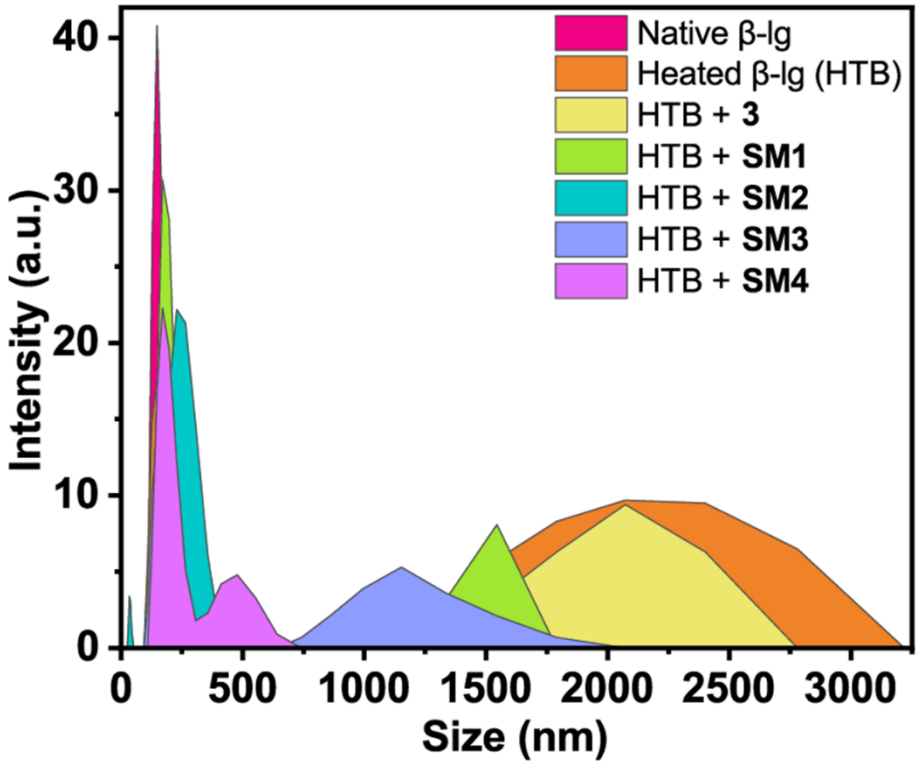

Fig. 10: Number particle size distribution spectra in DLS studies β-lg at native form and β-lg incubated at 75 °C for 1 h in the absence and presence of different coumarin derivatives (1: 1) at pH = 7.4.

3-9. Monitoring the changes in secondary structure of β-lg during thermal incubations with circular dichroism spectroscopy

The far-UV CD technique was used to investigate the potential secondary structural transformations of β-lg induced by different coumarinoid compounds. A CD spectroscopy analysis was performed on the β-lg solutions with or without various coumarin derivatives for one hour at 78°C. A CD spectrum within the range 190-250 nm was scanned for one hour. A CD spectrum between 190-250 nm is shown in Fig. 11. A localized negative band of 208 nm and a circular negative band of 215 nm was observed in native β-lg. A-helix and b-sheet are found to be present in both of these bands of β-lg. Incubated β-lg has more beta structure (61%) but less alpha structure (9%) than native β-lg.

Table 1: Structural integrity of native and heat stressed β-lg at 78 °C for 1h (in the absence and presence of various coumarin derivatives) on determined by CD calculations. [a]

β-lg Samples	% of α-Helix	% of β-Sheet	% of β-Turn	% of Random coil
Native	11.80	45.00	11.10	32.10
Heat stressed in absence of Coumarin derivatives	10.10	48.20	12.30	29.40
Heat stressed in presence of 0	11.50	47.15	11.25	30.10
Heat stressed in presence of 1	11.84	47.10	10.65	30.41
Heat stressed in presence of 2	13.05	45.15	10.48	31.32
Heat stressed in presence of 3	11.15	46.25	10.55	32.05
Heat stressed in presence of 4	11.00	46.00	10.20	32.80

[a] : Calculated by CDNN 2.1 Software

The analysis of this data revealed that greater b-sheet structures were formed when the protein was thermally aggregating. A significant shift in the band positions was also observed in the CD spectrum of β-lg. In the presence of different coumarin compounds, we investigated the secondary structure change of heat-treated β-lg. Compared to heated β-lg, there was a all change in peak position with a decrease in ellipticity value. β-lg CD spectra at 215 and 207 nm showed a decrease in MRE values with the presence of **SM1**. It appears that in both cases with **SM3** and **SM1**, fewer β structures have formed in comparison to heat-treated β-lg alone, indicating α structural transition that leads to the disaggregation. In presence of coumarin derivative **SM2**, the CD signal of β-lg looks similar to that of β-lg natively, but has decreased negative ellipticities around 208 and 215 nm. It is possible that the structure of the derivatives is responsible for the difference in MRE values. The amount of different β-structures decreased most significantly compared to heat-stressed β-lg alone showing maximum inhibitory power **SM2** against the thermal aggregation of β-lg. In this region, **SM3** and **SM4** display almost identical CD spectra, however CD results demonstrate that **SM4** inhibits fibrillation of β-lg better than **3**. This study showed that all coumarin compounds can suppress the thermal aggregation of β-lg, and that **SM2** inhibits **SM4** > **SM3** > **SM1** > **3** coumarin compounds. A comparison of secondary structural changes in β-lg when coumarin compounds are present and absent is shown in Table1.

3-10. Morphological studies with atomic force microscope (AFM)

AFM was also used to gain insight into fibril formation; it has been proven to be an important tool in studying fibril formation.27 In order to visualize the extent of disruption of β-lg samples aggregated alone or together with coumarin derivatives at molar concentration ratios of 1:1, AFM analysis was conducted.

β-lg aggregate morphology is shown in Fig. 11 under different conditions. An AFM image of the aggregates shows that they are globular rather than fibrillar. AFM images of β-lg reveal them to be all globular particles (Fig.11). Incubation of the β-lg solution alone at 78^{o}C for 1 h resulted in the formation of large globular aggregates of β-lg (Figure 12a). As a result of the change in conformation, the β-lg monomers swell during thermal incubation, forming aggregates.

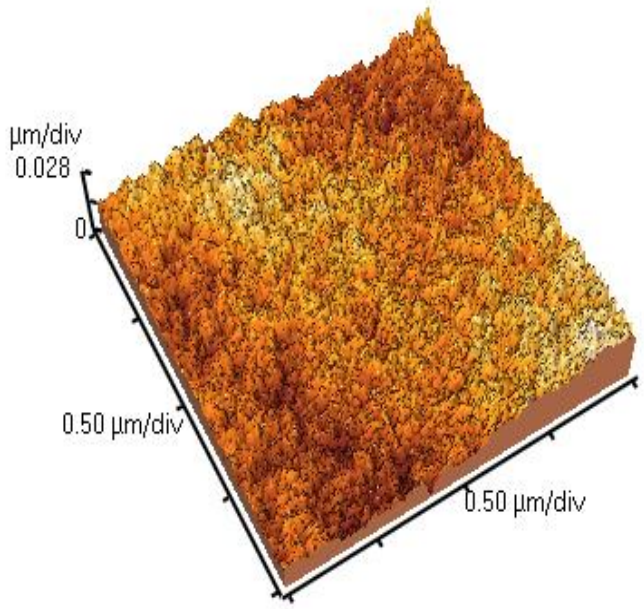

Fig. 11: AFM images representing the morphology of native β-lg

We investigated the aggregation mechanism of the β-lg extract by incubating it for 1 h at 78^{o}C in the presence of different coumarin derivatives at a molar ratio of 1:1. It was found that compound **3** was ineffective in inhibiting protein aggregation, since aggregates obtained after heat treatment are similar in size and nature to the ones produced by compound 3. In the case of 1, large aggregates of the globular type (Fig. 12c), similar results were obtained. When β-lg was thermally incubated with coumarin compound **SM2**, the larger, more numerous globular aggregates were evidently destroyed (Fig. 12d). Despite the low density of the aggregates, the aggregates were very large. Therefore, inhibiting, and arresting β-lg aggregation was found to be most effective. However, 3 has a lower efficiency than both SM2 and SM4, but is more

efficient than other compounds (Fig. 9e). The size of the aggregate particles formed with 3 is sufficiently aller than with heat-treated β-lg (Fig. 9a) or with heat-treated β-lg in presence of 3 (Fig. 9b). In this way, 3 is also effective for suppressing the formation of larger aggregates of β-lg. All the previous experiments have also demonstrated that **SM2** > **SM4** > **SM3** > **SM1** > **3** is the order in which the inhibition efficiency of the coumarin derivatives is the highest.

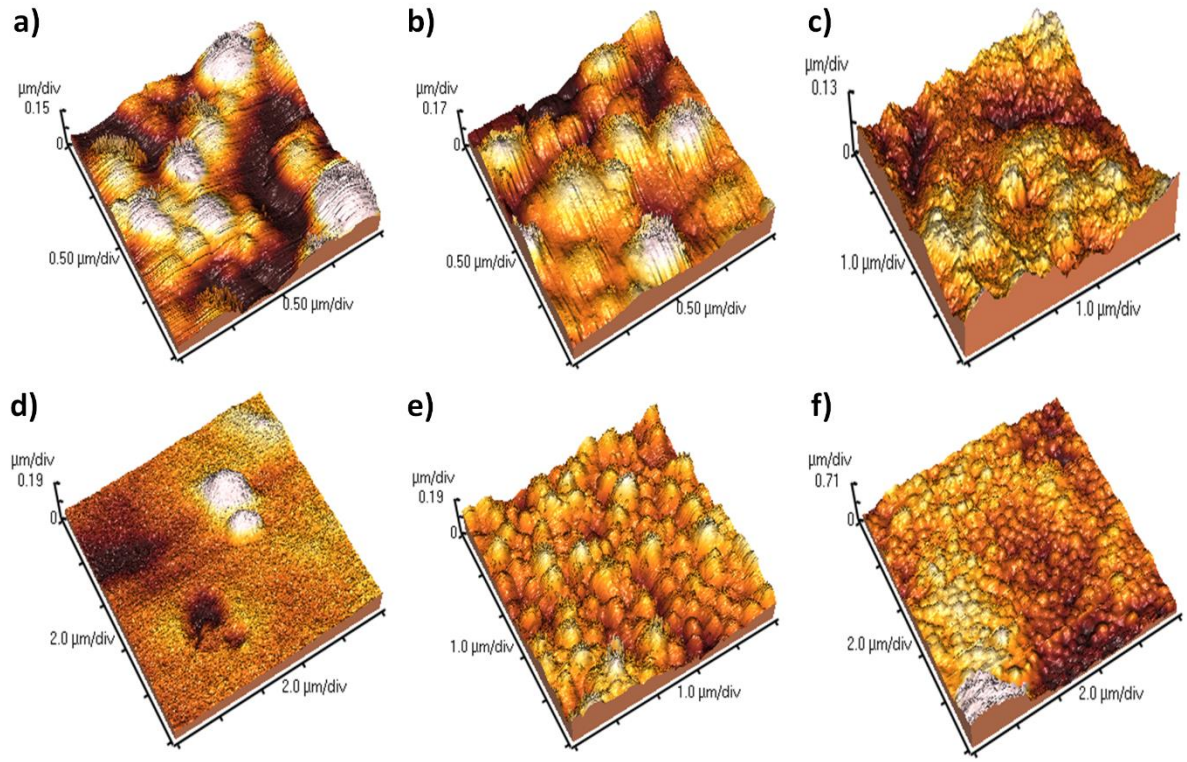

Fig. 12: AFM images representing (a) the aggregate morphologies of heat treated β-lg and(b-f) heat treated β-lg in the presence of coumarinderivatives: (b)with **3**, (c) with **1**, (d) with **2**, (e) with **3**, (f) with **4**.

3-11. Docking studies

This is due to the anti-protein aggregation effect of these all molecules, which can interact with the protein and stabilise its native conformation. Docking studies were conducted in order to understand how coumarin derivatives affect β-lg's native structure. Three, 1, 2, 3, and 4 have binding energies (DG) of -5.6, -5.8, -7.6, -6.3, and -6.9 kcal mol-1, respectively. 2 > 4 > 3 > 1 > 3 appear to be the order of stabilization by these molecules. Molecular order of stabilization with these molecules is consistent with their order of inhibition of protein aggregation. So coumarin molecules play a key role in preventing protein aggregation by stabilizing β-lg conformations and locking a protein structure.

Molecular interaction analysis can provide insight into how a protein conformation freezes by analysing docking results. As shown in Fig. 13a, the 2 binds to the narrow end of the barrel of the β-lg. An encapsulated molecule is contained in this cavity (Fig. 13b). Hydroxyl groups and methoxy oxygen atoms are involved in hydrogen bonding with amino acid residues N88, N98, and S116. Similarly, coumarin ring C=O and S116 also exhibit nonconventional hydrogen bonds. Hydrophobic interactions were observed between the methyl of methoxy and L39 in Fig. 13.

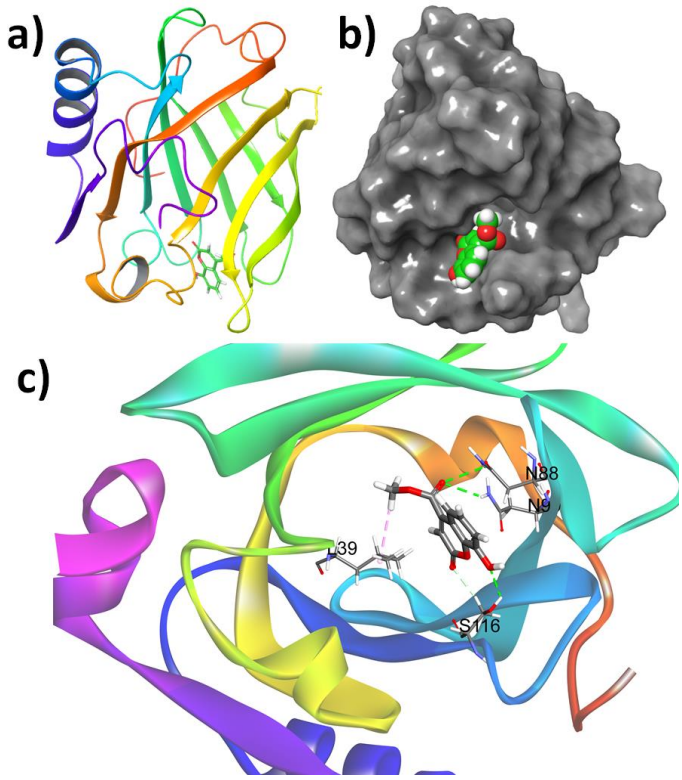

Fig. 13: (a) The structures of the most stable β-lg-coumarin 2, bind at the terminal of the barrel, (b) binding of 2 at the hydrophobic pocket, (c) different of non-covalent interactions in the binding site of β-lg with 2.

Protein binding is influenced by the positions of OH groups according to docking studies. As far as hydrogen bonding is concerned, C4 and C7 OH groups cannot form hydrogen bonds with amino acid residues. Nevertheless, hydroxyl groups located at C4 and C5 are capable of interacting with amino acid residues within their respective active sites. The stability of protein– complexes is therefore influenced by hydrogen bonding.

It is possible for the protein to exist in native form as dimers. It is possible to make this protein in quaternary form by adding an additional binding pocket. The aforesaid effect may be caused by coumarin derivatives that are able to bind to this site. Such binding possibilities were discovered through molecular docking. The docking process is carried out using energy-

minimized dimeric proteins. Based on the results of this study, it is found that 3, 1, 2, 3 and 4 have binding energies (DG) of 3.2, -3.9, -5.2, -4.3 and -4.7 kcal mol^{-1}, respectively. The binding energies obtained from the monomeric form of the protein are considerably lower than those obtained from this form.

3-12. Experimental

3-12.1. Synthesis of Coumarin derivative[19]

To a round bottomed flask a mixture of resorcinol (110 mg, 1.0 mmol) and dimethyl acetylenedicarboxylate (170 mg, 1.2 mmol) were taken in 1.3 mL dry toluene and stirred magnetically in a 10 mL round bottom flask. 4 mg CuO (0.05 mmol) catalyst was added to the mixture and allowed the stirring for 3 h at 110 °C. Thin layer chromatography was used to determine the degree of product formation. Under low pressure, the solvent was removed from the reaction mixture, and silica gel was used for column chromatography and purification. ^{1}H NMR and ^{13}C NMR spectroscopy were used to characterize the pure compounds. The ESI contains all the data.

NMR data:

Methyl 5-hydroxy-2-oxo-2H-chromene-4-carboxylate (SM1). Yellow semi solid (42%); 1H NMR (300 MHz, CDCl3) δ 3.95 (s, 3H), 6.57 (d, J = 7.8 Hz, 1H), 6.67 (d, J = 8.5 Hz, 1H), 6.79 (s, 1H), 7.35 (t, J = 8.4 Hz, 1H), 11.86 (brs, 1H); 13C NMR (75 MHz, CDCl3) δ 53.9, 102.0, 108.3, 114.9, 119.2, 132.9, 136.6, 156.9, 157.5, 167.4, 169.8.

Methyl 8-hydroxy-2-oxo-2H-chromene-4-carboxylate (SM2). White solid (65%); mp 78-82 °C; 1H NMR (300 MHz, CDCl3) δ 3.81 (s, 3H), 6.47 (s, 1H), 7.13-7.16 (m, 2H), 7.18-7.21 (m, 1H), 7.27 (dd, J = 1.2, 6.0 Hz, 1H); 13C NMR (75 MHz, CDCl3) δ 51.9, 106.1, 116.9, 117.4, 124.7, 125.9, 138.4, 138.6, 145.2, 154.1, 163.5.

Methyl-5,7-dihydroxy-2-oxo-2H-chromene-4-carboxylate (SM3). Yellow solid (75%); mp 216-218 °C; 1H NMR (300 MHz, DMSO-d6) δ 3.77 (s, 3H), 6.06 (d, J = 1.5 Hz, 1H), 6.14 (d, J = 1.5 Hz, 1H), 10.60 (br s, 1H), 6.52 (s, 1H), 11.28 (br s, 1H); 13C NMR (75 MHz, DMSO-d6) δ 52.8, 91.5, 99.2, 101.0, 117.9,168.4, 128.0, 157.3, 157.4, 164.4, 168.3.

Methyl-7-hydroxy-2-oxo-2H-chromene-4-carboxylate (SM4). Red solid (22%); mp 152-154 °C; 1H NMR (300 MHz, DMSO-d6) δ 3.77 (s, 3H), 6.46 (s, 1H), 6.64-6.68 (m, 2H), 8.36 (d, J = 8.4 Hz, 1H), 10.92 (br s, 1H); 13C NMR (125 MHz, DMSO-d6) δ 52.1, 98.4, 111.7, 112.1, 118.4, 130.0, 133.1, 157.7, 163.4, 165.4, 168.0.

3-12.2. UV-visible spectroscopy.

This measurement was conducted through the use of a UV-Visible JASCO V700 Spectrophotometer, model no V-730, Serial No. B184461798 and JASCO Spectra Manager software. A binding affinity and binding constant were determined by recording absorbance spectra at room temperature (25°C). A reference and sample Perkin Elmer quartz cell with a path length of 1cm was used for this experiment. Spectra of intensity versus wavelength were recorded from 200 to 600 nm for the absorbance measurement. It was taken a 10 mm phosphate buffer with a pH of 7.4 for use as a reference cell. Concentration of the sample solution were 13µm.

3-12.3. Intrensic fluoroscence:

Using a Horriba Fluorometer (FLUOROMAX-4C, Serial No 1734D-4018-FM), fluorescence measurements were conducted. Stock solution of β-lg and others solution of 13µm were taken in a fluorescence quartz cell of path length of 1cm and excited at 285 nm. An emission spectrum was recorded between 295 and 550 nm. An emission slit of 5 nm and an excitation slit of 5 nm were used. Scan rates of 100nm/s were used to record the data.

3-12.4. Th-T Assay:

A large percentage of ThT binds to aggregates of β-lg and other amyloid fibrils. The fluorescence at 480 nm is enhanced after binding. In this experiment, stock samples of 54.3 mg were mixed with 3.13 mM ThT solution. With Horriba Fluorometer (MODEL: FLUOROMAX-4C, Serial No 1734D-4018-FM) and Fluoromax Software, the emission range was measured from 460 to 600 nm using the assay solution excited at 450 nm2. The slit widths were both kept at 5 nm for excitation and emission.

3-12.5. ANS:

In order to determine the hydrophobicity of protein molecules, fluorescent probes were used that bind to hydrophobic packets on the surface of the protein.3 One of the most commonly used polarity sensitive probes is 1-anilinonapthalene-8-sulfonate (ANS). The hydrophobicity of the samples is determined by adding a stock solution of ANS (2 ml volume) to each sample. We maintain a final ANS concentration of 30M in each sample. In order to determine fluorescence spectra, Shimadzu spectrofluorometers (Shimadzu

5301 PC) were used to excite the cells at 380 nm and record emission spectra at 390 to 550 nm. One cm was the length of the path. The emission and excitation slits were both 5 nm.

3-12.6. Raleigh Light Scattering:

The formation or change in the presence of Turbidity or aggregates after the incubation of β-lg in absence and presence of different coumarin samples (0 – 4) with respect to the native β-lg were quantitively measured using Raleigh Light Scattering Study. Using a Horriba Fluorometer (MODEL: FLUOROMAX-4C, Serial No 1734D-4018-FM) and Fluoromax Software, samples were excited at 350nm and emission intensity was recorded at 350nm. Samples were prepared using 10mM phosphet buffer (pH – 7.4). Two milliliters of 13m solution of each sample were taken into a quartz cell of 1 cm path length and a 5nm excitation slit was used for the experiment.

3-12.7. CD spectroscopy

Jasco spectropolarimeter (J-815) (Jasco, Tokyo, Japan) CD spectra were recorded using Jasco Spectra Manager Software in presence of various coumarin compounds. The far UV (190-260nm) experiments were conducted in a parkin Elmer quartz cell with a path length of 0.2cm, and then data was recorded at the wavelength range from 190-260nm in nitrogen inside the instrument. Temperature was 30°C and scan speed was 100nm/s. Jasco Spectra Analysis tool was used to record spectra. Software CDNN 2.1 was used to calculate primary structural elements and secondary structural elements.

3-12.8. Dynamic light scattering (DLS)

Generally, in the biological Laboratories Dynamic light scattering (DLS) study is used to determine the size of proteins, nucleic acids, and complexes or to monitor the binding of ligands. Also, diffusion of tiny particle of Nano sized range alters the intensity of the scattered light. And DLS study is used to determine the presence of different molecules and supra molecules as it is very sensitive to particle size.[24] Using Zetasizer Nanos (Malvern Instrument, U.K.) equipped with 633 nm laser and using 2 ml rectangular helma cuvette of 1 cm path length DLS Measurements were done at 20°C taking 250 ml of samples (β-lg, heat treated β-lg, and heat treated β-lg in presence of different

coumarin compounds) in 1.75 ml 3 mM glycine– KOH buffers of pH 10.5. The time-dependent auto correlation function was acquired with twelve acquisitions for each run.

3-12.9. AFM:

It is necessary to prepare AFM Grid slides for this experiment. Grids are prepared by dropping various samples (each with 5 mg/ml 1-LG) onto a glass slide, spreading them over the entire slide, and then drying them overnight. An AFM microscope image was created using the DICP II auto probe (Model AP 0100) of Light Gray, Heat treated Light Grey and Light Grey in the presence of different Coumarin Compounds.

3-12.10. Molecular docking study:

The AutoDock 4.2.0 based docking studies of coumarin derivatives molecule with β-lg (2BSY) were carried out. The structure of coumarin derivatives used in docking after minimized its energy by DFT optimization using Gaussian 09W. Lamarckian genetic algorithm (LGA) was utilized for molecular docking. In this calculation, 126 x 126 x 126 grid box was used.

3-13. Conclusions

In summary, the study presents the application of coumarin derivatives for inhibiting β-lg aggregation. A particular protein conformation can cause protein aggregation due to protein-protein interaction. By stabilizing a protein conformer, the synthesized coumarin derivative prevents protein-protein interaction by preventing it from reaching the conformation required. In order to see if coumarin derivatives can prevent protein aggregation, we have tested five of them. The results show that coumarin derivatives have an order of inhibition ability of **2** > **4** > **3** > **1** > **0**. In our study, we used UV-visible, fluorescence, and CD spectroscopy, Raleigh Light Scattering, dynamic light scattering (DLS), and atomic force microscopy (AFM) to identify the order and understand the mechani that inhibits protein aggregates. Thus, proteins can be locked at a native-like conformation if they interact with a all molecule to inhibit protein aggregation by protein-protein interactions.

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Chapter:4

Review of literature on Chalcones

4-1. Introduction:

As a chemical scaffold for many naturally occurring compounds, chalcones are found in a wide range of plant tissues. They are found in vegetables, teas, fruits, and other plants throughout the world [1-3]. The word chalcone derives from the Greek word chalcos, which means "bronze", which is evident from the colors of most natural chalcones, which result from the process of formation [2]. It has been proved that chalcone compounds have a common chemistry. These compounds possess a common scaffold of 1,3-diethyl-2-propen-1-one, also known as chalconoid, simple molecule with two aryl rings (A and B) joined by α, β unsaturated carbonyl (Figure 1), which exists in both trans and cis analogs, with the trans isomer being thermodynamically more stable than the cis isomer [3,4]. During the last few decades, the chalcone family has attracted much attention in many different areas, not only from the perspective of synthetics and biosynthesis, but also due to its broad range of biological activities. Thousands of years ago, plants and herbs were used for treating a variety of medical

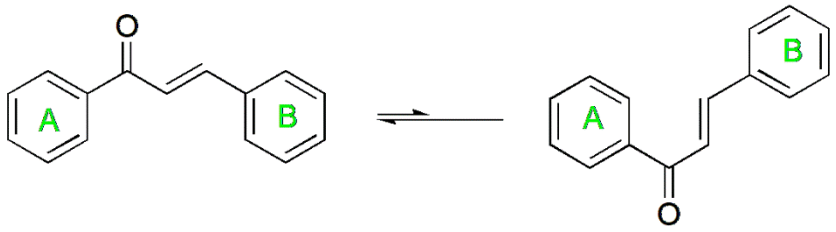


Fig. 1: Structures of chalcone, the phenyl ring attached to the carbonyl group is defined to be the A ring and the other benzene ring is named as the B ring

conditions, including diabetes, inflammation, tuberculosis, and cancer, with chalcones. [1-3, 6 - 12]. As a potential Michael acceptor, the unsaturated carbonyl functional group on chalcone molecule allows it to interact with sulfhydryls of cysteine residues. This interaction is considered responsible for their biological activities [13-16]. Several chalcone-based compounds have been approved for clinical use. For example, sofalcone was previously used

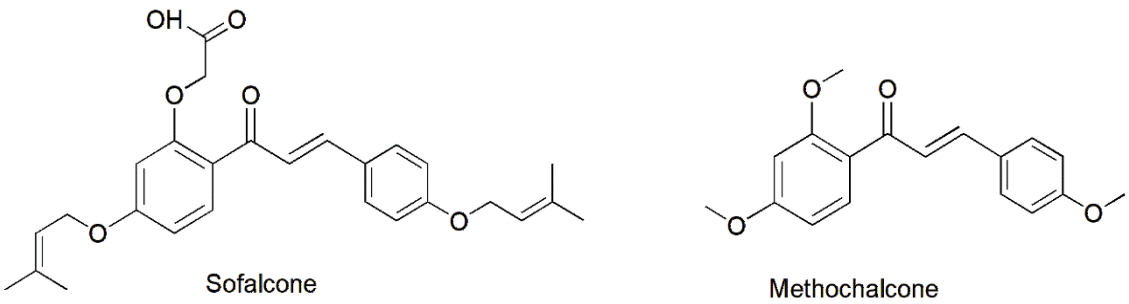


Fig. 2: Structures of chalcone, the phenyl ring attached to the carbonyl group is defined to be the A ring and the other benzene ring is named as the B ring

as an antiulcer and mucoprotective drug [2], metochalcone was once marketed as a choleretic drug (Figure 2) [17], while hesperidin methyl chalcone is used as vascular protective (Figure 2) [18]. In recent years, many minireviews of chalcones have been published, which have

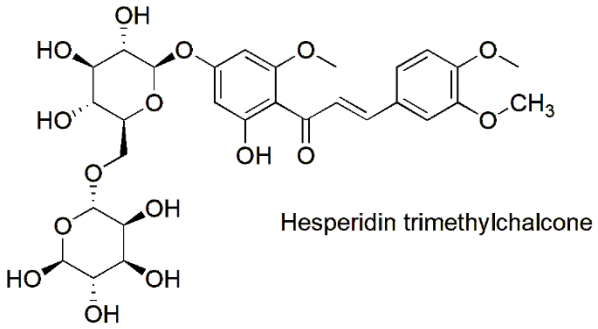


Fig. 3: Structures of chalcone, the phenyl ring attached to the carbonyl group is defined to be the A ring and the other benzene ring is named as the B ring

covered the extensive biological activities of chalcones. However, there is still a considerable amount of uncertainty about the precise mechanism of action for the wide range of biological activities attributed to chalcones. Chalcones have gained significant attention in drug discovery for central nervous system diseases (CNS), including Alzheimer's Disease (AD), because of their low molecular weight, easy synthesis, and easy optimization of lipophilicity (logP) by substituents. This simple scaffold can be modified in several ways. Some of the most prominent modifications include substituting ring A and B with heteroaryls, (ii) substituting phenyl rings with functional groups such as hydroxyls, methoxy, halogens (Cl, F, Br), and amines. (iii) fusion of ring A and α-carbon and various combinations of the above modifications. Condensation reactions catalyzed by acids or bases are used to prepare it synthetically. Chalcone(s) are most commonly prepared by the Claisen-Schmidt reaction [19,20].

Furthermore, chalcones have a variety of biological potential and are considered important pharmacophores of bioactive natural products. A representative example of a naturally occurring bioactive chalcone is cardamonin, a hydroxychalcone found in Zingiberous plants, which has antimutagenic, vasorelaxant, and anti-inflammatory properties, as well as xanthohumol, a prenylated flavonoid derived from hop plants, which acts as a broad-spectrum cancer chemopreventive agent in vitro [21, 22].

4-2.1 Chemical Structure

A chalcone is an unsaturated ketone containing a ketoethylenic group (–CO-CH=CH-). Additionally, these compounds are referred to as benzalacetophenones and benzylideneacetophenones. The chemical formula of chalcones is 1,3-diaryl-2-propen-1-one,

a compound in which two aromatic rings are linked by an aliphatic three-carbon α, β-unsaturated carbonyl system (Figure 1). On both benzene rings, chlorocones possess conjugated double bonds and delocalized electrons. The three-carbon aliphatic system serves as an adjunct between two aromatic rings A and B in open-chain flavonoids. Non-chiral molecules with relatively high lipophilicity (Log P ≈ 5–7), chalcones are small molecules with a low molecular weight (300–600 g/mol). As a result of the presence of the chromophore -CO-CH=CH-, chalcones are colored compounds. Chalcones may exist as either cis (E, 1) or trans (Z, 2) isomeric forms. The trans form is thermodynamically more stable than the cis form [15].

4-2.2. Nomenclature

There are a number of significant biological compounds composed of chalcones or chalconoid. Chalcones and chalconoid are enones and aromatic ketones, which form the core of the compounds. In the chalcone series, benzylideneacetophenone is the parent compound. A number of alternatives to chalcone have been developed, including benzalacetophenone, α-phenyl-β-benzoylethylene, phenyl styryl ketone γ-oxo-α, γ-diphenyl-α-propylene, and β-phenylacrylophenone. Different nomenclatures are used to refer to Chalcone. For example, here the chemical structure and nomenclatures have been adopted by the "Chemical Abstracts" published by American Chemical Society (I) and the IUPAC name of chalcone is 1,3-diphenyl-2-propen-1-one [15].

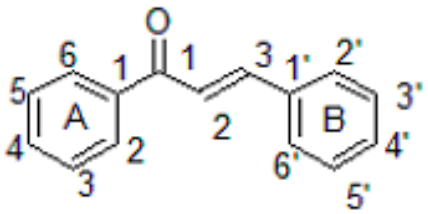


Fig. 4: chalcone nomenclature

4.2.3. Occurrence of Chalcones

A significant fraction of edible plants, particularly fruits and vegetables, contain chalcones, a type of secondary plant metabolite belonging to the flavonoid family. Chalcones, therefore, are plant flavonoids (C6-C3-C6) (Figure 1c). During flavonoids biosynthesis, chalcones and their derivatives play an influential role. Among the bioactive compounds found in nature, flavonoids play a crucial role. In nature, chalcones are polyhydroxylated aromatic compounds that can be found abundantly in fruits, grains, legumes, vegetables, and beverages such as tea, coffee, red wine, and beer. The medicinal benefits of polyhydroxylated chalcones are mainly attributed to their free radical scavenging activity (antioxidant property), which in turn mitigates oxidative stress-induced tissue damage associated with some chronic disorders such

as cardiovascular diseases, inflammatory diseases and neurological disorders, and certain infectious diseases [22-24].

4-3. Biosynthesis of Chalcones

There are many polyphenolic compounds that are synthesized from chalcone, including flavonoids, isoflavonoids, anthocyanidins, and proanthocyanidins [22]. In the biosynthesis of chalcones, Chalcone synthase (CHS) plays a vital role [23, 24]. A chalcone synthase (CHS) has two active sites, which is what makes it effective as an enzyme for chalcone synthesis. The upper domain consists of four amino acids and is one of the active sites. The second active site referred as the lower domain is also essential for chalcone formation [22]. In the biosynthesis

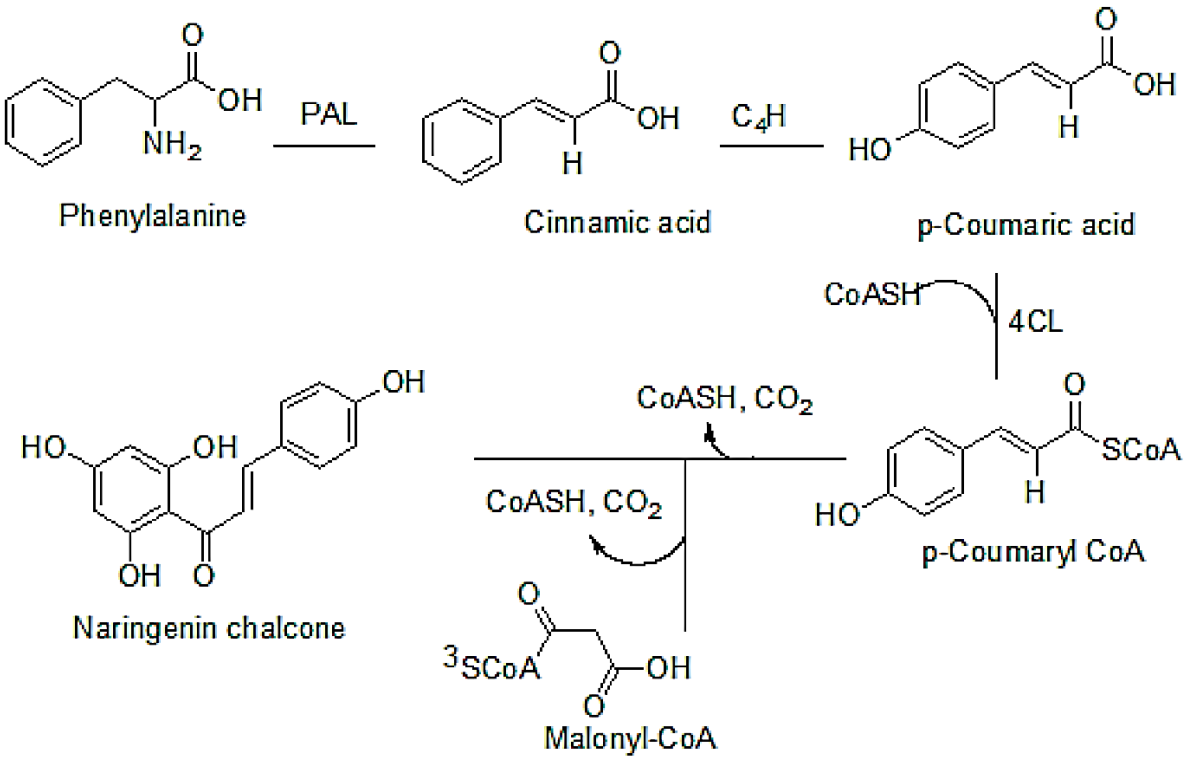


Fig. 5: Biosynthesis of chalcone. PAL: phenylalanine ammonia-lyase, C4H: cinnamate 4 -hydroxylase, 4CL: 4-coumarate-CoA ligase.

of chalcones, phenylalanine serves as a major precursor (phenylalanine is derived from chorismate). The biomolecules p-coumaroyl CoA and malonyl CoA are also necessary for the formation of chalcones. Nevertheless, p-coumaroyl CoA is formed from phenylalanine [24]. The aliphatic chain of phenylalanine undergoes deamination to form cinnamic acid. In this reaction, phenylalanine ammonia-lyase (PAL) catalyzes hydroxylation of the phenylalanine aromatic ring at the para position, followed by cinnamate-4-hydroxylase to form p-coumaric acid. The enzyme 4-coumaroyl-coenzyme A ligase substitutes the hydroxyl group of p-coumaric acid with a succinyl-CoA group to yield p-coumaroyl CoA. The CHS catalyzes the

sequential condensation of three molecules of malonyl CoA and p-coumaroyl CoA (one molecule). Additionally, malonyl CoA undergoes decarboxylation, cyclization, and aromatization due to the four amino acids found in the active site of CHS (Asn 336, His 303, Phe 215, and Cys 164) [24]. Figure 5 shows the biosynthesis of chalcones. As a result, chalcone can be synthesized into various polyphenolic natural products, including flavanones, flavonols, flavanols, dihydroflavonols, isoflavones, flavones, flavonoids, aurones, and anthocyanidins [22]. Figure 6 depicts the biosynthesis of various chalcone bioprecursors.

4-4. Naturally Occurring Chalcones

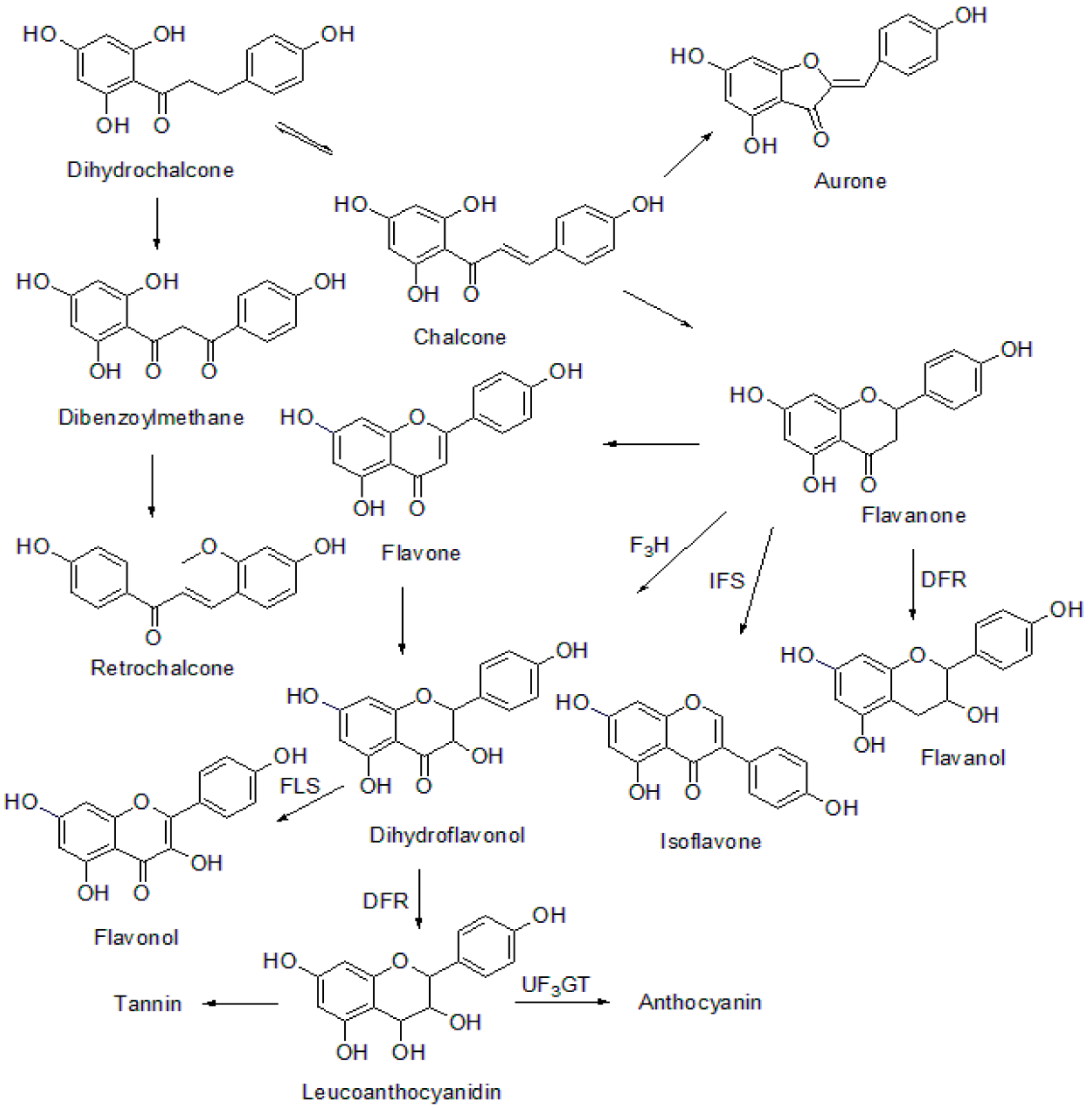


Fig. 6: Biosynthesis of chalcone precursors. DFR: dihydroflavonol-4-reductase, IFS: isoflavonone synthase, F3H: flavanone-3-hydroxylase, FLS: flavonol synthase, UF3GT: UDP-glucose flavonoid-3-O-glucosyltransferase.

Plants are the primary source of naturally occurring chalcones. Generally, they are found in medicinal plants or in dietary plants. Chalcones are found in nature as derivatives of chalcones and flavonoids [26]. In the laboratory, chalcone derivatives of medicinal importance can be chemically synthesized by modifying the parent scaffolds with a variety of structural substitutions [27].

Many medicinal and potential medicinal plants contain chalcones with therapeutic effects. In 1978, Star et al. isolated 2,6-dihydroxy-4-methoxy-3-methyl chalcone from Pityrogramma triangularis [28] exudate, which was reported as a new compound. A number of compounds have been isolated from liquorice (Glycyrrhiza glabra), which has medicinal properties against many human diseases [29], including isoliquiritigenin, isoliquiritin, neoisoliquiritin[29], licochalcone A, licochalcone B[30], echinatin [31], licuroside [29], and neolicurosid [33]. In 1978, Uyar et al. described two dihydrochalcones from Myrica gale, 2,6-dihydroxy-4-methoxy-3,5-dimethyldihydrochalcone and 4,4,6-trimethyl-2-(3-phenylpropionyl)-cyclohexane-1,3,5-trione [33]. Isolated crotaoprostrin was found to be produced by Crotalaria prostrata, an Indian medicinal plant [34]. Bavachromanol, a novel natural chalcone, is also found in Psoralea corylifolia, an Indian and Chinese traditional medicine [35]. Extracts of Lonchocarpus xuul root have been reported to contain dihydrochalcone and dihydroisocordon [36]. There have been reports of 42 chalcones isolated from licorice in a comprehensive review by Wang et al. (2020) [37]. An Ochnaceae plant called Brackenridgea zanguebarica produces a dimeric dihydrochalcone called Brackenin [38]. Column chromatography was used to isolate six chalcones from Angelica keiskei extracts [39]. In addition to mixtecacin, oaxacin have also been isolated from Tephrosia woodii [40] and epoxychalcone been isolated from Tephrosia carrollii [41]. As chalcone constituents of Pongamia pinnata, ponyanones I and II were identified [42]. Bidens tripartitus also contains 2′-hydroxy-4,4′-dimethoxychalcone [43]. It has been reported that the chalcone constituents of Zuccagnia punctata are 2′,4′ -dihydroxy-3′-methoxychalcone and 2′,4′-dihydroxychalcone [44]. A dihydroxy-3′,5′-dimethyl-6′-methoxychalcone was reported from Dalea versicolor [45]. The fruits and seeds of Cedrelopis grevei contain cedreprenone, 2′-methoxy helikrausic chalcone, cedrediprenone, 5,7-dimethylpinocembrine, flavokawin B, and uvangoletin. Anneslea fragrans var [46]. There are also two dihydrochalcones isolated from the aerial parts of Boronia inconspicua, 2′,4,4′, 6′-tetrahydroxy-5-(E-3,7-dimethylocta-2,6-dienyl)-3-(3-methylbut-2-enyl) dihydrochalcone, and 2′,4,4′,6′-tetrahydroxy-3,5-di(3-methylbut-2-enyl) dihydrochalcone [47]. Fissistigma

lanuginosum ethyl acetate extract has been found to contain a variety of condensed chalcones, including pedicin as well as fissistin and isofissistin. [48]. It has been established that Litseaone

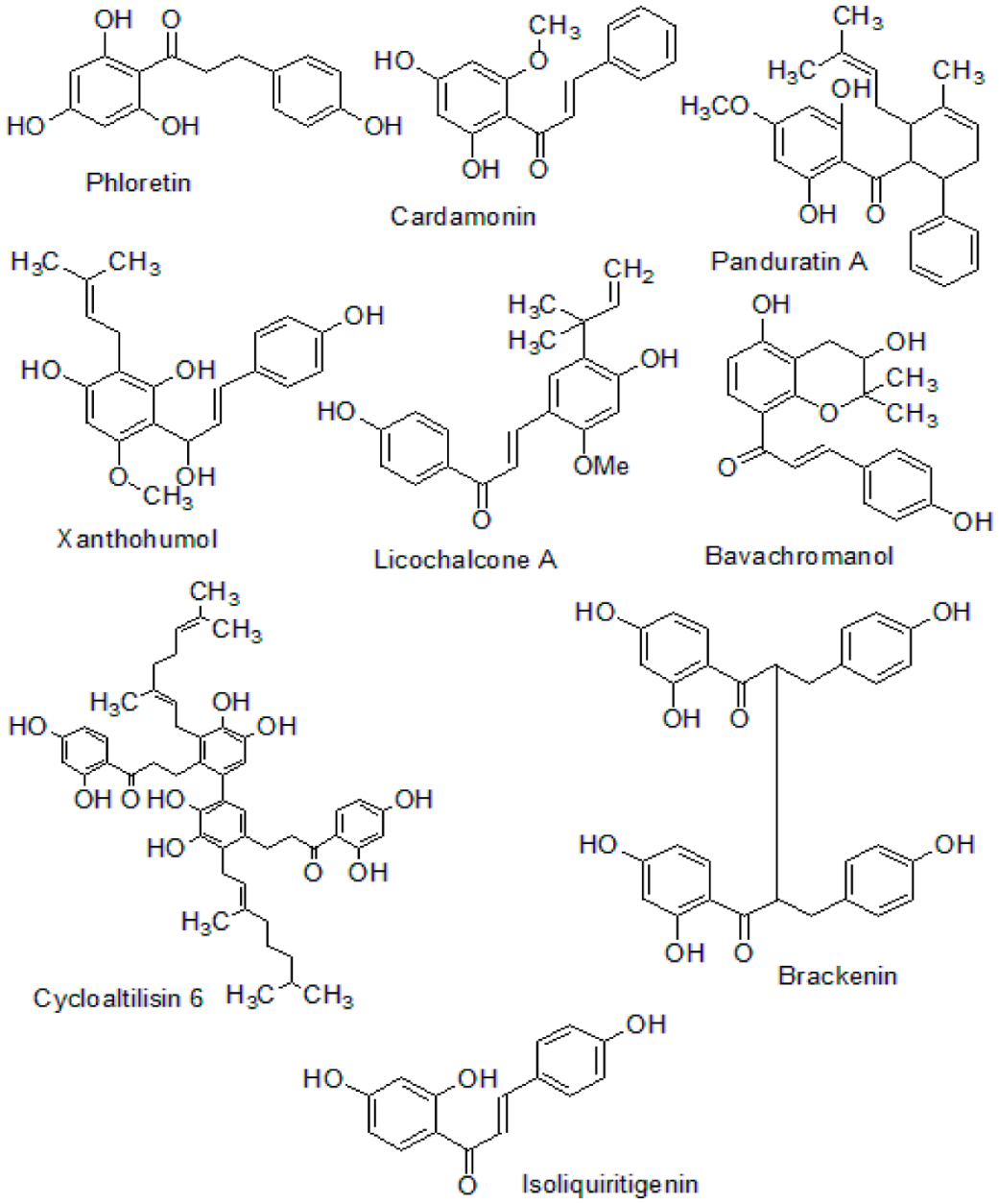


Fig. 7: The structures of naturally occurring chalcones

A and B can be isolated from Litsea rubescens and Litsea pedunculata stem bark [49]. There has been some evidence that cyclohexanyl chalcone and panduratin are present in Boesenbergia rotunda [50]. The plant Crotalaria trifoliastrum yielded munchiwarin, which is composed of 2,2,6-triisoprenyl-cyclohex-5-ene-1,3-dione [51]. Glycyrrhiza inflate has been reported to contain licochalcone A, D, and G, kanzonol, licoagrochalcone A, 5-prenyl butein, and echinantin, isoliquiritigenin [52]. Xanthohumol has been reported from Humulus lupulus [53]. Another report proved the presence of licochalcone A, Glabridin, isoliquiritigenin,

glycycoumarin, glycerol, glycerin, and liquiritigenin in Glycyrrhiza uralensis [54]. α-Hydroxy dihydrochalcones, novel isoflavanone, norisojamicin have been found in the Millettiaus aramensis stem bark [55]. Tuchinda et al. investigated some newer chalcones such as (-)-hydroxypanduratin A, dihyro-5,6-dehydro kawain, a cyclohexenyl chalcone derivative, pinocembrin, pinostrobin, panduratin A, and sakuranetin [56]. Six flavonoids with the chalcone moieties α,4,4′-trihydroxydihydrochalcone-2′-O-β-d-glucopyranoside, α,3,2′,4′-tetrahydroxy-4-methoxy-dihydrochalcone, 2′,4′-dihydroxychalcone-6′-O-β-d-glucopyranoside, and 3′-C-β-glucopyranosy-6′-O-β-d-glucopyranoside have been reported from the bark of Eysenhardtia polystachiya [57]. Chalcone has been identified as one of the vital constituents of some edible plants [58]. Phenolic chalcones in edible plants are essential to maintaining good health for humans, serving as antioxidants as well as antimicrobial compounds, among other functions. [59]. chalconaringenin and a dihydrochalcone Phloretin-3′,5′-di-C-β-glucopyranoside, have been reported from Solanaceae specie of tomatoes [60]. In tomatoes (Solanum lycopersicum), eriodictyol chalcone was found by Iijima et al. (2008). In addition, narigenin chalcones were reported [61]. According to Slimestad and Verheul (2011), fresh cherry tomatoes contain chalconarigenin [62]. From the peel of sweet orange (citrus sinensis), two hydroxylated polymethoxychalcones have been isolated [63]. There is evidence that apple fruit (Malus domestica) contains phloridzin, seboldin, and trilobatin [64].

4-5. Chemical Synthesis of chalcones

The preparation of chalcones generally involves condensation reactions catalyzed by bases or acids. Because of their interesting biological activities and the development of various catalysts and reaction conditions, chalcones are among the easiest ketone compounds to synthesize. Chalcone scaffold synthesis strategies, general methodologies, catalysts, and conditions are summarized below.

4-5.1. Claisen−Schmidt Condensation.

Claisen-Schmidt reaction is named for two pioneering investigators, R. L. Claisen [65] and J. G. Schmidt [66], and is characterized by the condensation of benzaldehyde with methyl ketone in the presence of catalysts.

Organic chemistry considers this reaction to be one of the most classical reactions [67]. Strong bases or acids serve as catalysts. The chalcone is produced from the aldol product by dehydration in an enolate mechanism in base catalysis, whereas it is produced via an enol mechanism in acid catalysis [68]. Its main disadvantage is its slow reaction rate; it typically

takes several days to complete the reaction. Also, a complex mixture made up of the desired product, its byproducts, and sometimes the starting material may result from the reaction. Accordingly, the yields could vary dramatically depending on the catalysts and reactants, ranging from 10% to almost 100%. [68, 69], However, most publications have used this reaction because of its simplicity of experimentation and high efficiency in forming double bonds with little limitation to the complexity of molecules. Széll and co-workers synthesized a series of nitrochalcones and demonstrated that the presence of electron-donating groups in the aldehyde favors condensation by acids, while electron-withdrawing substituents favor condensation by bases [70] Chalcone synthesis generally takes place under base conditions.

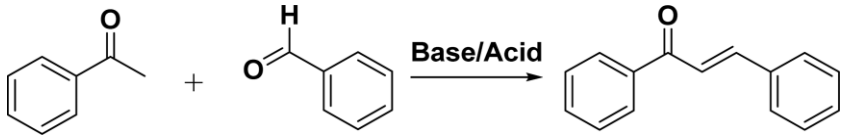


Fig. 8: Claisen−Schmidt Condensation.

Using common base (sodium hydroxide) condition, a group of ferrocenylchalcones has been synthesized [71]. There have also been many studies that have shown acid catalysts to be effective in synthesising chalcones, in addition to the conventional base catalysts. Brønsted acid [70,72] Lewis acids [73−75] and solid acids [76] have also been utilized as acid catalysts. Only a 10-40% yield is achieved with ethanol saturated with Bronsted acid HCl. In this reaction, dry HCl gas has been shown to be more favorable because it both acts as a catalyst and an absorbent for water [70, 72].

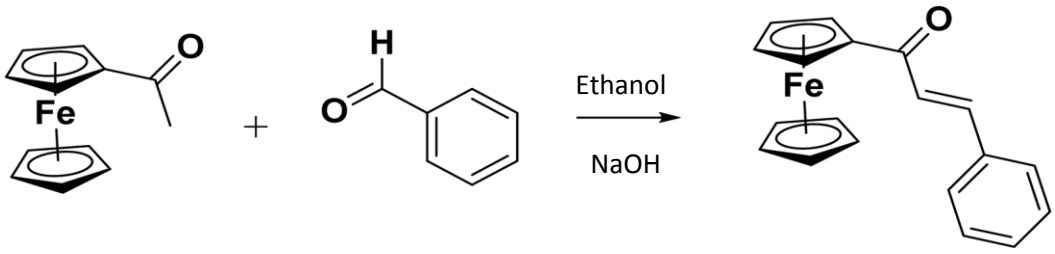


Fig. 9: synthesis of ferro chalcone under common base

4-5.2. Cross-Coupling for the synthesis of chalcones

4-5.2.1. Suzuki Coupling

The palladium-catalyzed cross couplings termed as Suzuki Coupling [77-78] were first reported in 1979 by Akira Suzuki, who received the 2010 Nobel Prize in Chemistry along with Richard F. Heck and Ei-ichi Negishi. It is a powerful palladium-catalyzed reaction that forms carbon-carbon bonds, first used in 2003 [79]. According to the retrosynthetic analysis, two

approaches [79] could be used for the synthesis of chalcones: coupling cinnamoyl chloride with phenyl boronic acid (fig10 – scheme A) and benzoyl chloride with phenyl vinyl boronic acid (fig10 – scheme B). It has been demonstrated that the electronic properties of the substituents on benzene rings have a minimal influence on the Suzuki coupling reaction's ability to

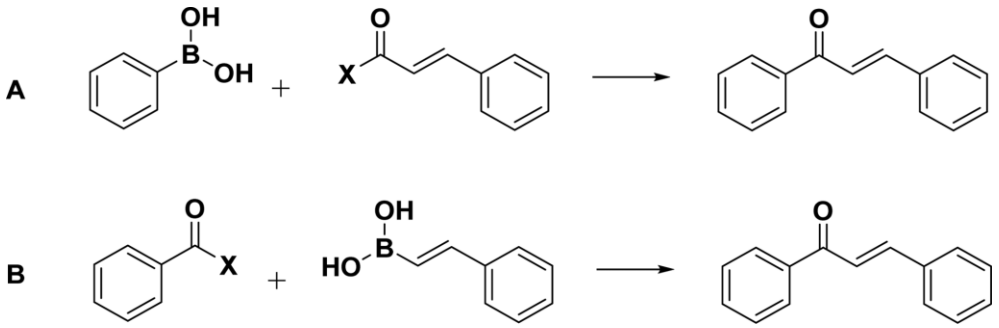


Fig. 10: Suzuki Coupling for Chalcone Synthesis

synthesize chalcones with electron-withdrawing or electron-donating substituents. Using Suzuki-Miyaura couplings, Buszek et al. reported high yields of chalcones from N-vinylpyridinium tetrafluoroborate salt [80].

4-5.2.2. Wittig Reaction

Alkene compounds can also be created by the Wittig reaction or Wittig olefination. Chalcone is an appropriate alkene template for the Wittig reaction strategy (Scheme 6). An initial attempt

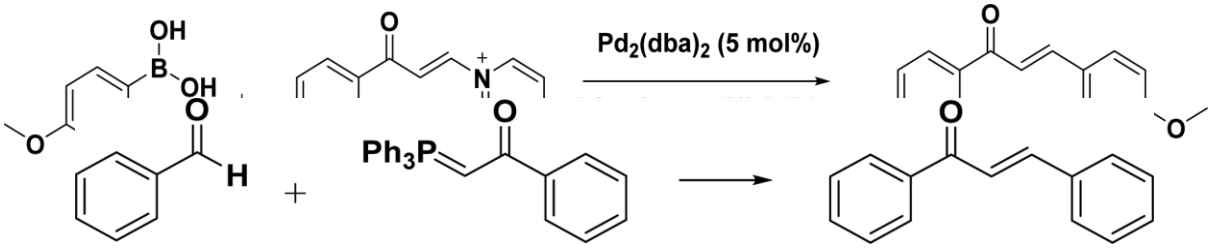


Fig. 11: Wittig Reaction for Chalcone Synthesis

was conducted using triphenyl benzoyl methylene phosphorane and benzaldehyde with a good yield of 70% after 3 days in benzene or 30 hours in THF. The reaction rate has been significantly enhanced by microwave irradiation, according to further development [81, 82]. Huang et al. synthesized various chalcones using eight aromatic aldehydes. Microwave irradiation provided good yields (>80%) for all substrates studied, and the reactions were completed in 5 to 6 minutes [83].

4-5.2.3. Heck reaction

As a stilbene, chalcone can be obtained by the classical Heck reaction of an aryl boronic acid or an aryl iodide with an unsaturated ketone in the presence of palladium catalyst and a base (fig. 12.) [84-88]. According to Cavarischia et al., the synthesis of aryl vinyl ketones and its direct coupling with aryl iodides readily produces chalcone derivatives in excellent yields (75−96%) under catalytic conditions (Pd(OAc)2, Ph3P, CH3CN, TEA) [89]. Furthermore, it is possible to perform Heck coupling through the formation of a carbon-carbon bond generated by the rhodium-catalyzed reaction, which is a competitive side reaction of the conjugate addition resulting from the phosphine−rhodium catalyzed reaction [87]. It has also been reported by Beller et al. (2010) that carbonylative Heck coupling can also be used to produce chalcones [90].

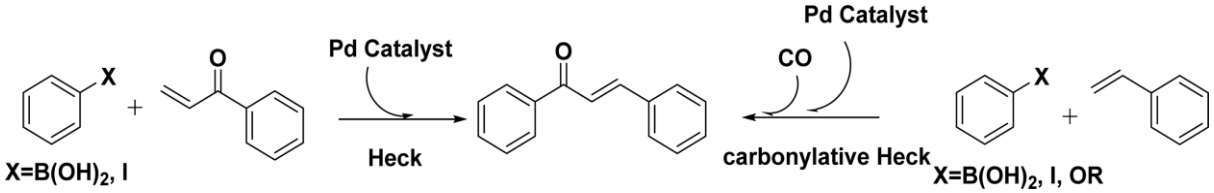


Fig 12: Heck Coupling and Carbonylative Heck Coupling for Chalcone Synthesis

4-5.3 Other Strategies.

4-5.3.1. Friedel−Crafts Acylation with Cinnamoyl Chloride

It is possible to synthesize chalcones using a Lewis acid catalyst, such as aluminum trichloride, by Friedel−Crafts acylation of an aromatic ether and cinnamoyl chloride (fig 13). In 1978, Shotter et al. reported making four chalcones in good yields.[91] However, this method is not widely used.

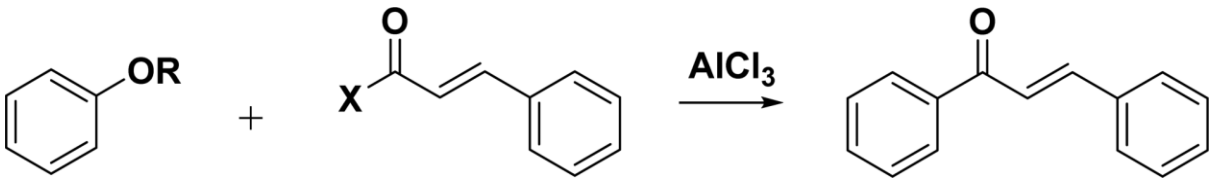
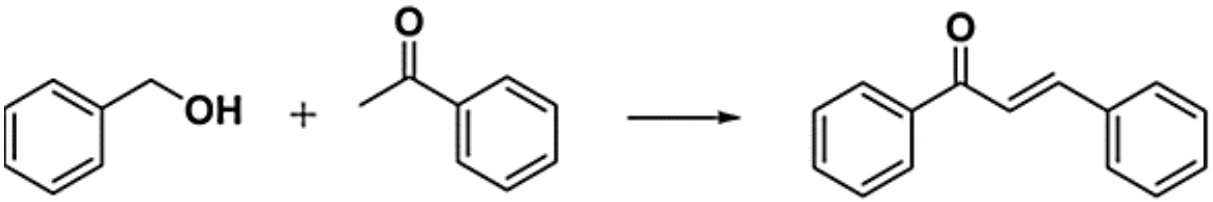


Fig. 13: Friedel−Crafts acylation of an aromatic ether and cinnamoyl chloride

4-5.3.2. One-Pot Synthesis of Chalcones.

A one-pot synthesis saves time and increases yield by improving the efficiency of a reaction and avoiding the purification of intermediates. Recent work has shown that the chalcone scaffold can be synthesized through a one-pot synthesis using alcohol and ketone. Xu et al. reported the one-pot reaction of an alcohol and different ketones by changing the reaction temperature from −10 to 100 °C for 10−96 h with a catalyst consisting of copper iodide, 2,2′-bipyridine, and 2,2,6,6- tetramethylpiperidine-1-oxyl (TEMPO).



Condition I: CrO_3, 58°C [92]
Condition II: CoI, 2,2'-bipyridine, TEMPO, -10-100°C [93]
Condition III: nano-Pd-V, $Ba(OH)_3$, O_2, H_2O, 80°C [94]
Condition IV: toluene, 1.0 mol% Au, Cs_2CO_3, rt, O_2 [95]

Fig. 14: One-Pot Synthesis of Chalcones

4-6. Bioactivities of Naturally Occurring Chalcones

There are a wide range of biological activities associated with chalcones: antioxidant, antimalarial, anti-inflammatory, antimicrobial, antiosteoporosis, antiplasmodial, anticancer, antifungal, and antihyperglycemic [58]. In particular, chalcones from medicinal plants exhibit these biological activities, and as a result, they are used as therapeutic agents in various diseases. A number of plants containing chalcones have been shown to inhibit the growth of cancer cells. [96–97] Anticancer activity has been reported for licochalcone A, 4-hydroxyderricin, butein, phloretin, garcinol, flavokawain A, B, and C, broussochalcone, dimethyl amino chalcones, cardamonin, and 2´-hydroxy-2,3,4´,6´-tetramethoxy chalcone [98]. Antifungal properties have been demonstrated for Chalcones from Maclura tinctoria and Mallotus hilippinensis [99]. A substantial amount of hyperglycemic activity has been reported for xanthoangelol and 4-hydroxyderricin, which are constituents of ashitaba [100]. There is a significant role played by protein tyrosin phosphatase IB (PTBIB) in regulating hyperglycemia [101].

A number of biological activities are also exhibited by chalcones derived from dietary sources. Anticardiovascular, antidiabetic, and anticancer properties have been reported for tomatoes [102–104]. It has been reported that naringenin chalcone has anti-inflammatory [105], antiallergic [106], and antiobesity properties [107]. Tomatoes are rich in it [106]. It has also been reported that phloretin-3´,5´-di-C-glucoside, found in tomatoes, is an antioxidant [96].

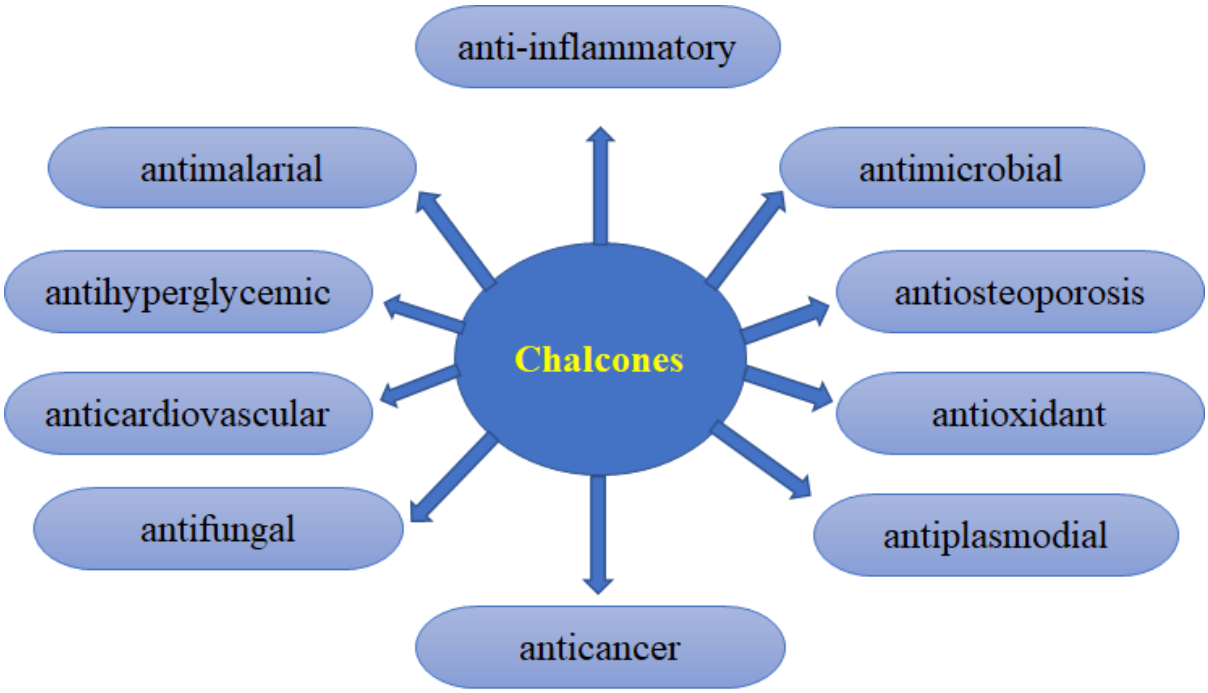


Fig. 15: Diverse biological activities of chalcones.

The compounds pinostrobin chalcone, boesenbergin A, and panduratin A in tomatoes have been reported to have aphrodisiac properties (107). Several antimicrobial, antioxidant, antiobesity, and anti-inflammatory effects of panduratin A have also been described [108-110]. Despite its hepatotoxic properties, boesenbergin is anti-inflammatory, antioxidant, and anticancer [111]. There are a variety of antipyretic, anticancer, and protease-inhibiting properties associated with cardamonin [112]. Phytopanduratin A, pinostrobin, and panduratin chalcone have been shown to have antiretroviral activity [113]. Licochalcone A, a constituent of licorice, has been reported to have a good inhibition of TNF-α, IL-β, and IL-6 inflammatory markers [114,115]. This chalcone along with licochalcone B, C, and D has been associated with antiviral [21], antidiabetic [116], antiinflammatory [117], anticancer [118], antitrypanosomal [119], and antibacterial [120] activities. Many diseases can be prevented by apple products containing dihydrochalcone constituents [121. 122]. In apple, phloretin is the most important chalcone. It has been shown that phoretin is an antioxidant, an anticancer, and

an antiinflammatory [123,124]. By inhibiting GLUT2, it acts as an anticancer agent. Furthermore, it inhibits anti-inflammatory markers such as NF-κβ, TNF-α, etc. [123].

4-7. Amyloid inhibition of chalcones

It has been reported that deposition of Aβ in brains due to imbalance in production and clearance results in Alzheimer's disease. Insoluble amyloid plaques formed along with neurofibrillary tangles promote neuronal degeneration and cognitive decline. Aβ was produced from integral membrane protein amyloid precursor protein (APP) by the action of two proteases β- and γ- secretase [125]. A major strategy for developing disease-modifying AD therapies has been inhibiting amyloid plaque formation. Recent research has focused on developing inhibitors of α- and β-secretase, the enzymes responsible for Aβ production. However, due to involvement of α- secretase in Notch signaling and severe toxicity associated, β-secretase (beta-site amyloid precursor protein cleaving enzyme 1; BACE-1) has gained much attention as an optimum therapeutic target for AD.

4-7.1. BACE-1 inhibitors

BACE-1, present primarily in neurons, is an aspartyl protease that cleaves APP at the beta site and is considered a rate-limiting step in the generation of Aβ. Its predominant location in CNS, role in Aβ production, encouraged many researchers to develop its inhibitor as a disease modifying agents for AD. Several large companies developed several potent non-peptidomimetic BACE-1 inhibitors such as atabecestat (JNJ-54861911), lanabecestat (AZD3293; LY3314814), elenbecestat (E2609), verubecestat (MK-8931), Umibecestat (CNP-520) and were tested in clinical trials for treatment of AD [126 –130]. Unfortunately, most of these compounds failed and the studies have been stopped due to low efficacy and/or adverse side effects [131]. It has been reported by Youn et al. that three flavonoids including a chalcone, cardamonin from Boesenbergia Rotunda inhibit BACE-1 [132]. By relying on naturally inspired small molecules, Rampa et al. developed several chalcone derivatives. In house screening of small molecules identified two hit compounds, one chalcone derivative and benzophenone derivatives (Fig 16). Based on computational modeling, it was found that the N, N'-benzyl methylamine groups of the benzophenone derivative interact with the catalytic dyad of the BACE-1 enzyme [133].

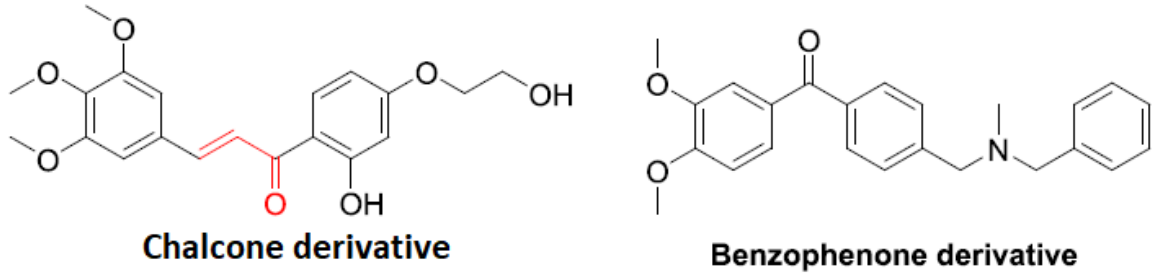


Fig. 16: chalcone derivative and benzophenone derivatives

4-7.2. Amyloid beta aggregation inhibitors

To prevent amyloid plaque formation, there is another approach that can be used to prevent the aggregation of Aβ into pathogenic oligomers and fibrils. This approach is believed to be more beneficial than inhibition of physiologically relevant Aβ production as it may avoid the mechanism-based toxicity. Therefore, development of disease modifying Aβ aggregation inhibitors has emerged as an attractive area of AD drug discovery [134, 135]. Regions of Aβ such as hydrophobic core, N-terminus, hinge or turn regions and C-terminus contribute differently and are critical for Aβ aggregation. central hydrophobic core, His13-Lys16 (HHQK), and hydrophobic C-terminus are the main sites responsible for nucleation of β-sheet rich conformation [136, 137]. Electrostatic and hydrophobic interactions are important in Aβ aggregation. Moreover, it has been identified that the C-terminus of Aβ contributes crucially to the process of aggregation, and this has been well documented. Two additional hydrophobic

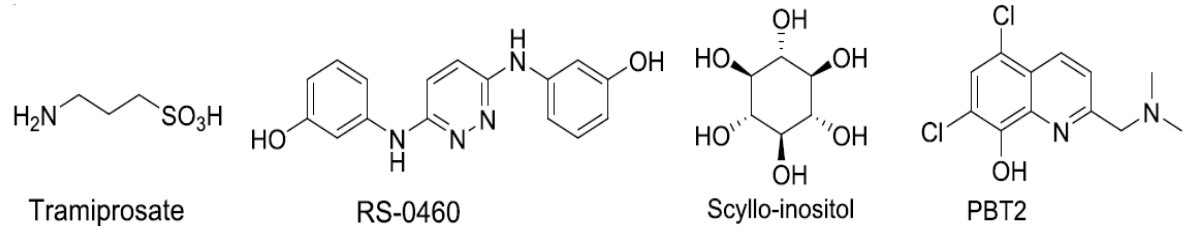


Fig. 17: Aβ aggregation inhibitors: compounds that entered clinical trials

residues at C-terminus of Aβ42 is responsible for its higher tendency for aggregation and neurotoxicity as compared to Aβ40 [138]. Several compounds, including Scyllo-inositol, RS-0406, and Tramiprosate, bind specifically to the HHQK region (N-terminus), central hydrophobic region, and C-terminus of the Aβ peptide, are under clinical trials against the aggregation of Aβ [139-141]. There have been reports that the natural product curcumin, which is closely related to chalcone, can prevent oligomerization of Aβ and disaggregate preformed

Aβ fibrils [142]. In a study Pal et. al. reported that curcumin inhibit the metal induced induced amyloid fibrillation of b-lactoglobulin [143]. A study by Hirohata et al. [144] has examined the mechanism by which flavonoids prevent amyloid production. It was observed that Myricetin bound reversibly to the Aβ fibrils rather than the monomers.

The hydroxylated chalcones prepared by Cong et al. [145] were tested for their dual inhibitory effects on Aβ aggregation and ferroptosis. They observed that trihydroxy compounds (Fig. 18) showed better Aβ aggregation inhibition than other compounds. $A\beta_{1-42}$ induced neurotoxicity is better protected by trihydroxy substituted chalcones than EGCG or curcumin in vitro.

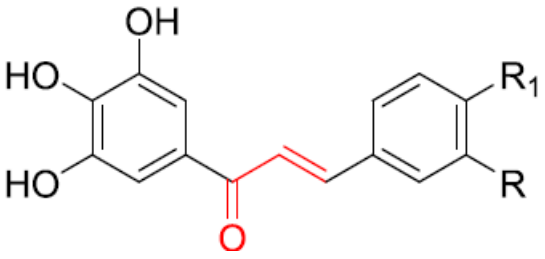


Fig. 18: Aβ aggregation inhibitors: trihydroxy substituted chalcones

4-8. Toxicities of Chalcones

In addition, natural chalcones have also been found to affect the pharmacokinetics of drugs when administered simultaneously. According to Choi et al. (2014), licochalcone A adversely affected nifedipine's and its metabolite's pharmacokinetics in rats. Hepatic CYP3A4 metabolizes nifedipine. In MCF-7/ADR cells overexpressing P-gp, oral nifedipine with licochalcone A inhibits CYP3A4 and induces the cellular accumulation of rhodamine-123, leading to a higher peak plasma concentration (Cmax) [146]. According to Boonnop et al. (2017), co-administration of Boesenbergia rotunda extract with therapeutic drugs may cause herb–drug interactions, altering efficacy and toxicity. It has been reported that panduratin A isolated from Boesenbergia rotunda alters renal cationic drug clearance by inhibiting organic cation transporters (OCT2), which are responsible for renal cationic drug excretion [147].

Isobavachalcone, a natural chalcone obtained from Psoralea corylifolia, has also been studied metabolically and as an inhibitor of efflux transporters, cytochrome P450 enzymes, and UDP-glucuronosyltransferases by Qin et al. (2021). Three glucuronides have been produced by the glucuronidation of isobavachlacone in human liver microsomes and human intestine microsomes. In addition, UGT1 A9, 1A8, 1A7, 1A3, and 1A1 were the major contributors to glucuronidation. Glucuronide excretion is predominantly mediated by MRP1, MRP4, and

BCRP transporters. There is evidence that isobavachalcone inhibits UGT1A9, UGT2B7, UGT1 A1, CYP2D6, CYP2E1, CYP2C19, CYP2B6, and CYP2C9 [148].

4-9. Conclusion:

Many researchers, including medicinal chemists, are exploring various chemical scaffolds in search of a potential therapeutic for AD due to the limited efficacy of FDA-approved drugs for AD treatment and the lack of disease-modifying agents. Chalcone is one of the privileged scaffolds with diverse biological functions being extensively studied as a therapeutic and molecular probe for Alzheimer's disease. In addition to drug potency, BBB permeability is a crucial factor in the development of CNS drugs. For the treatment of AD, researchers have focused on developing novel agents that target other factors, such as neuroinflammation and mitochondrial dysfunction. The anti-neuroinflammatory activities of several natural chalcones and their analogs (e.g., butein, licochalcone, isoliquiritigenin) have been demonstrated in vitro and in vivo. Furthermore, Alzheimer's disease is a complex, multifactorial condition. A single protein target/mechanism that plays a role in AD's pathogenesis would not be sufficient to treat the disease. There is a structure similarity between chalcone and curcumin, and studies found that several chalcone analogs inhibit Aβ aggregation. Development of NIR fluorescence theragnostic agents (for AD) can be achieved by exploring and optimizing chalcone and its analogs.

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Chapter:5

Hydroxychalcones promote the thermal aggregation of β-lg

5-1. Introduction

Privileged compound chalcone, 1,3-diphenyl-2E-propene-1-one, contains two benzene rings and one α,β-unsaturated ketone functional group and used as an efficient template in drug discovery. The moiety is of interest for their synthesis, biosynthesis, and broad biological activities to organic chemist and biochemist for many decades. It has more than thousand years of therapeutics history [1].Derivatives of chalcone, found a wide range of plants vegetables, fruits, and teas, are the root chemical of various natural products [2,3].Chalcone containing plants and herbs were used for treatments of different biological disorders like inflammation, cancer, diabetics etc. [4]. In the case of their applications, there are several compounds with chalcone moiety are drug candidate (metochalcone, sofalcone).

Aggregation of protein causes different adverse physiological effect in human body [5]. For example, aggregation of Aβ peptide, α-synuclein, insulin etc. cause several neurodegenerative disease like Alzymear's, Parkinson disease and diabetics type II, respectively [6,7]. Many proteins have an alpha helix, a spiral coil with a right-handed orientation, as their most prominent structural motif in their native conformation [8]. Proteins that become toxic undergo extensive conformational changes and acquire a beta-sheet motif. In addition to native proteins, beta-sheets are also found in many functional proteins, such as immunoglobulins. However, amyloid deposits generally change from an alpha-helix conformation to a beta-sheet conformation. Formation of cross β-sheet structure is prerequisite condition of protein aggregation and thus amyloid fibril formation. Misfolding of the protein creates fibrillar structure depending upon the physical and chemical stimulants. Binding of small molecules to the protein can change the protein structure causing its misfolding. Hence preventing the formation of these soluble aggregates during the fibrillation procedure could serve as the key to the non-occurrence of many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, type II diabetes and the spongiform encephalopathies etc. diseases.

The underlying mechanism of fibril formation is facilitated with the denaturation of proteins suggesting that it is a property of unfolded or non-native proteins. Hydrophobicity is also a dominant factor determining the amyloidogenicity of proteins. Formation of the cross β-sheet structure with the hydrogen bond network is essential for the fibril formation and also the propensity of side chains as well as the main chains to form hydrogen bonds is also important.

Protein aggregation occurs when amino acid residues are exposed and the alpha-helix to beta-sheet transition become hydrophobic. Toxic configurations can often catalyze the transition of

native copies of the same protein into the toxic state by interacting with them. They are thus called infective conformations because of their ability to spread infection. It is believed that the newly formed toxic proteins continually repeat the cycle, amplifying the toxic effect until the cells are killed or their function is impaired.

5-2. Synthesis of chalcones

Reaction of 2-hydroxybenzaldehyde (1) and the substituted acetophenone compounds (2) in 1:1 mixture of aqueous ethanol medium in the presence of 10% NaOH gives derivatives of (E)-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one (HC). The reaction is presented in the Fig. 1. Using this method, four compounds were synthesized with good yield. Among these synthesized compounds, the hydroxychalcone HC1 is the basic compound. The methyl substitution on the acetophenone part of HC has produced HC2 which is more hydrophobic than HC1. Replacement of methyl with nitro group gives HC3 which is not only more polar but also electron deficient with respect to both HC1 and HC2. Attachment of hydroxyl group at the 2-position of the acetophenone part givesHC4. This molecule is polar and electron rich with respect to other molecules. Therefore, in this study, substituents having different electronic and steric effects have been introduced on hydroxychalcone nucleus. This substitution will influence on the polarity and hydrophobicity of the resulting compounds. Thus, we in this study, we aimed to investigate the effect of the hydroxychalcone and their derivatives on the aggregation property of a model protein bovine beta-lactoglobulin (β-lg) under thermal conditions.

Bovine beta-lactoglobulin (β-lg), a well-known globular whey protein (MW ~ 18.3 kDa), in ruminant milk, has been chosen as a model protein for studies of protein aggregation and amyloid formation [9]. Bovine b-lg has been classified as an effective carrier-protein of oxidation-sensitive hydrophobic drugs, retinol, fatty acids, and nutraceuticals and have a unique acidic pH resistivity and potential encapsulating property [10]. At elevated temperature (above 600C), it undergoes a conformational change, with exposure of buried hydrophobic residues and the thiol (–SH) group (Cys-121). However, the protein (β-lg) is found to form amyloid fibrils when heated above 750C at physiological pH having all the characteristic features observed in many amyloid diseases [11].

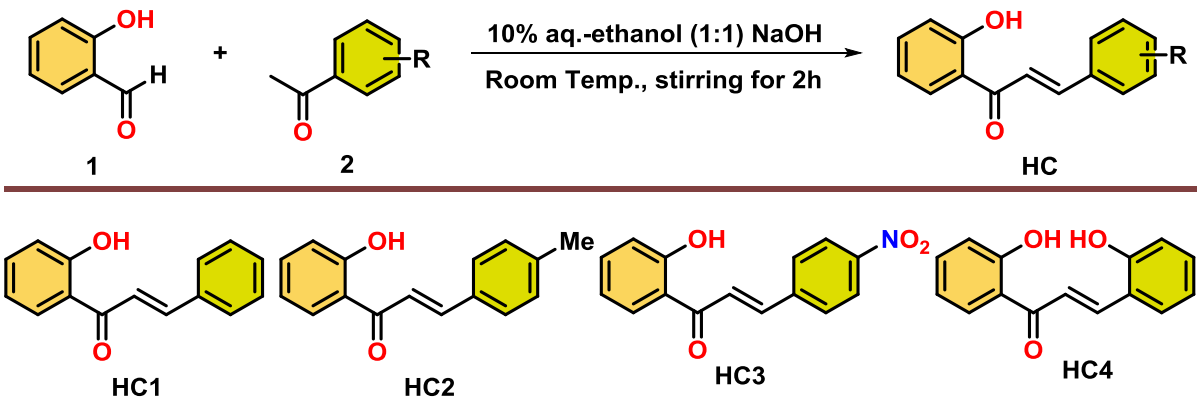

Fig. 1: Synthesis of different hydroxychalcone derivatives.

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5-3. UV-spectral characterization

The UV-vis spectroscopic study gives a preliminary understanding of the change of microenvironment of chromophore in the protein during structural change. After incubation of native β-lg solutions at 78 °C (to accelerate aggregation process) in the absence and presence of chalcones (**HC1-HC4**), the system was investigated with UV-vis spectroscopy (Fig 2). Native β-lg showed λ_{max} at 280 nm, characteristic absorption band of a protein. After the thermal exposure, the characteristic peak at λ_{max} at 280 nm of heat treated β-lg was disappeared due to exposure of its hydrophobic groups and resulting aggregation.

The intensity and position of peaks of the protein has changed on the heating of protein in the presence of HC1-HC4. The protein β-lg showed two peaks at λmax = 258 and 288 nm after thermal incubations with the chalcones (HC1-HC4).The results thus indicate that the chalcone-derivatives have interacted with β-lg molecule and have potential to change the positions of the chromophores.

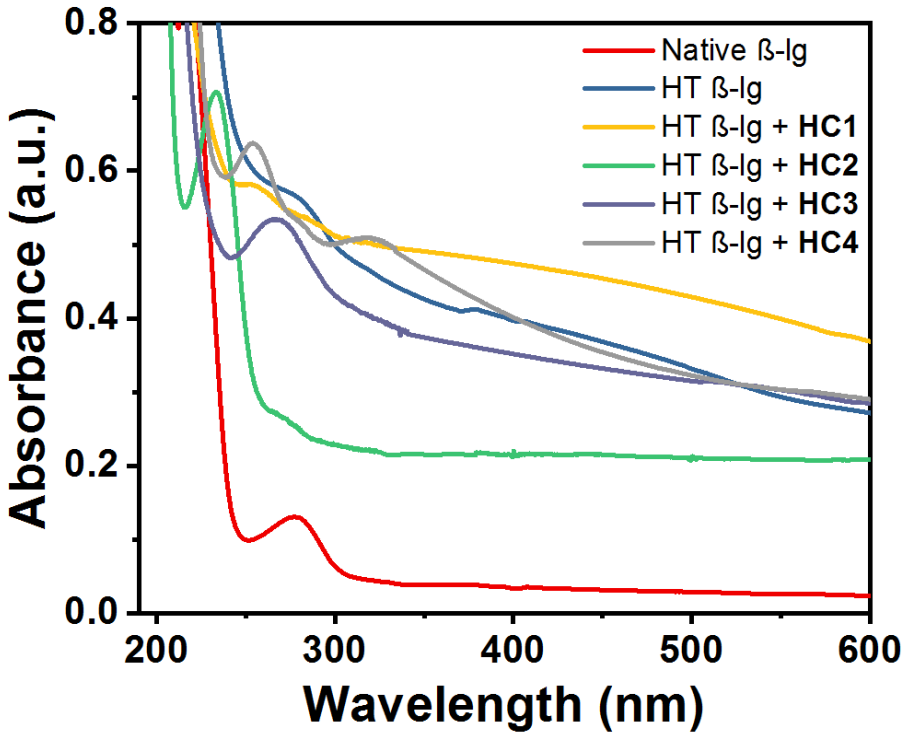


Fig. 2. Absorption spectra of native β-lg and β-lg incubated at 78^0C for 1 h in the absence and presence of different hydroxychalcone compounds (**HC1-HC4**). Concentration of β-lg was 13.6 μM where the concentrations of hydroxychalcone compounds (**HC1-HC4**) kept at10 μM.

5-4. Intrinsic fluorescence

The β-lg has two Trp residues which are responsible for the fluorescence properties of the protein. The fluorescence of a protein is highly sensitive on its structure. Change in the microenvironment of the Trp residues alters the fluorescence properties of the protein. The native β-lg shows fluorescence emission peak at 335 nm after excitation at 295 nm (Fig.3). The fluorescence intensity of the heat-treated β-lg was increased with respect to native protein due to exposure of the fluorophores (Fig. 3) during the heating. In the presence of all the hydroxychalcones (HC1-HC4), the emission intensities of the protein have been shown to increase more than that of the aforesaid heat-treated protein having slight bathochromic shifts of the peaks. So, it is very interesting that all the compounds may have ability to interact with the protein structure in such a way which triggers protein aggregation.

From this experiment, it becomes clear that the compound HC1 may have highest ability to expose the fluorophores due to change in structure leading to the protein aggregation process. However, the effect was found to be minimum in the case of HC4 and thus it interacts minimally with the protein. In the case of compounds HC2 and HC3, such effects are moderate. The order was found to be HC1>HC2>HC3>HC4. To confirm this observation, other experiments were performed.

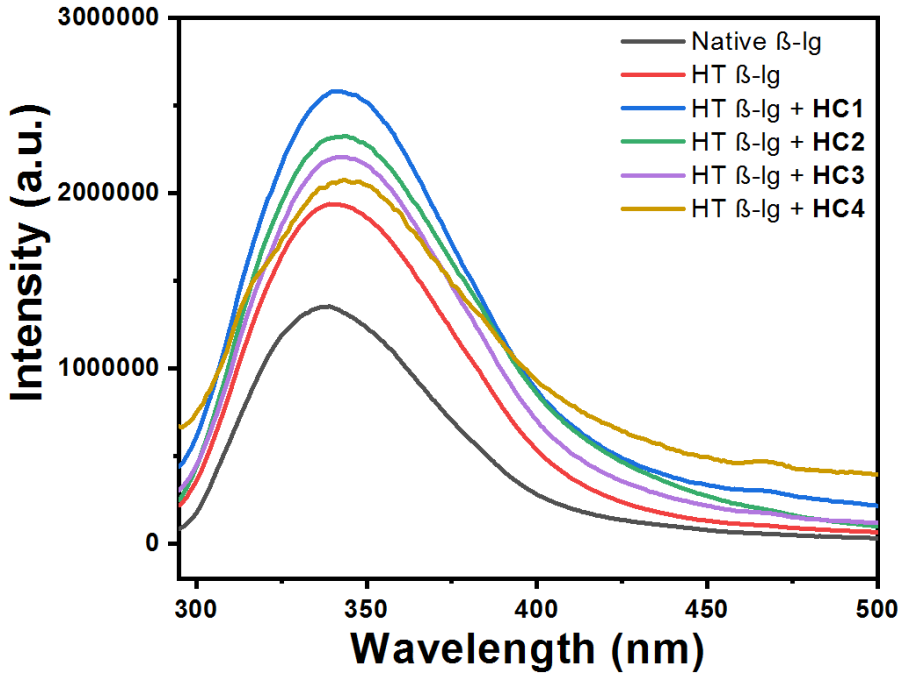


Fig.3. Intrinsic fluorescence spectra of native β-lg and thermally incubated β-lg (at 78ºC for 1 h) in the absence and presence of hydroxychalcones **HC1-HC4** in 10 mM phosphate buffer, pH 7.4 at room temperature. The excitation wavelength was 295 nm and emission spectra were recorded between 300 nm and 550 nm. Both the excitation and emission slits were set at 5 nm. Concentration of β-lg and hydroxychalcones were 13.6 µM and 10 µM respectively. Results are mean of three independent experiments (n = 3).

5-6. ANS-Assay

8-Anilino-1-naphthalenesulfonic acid (ANS), is a yellow coloured organic fluorescent dye, shows emission peaks at 480 nm on excitation at 370 nm. The speciality of the compound is that they can bind at such pockets of the protein surface which are hydrophobic in nature. Effect of the chalcone compounds on the β-lg structure and its aggregation mechanism can be investigated using the dye.

The protein β-lg has two hydrophobic binding sites for small molecules. Inner part of the β-barrel is the first site where various natural nutrients can bind. However, ANS dye cannot bind

at this site. The second hydrophobic binding sites of β-lg are the channel between the barrel and the alpha helix. In aqueous medium, ANS shows a very low emission property but it increases many folds after binding with the hydrophobic binding site of the protein.

If the binding of the hydroxychalcones (**HC1-HC4**) with the unfolded β-lg occurs through the exposed hydrophobic sites in the protein surface, then the ANS fluorescence intensity will be increased. Incubation of β-lg with **HC1** shows the highest hydrophobic sites exposure resulting the highest ANS emission intensity (Fig. 4). This effect gradually decreases from **HC1** to **HC4**. It should be mentioned here that the **HC4** has least ability to change protein conformation and thus to expose hydrophobic amino acids residues. The effect is almost similar to that obtained after thermal incubation of the β-lg solutions without hydroxychalcons. Therefore, during protein aggregation, protein-protein interaction facilitates due to the exposure of more hydrophobic amino acids on the protein surface in the presence of hydroxychalcons. In the present study, the efficiency order of the hydroxychalcons to expose the hydrophobic residues is **HC1** > **HC2** > **HC3** > **HC4**. However, this order is in agreement with their ability to assist the aggregation of β-lg as evident from the results obtained from Th T and TEM studies.

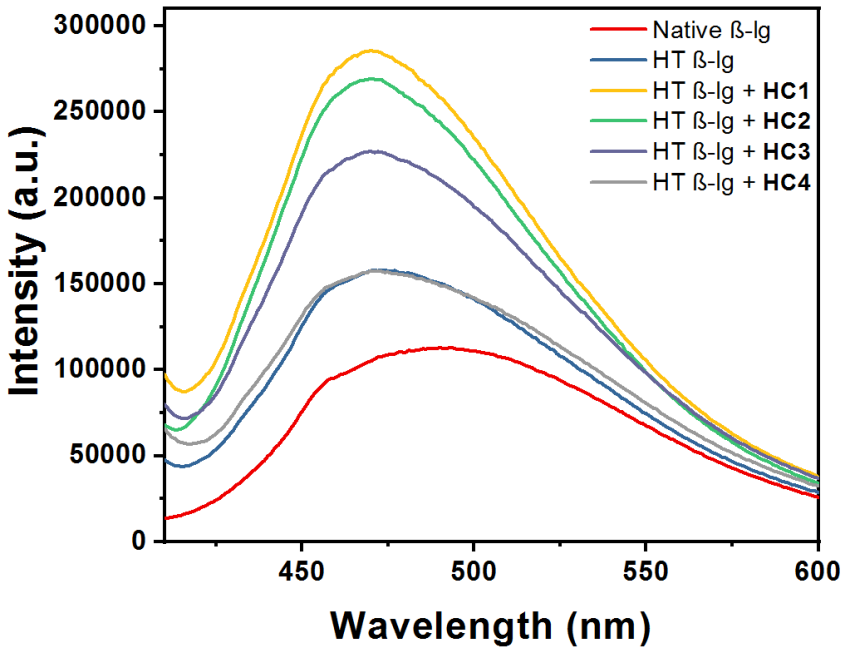


Fig.4 ANS-fluorescence emission spectra of native β-lg and thermally incubated β-lg (at 78°C for 1 h) in the absence and presence of hydroxychalcones **HC1-HC4** (10 μM**)** in 10 mM phosphate buffer, pH 7.4. The excitation was done at 380 nm and emissions were measured between 390 nm and 600 nm. Both the excitation and emission slits were set at 5 nm. Concentration of β-lg was 13.6 μM through the experiment. Results were the mean of three different experiments.

5-7. Th-T assay

Thioflavin T, a benzothiazole dye, is very effective to identify protein aggregation through fibrillation. The dye preferably binds to the β-sheets of the fibrillar aggregates of protein and show high fluorescence emission at 485 nm on excitation at 440 nm. The fluorescence intensity of the Th-T is proportional to the extent of fibrillar aggregates formation of protein. Therefore, high emission intensity of protein in the presence of Th-T indicates high degree of protein aggregation. Thus, the increase of Th-T intensity of a protein solution incubated with a small-molecule shows the compound is a fibrillating agent.

Th-T have very low fluorescence intensity in the presence of β-lg in native state. The protein β-lg was incubated alone at 85 °C and treated with Th-T. The fluorescence intensity of the system is many folds higher than that of the first system (Figure 4). It shows that the β-lg forms a fibrillar aggregates after incubation under thermal conditions.

The protein β-lg was also incubated at 85 °C in the presence of each hydroxychalcones (**HC1-HC4**), separately. Fluorescence spectra of these solutions were recorded after mixing Th-T solution. The results shows that each compounds have more ability to aggregate the protein with respect to the chalcones free heat-treated protein which is very clear from corresponding Th-T spectra in figure 4. The highest and lowest intensity was observed for **HC1** and **HC4**, respectively. Here, the emission intensity of the systems of **HC1**, **HC2**, and **HC3** are close to each other. These results indicate that these chalcones have similar structure of the aggregates. In the case of **HC4**, degree of fibril in the self-assembly of the hydroxychalcone bound β-lg is lower than that of other investigating molecules.

Considering both ANS and Th-T assay, it may be comment that the all the chalcones can hamper native conformer of the protein. They can expose more hydrophobic amino acids in the protein surface which may destabilise protein in the solution. As a results more similar protein system combined each other which leads to their self-assembly. In this way, the protein may form fibril type aggregates in the presence of hydroxychalcones (**HC1-HC4**).

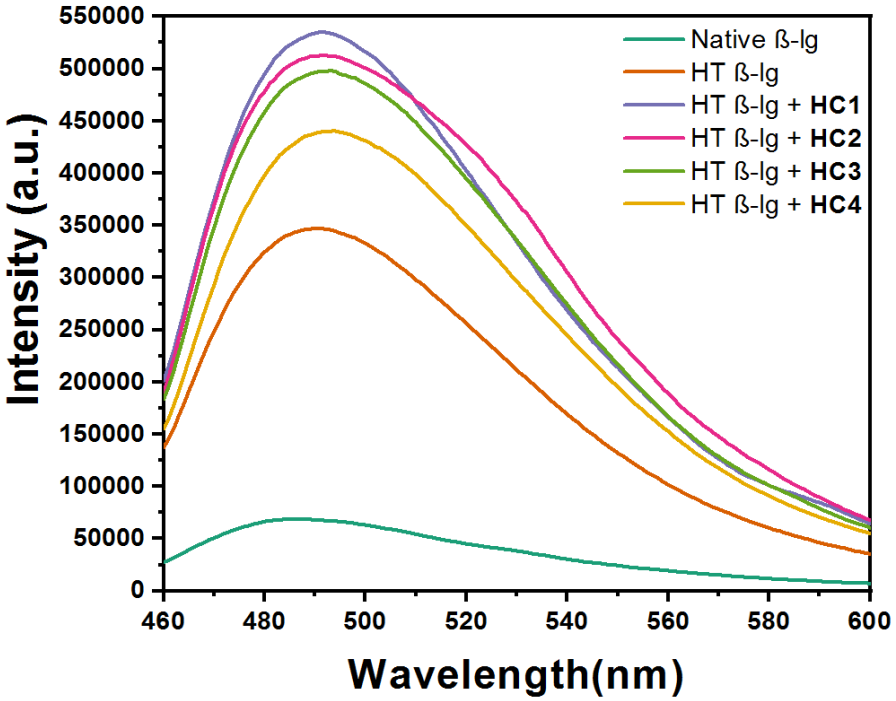


Fig.5. Th-T fluorescence emission spectra of native β-lg and thermally incubated β-lg (at 78ºC for 1 h) in the absence and presence hydroxychalcones **HC1-HC4** (10 µM**)** in 10 mM phosphate buffer at pH 7.4. Concentrations of β-lg were kept at 13.6 µM in all the cases. Fluorescence emissions were monitored in the wavelength range 460–600 nm after excitation at 450 nm. Results are mean of three independent experiments (n = 3) and the error bars show the standard deviation.

5-8. FT-IR

To support the observations from Th-T binding studies and TEM in presence and absence of different hydroxychalcones (HC1-HC4), FT-IR technique was used. It is the second most popular method to confirm the β-sheet structure formation in the course of fibrillation. During fibril growth, the structural transition and behaviour of β-lg can be monitored using this technique through the observation of changes in the shape and frequency of the amide C=O (amide I) stretching and combined of N-H bending and C-N (amide II) stretching bands. These two bands are highly sensitive to conformational changes of protein. These peaks in FT-IR spectra of β-lg in the absence and presence of hydroxychalcones incubated at 75 °C. The FT-IR spectra of β-lg with and without heating were considered as control experiment.

Native β-lg at room temperature showed an amide I peak around 1640 cm-1, which is the characteristic of β-sheet major protein. It is confirming the native of β-lg in the experimental conditions. After incubation of β-lg at 75 °C, the FT-IR spectrum shows the shift of the said band from 1640 cm-1 to 1638 cm-1 which supports the formation of protein aggregation. In

the case of HC1, the peak was appeared at 1634 cm-1 which is a confirmatory band for β-sheet enriched amyloid fibrils formation. This result thus confirms the formation of β-lg amyloid fibrils. The FT-IR band for HC2 and HC3 was found to be at 1637 and 1636 cm-1 which is also indicates fibrils formation. However, for HC4, the band appeared at 1633 cm-1 which is also favour the formation of amyloid fibrils of β-lg. (Fig. 5)

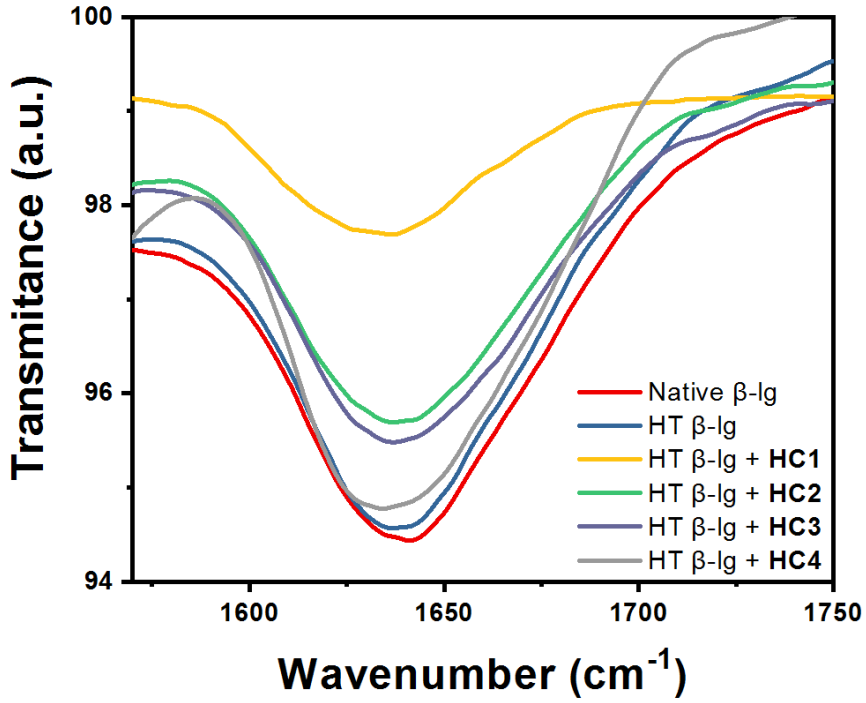


Fig. 6: FT-IT spectra of native β-lg and thermally incubated β-lg (at 78°C for 1 h) in the absence and presence hydroxychalcones **HC1-HC4** (10 μM**)** in 10 mM phosphate buffer at pH 7.4. Concentrations of β-lg were kept at 13.6 μM in all the cases.

5-9. Investigation of the morphology of β-lg aggregates with high resolution transmission electron microscopy (HRTEM) after thermal incubations with hydroxychalcones

All the structural effects discussed above on the β-lg by the hydroxychalcones (**HC1-HC4**) can be visualized using transmission electron microscope images of corresponding systems. Under TEM, native β-lg has very tiny irregular structures (Fig. 7a) which is commonly found for native β-lg and reported in the literature. The protein forms aggregates having fibrillar net-work upon incubation at 78°C for 1 h(Fig.7b). Using the same procedure of thermal incubation of protein with the hydroxychalcones (**HC1-HC4**) separately, very interesting results were observed. In the case of **HC1**, β-lg forms fibrils through its self-assembly which was subsequently converted to higher order aggregates showing small spherical morphology (Fig.

7c). Again, smaller and less fibrillar aggregates were formed during the incubations of β-lg with **HC2** than **HC3**(Fig 5d and Fig.7e). The size and number of the fibrils were reduced more in **HC3**than **HC2** and **HC1**. The morphology of the aggregates are different for **HC4** (Fig. 5f). **HC4** prevents the formation of higher order aggregates rather it helps to form the fibrillar aggregates similar to that obtained during the thermal incubation of native β-lg alone. Hence the hydroxychalcones promotes the thermal aggregation of β-lg in the following order**HC4<HC3<HC2<HC1.**

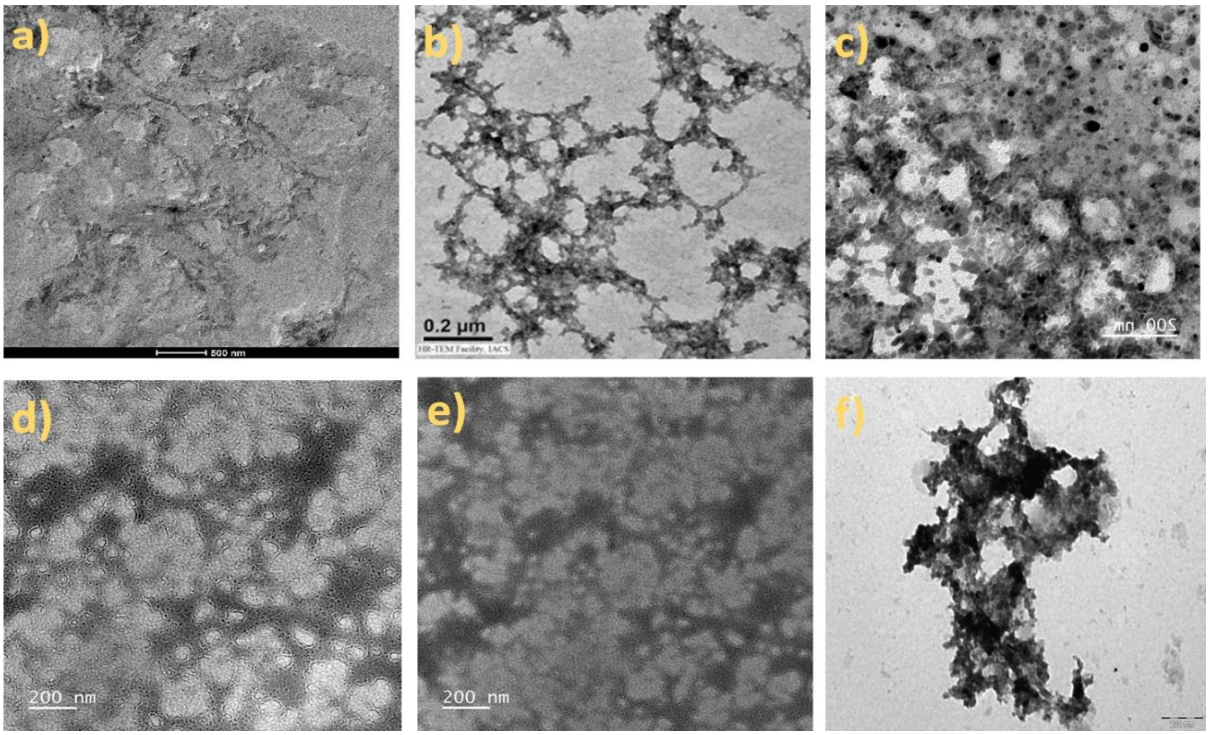


Fig. 7: Transmission Electron Microscopy images at 25°C of β-lg alone (a) of heat-treated β-lg incubated alone at 78°C for 1 h (b), (c) heat-treated β-lg incubated in presence of HC1, (d) heat-treated β-lg incubated in presence of HC2, (e) heat-treated β-lg incubated in presence of HC3, (f) heat-treated β-lg incubated in presence of HC4.

5-10. Theoretical study

Stability of a protein is highly depending on the conformation of the protein. Binding of small molecules to a protein can change protein conformations which may a cause of hydrophobic residue exposer of the solvent through unwinding of the protein. In such cases, stability of formed protein decreases due to the hydrophobic interactions which some time causes self-assembly through protein-protein interactions. In this way, the binding of the hydroxychalcones to β-lg is able to stabilize a conformation in which there are more hydrophobic amino acids on the surface of the protein. This may be responsible for protein aggregation.

To identify this effect, molecular docking of these compounds on the energy minimized β-lg can be very helpful. The crystallographic structure of the protein was energy minimized after fixing all the missing atoms and surface charge. Protein surface was generated with mapping of hydrophilic and hydrophobic regions. The study shows that the ligand free protein have four zones with different polarity (Fig 9). The zone I is fully hydrophilic (blue). Remaining three zones are mostly hydrophobic (gray).

Binding of hydroxychalcones was simulated with the help of molecular docking. To know the effect of the binding of these compounds to the protein structure, each HC-β-lg systems have been energy minimized. Overly of all the β-lg structures (ligand free β-lg (green), HC1-β-lg (blue), HC2-β-lg (purple), HC3-β-lg (yellow), and HC4-β-lg (cyan)) shows that each structure

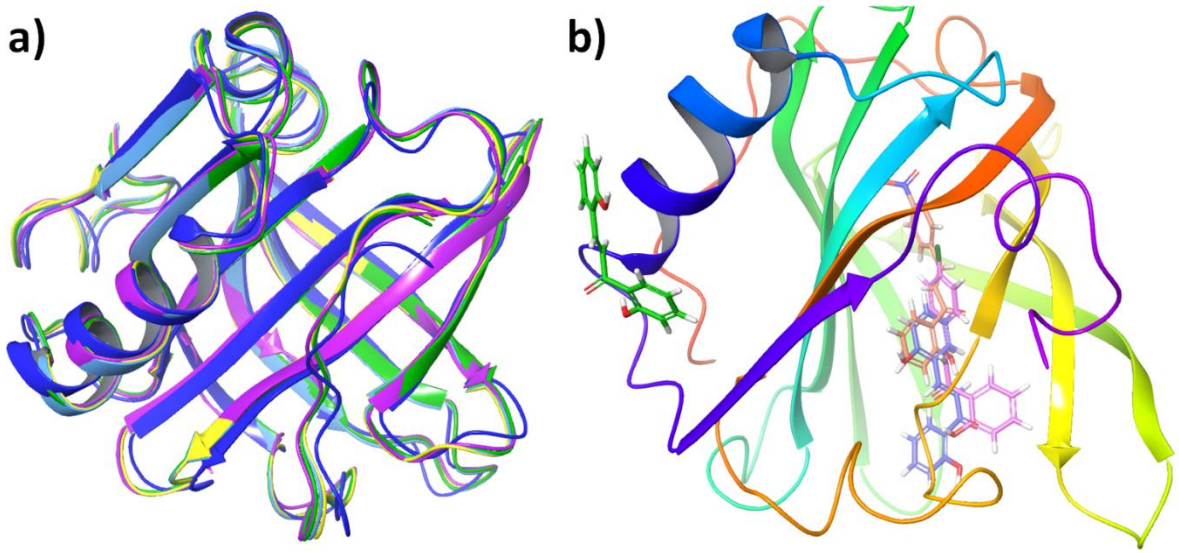


Fig.8: Simulated picture of molecular docking of hydroxychalcones. Almost no change of hydrophilic region (Zone I) with respect to the native protein (Fig 8a). the hydrophobic regions (Zone II-IV) spreads more and become more intense here (Fig 8b)

differs from the ligand free β-lg (Fig 7a). The deviation of these structures is minimum for HC4-β-lg system and maximum for HC1-β-lg. It is interesting to note that all the compounds HC1-3 bind inside the beta-barrel whereas HC4 prefers outer surface of the barrel (Fig 7b).

The effect of the binding of **HC1-4** with β-lg is clear from the energy minimized **HCs**-β-lg system after generation of their protein surface. In all the case of **HCs**-β-lg complex, there are almost no change of hydrophilic region (Zone I) with respect to the native protein (Fig 8 and S). However, in the case of **HC1**-β-lg, the hydrophobic regions (Zone II-IV) spreads more and become more intense here (Fig 8b) than that of free β-lg (Fig 8a). Such type of observation are common for all other compounds. It is interesting to note that the degree of spreading and intensification of hydrophobic regions (Zone II-IV) decreases gradually from **HC1**-β-lg to **HC4**-β-lg system. For example, in the case of **HC2**-β-lg system, the area and intensity of hydrophobic regions in Zone II-IV are in between free β-lg and **HC1**-β-lg. These results indicate that more surface hydrophobicity increases in the presence of hydroxychalcones which facilitate the more aggregation of β-lg.

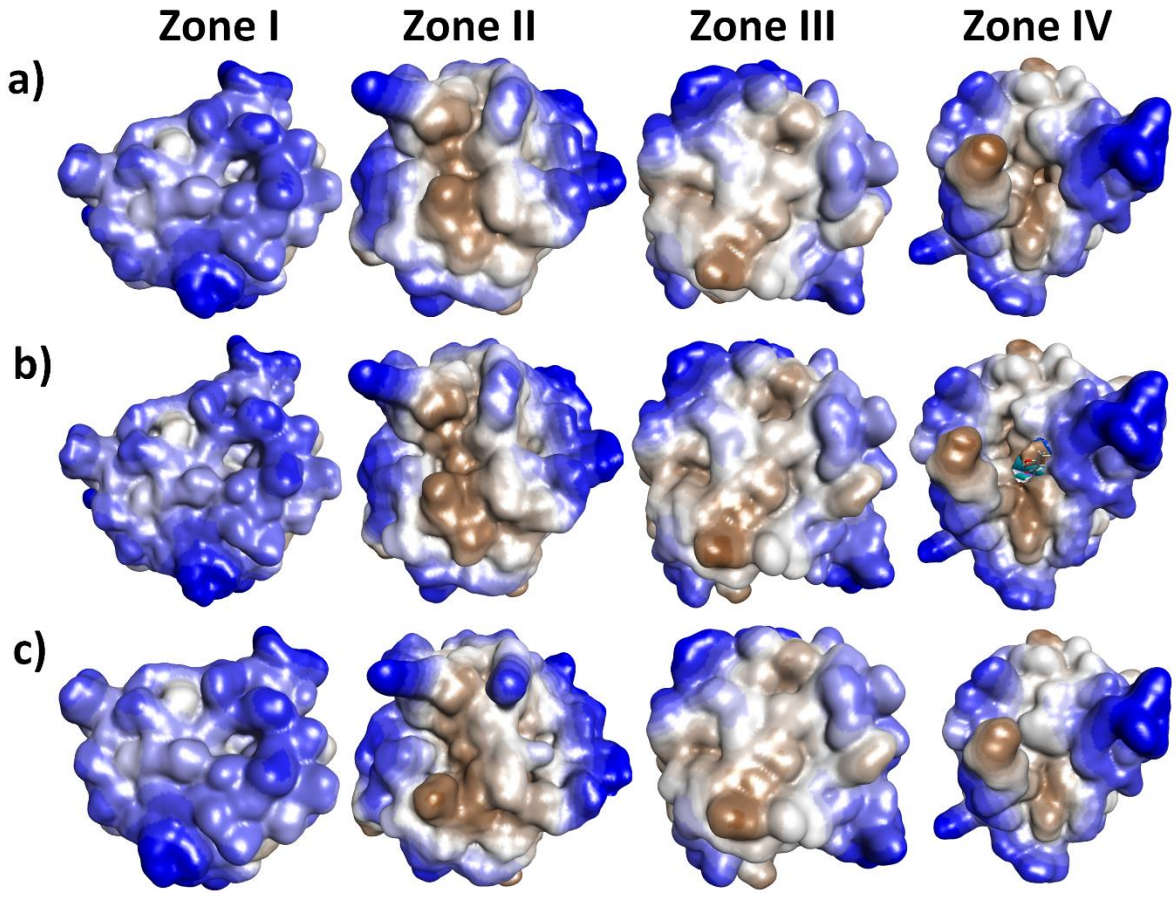
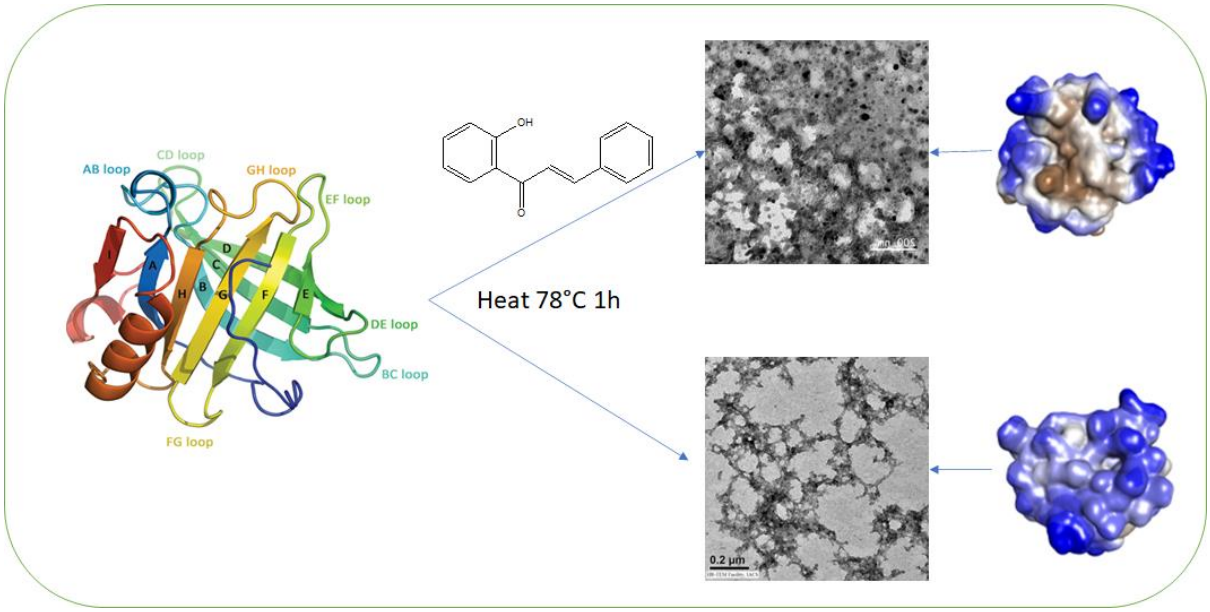


Fig.9 Different pose of β-lg with Hydroxychalcone showing Hydrophobic Zones of the protein

5-11. Schematic representation



5-12. Conclusion

In summary, present study demonstrates the synthesis of the hydroxychalcones having different substituents. Chalcones are αβ-unsaturated ketone, which includes a variety of important biological compounds collectively known as chalcones or chalconoids. Chalcones and their derivatives demonstrate a wide range of biological activities including anti-inflammation. These compounds were tested for the study of conformational change of β-lg leading to its amyloid fibrillation process. Depending upon electronic property of the substituent, chalcones derivatives can interact differently with the protein and thus can affect their conformation change in different extents. Such binding of the compounds to the β-lg changes the protein conformation which exposes hydrophobic amino acids to the protein surface. It was also observed in the hydrophobic site specific ANS-assay. Exposure of hydrophobic amino acids to the protein surface, leads to the aggregation of the proteins. All the studies including Th-T binding assay, FT-IR, TEM and molecular docking studies show that the order of chalcone induced β-lg aggregation is **HC1 > HC2 > HC3 > HC4.**

5-13. Experimental

5-13.1. Synthesis

In a round bottom flask, equipped with magnetic fly, 1mM of 2 hydro acetophenone and 1mM benzaldehyde and its derivatives were taken in a 10% aqueous- ethanolic (1:1) NaOH Solution. The mixture was stirred for 1h. at Room temperature a bright yellow to red precipitate of Compounds (HC1 – 4) were obtained after neutralization of the solution. The crude product was filtered and crystallized with ethyl acetate-petroleum ether (20%). Crystallized compounds were filtered off, dried, and characterised with 1H, 13C NMR, and Mass spectroscopy.

NMR data

(E)-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one (HC1). Yellow solid (71%); 1H NMR (300 MHz, CDCl3) δ 6.82 (d, J = 7.2 Hz, 1H), 7.33-7.54 (m, 7H), 7.60 (d, J = 15 Hz, 1H), 8.02 (d, J = 15 Hz, 1H), 8.07 (d, J = 7.2 Hz, 1H), 13.4 (s, 1H).

(E)-1-(2-hydroxyphenyl)-3-(p-tolyl) prop-2-en-1-one (HC2). Yellow solid (62%); 1H NMR (300 MHz, CDCl3) δ 2.4 (s, 3H), 6.79 (d, J = 7.2 Hz, 1H), 7.35 (d, J = 7.1 Hz, 2H), 7.43-7.57 (m, 4H), 7.58 (d, J = 15 Hz, 1H), 8.04 (d, J = 15 Hz, 1H), 8.08 (d, J = 7.2 Hz, 1H), 13.4 (s, 1H).

(E)-1-(2-hydroxyphenyl)-3-(4-nitrophenyl) prop-2-en-1-one (HC3). Yellow solid (75%); 1H NMR (300 MHz, CDCl3) δ 6.9 (d, J = 7.2 Hz, 1H), 7.46 (d, J = 7.1 Hz, 2H), 7.91 (d, J = 15 Hz, 1H), 8.03 (d, J = 7.2 Hz, 1H), 8.19 (d, J = 15 Hz, 1H), 8.33 (d, J = 7.1 Hz, 1H), 8.09 (d, J = 7.2 Hz, 1H), 13.6 (s, 1H).

(E)-1,3-bis(2-hydroxyphenyl) prop-2-en-1-one (HC4). Red solid (59%); 1H NMR (300 MHz, CDCl3) δ 6.65 (d, J = 7.2 Hz, 1H), 6.90-7.07 (m, 3H), 7.46-7.51 (m, 3H), 7.41 (d, J = 15 Hz, 1H), 8.03 (d, J = 7.2 Hz, 1H), 8.27 (d, J = 15 Hz, 1H), 10.24 (s, 1H), 13.6 (s, 1H).

5-13.2. Isolation, Purification, and preparation of Beta – lactoglobulin Solution:

As per the method described by Aschaffenburg and Drewry, the bovine β-lg was isolated and purified from cow milk.1 After purification the final product was Lyophilized (Eyela) and stored at 4°C. For Spectroscopy measurements stock solution of 5 mM βlg sample is prepared. At first B-lg was weighed and dissolved in 10mM Sodium-Phosphate buffer solution containing 2% ethanol of pH 7.4 [9]. As the extinction co efficient of β-lg (0.959 mg-1 mL-1 cm-1 at 280 nm) is known, different concentration of protein samples was prepared by

dissolving the β-lg stock solution in MilliQ-Water followed by absorbance measurement at 280 nm.

5-13.3. UV – Vis spectroscopy:

To understand the Binding affinity and binding constant, absorption spectra of samples of different β-lg, heat incubated β-lg in presence and absence of chalcone derivative were recorded Using JASCO V700 UV – visible spectrophotometer (Serial no: B184461798, model no: v-730) and JASCO spectra manager Software at room temperature(25°C). For this experiment two PerkinElmer Quartz cell of path length 1cm were used for both sample cell and reference cell. In the reference cell 10 mM phosphate buffer pH 7.4 containing 2% ethanol were taken as reference. β-lg concentration of the sample solution were 14µm.

5-13.3. Intrinsic Fluorescence:

Utilizing Horriba Fluorometer (Serial No: 1734D-4018-FM, Model: Fluoromax-4C) Intrinsic Fluorescence Measurement were Carried Out. To perform this experiment sample solutions containing β-lg concentration of 13.6 µm were taken in a four-side transparent quartz rectangular cell of 1cm pathlength. The sample solutions were excited at λmax 295 [7]. Both the excitation and emission slit were set at 5 nm and emission spectra were recorded in the range of 300 – 550 nm at a scan speed of 100 nm s-1.

5-13.4. ANS Assay:

To understand the change of surface hydrophobicity of β-lg molecule in presence and absence of different chalcone molecules, ANS Binding assay was performed. 1 – anilinonaphthalene – 8- sulfonate (ANS) is a polarity sensitive fluorescent probe [12]. In our study the final concentration of ANS in each sample was 30 µm to maintain 50 molars excess ANS than the protein concentration [13]. Utilizing Horriba Fluorometer (Serial No: 1734D-4018-FM, Model: Fluoromax-4C) ANS Fluorescence Measurement were performed by exciting each sample at λ_{max} 385 nm in quartz cuvette of 1 cm pathlength. Emission and excitation slit were 5 nm and ANS – fluorescence emissions were recorded at 395 to 550 nm using Fluoromax Software [14]

5-13.5. Thioflavin T(Th-T) fluorescence:

Thioflavin T is an organic dye, binds with the beta sheeted structure of the aggregates and shows enhanced fluorescence emission intensity at around 485 nm [15]. Hence comparison of

amount of presence of aggregate in a solution can be carried out by this Th-T binding assay. To perform this comparison, β-lg, heat exposed β-lg in presence and absence and Prescence of different chalcone derivatives were mixed with 30 μm Th T solution. At first 5mM stock Th-T solution was prepared by dissolving Th-T in 10mM sodium phosphate Buffer. 20 μl of this stock solution was added in each sample and mixed thoroughly and incubated for 30 minutes at room temperature 25°C. Using Horriba Fluorometer (Serial No: 1734D-4018-FM, Model: Fluoromax-4C) Th-T fluorescence assay was performed by exciting each sample at λmax 450 nm [16]. Slit widths were maintained at 5 nm for both excitation and emission slit. Emission spectra were recorded between the range of 460 – 600 nm. The data are reported were means of three replicates.

5-13.6. FT-IR absorption spectra:

To find out the structural changes in the finger print region of the β-lg in presence and absence of different chalcone derivatives, Infra-red absorption spectra of the samples were measured at room temperature (25°C) utilizing a Spectrum 100 FT-IR spectrometer (Perkin-Elmer) at a nominal resolution of 2 cm-1. 500 μm β-lg concentrations were maintained in each sample for these measurements. Data are recorded in the range of 1400-1800 cm-1[16].

5-13.7. Transmission Electron Microscopy:

Imaging of the aggregates upon thermal incubation of β-lg in presence and absence of different chalcone derivatives were captured using High resolution Transmission Electron Microscopy (HR TEM) (JEOL – HRTEM- 2011, TOYKO, JAPAN) under an accelerating voltage of 80 - 85kv in different magnifications.[17]. For this study all the sample solutions were centrifuged and diluted. These diluted solutions were drop casted on a carbon coted copper grid of mesh size 300C (Pro Sci Tech). After 20s the droplet was removed from the mesh grid by shocking it on a filter paper by the side of the grid followed by a droplet of 2% uranyl acetate (Sigma) was added in it to stained. Next the grids were air dried in a desiccator overnight. After incubation for few hours the grids were used for TEM imaging at a magnification 12 - 30000.

5-13.8. Molecular Docking Study:

To understand the interaction of Chalcone derivatives with β-lg molecules(1BSY) molecular modelling was designed using Auto Dock 4.2 software [18]. Using Gaussian 0.9w DFT optimization is carried out to minimized the energy of the chalcone derivatives. Lamarckian genetic algorithm (LGA) with grid box 126x126x126 was used for molecular docking.

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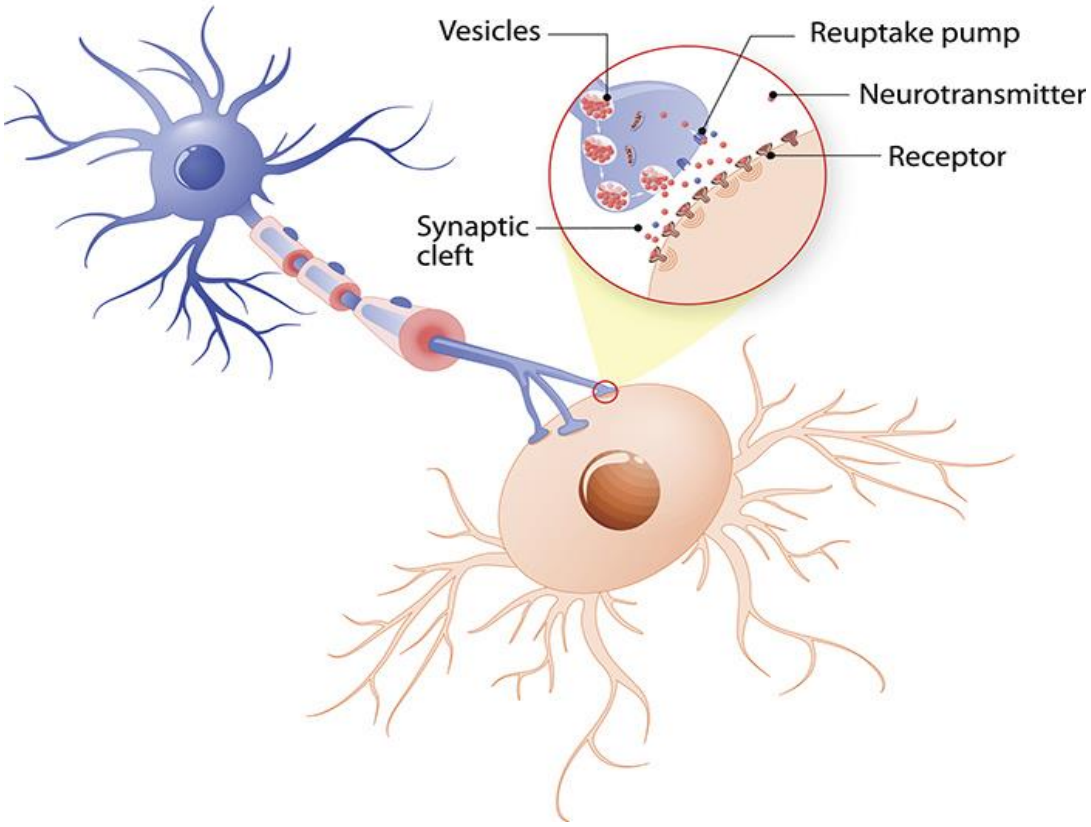
Chapter:6

Anti-amyloid aggregation activity of the neurotransmitters dopamine, phenylethyl amine and γ-aminobutyric acid

6-1. Introduction

Neurotransmitters are called as the chemical messenger of the body. In the nervous system messages are transmitted between neurons or neurons to muscles by these molecules. Two neurons communicate with them through a synaptic cleft (a very small gap between the synapses of the neurons). The electrical signals passing through the axon is transformed into the chemical signal through the release of neurotransmitters and causes a specific response at the receiving neuron.



Schematic presentation of nervous system

Mostly neurotransmitters are amino acids, small neuropeptides or amine molecules. More than 100 different neuropeptides and about a dozen known small-molecules are known to act as neurotransmitters while scientists are still engaged to discover more about the chemical messengers.

6-1.1. Key neurotransmitters

Acetylcholine was discovered first as neurotransmitter, having an important role in the peripheral nervous system, where the motor neurons release. It also plays the important role to maintain the cognitive function in the central nervous system (CNS). In the CNS glutamate acts as the primary excitatory transmitter. Conversely, its derivative γ-aminobutyric acid (GABA) acts as a major inhibitory transmitter while the amino acid glycine, found mainly in the spinal cord also functions as inhibitory neurotransmitter. The monoamines like dopamine function as neuromodulators. Several dopamine pathways are found in the brain involving in many functions like motor control, motivation, reward and reinforcement. Another monoamine, noradrenaline or norepinephrine, function as the primary neurotransmitter in the sympathetic nervous system. It works there on the activities of the various organs in the body to control heart rate, blood pressure, liver function and various other functions.

6-1.2. Types of neurotransmitters

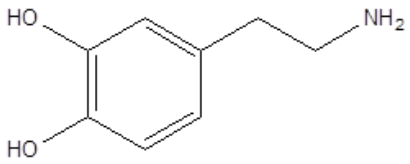
Generally depending on the specific neurotransmitter, it can transmit any one of the following three possible actions.

- **Excitatory-** The excitatory neurotransmitters "excite" the neuron and make it possible to "fire off the message," which means that the message continues to be passed along the next cell. Glutamate, epinephrine and norepinephrine are the examples of excitatory neurotransmitters are
- **Inhibitory-** Chemical messages are prevented or blocked by Inhibitory neurotransmitters for being passed along any farther. Inhibitory neurotransmitters include the examples of gamma-aminobutyric acid (GABA), glycine and serotonin.
- **Modulatory-** Modulatory neurotransmitters influence on the effects of other chemical messengers. They control the communications of the cells at the synapse. Larger numbers of neurons are also affected at the same time by them.

6-1.3. Dopamine: Dopamine (3,4-dihydroxyphenethylamine) is a neuro modulatory compound, has several important roles in body. Dopamine belongs to the catecholamine and phenyl amine families. More than 70% of the catecholamine content of the brain are constituted by them. It is an amine type compound having synthesized by the removal of carboxyl group from the precursor compound L-Dopa, which is synthesized in the brain and kidneys. Dopamine is also present in plants and animals. As a neurotransmitter dopamine plays an

important role and it is a chemical released by neurons to send signals to other nerve cells. Neurotransmitters are synthesized in the specific regions of the brain, but may affect many other regions of the body. The brain involves several distinct dopamine pathways, one of which plays a major role in the motivational component of reward-motivated behavior. This type of reward-motivated pathway increases the level of dopamine in the brain [1] and many drugs can increase dopamine release or block its reuptake into neurons following release [2]. Other brain dopamine pathways are involved in motor control and also the release of various hormones.

Dopamine functions primarily as a local paracrine messenger outside the central nervous system. It inhibits norepinephrine release and also acts as a vasodilator in the blood vessels. It increases urine output and sodium excretion and it reduces insulin production in the pancreas. It reduces the activity of lymphocytes. It reduces also the motility of stomach and protects the mucus cell of the intestine in the digestive system.



Dopamine

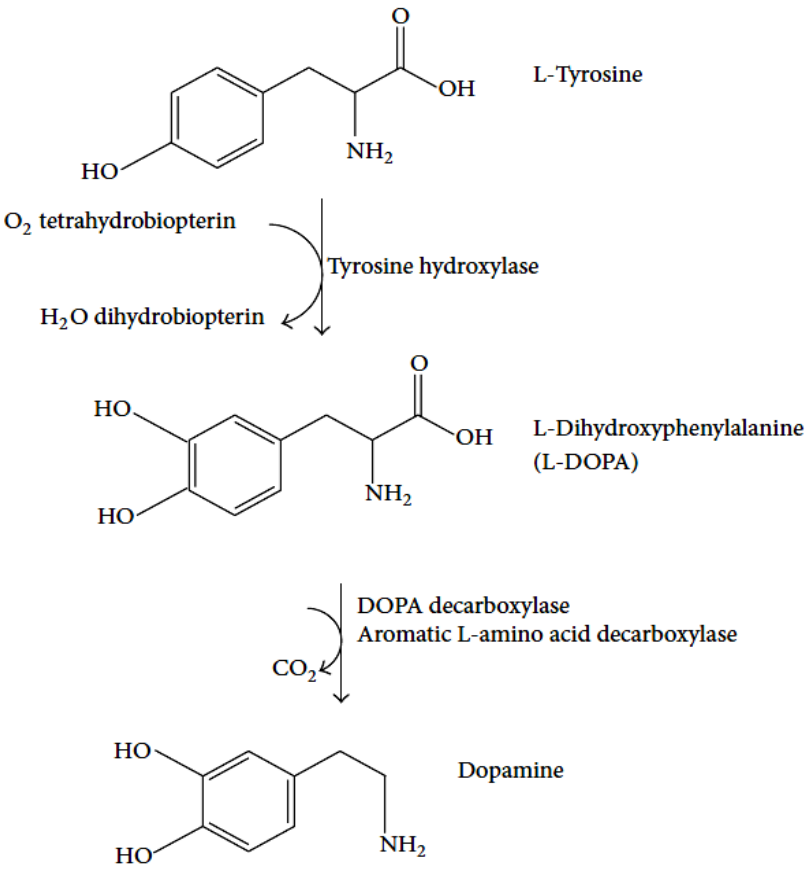
Dopamine is often portrayed as the main chemical of pleasure in popular culture and media, dopamine instead confers motivational salience according to the current opinion in pharmacology [3,4] conversely, dopamine signals the perceived motivational prominence (i.e., the desirability or aversiveness) of an outcome which thus propels the organism's behavior away or toward from achieving that particular outcome. Dysfunction of the dopamine system brings various important diseases of the nervous system, and altering the effects of dopamine some of the key medications are used to treat them. Parkinson's disease, a neurodegenerative condition involving tremor and motor impairment, is probably caused by the loss of dopamine-secreting neurons in the midbrain area called the substantia nigra.

6-1.3.1. L-Dopa

Dysfunctions of the dopamine system are associated with several important diseases. In the neurodegenerative disease called Parkinson’s disease, tremor and motor impairment are commonly observed due to loss of dopamine-secreting neurons in the midbrain area known as the substantia nigra. *Levodopa*, known as L-DOPA, the precursor of dopamine is most widely used treatment for Parkinson's. The disease schizophrenia involves altered levels of dopamine

activity, and the most antipsychotic drugs used to treat this are the dopamine antagonists that reduce the dopamine activity [5]. Similar dopamine antagonist drugs are also effective and used as anti-nausea agents. Decreased dopamine activity causes attention deficit hyperactivity disorder (ADHD) and restless leg syndrome [6]. Dopamine is also available as an intravenous injection though it cannot cross the blood brain barrier, its peripheral effects make it helpful in the treatment of heart failure or shock especially in newborn babies.

6-1.3.2. Biosynthesis of dopamine



Sceme-1: Biosynthesis of dopamine

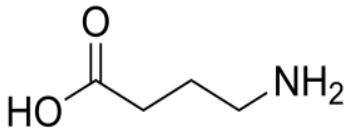
6-1.4. Gamma Aminobutyric Acid (GABA)

Gamma aminobutyric acid (GABA) plays an important role as principal inhibitory neurotransmitter in the central nervous system (CNS). After the discovery of GABA in biological tissues in 1910, its neurological role in mammalian system remained unknown until the late 1950s. Cortical neuron studies in the late 1960s concluded that GABA was unequivocally inhibitory. Later subsequent studies were conducted to elucidate the mechanisms of GABA-induced inhibition and its role in GABA-related pathologies, including

anxiety disorders, alcohol induced disorder, spastic diseases, epilepsy, and idiopathic hypersomnia [7]. The action of most antiepileptic drugs, anxiolytic drugs, and anesthetic drugs serves as GABA agonists [8,9]. Some GABA antagonists are useful as antidotes against GABA agonist overdoses [10]. Scientists also call GABA a non-protein amino acid neurotransmitter.

6-1.4.1. Function of GABA in our body

- antidepressant
- sedative
- antihypertensive, which means it can reduce blood pressure
- antidiabetic
- anticancer
- immune system enhancer



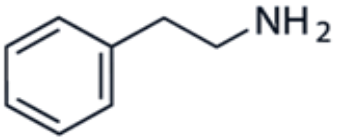
Gamma Aminobutyric Acid (GABA)

6-1.5. Phenylethyl amine (PEA)

Phenethylamine (PEA) is a natural monoamine alkaloid, and a trace amine, that can act as a central nervous system stimulant in humans. Phenethylamine, in the brain, regulates monoamine neurotransmission by binding to trace amine-associated receptor 1 (TAAR1) and inhibiting vesicular monoamine transporter 2 (VMAT2) in monoamine neurons. To a lesser extent, it also acts as a neurotransmitter in the human central nervous system (CNS) [11]. Phenethylamine, in mammals, is produced from the amino acid L-phenylalanine by the enzyme L-amino acid decarboxylase. Substituted phenethylamines are a chemical class of organic compounds based upon the phenethylamine structure. Many substituted phenethylamines are psychoactive drugs, which belong to a variety of different drug classes, including CNS stimulants (e.g., amphetamines), hallucinogens (e.g.,2,5-dimethoxy-4-methylamphetamine) entactogens, appetite suppressants (e.g., Phentermine), nasal decongestants and bronchodilators (e.g., pseudoephedrine), antidepressants (e.g., bupropion), anti-Parkinson agents (e.g., selegiline), and vasopressors (e.g., ephedrine) among others. Many

of these psychoactive compounds exert their pharmacological effects primarily by modulating monoamine neurotransmitter system.

Schizophrenia is rare mental disorder that could potentially be related to PEA. About 1% of the world's population is affected by schizophrenia, which is characterized by problematic thinking and perception. Phenylethylamine is thus a small molecule with large impact.



2-Phenylethylamine

6-1.5.1. Natural Occurrence and Biological Synthesis of PEA

The occurrence of PEA and its derivatives has previously been reviewed. PEA can be found in many algae, fungi, and bacteria [12] as well as a variety of different plant species [13]. PEA is the decarboxylation product of phenylalanine.

Tryptophan Phenylethylamine

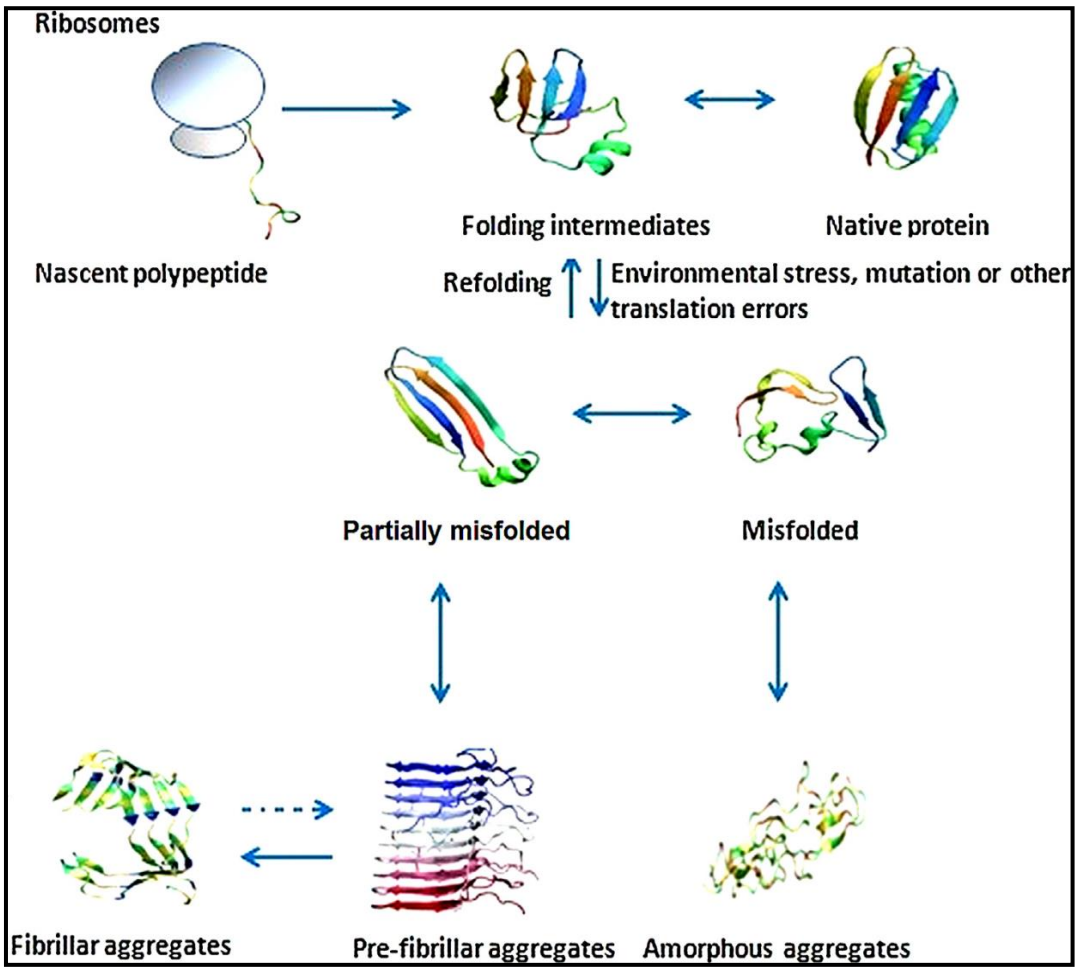
In several bacterial species, the above reaction is catalyzed by the enzyme tryptophan decarboxylase.

6-1.6. Protein aggregation and amyloid diseases

Many proteins and peptides have been found to form the stable self-assembly leading to insoluble aggregates termed as amyloid fibrils. Amyloid fibrils, generated due to protein aggregation, play an important role in a range of amyloid diseases, including Alzheimer's disease, dialysis-related amyloidosis, and type II diabetes mellitus [14, 15]. In addition, various peptides and proteins not related to amyloidosis form similar fibrillar aggregates *in vitro* [16]. Many other proteins can also form amyloid like fibrils in vitro under well-defined conditions of pH, ionic strength, and temperature [17]. The structural hallmark of amyloid fibrils is the presence of high content of intermolecular β-sheet having the β-strands running perpendicular to the fibril axis [18]. Fibrillation may also involve conversion of α-helical secondary structure

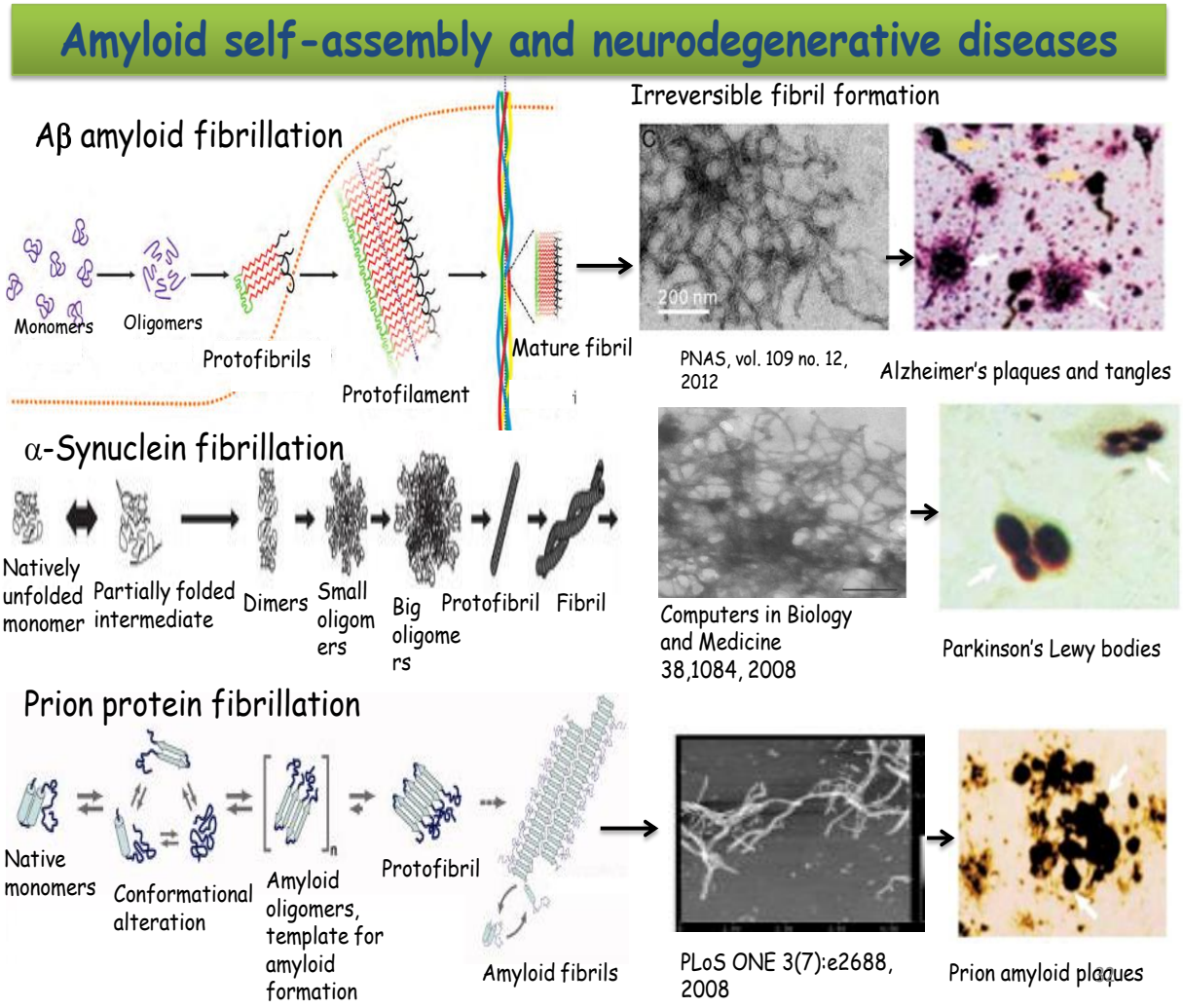
in to β-sheet. The situation that favors the formation of such fibrillar aggregates is involved with the perturbation of native structure followed by misfolding of the native protein structure [19, 20]. Researchers have stressed the involvement of early structural intermediates to initiate the protein aggregation process yielding the small soluble oligomers. Reorganization of the structure of these oligomers produce small wormlike assemblies called protofibrillar intermediates which have less organized structures than the final fibrillar state. In vitro studies have shown that the spherical oligomers and the small worm-like protofibrillar intermediates, formed in early aggregation process, are more toxic than the mature amyloid fibrils [20, 21].

Hence preventing the formation of these soluble aggregates during the fibrillation procedure could serve as the key to the nonoccurrence of many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, type II diabetes and the spongiform encephalopathies etc. diseases.



Sceme-2: Steps in Amyloid Fibril Formation

The underlying mechanism of fibril formation is facilitated with the denaturation of proteins suggesting that it is a property of unfolded or non-native proteins. Hydrophobicity is also a dominant factor determining the amyloidogenicity of proteins. Formation of the cross β-sheet structure with the hydrogen bond network is essential for the fibril formation and also the propensity of side chains as well as the main chains to form hydrogen bonds is also important.



Sceme-3: Amyloid of different proteins

Table-1: Amyloid of different proteins

CLINICAL SYMPTOM	PROTEIN INVOVED	FIBRIL COMPONENT	AFFECTED ORGANS
Alzheimer's Disease (AD)	β-Amyloid	β-Peptide 1-40/1-42	Cortex, hippocampus
Parkinson's Disease (PD)	α-Synuclein	Lewy bodies	Basal ganglia, substantianigra
Primary systemic amyloidosis	Immunoglobulin light chain	Intact chain or fragments	Kidneys, Heart, liver, GI tract, peripheral and autonomic nervous system,
Secondary systemic amyloidosis	Serum amyloid A	Amyloid A (76 residue fragment)	Heart, Kidneys, GI tract,
Senile systemic amyloidosis	Transthyretin	Transthyretin or fragments	Heart
Familial amyloid polyneuropathy I	Transthyretin	Over 45 transthyretin variants	Autonomic nervous system
Haemodialysis-related amyloidosis	β_2-Microglobulin	β_2-Microglobulin	Joints
Familial amyloid polyneuropathy III	Apolipoprotein A1	Fragments of apolipoprotein A1	Liver, Heart, kidneys, peripheral nervous system
Type II Diabetes	Islet amyloid polypeptide (IAPP)	Fragment of IAPP	Pancreatic islets cell
Medullary carcinoma of thyroid	Calcitonin	Calcitonin fragments	Thyroid

CLINICAL SYMPTOM	PROTEIN INVOVED	FIBRIL COMPONENT	AFFECTED ORGANS
Spongiform encephalopathies	Prion protein	Prion or fragments	Cortex, brain stem, cerebellum
Atrial amyloidosis	Atrial natriuretic factor (ANF)	ANF	Heart
Cataract	Crystallin	γ-crystallin	Kidneys, liver
Injection-localized amyloidosis	Insulin	Insulin	Injection site (subcutaneous)
Cancer	P53		Any organ affected
Huntington's Disease	Huntingtin	CAG-repeat extension	Striatum, other basal ganglia
Cystic fibrosis	Cystic fibrosis trans-membrane regulator	Mutated CF trans membrane regulator	Pancreas, intestines, Lungs
Sickle-cell anaemia	Haemoglobin	HbS	Heart, Kidney, Liver, GI tract.
α1-Antitrypsin deficiency	α1-Antitrypsin protein	C-terminal stretch of α1-antitrypsin	Liver, Lungs, skin
Phenylketoneuria	Phenyalalanine hydroxylase (PAH)	Mutated PAH	Plasma, cerebrospinal fluid, brain tissue
Tay-Sach Disease	β-Hexoseaminidase	β-Hex A mutants	Brain
Creutzfeldt-Jakob Disease	Prion protein	PrP and non-protein components	Cortex, brain stem, cerebellum

6-1.7. Development of Therapeutic agents

Efforts have been given devoted towards the discovery and development of novel anti-amyloidgenic agents [22], to combat with the amyloid diseases like Alzheimer's disease, dialysis-related amyloidosis, and type II diabetes mellitus etc. However, drugs developed for Alzheimer's disease were reported to be unsuccessful at the different phases of clinical trials. Small molecules capable of crossing cell membranes and blood brain barrier are well thought-out to be potential drug candidates comparatively, including small aromatic molecules that were reported as efficient inhibitors against the in vitro amyloid fibrils formation [23].

6-1.7.1. Inhibition by denaturants and chaperones

Urea and guanidine hydrochlorides are the commonly known denaturants acting as additive that facilitate protein folding also act as aggregation suppressors. Besides, sodium dodecyl sulfate (SDS), an anionic surfactant also inhibits aggregation in proteins where amyloids are induced at micellar concentrations. The peptide-denaturant interaction prevents directed protein aggregation and becomes inhibitory at higher concentrations. This has been observed at >4.5M urea and >4M SDS [24]. Also, in acidic conditions (pH 2), where the protein adopts a molten globule state, the effect of a denaturant (either urea or GdnHCl) was shown to decrease fibril formation even at low concentrations [25].

Formulation of artificial chaperone is another important technique which can be employed to inhibit protein aggregation and accelerates the refolding of the proteins like serum albumin, lysozyme and insulins. This effectively involves a non-protein system showing chaperone like activity, e.g., GroEL, a bacterial chaperone binds to non-native proteins and competes for hydrophobic sites that act as protein aggregation inducing site [26]. Beside these various ligands can bind to peptides/proteins and prevent them from their pathway of amyloid formation.

6-1.7.2. Inhibition by natural and synthetic aromatic polyphenolic compounds

Various natural or synthetic polyphenolic compounds have been observed as inhibitors of protein fibril formation [27]. These lists include well-studied compounds resveratrol, catechin, tannic acid, curcumin, and epigallocatechin gallate. These compounds are potent food-grade antioxidants and make their possible aromatic interactions with peptides [28].

L-Dopa (L-3,4-dihydroxyphenylalanine) is the precursor of neurotransmitters dopamine, epinephrine (adrenaline) and norepinephrine (noradrenaline) in human. They are collectively

known as catecholamines. L-Dopa is also found in some plants like Mucuna pruriens, Tamarindus indica and Canavalia gladiate [29]. Unlike dopamine, L-Dopa can also cross blood–brain barrier [30]. The dopamine metabolism includes the tyrosine hydroxylation by tyrosine hydroxylase that forms L-Dopa or Levodopa. Numerous reports are available to support the in vitro amyloid fibril formation [31, 32]. L-Dopa have been reported earlier to inhibit the fibrillation of model proteins like human serum albumin (HSA) [33], amyloid β-peptide (Aβ) and α-synuclein aggregation in the central nervous system as a striking therapeutic aiming for the treatment of Parkinson disease (PD) as well as Alzheimer's disease (AD) [34, 35].

6-1.7.3. Inhibition of amyloid fibrillation by Gamma Aminobutyric Acid (GABA)

Gamma-aminobutyric acid (GABA) is a ubiquitous non-protein amino acid widely occurring in fungi, bacteria, animals and plants. GABA is formed through the glutamate decarboxylase-catalyzed decarboxylation of L-glutamic acid [36]. GABA acts as the principal inhibitory neurotransmitter in the central nervous system. In addition, GABA exhibits anti-hypertensive, anti-inflammatory [37] anti-diabetic [38], anti-hyper chloesterolemic [39] and antioxidant property [40].

Studies suggest that aggregation of Aβ peptides in the hippocampus interfere with GABA inhibitory interneuron function and promote memory impairment [41]. Again, defects in the cholinergic system can reduce the ACh levels in the brain due to the loss of cholinergic neurons and neuro transformation. Based on the cholinergic hypothesis, the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) and increment in ACh levels can improve the memory and cognitive functions in AD patients [42]. Researchers have synthesized β-alanine- and GABA-substituted peptides as inhibitors of disease-linked protein aggregation. They have synthesized small peptides by replacing alanine with β-alanine and GABA. These short peptides inhibit the aggregation of α-synuclein in Parkinson disease [43].

In this work, we have demonstrated the inhibitory effects of three important compounds dopamine, phenylethyl amine and γ-aminobutyric acid (GABA) related to the neurotransmission on the thermal aggregation of bovine beta-lactoglobulin (β-lg) in vitro with the help of intrinsic Trp and Thioflavin T (Th-T) fluorescence, ANS fluorescence, FTIR and CD spectroscopy measurements and transmission electron microscopic (TEM) studies.

Bovine beta-lactoglobulin (β-lg), a well-known globular whey protein (MW ~ 18.3 kDa), in ruminant milk, has been chosen as a model protein for studies of protein aggregation and

amyloid formation. Bovine β-lg has been classified as an effective carrier-protein of oxidation-sensitive hydrophobic drugs, retinol, fatty acids and nutraceuticals and have a unique acidic pH resistivity and potential encapsulating property [44]. At elevated temperature (above 60^0C), it undergoes a conformational change, with exposure of buried hydrophobic residues and the thiol (–SH) group (Cys-121). However, the protein (β-lg) is found to form amyloid fibrils when heated above 75^0C at physiological pH having all the characteristic features observed in many amyloid diseases [45].

6-2. MATERIALS AND METHODS

6-2.1. Reagents and chemicals

Bovine β-lactoglobulin (β-lg) was isolated and purified from cow milk as described by Aschaffenburg and Drewry [46]. The final product was lyophilized and stored at 4^0 C. Since the extinction coefficient of β-lg (0.96 mg^{-1} mL^{-1} cm^{-1} at 280 nm) is known, different concentrations of protein samples were prepared by dissolving β-lg samples in 10 mM pH 7.4 sodium phosphate buffers and centrifuging the resulting solution.

Sodium dihydrogen phosphate (AR grade) was purchased from Merck (Mumbai, India). Dopamine and GABA were purchased from Sigma Chemical Company having lot numbers BCVB 9268 and BCBV 0838 respectively. PEA (2-Phenyl ethylamine) was purchased from E. Merck, India having CAS No.: 64-04-0. Different fluorescent probes, viz., 8-anilinonaphthalene, 1-sulfonic acid ammonium salt (ANS) and Thioflavin T (Th-T) were obtained from Sigma Chemical Co. (St. Louis, USA) and used as received without further purification. The other chemicals used were of highest purity available. We used purified bovine β-lg samples in all the experiments.

6-2.2. Sample preparation

Stock solution of β-lg was made in sodium phosphate buffer (10 mM, pH =7.4) and was subjected to dialysis against the same buffer. Th-T and ANS stock solutions were achieved in HPLC grade ultrapure water and were further filtered by using 0.2μm syringe filter. The concentration of Th-T and ANS were estimated spectrophotometrically using $\varepsilon^{1\%}_{412nm}$= 36,000 $M^{-1}cm^{-}1$and $\varepsilon^{1\%}_{350nm}$= 5000 $M^{-1}cm^{-1}$, respectively.

For the formation of β-lgaggregates,13.6 μM of β-lg was incubated without and with a concentration series of dopamine (DOPA), phenylethylamine (PEA) and GABA (0–150 μM)

at 75^0C for 2 h, in water bath shaker.13.6 μM of β-lg was used for all the experiments performed and the concentration of neurotransmitters were ranging from 0 to 150 μM.

6-2.3. Intrinsic fluorescence study

Fluorescence measurements were monitored using Horiba Fluorometer (Model: FLUOROMAX-4C, Serial no. 1734D-4018-FM). Stock solutions of β-lg in Na-phosphate buffer at pH 7.4 were taken in a fluorescence quartz cell of path length of 1cm keeping the protein concentration at 13.6 μM and excited at 295 nm. Emission spectra were recorded in the range of 300 to 550 nm. Excitation and emission slit were set at 5 nm. Data were recorded at a scan rate of 100 nm s^{-1}. The temperature was maintained at 25ºC. Intrinsic fluorescence measurements were done with native β-lg and β-lg incubated at 78ºC for 1 h in the absence and presence of DOPA, PEA and GABA of varying concentrations.

6-2.4. ANS-fluorescence measurements to monitor the hydrophobicity changes

Exposure of hydrophobic patches in protein during the aggregation process was monitored using polarity sensitive fluorescent probe 1-Anilinonapthalene-8-sulfonate (ANS)which binds with the hydrophobic packets of protein surface [47].To measure the hydrophobicity a stock solution of ANS is prepared and added to the each samples (2 mL volume). The final concentration of ANS in each sample is maintained 30 mM. Fluorescence spectra were measured at 390 to 550 nm after excitation at 380 nm and emission spectra were recorded using Shimadzu spectrofluorometer (Shimadzu 5301 PC). Path length was 1 cm. Excitation slit was 5 nm and emission slit was also 5 nm. ANS-fluorescence were studied with native β-lg and β-lg incubated at 78ºC for 1 h in the absence and presence of DOPA, PEA and GABA of varying concentrations.

6-2.5. Identification and quantification of the aggregates by Th-T binding assay

Thioflavin T (Th-T) is a dye which shows enhanced fluorescence at around 480 nm when bound to amyloid fibrils [48]. Thus, to investigate and compare the aggregates formed by native β-lg and β-lg incubated thermally at 78ºC for 1 h in the absence and presence of DOPA, PEA and GABA the following Th-T assay was employed. Stock β-lg samples of 54.3 mM were mixed with 3.13 mM Th-T solution (stock 1 mM Th-T in 20 mM sodium phosphate buffer, pH 7.5). The assay solution was excited at 450 nm [48] and the emissions were measured over the range 460 to 600 nm using Horriba Fluorometer (Model:FLUOROMAX-4C) and Fluoromax

Software. Slit widths for both excitation and emission were kept at 5 nm. All the samples were blank corrected. Three replicates were performed and the data were averaged.

6-2.6. Analysis of secondary structures by CD spectroscopy

Using Jasco spectropolarimeter (J-815) (Jasco, Tokyo, Japan) CD spectra of native β-lg, heat treated β-lg and heat treated β-lg in presence of DOPA, PEA and GABA were recorded with the help of Jasco Spectra Manager Software. For this experiment in the far UV region (190–260 nm), each sample containing 13.6 mM β-lg was taken in a Perkin Elmer quartz cell of path length of 0.2 cm and then data was recorded in the inner nitrogen atmosphere of the instrument at 190 nm to 260 nm. Scan speed was maintained at 100 nm min^{-1} and temperature was kept 30^0 C. The results were expressed as mean residual ellipticity (MRE) in deg cm^2 $dmol^{-1}$ which is defined as:

$MRE = \theta_{obs}$ (mdeg)/10 x n xCp x l

Where, θ_{obs} is the CD in milli degree, n is the number of amino acid residues in one subunit (162 for β-lg), 'l' is the path length of the cell in centimeters and Cp is the molar fraction of proteins. Spectra were recorded using Jasco Spectra Analysis tool and the secondary structural elements were calculated using CDNN 2.1software.

6-2.7. Monitoring the secondary structural changes of β-lg during thermal incubation by FTIR spectroscopy

In the FTIR measurements, β-lg solutions (native and thermally incubated in presence and absence of DOPA, PEA and GABA) having concentrations 1087 mM were taken in a micron filter device and diluted with 200 mL of D_2O. It was then quickly centrifuged at 4000 x g for 10 minutes until the volume reduced to~50 mL. 200 mL of D_2O was added again and centrifuged for another 8–10 min. This process of D_2O exchange was repeated 3–4 times [49]. Finally, the D_2O exchanged β-lg samples were placed between two CaF_2 windows separated by a 50 mm thick Tefon spacer. FTIR scans were collected in the range of 1400–1800 cm^{-1} at a resolution of 2 cm^{-1} in N2 environment using a Spectrum 100 FTIR spectrometer (PerkinElmer). Spectrum of D_2O at pD 7.5 was collected and subtracted from sample spectrum.

6-2.8. Morphological studies with Transmission electron microscopy (TEM)

The size and morphology of the β-lg aggregates (thermally incubated at 78^{o}C for 1 h in the in presence and absence of DOPA, PEA and GABA) were investigated by high resolution

transmission electron microscopy (Jeol-HRTEM-2011, Tokyo, Japan) with an accelerating voltage of 80–85 kV in different magnifications. The sample solutions were diluted 50 times in 10 mM phosphate buffer of pH 7.4. A droplet of the diluted sample was put on a carbon coated copper grid of mesh size 300C (Pro Sci Tech). After 20 s the droplet was removed with a filter paper followed by a droplet of 2% uranyl acetate (Sigma, Steinheim, Germany) solution put on the grid and finally removed after 15 s and left for air-dry and used for imaging purpose.

6-3. Results and Discussion

6-3.1. Microenvironment change of β-lg in presence of DOPA, PEA and GABA investigated by intrinsic fluorescence

The compounds DOPA, PEA and GABA, having influence on neurotransmission, were employed to study their interactions with the native and thermally incubated β-lg. The fluorescence technique, a highly effective and sensitive method, is widely used to monitor the structural change of protein and its interaction with the small molecules. Generally, Trp and Tyr are the amino acid residues that absorb the light of corresponding wavelengths and show the fluorescence properties for most of the proteins. β-lg has four Tyr (Tyr20, Tyr42, Tyr102, and Tyr99) and two Trp (Trp19 and Trp61) residues which are responsible for the intrinsic fluorescence of the protein [50]. It can be used to investigate the alteration of the polarity around the microenvironment of the fluorophore residue to the structural change of beta-

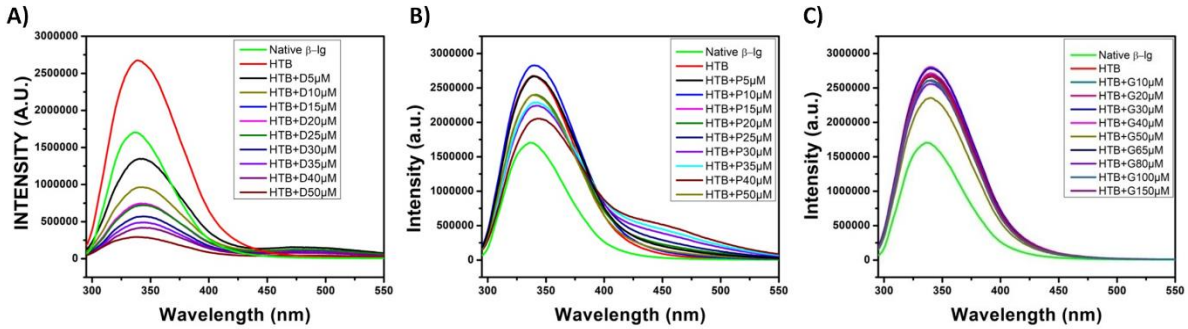


Fig.1. Intrinsic fluorescence spectra of native β-lg and thermally incubated β-lg (at 78°C for 1 h) in the absence and presence of DOPA, PEA and GABA in 10 mM phosphate buffer at pH 7.4 at room temperature. The excitation wavelength was 295 nm and emission spectra were recorded between 300 nm and 550 nm. Both the excitation and emission slits were set at 5 nm. Concentration of β-lg was 13.6 μM. (A) In the absence and presence of DOPA (0-50μM). (B) In the presence and absence of PEA (0-50μM). (C) In the absence and presence of GABA (0-150 μM). Results are mean of three independent experiments (n = 3).

lactoglobulin. In the case of aggregation of β-lg, solvent exposure of fluorophores (mainly tryptophan) decreases which is reflected in their emission spectra with the change in fluorescence intensity. The intrinsic fluorescence spectra of native β-lg and β-lg in presence DOPA, PEA and GABA were shown in the Fig.1 (A-C).

Results indicate that the fluorescence intensities of samples containing thermally incubated β-lg in presence of DOPA, PEA and GABA are lower than the thermally incubated β-lg alone. This indicates the possible interactions of β-lg with these compounds, resulting the structural alteration of the protein and thus leading to the change of the position of fluorophore.Fig.1A shows the fluorescence intensity of β-lg decreased regularly with gradual addition of DOPA into a fixed concentration of β-lg solutions. This suggested a concentration-dependent change in the intrinsic fluorescence of the heat-exposed β-lg in presence of DOPA. Such lowering of fluorescence intensities of β-lg is less and not regular with PEA (Fig. 1B) than with DOPA. This indicates that lesser interaction of PEA with of β-lg occurs than DOPA. PEA alters the structure and position of tryptophan fluorophoreless efficiently than DOPA due to difference in their structures. On the other hand, GABA interacts poorly with heat treated β-lg (Fig. 1C) and thus fails to bring about the much changes in the conformation and polarity of microenvironments around the tryptophan moieties at much higher concentrations of GABA (150 μM) compared to that used for DOPA (50μM). Such anomalous behavior of GABA compared to DOPA and PEA may be due to the lack of aromatic nucleus in their structure. Due to the presence of both aromatic nucleus and hydroxyl group, DOPA interacts strongly with β-lg.

6-3.2. ANS-fluorescence study to monitor the hydrophobicity change of β-lg in the presence of DOPA, PEA and GABA

The effects of DOPA, PEA and GABA on the surface hydrophobicity of β-lg were investigated by ANS-fluorescence emission measurements. To monitor hydrophobic interactions involved in the aggregation processes, ANS fluorescence experiment was performed. ANS is extensively used to demonstrate the protein unfolding intermediates and to identify the existence of hydrophobic patches on the protein surface. ANS on interacting with hydrophobic patches exposed on the protein causes a remarkable enhancement in the fluorescence intensity [51]. As revealed in Fig. 2(A-C), the ANS-fluorescence intensity of native β-lg at 25^0C was observed to be the lowest, suggesting a negligible surface hydrophobicity compared to other β-lg solutions, as in native protein hydrophobic patches are buried in compactly folded

conformation. But a noticeable increment in ANS-fluorescence intensity was observed in case of heat-treated β-lg on incubationat 78^0C for 1has a result of exposure of hydrophobic patches due to amyloid fibrillation [52]. In contrast, a concentration dependent decrease in ANS-fluorescence intensity was observed when β-lg samples were co-incubated with increasing concentrations DOPA (Fig.2.A). The results thus suggested that the hydrophobic patches exposure was notably decreased when treated with the DOPA, indicating the stabilization of thermally unfolded structure of β-lg by DOPA. Thus, DOPA can prevent the aggregation of β-lg. Similar stabilization action of a closely related molecule L-Dopa was reported in connection with the thermal aggregation of the protein human insulin [53]. The plausible cause for this reduced ANS-fluorescence intensity that DOPA could have interacted with β-lg through non-covalent interactions with its two –OH groups and thus hindered amyloid fibrillation process. But the ANS-fluorescence intensity was reduced but not in a concentration dependent manner during incubation of β-lg with PEA, at lower concentrations (up to 30 μM) it only resists the exposure of hydrophobic patches showing lower fluorescence intensities (Fig.2.B). Thus, PEA is less efficient to stabilize the thermally unfolded state of β-lg probably due to its inability to form the non-covalent bonds as it lacks two –OH groups in its structure. On the other hand, due to absence of both –OH groups and phenyl nucleus, GABA fails to stabilize the heat exposed β-lg structure leading to its aggregation.

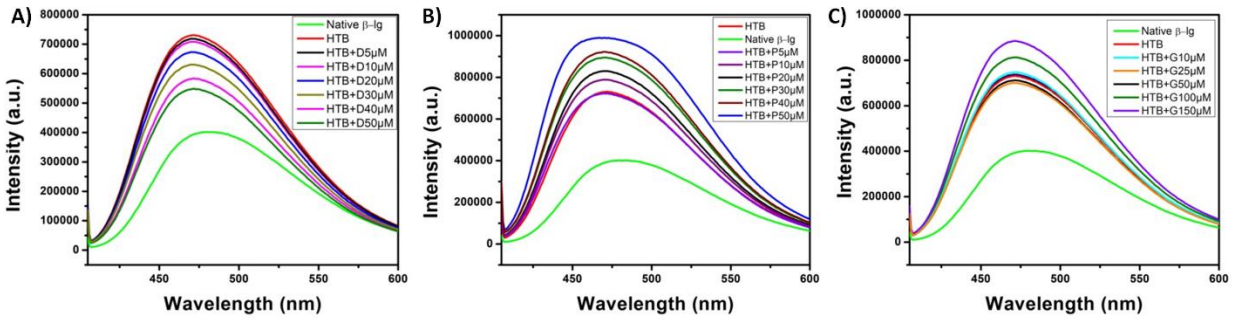


Fig.2. ANS-fluorescence emission spectra of native β-lg and thermally incubated β-lg (at 78oC for 1 h) in the absence and presence of DOPA, PEA and GABA in 10 mM phosphate buffer at pH 7.4. The excitation was done at 380 nm and emissions were measured between 390 nm and 600 nm. Both the excitation and emission slits were set at 5 nm. Concentration of β-lg was 13.6 μM through the experiment. (A) In the absence and presence of DOPA (0-50μM). (B) In the presence and absence of PEA (0-50μM). (C) In the absence and presence of GABA (0-150μM). Results were the mean of three different experiments.

6-3.3. Inhibition of β-lg aggregate formation monitoring through Th-T fluorescence assay

A benzothiazole dye, Thioflavin T (Th-T), is a good fluorescent ligand that binds selectively with the hydrophobic site of the fibrillar aggregates of the protein and leads to an increase in fluorescence intensity at around 480 nm upon excitation at 480 nm [54]. Here, the native β-lg and the other heat-treated β-lg samples in absence and presence of three neurotransmitters DOPA, PEA and GABA produced different Th-T emission intensities which were proportional to the amount of aggregates formed in the different protein solutions. Lower intensity of the Th-T will indicate a lower degree of aggregate formation due to the presence of the neurotransmitters like DOPA, PEA and GABA having anti-fibrillating properties. Fig.3 (A-C) shows a comparative study of the aggregation patterns of native and heat-treated β-lg in the absence and presence of DOPA, PEA and GABA with a fixed at β-lg concentration at pH 7.4 in Th-T assay. Owing to self-assembly formation, the heat-treated β-lg shows the highest Th-T intensity as shown in all the panels of Fig. 3. Th-T intensities were lower than heat incubated native β-lg in all other cases.The DOPA inhibited the amyloid fibril formation of β-lg in a concentration dependent manner, as shown in the Th-T spectra of β-lg with DOPA (Fig. 3A).Th-T fluorescence intensity of β-lg was decreased by almost 80% compared to thermal incubation of β-lg alone when 50 μM DOPA has been added. Similar result has been reported during anti-aggregation study of human lysozyme with L-Dopa [53].

Concentrations of β-lg were kept at 13.6μM in all the cases. Fluorescence emissions were monitored in the wavelength range 460–600 nm after excitation at 450 nm. Results are mean of three independent experiments (n = 3) and the error bars show the standard deviation.

The β-lg in the presence of PEA, another monoamine neurotransmitter, under identical heating conditions also exhibits lesser intensity in Th-T fluorescence measurement than heat-treated β-lg alone. Here 50% lowering of Th-T intensity was observed after the thermal incubation of β-lg solutions with 60μM PEA. Therefore, PEA can also prevent the self-assembly formation of β-lg and can be used as potent anti-fibrillating agent but having lesser efficiency than DOPA. In contrast, GABA having completely different structures than DOPA and PEA, lowers Th-T intensity in lesser extent than DOPA and PEA compared to thermal incubation of β-lg alone and it is effective to inhibit the oligomerization of β-lg only in lower concentrations. Therefore, the neurotransmitters DOPA, PEA and GABA act as effective anti-fibrillating agents to inhibit the oligomerization of β-lg. In this case, the inhibition of aggregation of β-lg follows the order as DOPA> PEA > GABA.

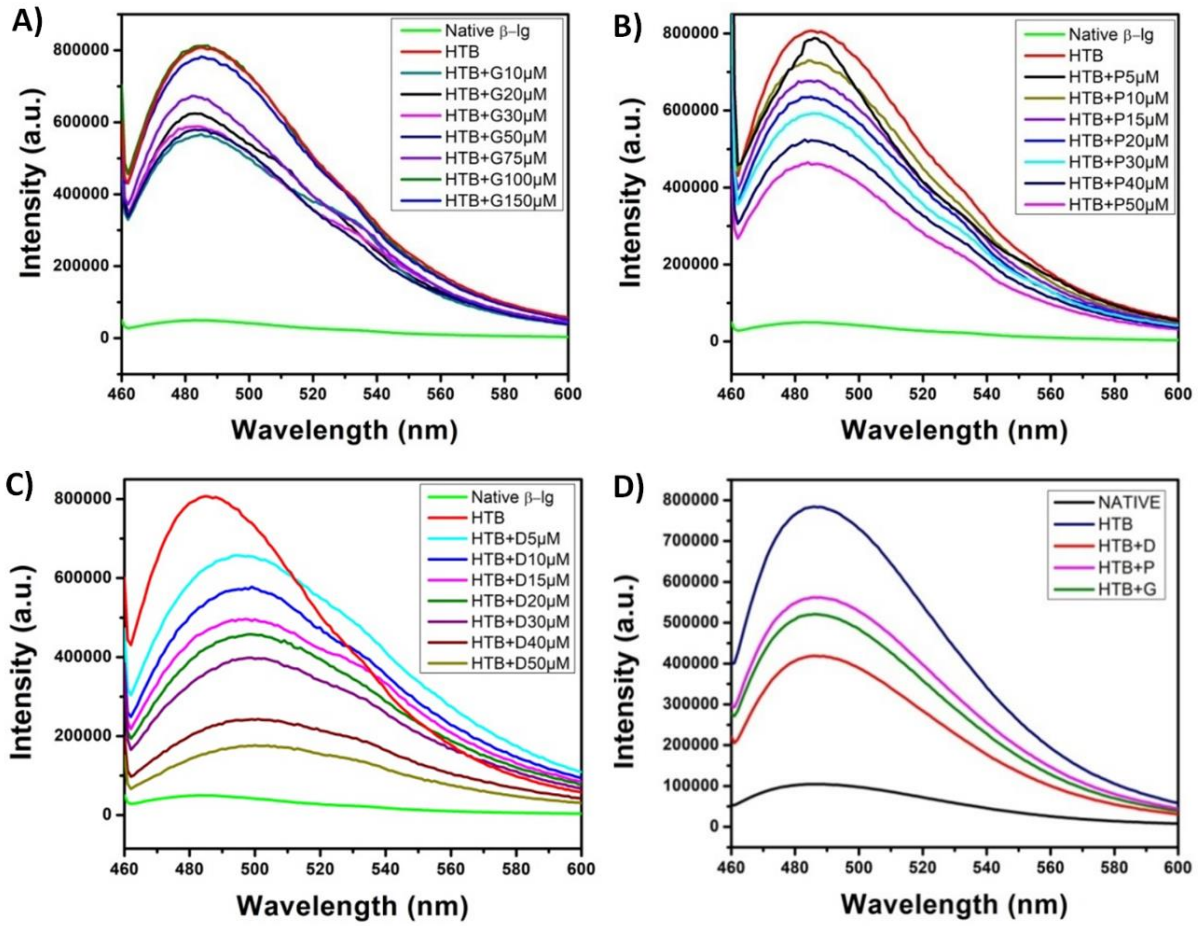


Fig.3 Th-T fluorescence emission spectra of native β-lg and thermally incubated β-lg (at 78ºC for 1h) in the absence and presence of DOPA, PEA and GABA in 10 mM phosphate buffer at pH 7.4 (A) Th-T fluorescence spectra of β-lg in the absence and presence of DOPA (0-50 μM) (B) Th-T fluorescence spectra of β-lg in the absence and presence of PEA(0-60μM) and (C) Th-T fluorescence spectra of β-lg in the absence and presence of GABA(0-150μM) (D) Relative Th-T fluorescence spectra of β-lg with fixed concentration of DOPA, PEA and GABA (30μM).

6-3.4. Monitoring the changes in secondary structure of β-lg with circular dichroism spectroscopy

Far-UV CD technique has been employed to investigate and compare the potential effect of the three neurotransmitters DOPA, PEA and GABA used in this study on the secondary structural transformation of β-lg. The β-lg solutions were incubated with or without DOPA, PEA and GABA at 78^0C for 1 h and the different secondary structures were determined by CD spectropolarimeter. CD measurements were done by scanning the spectra in the region 190–260 nm and represented in Fig. 4. Native β-lg gave two negative bands at 209 nm and 215 nm.

These two peaks represented the existence of ordered secondary structure that contained both α-helix and β-sheet structures [55]. The CD spectrum of heat-treated β-lg showed greater negative ellipticity value and a significant shift in the band positions (red curve). The self-assembled oligomeric structure of heat incubated β-lg found to have greater amount of beta structures(62%) with lesser alpha helical structural content (9.0%) than the native (Table-2). This data indicated the formation of greater β-sheet structures arising out of the thermal aggregation of β-lg. We also analyzed the change of secondary structures of heat-treated β-lg in presence of DOPA, PEA and GABA. With DOPA, a change in peak position along with decrease of negative ellipticity value differing from heat-treatedβ-lg was observed (green curve). CD spectra of β-lg in presence of PEA showed lower MRE values at 207 and 215nm blue line). Thus, both the DOPA and PEA influenced to form lesser beta structures compared to heat-treated β-lg alone, indicating structural transitions leading to the disaggregation in the presence of DOPA and PEA. It is interesting to mention that the shape of CD signal of β-lg in presence of DOPA and PEA are similar to that of the nativeβ-lg in the vicinity of 208 nm and 215 nm. The difference in MRE values can be related to the differences in their structure. On the other hand, GABA, having completely different structure, modifies the secondary structure of heat-treated β-lg. CD spectrum of β-lg with GABA shows greater negative ellipticity and having similarity with heat-treated β-lg alone. The amount of decrease in different β-structures can be compared to that of native β-lg alone showing maximum inhibitory power of DOPA against the thermal aggregation of β-lg. PEA also displayed similar CD signal in this region. Hence the effectiveness in the suppression of thermal aggregation of β-lg follows the order DOPA > PEA > GABA.

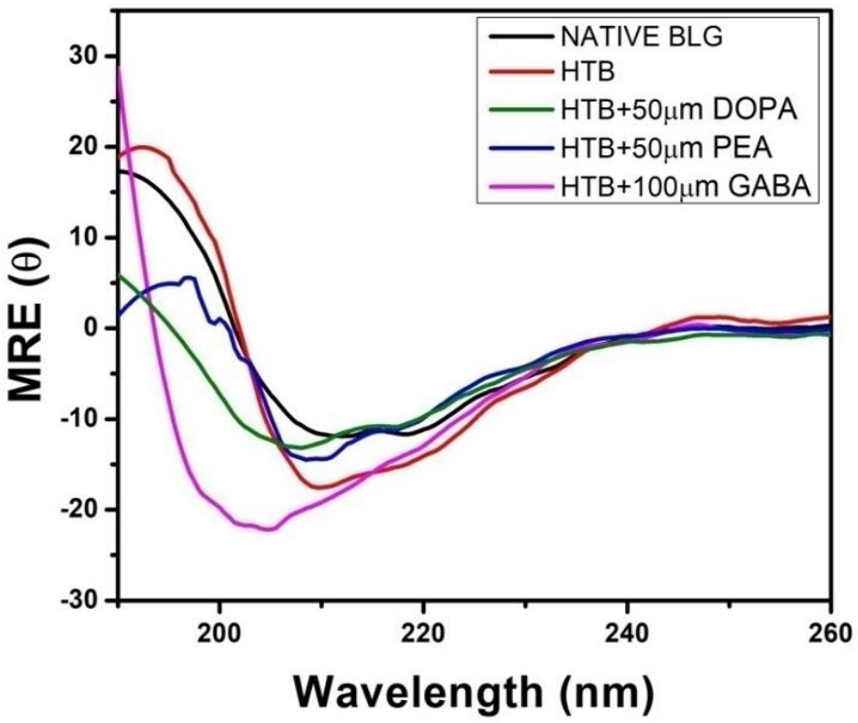


Fig.4. Far-UV CD spectra (190–260 nm) of native β-lg, heat treated β-lg (78^0C, 1h) and β-lg incubated (78^0C, 1h) separately with three neurotransmitters DOPA, PEA and GABA in 10 mM phosphate buffer at pH 7.4 at room temperature showing secondary structural changes during thermal aggregation. The sample concentrations were kept at 13.6 mM.

6-3.5. Secondary structural changes with DOPA, PEA and GABA revealed by ATR-FTIR analysis

FTIR technique is commonly exploited to understand the secondary structural composition of protein. Thus, this technique was employed to investigate the β-sheet structure formation during fibrillation of β-lg at higher temperature (78°C, 1h) in absence and presence of DOPA, PEA and GABA. The secondary structures of β-lg can be acquired through the assignment of the amide-I band ranging from 1600 to 1700 cm^{-1} of ATR-FTIR spectra. The amide-I band of native β-lg appears at around 1632 cm^{-1} (Fig.4) which is the characteristic feature of the protein like β-lg having predominant β-sheet structure [56]. In the absence of neurotransmitters (DOPA, PEA and GABA) the IR spectrum of heat-treated β-lg exhibited a band roughly at

1638 cm^{-1} having greater intensity of amide-1band. It indicates the formation of increased amount of β-sheet structure in comparison to native β-lg owing to its self-assembled structure formation at elevated temperature. Presence of DOPA (50μM) and PEA (50 μM) during thermal incubation separately with β-lg, reduced the intensity and changed the peak position of amide-1 band compared to that heat-treated β-lg alone. FT-IR showed a peak centred on 1629

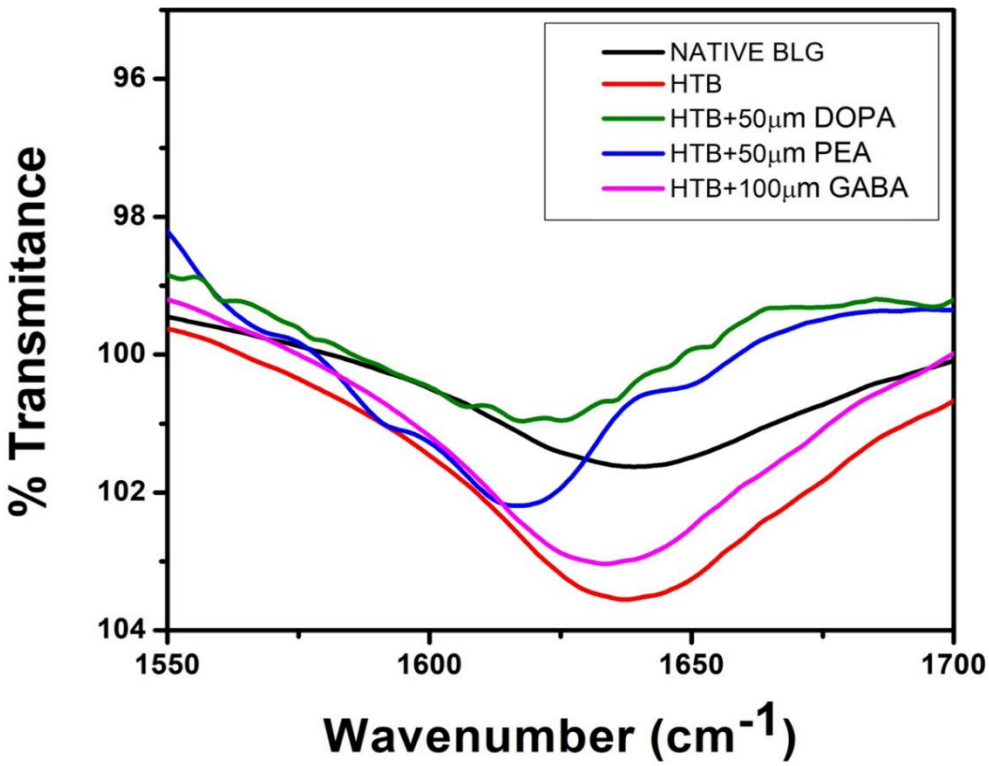


Fig.5. FTIR spectra of nativeβ-lg and heat-treated β-lg in absence and presence of DOPA, PEA and GABA in the amide region (1550–1700 cm-1). Sample solutions were incubated at 78°C for 1h in the absence and presence of DOPA, PEA and GABA in 10 mM phosphate buffer at pH 7.4. Concentrations of β-lg were 1060μM in D2O. Reported every spectrum was mean of 32 scans measured at 25°C.

cm^{-1} for DOPA and 1626 cm^{-1} for PEA, which indicated lesser amount β-sheet structure formation with restoration of native-like β-structure formation of β-lg in presence of DOPA and PEA. Thus DOPA and PEA inhibit the thermal aggregation of β-lg. Fig. 4 also reveals that the GABA at higher concentration (150 μM), shows lesser decrease of intensity and peak position of β-sheet structure of thermally incubated β-lg alone. Thus, GABA inhibits the thermal aggregation of β-lg less effectively than DOPA and PEA. FTIR results were in harmony with the conclusion obtained from our CD spectroscopy measurements.

6-3.6. Morphology of β-lg aggregates with high resolution transmission electron microscopy (HRTEM)

The HR-TEM study was also performed to ascertain how the neurotransmitters DOPA, PEA and GABA can affect the heat induced aggregation and fibril formation of β-lg (Fig.6). The heat-treated β-lg (at78^{o}C for 1 h) formed the self-assembly without the neurotransmitters (Fig.6A) and the morphology is totally fibrillar. However, the TEM image of β-lg in the presence of DOPA showed that the aggregation has been reversed to a different extent. The fibrillar network has been destroyed completely in presence of DOPA. Thus, DOPA inhibits the amyloid fibrillation of β-lg (Fig.6B). Similarly, PEA also can inhibit amyloid fibrillation of β-lg but to a lesser extent than DOPA. TEM image (Fig. 6C) in presence of PEA shows the formation of smaller fibrils along with some small spherical aggregates compared to heat-treated β-lg alone. Conversely, Fig. 5D. demonstrates the formation of aggregates of different morphology having larger spherical aggregates along with some fibrillar species when β-lg was co-incubated with GABA. These results signified that DOPA constrained maximally the fibril formation process of β-lg. PEA has also some potentiality against fibril formation. Their anti-amyloidogenic properties can be explained on the basis of their similarities in the structures. The TEM data is consistent with the previous reported data.

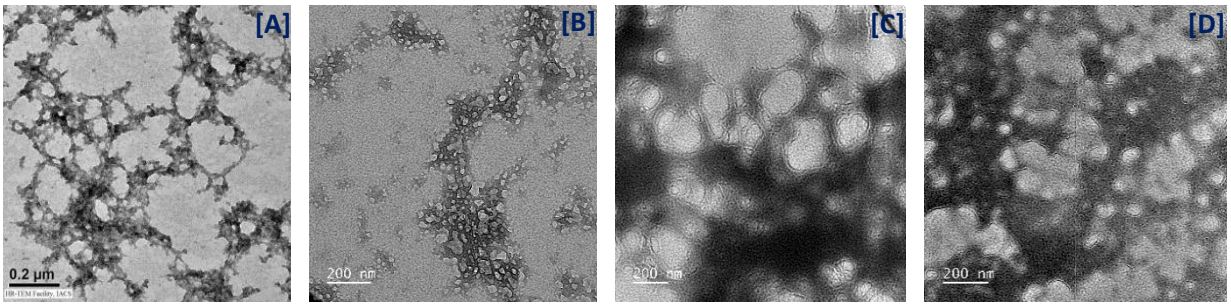


Fig.6. Transmission Electron Micrographs at 25°C of heat-treated β-lg incubated alone at 78°C for 1 h (A) incubated the in presence of 50 µM DOPA (B) incubated in the presence of 50µM PEA, (C) incubated in the presence of 150µM GABA, (D) During incubations β-lg concentrations were kept at 135µM.

6-3.7. Result of docking studies

Molecular docking is one of the most utilised studies to find the conformation of small molecules in inside the bio-macromolecules. Here, the molecular docking has been used to understand the effect of three neurotransmitters on the structure of β-lg. Dopamine is the hydroxylated form of the phenylethyl amine. Therefore, the phenyl ring of the dopamine is

more electron rich with respect to the phenylethyl amine. Molecular docking results shows that the order of the binding of the investigating molecules is Dopamine > phenylethylamine> GABA and the binding energy was found to be -7.1, -6.6, and -5.1 kcal/mol. It was also found that all the three compounds can bind at the calyx of the protein. Both the compounds dopamine and phenylethylamine showed hydrophobic interactions with the calyx amino acids. Since the electron density over the dopamine phenyl ring, it is responsible for stronger C-H…π interactions than that of phenylethylamine which facilitate the strong binding of dopamine with respect to the phenylethylamine.

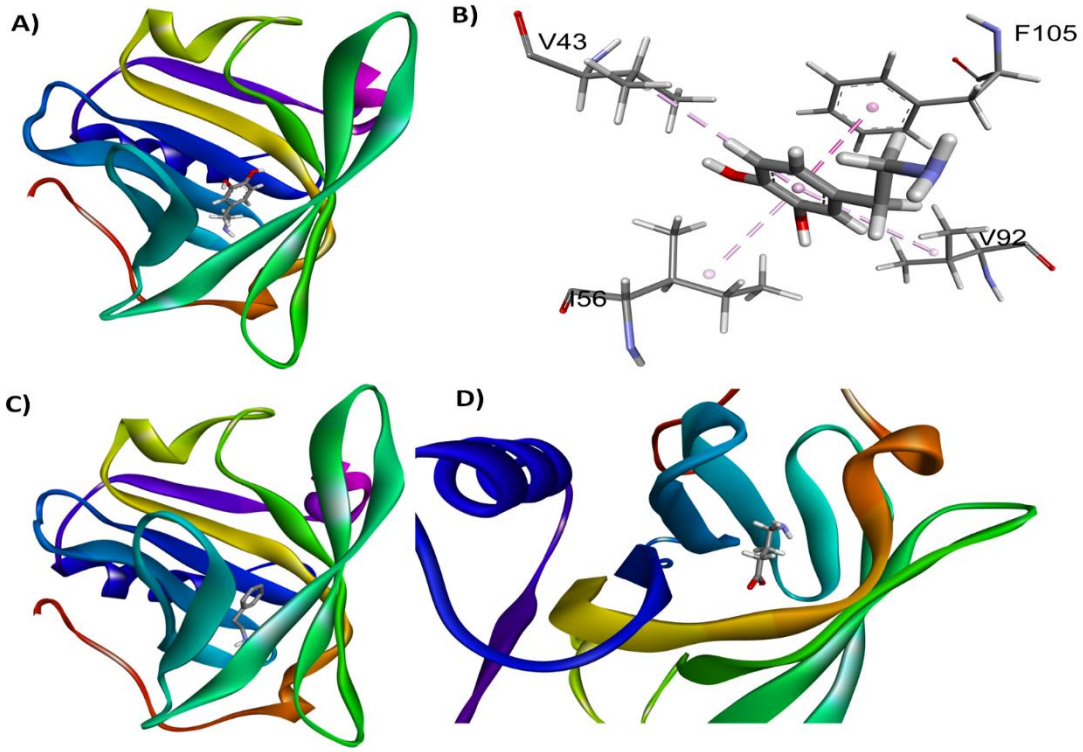


Fig. (A) Schematic representation of docked conformation of DOPA with interacting residues of β-lg. (B) mode of interaction of dopamine with amino acid residues of β-lg. (C) Docking pose of β-lg in presence of PEA (D) Docking pose of β-lg in presence of GABA.

6-4. Schematic representation

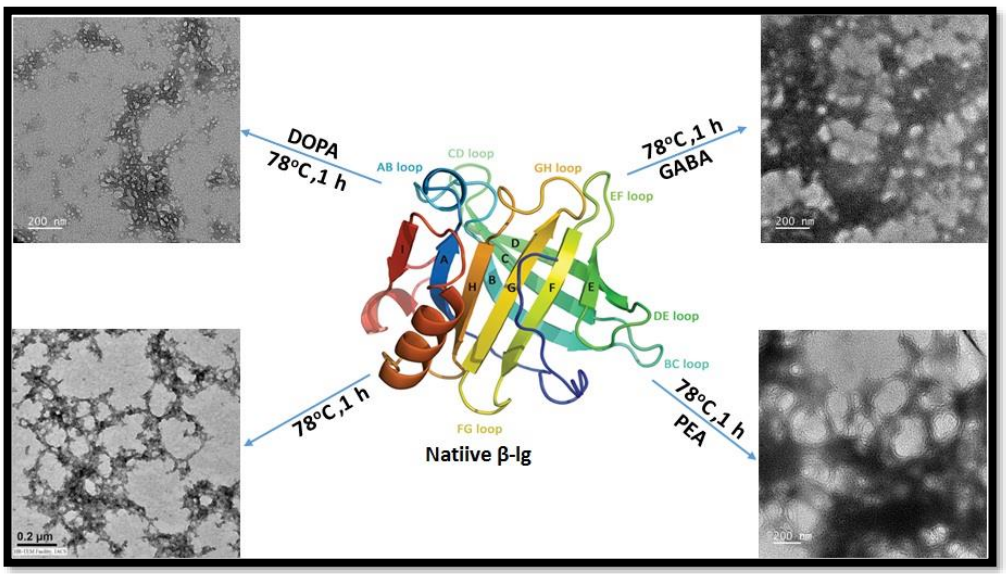


Fig.7.Schematic representation of β-lg amyloid fibrillation in absence and presence of DOPA, PEA, GABA at 78^{o}C for 1 h.

6-5. Conclusion

Our present studies address the potential application of the three therapeutic compounds dopamine (DOPA), phenylethyl amine (PEA) and gamma aminobutyric acid (GABA) related to neurotransmission, in the inhibition and depolymerization of amyloid fibrils formed by bovine β-lactoglobulin (β-lg) under thermal condition. Our investigation involves in vitro studies of aggregation mechanism using multi spectroscopic, microscopic and computational approaches. Th-T assay and TEM study clearly shows that DOPA inhibits completely the fibrillation of β-lg by preventing the structural change of native β-lg into the β-sheet rich fibrillar structure in a concentration dependent manner. Other two compounds PEA and GABA can also be used as anti-fibrillating agent to inhibit the aggregation process, but their effectiveness is less than DOPA. The effectiveness in the suppression of thermal aggregation of β-lg follows the order DOPA > PEA > GABA. Results of ANS fluorescence studies confirmed that DOPA is opposing the thermal exposure of hydrophobic patches and thus stabilizes the heat-treated β-lg structure. CD spectra confirm the transformation of β-sheet towards native-like structure in the presence of DOPA. Hydrogen bond formation and the hydrophobic interactions are the key forces involved in the stabilization of heat-exposed β-lg by DOPA. DOPA forms hydrogen bonds through its two –OH groups at C2 and C3 positions (as shown by molecular docking). ANS study for hydrophobic measurements shows PEA is

less efficient to stabilize the thermally unfolded state of β-lg probably due to its inability to form the non-covalent bonds as it lacks two –OH groups in its structure. On the other hand, due to absence of both –OH groups and phenyl nucleus, GABA fails to stabilize the heat exposed β-lg structure leading to its aggregation. Based on our present findings we come to the conclusion that DOPA and PEA may have potential use for pharmaceutical application against beta lactoglobulin related amyloidosis and amyloid-related diseases.

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Poster Presented in academic Seminar

1. "Coumarin Derivatives inhibit the aggregation of bovine β-lactoglobulin" – National Seminar on Emerging Trends in Chemical Science under Centre for Advance Studies Department of chemistry, Jadavpur University, Kolkata – 700032 – January 07, 2020.

2. "Dopamine inhibits the fibrillation of bovine β-lactoglobulin" – National Seminar on CHEMICAL SCIENCES: TODAY AND TOMORROW (CSTT-2019) under Centre for Advance Studies Department of chemistry, Jadavpur University, Kolkata – 700032 – March 14, 2019.

3. "Methionine Oxidation reduces thermal stability of bovine beta-lactoglobulin and modulates the route of amyloid fibril formation" – International Conference on Chemistry for Human Development (ICCHD – 2018) – January 8-10th, 2018.

Workshop Attended

1. National Workshop on Electron Microscopy and its Applications in Material Science and Biological Science – Organised by DEPARTMENT OF INSTRUMENTATION SCIENCE, JADAVPUR UNIVERSITY, KOLKATA-700032, January 29-30, 2018.

Seminar attended

1. National Seminar on CURRENT DEVELOPMENTS IN CHEMICAL SCIENCES (CDCS-2018) under Centre for Advance Studies Department of chemistry, Jadavpur University, Kolkata – 700032 – March 7, 2018.

2. National Seminar on Emerging Trends in Chemistry (ETC-2017) under Centre for Advance Studies Department of chemistry, Jadavpur University, Kolkata – 700032 – February 15, 2017.

National Seminar on

Emerging Trends in Chemical Sciences
(January 07, 2020)

under

Centre for Advanced Studies II Program

Organized by

Department of Chemistry, Jadavpur University, Kolkata 700 032

Certificate of Participation

This is to certify that Hasan Parvej

of Jadavpur University *has ~~taken part~~/presented a poster in the seminar organized by the Department of Chemistry, Jadavpur University, Kolkata 700 032 on January 07, 2020.*

Date: 07-01-2020
Place: Kolkata

KAJAL KRISHNA RAJAK
Convener

SAMIT GUHA
Co-Convener

National Seminar on

CHEMICAL SCIENCES: TODAY AND TOMORROW (CSTT-2019)

(Thursday, March 14, 2019)

under

Centre for Advanced Studies II Program

Organized by

Department of Chemistry, Jadavpur University, Kolkata 700 032

This is to certify that HASAN PARVEJ *of* Dept. of Chemistry

JADAVPUR UNIVERSITY *has delivered an invited talk /participated/ presented a poster in the seminar organized by the Department of Chemistry, Jadavpur University, Kolkata 700 032 on Thursday, March 14, 2019.*

Date: 14-03-2019
Place: Kolkata

Partha Roy
Co-Convener

S Bhattacharya.
Swapan Kumar Bhattacharya
Convener

International Conference on Chemistry for Human Development (ICCHD-2018)

January 8–10th, 2018

Certificate

This is to Certify that Prof/Dr./Mr./Mrs. Hasan Parvej

of Jadavpur University

has participated and presented a Paper (Oral/Poster)~~Delivered Talk~~ in the International Conference on Chemistry for Human Development (ICCHD-2018) held at Heritage Institute of Technology, Kolkata

Jt. Convenor

Convenor

National Workshop on
Electron Microscopy and Its Applications in Material Science and Biological Science

January 29 - 30, 2018

Organized by
DEPARTMENT OF INSTRUMENTATION SCIENCE
JADAVPUR UNIVERSITY, KOLKATA – 700032

Certified that *Hasan Parvej of Jadavpur University* has actively participated and successfully completed the National Workshop on 'Electron **Microscopy and Its Applications in Material Science and Biological Science'** held during January 29- 30, 2018, in the Department of Instrumentation Science, Jadavpur University.

(Dr P.P.Lahiri)	(Dr Sankar Narayan Patra)	(Prof. R. Bhar)
Registrar	*Head*	*Convener*
Jadavpur University	*Dept. of Instrumentation Science, JU*	*Dept. of Instrumentation Science, JU*

Date: 30.01.2018

National Seminar on

CURRENT DEVELOPMENTS IN CHEMICAL SCIENCES (CDCS-2018)

(Wednesday, March 7, 2018)

under

Centre for Advanced Studies II Program

Organized by

Department of Chemistry, Jadavpur University, Kolkata 700 032

This is to certify that Hasan Parvej *of* Jadavpur University *has participated/presented a poster in the seminar organized by the Department of Chemistry, Jadavpur University, Kolkata 700 032 on Wednesday, March 7, 2018.*

Date: 07-03-2018
Place: Kolkata

SAUBHIK HALDAR **PARTHA MAHATA**

Conveners

National Seminar on

EMERGING TRENDS IN CHEMISTRY (ETC-2017)

(Wednesday, February 15, 2017)

under

Centre for Advanced Studies II Program

Organized by

Department of Chemistry, Jadavpur University, Kolkata 700 032

This is to certify that HASAN PARVEJ *of*

.....*has participated/~~presented a poster~~ in the seminar organized by the Department of Chemistry, Jadavpur University, Kolkata 700 032 on Wednesday, February 15, 2017.*

Date: 15-02-2017
Place: Kolkata

KAUSIKISANKAR PRAMANIK
Convener

SUMAN DAS
Co-Conveners

A. Saha
AMRITA SAHA

Lists of Publications

List of Publications

1. "Coumarin derivatives inhibit the aggregation of beta-lactoglobulin"- **H. Parvej**, S. Begum, R. Dalui, S. Paul, B. Mandal, S. Sardar, N. Sepay, G. Maiti, U. C. Halder, RSC Adv., 2022, 12, 17020-17028.

2. "New insight into the alcohol induced conformational change and aggregation of the alkaline unfolded state of bovine b-lactoglobulin" S. Maity, S. Sardar, S. Pal, **H. Parvej**, J. Chakraborty, U. C. Halder, RSC Adv., 2016, 6, 74409 – 74417.

3. "Facile synthesis and characterization of beta lactoglobulin-copper nanocomposites having antibacterial applications"- S. Sardar, S. Maity, S. Pal, **H. Parvej**, U.C. Halder, RSC Adv., 2016, 6, 85340.

4. "Curcumin inhibits the Al(III) and Zn(II) induced amyloid fibrillation of β-lactoglobulin in vitro"- S. Pal, S. Maity, S. Sardar, **H. Parvej**, N. Das, J. Chakrabort, U. C. Halder, RSC Adv., 2016, 6, 111299.

5. "Multispectroscopic Analysis and Molecular Modeling to Investigate the Binding of Beta Lactoglobulin with Curcumin Derivatives"- S. Maity, S. Pal, S. Sardar, N. Sepay, **H. Parvej,** J. Chakraborty, U. C. Halder, RSC Adv., 2016, 6, 112175.

6. "Inhibition of amyloid fibril formation of b-lactoglobulin by natural and synthetic curcuminoids"-S. Maity, S. Pal, S. Sardar, N. Sepay, **H. Parvej,** S. Begum, R. Dalui, N. Das, A. Pradhan New J.Chem., 2018, 42, 19260-19271.

7. "Silver nanoparticle modulates the aggregation of beta-lactoglobulin and induces to form rod-like aggregates"- S. Sardar, M. Anas, S. Maity, S. Pal, **H. Parvej,** S. Begum, R. Dalui, N. Sepay, U. C. Halder-International Journal of Biological Macromolecules 125 (2019) 596–604.

8. "Antioxidant ferulic acid prevents the aggregation of bovine b-lactoglobulin in vitro"-S. Pal, S. Maity, S. Sardar, S. Begum, R. Dalui, **H. Parvej,** K. Bera, A. Pradhan, N. Sepay, S. Paul, U. C. HALDER- J. Chem. Sci. (2020) 132:103.

9. Modulation of amyloid fibrillation of bovine β-lactoglobulin by selective methionine oxidation –S. Maity, N. Sepay, S. Pal, S. Sardar, **H. Parvej,** S. Pal, J. Chakraborty, A. Pradhan, U. C. Halder RSC Adv., 2021, 11, 11192- 11203.

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Coumarin derivatives inhibit the aggregation of β-lactoglobulin †

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The binding of a small molecule to a protein through non-covalent interactions mainly depends on its size and electronic environment. Such binding can change the stability of the three dimensional protein structure which sometimes may destabilize it to accelerate or to inhibit protein aggregation. Coumarin is a widely used fluorescent dye with several biological applications. Different substituents (electron-donating and electron-withdrawing) at different positions of the coumarin moiety can influence its molecular volume, physical and chemical properties. Here we investigate the effect of such substituents of coumarin on the aggregation of a model protein, beta-lactoglobulin (β-lg) through a multi spectroscopic approach. It was observed that coumarin methyl ester with an 8-hydroxyl group can inhibit the β-lg aggregation. This compound can bind the hydrophobic site of beta-lactoglobulin and stabilize a particular protein conformation through the formation of hydrogen bond and hydrophobic interactions. Thus a properly designed compound can inhibit protein–protein interactions through protein–small molecule interactions. Other coumarinoid compounds also are effective in the prevention of thermal aggregation of β-lg.

Introduction

The formation of insoluble amyloid fibrils from normal soluble proteins is well known. Amyloid fibrils are impervious to degradation.[1] Proteins can form amyloid fibrils and each case brings a different characteristic disease. Aggregation of Aβ, α-synuclein, or prion proteins causes neurodegenerative brain disorders Alzheimer's disease, Parkinson's disease, or mad cow disease, respectively. It is also found that diseases like diabetes type 2 are disorders due to amyloid fibril formation of insulin.[1] Since amyloid fibrils are insoluble in physiological conditions, they can deposit around the cell and tissues and cause a pathogenic effect.[2] Aggregation of such proteins gives very stable systems and facilitates the process. Structurally, fibrillar assemblies are predominantly cross-β conformation of the proteins. Interestingly, the formation of fibrils is independent of protein size, shape, and sources. Recently, hydrophobins, curli, and melanosomes are identified as the expression of functional amyloidogenesis.[1]

To prevent the amyloid fibrils formation, a number of therapeutic strategies have been developed. Site-specific glycosylation of protein[3] and protein engineering[4] are of some example of prevention techniques. Most of the proteins have a very stable conformational structure in fibrillar form because it is in a global free-energy minimum.[5–7] Therefore, once a protein starts partial degradation to form a fibril, it is very hard to stop the process. Stabilization of the native conformer of a protein can slow down the partial degradation of protein and can deem fibrillation.[8] It is the most acceptable therapeutic strategy to prevent fibrils formation now. Therefore, the design of new anti-fibrillating molecules is in demand.

Coumarin, an aromatic heterocyclic lactone compound, is an important natural product found in many plants. Coumarin derivatives or coumarinoids have a large family containing thousands of compounds. They have very interesting photophysical,[9] biological, and medicinal[10] properties. Coumarin absorbs light at ~280 nm wavelength and shows fluorescence property by the emission at 410 to 470 nm. This photophysical property is very much tunable with the substituents attached to the moiety.[11] For this reason, these compounds are widely used as dyes. Some coumarinoids were designed for blue-green tunable organic laser dyes.[11]

Whey contains an important lipocalin protein, *i.e.* β-lactoglobulin (β-lg), which is of immense interest due to its nutritional and small molecule carrier properties.[12] Structurally, the protein is β-sheet enriched (eight anti-parallel β-sheets forms a barrel-like structure) and forms self-assembly upon thermal exposure. The protein can be isolated and purified in high yield

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following the method described by Aschaffenburg and Drewry, 1957.[13] Hence it is a very good model protein for the protein aggregation studies.[14]

A 4-hydroxycoumarins derivative Warfarin can interact with proteins to inhibit their aggregation and it is used as an anti-coagulant of blood.[15] 4-Hydroxycoumarins are also anticoagulants and antagonists of vitamin K.[16] It was observed that the substituents of coumarin have a substantial effect on the aggregation process of the amyloid-β protein.[17] Some coumarinoids show inhibition of insulin fibrillation.[18] Some coumarin hybrid molecules are also be designed as a multi-targeting agent to stop the fibrillation process in neurodegenerative disorders.[19] All these facts encourage us to investigate some polyphenolic coumarinoids as anti-fibrillating agents. Here, we have synthesized and characterized some polyphenoliccoumarinoids and applied them to check their anti-fibrillation power on the model protein β-lg.

Results and discussion

Synthesis of coumarinoids

In this study, all the coumarin derivatives was synthesized except 4-hydroxycoumarins (**3**) which were collected from commercial source and used without further purification. Kayal *et al.*, showed that polyhydroxy benzenes can be reacted with electron-deficient alkynes to produce polyhydroxycoumarinoids (**SM1–SM4**) with the help of 5 mol% CuO in refluxing toluene.[20] Here, this method was used for the synthesis of different polyhydroxycoumarinoids and it has been shown in the Fig. 1. All these compounds were well characterized and used in the protein aggregation experiments.

It is interesting to note the distribution of hydroxyl groups around the coumarin nucleus in the compounds under investigation. In compound **3**, the hydroxyl group at C4 position. In the case of **SM1**, one methyl carboxylate is attached at C4 position and OH is shifted to the C5 position. The OH group is at C8 and C7 carbon in **SM2** and **SM4**, respectively. **SM3** contains two hydroxyl groups at C5 and C7 positions. Here, we investigate the effect of hydroxyl groups along with methyl carboxylate group on the protein aggregation.

UV-spectral characterization

UV spectroscopy is employed to get an intuition of structural change of β-lg due to interaction with small molecules.[21] Here we used absorption spectroscopy to investigate the structural change of β-lg in the presence of coumarin derivatives. Due to the presence of Trp and Tyr moiety a sharp characteristics peak is observed at λ_{max} 280 nm for β-lg. Coumarin derivatives have also light absorption properties and have λ_{max} around 270 nm (for **3**, **SM1**, **SM2**, and **SM4**) and 350 nm (for **SM3**) which is presented in Fig. S1a.†

In the presence of methyl 4-hydroxy-chromen-2-one (**3**) and methyl 8-hydroxy- 2-oxo-2*H*-chromene-4-carboxylate (**SM2**), the absorption intensity become lower than that of native β-lg and higher than that of heat-treated β-lg UV spectra (Fig. 2). However, β-lg with methyl 5-hydroxy-2-oxo-2*H*-chromene-4-carboxylate (**SM1**), methyl 5,7-di-hydroxy-2-oxo-2*H*-chromene-4-carboxylate (**SM3**), and methyl 7-methoxy-2-oxo-2*H*-chromene-4-carboxylate (**SM4**) shows a clear bathochromic shifts with low-intensity peaks compared to native. The contribution of the coumarin molecules in the aforesaid absorption spectra can be eliminated by their addition in the reference cell along with sample cell (both the cell have the same concentration of coumarin derivatives). In this experiment, we found only single peak at 280 nm for all coumarin derivatives (Fig. S1b†). The intensities of the spectra of the sample containing protein with coumarin derivatives are higher than that of native and heat-treated protein. All these observations indicate the probability of interactions of the small molecule with the protein β-lg.

Intrinsic fluorescence

The fluorescence technique, a highly effective and sensitive method, is widely used to monitor the structural change of

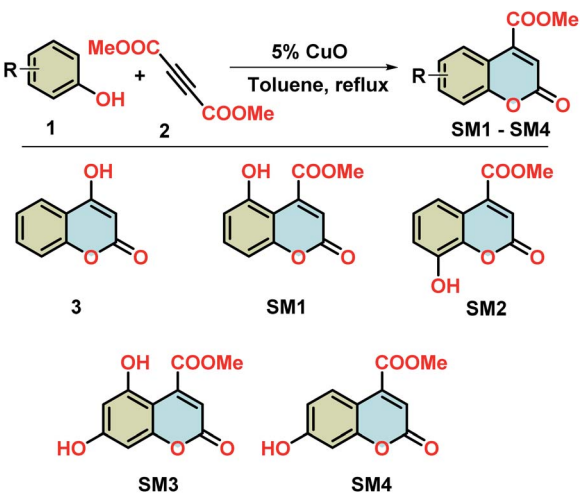

Fig. 1 Synthesis of coumarin derivatives.

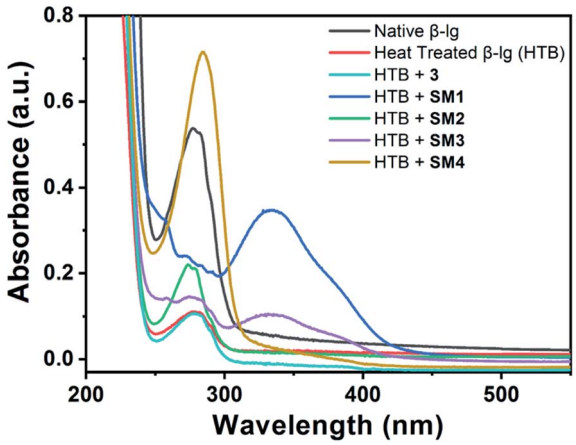

Fig. 2 Absorption spectra of native β-lg and β-lg incubated at 78 °C for 1 h in the absence and presence of different coumarinoid molecules.

protein and its interaction with the small molecules. Generally, Trp and Tyr are the amino acid residues that absorb the light of corresponding wavelengths and show the fluorescence properties for most of the proteins. β-lg has four Tyr (Tyr20, Tyr42, Tyr102, and Tyr99) and two Trp (Trp19 and Trp61) residues which are responsible for the intrinsic fluorescence of the protein.[22] It can be used to investigate the alteration of the polarity around the microenvironment of the fluorophore due to the structural change of beta-lactoglobulin. In the case of aggregation of β-lg, solvent exposure of fluorophores (mainly tryptophan) decreases which is reflected in their emission spectra with the change in fluorescence intensity. Here, aggregation of β-lg was investigated in the presence of coumarin derivatives.

The intrinsic fluorescence of β-lg increases after heating at 78 °C for 1 h due to the aggregation of the native protein (Fig. 3). In the case of **SM2** and **3**, the fluorescence intensities are slightly higher and lower with respect to heat-treated β-lg, respectively. Under the same aggregation conditions, **SM1** and **SM3** show higher fluorescence intensities than native β-lg but lower than heat-treated β-lg. However, in the presence of **SM4**, the intensity is lowered compared to native β-lg.

Thus coumarin derivatives interact with β-lg and are able to change the conformation of the protein during its thermal exposure. It should be mentioned here that compound **SM4** can interact with β-lg in such a way that Trp residue may expose more in the solvent. It may be the reason for the decrease in fluorescence intensity of β-lg with **SM4** under aforesaid conditions.

Aggregation of β-lg identification and quantification of the aggregates by ThT assay

Thioflavin T (Th-T), a benzothiazole dye, is a good fluorescent ligand that selectively binds with the hydrophobic site of the fibrillar aggregates of the protein leading to an increase in fluorescence intensity at around 480 nm upon excitation at 480 nm.[23] Upon heating at 78 °C, the native β-lg gets aggregated which can be measured in terms of intensity of the Th-T spectra. Here, all the other heat-treated β-lg samples with different coumarin derivatives produced different Th-T emission intensities which are proportional to the amount of aggregates formed in the different protein solutions. Therefore, lower intensity of the Th-T will indicate a lower degree of aggregate formation due to the presence of effective anti-fibrillating coumarin derivatives. Determination of equivalent molar ratio of the compounds against protein through UV and fluorescence spectra is very difficult as both of them (protein and **SMs**) have absorbance and emission peaks at the same region. In such case, we have monitored the change of fluorescence intensity of Th-T in the presence and absence of coumarin derivatives with different molar ratio at a fixed [BLG] which is given in Fig. S2.† The study showed that 1 : 1 protein and SM compound ratio is most effective for protein aggregation inhibition. In the Fig. 4, it shows a comparative study of the aggregation patterns of native and heat-treated β-lg in the absence and presence of different coumarin derivatives (1 : 1) at pH 7.4 in Th-T assay.

Owing to self-assembly formation, the heat-treated β-lg shows the highest Th-T intensity as shown in the Fig. 4. The β-lg in the presence of **3** under identical heating conditions exhibits slightly lesser intensity in Th-T fluorescence measurement than heat-treated β-lg alone. However, when β-lg was incubated at 78 °C separately with other coumarin compounds **SM1**, **SM2**, **SM3**, and **SM4** very much decreased intensities of Th-T fluorescence were observed due to the formation lower β-lg aggregates. Therefore, synthesized coumarin derivatives can act as effective anti-fibrillating agents to inhibit the oligomerization of β-lg. In this case, the inhibition of aggregation of coumarin derivatives follows the order as **SM2** > **SM4** > **SM3** > **SM1** > **3**.

ANS-fluorescence study to monitor the hydrophobicity changes

1-Anilinonaphthalene-8-sulfonate (ANS), a fluorescent probe, can bind at the surface of protein aggregates through the

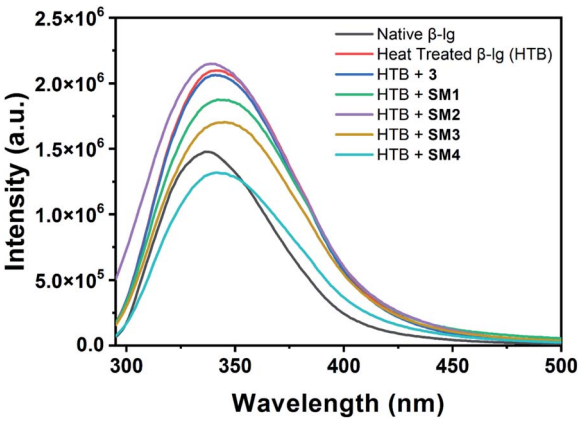

Fig. 3 Intrinsic fluorescence spectra of native β-lg and β-lg incubated at 78 °C for 1 h in the absence and presence of different coumarin derivatives (protein: coumarin derivatives = 1 : 1).

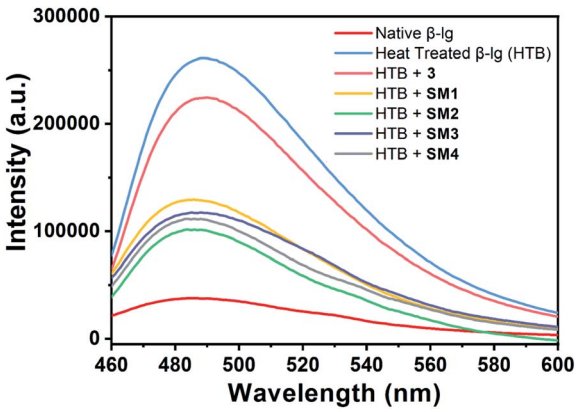

Fig. 4 Th-T (λ_{ex} = 440 nm and λ_{em} = 485 nm) assay of native β-lg and heat-treated β-lg (incubated at 78 °C for 1 h) in the absence and presence of different coumarin compounds (1 : 1) at pH = 7.4, excitation wavelength was set at 440 nm and emission wavelength was 480 nm.

hydrophobic and electrostatic interaction at the hydrophobic site.[24] To monitor hydrophobic interactions involved in the aggregation processes, ANS fluorescence experiment was performed.

The protein, β-lg, has two potential binding sites for binding with hydrophobic small molecules. The first site is the inner side of the beta-barrel and the second site is at the channel between the barrel and the alpha helix. The dye shows fluorescence emission around 480 nm with an increase of fluorescence intensity after binding with the hydrophobic site of the protein.[25]

The heat exposed β-lg (78 °C, 1 h) showed an enhanced ANS fluorescence intensity at around 480 nm. This increase in fluorescence intensity may be attributed to more access of ANS to the hydrophobic patches present in heat treated β-lg compared to native β-lg during its aggregation (Fig. 5). The incubation of the coumarin derivatives with β-lg slows down the aggregation process since the ANS intensity in each case is lower than that of the heat-treated sample. Additionally, it may be mentioned here that these aggregates have lower surface hydrophobicity. Therefore, the hydrophobic pockets of β-lg are opened up during thermal exposure implying a conformational change of native protein.

We obtained significant result when β-lg was incubated with **SM2** at 78 °C. The ANS fluorescence intensity was found to decrease considerably and it is very closer to that of native β-lg, indicating least disclosure of hydrophobic loops of β-lg demonstrating least binding of ANS, and thus **SM2** very efficiently decreased protein–protein interactions and thus inhibiting the thermal aggregation of β-lg.

For incubation of β-lg with **3**, **SM1** and **SM3**, such spectral changes are though very close to each other but ANS fluorescence intensities have been reduced to almost 55% compared to that of heat-treated β-lg alone. This suggests lesser opening of hydrophobic loops resulting decreased protein–protein interactions in the presence of **3**, **SM1** and **SM3** although greater ANS bindings and hence increased hydrophobicities compared to **SM2** were observed. Therefore, it may be concluded that the β-lg fibrillation proceeds through the exposer of hydrophobic sites to the solvent and the binding of coumarin derivatives to the hydrophobic site of the protein can stabilize it in such a way that the protein cannot proceed to the fibrillation mechanism. Therefore, lower ANS intensity indicates a higher ability of stabilization of protein conformation by coumarin derivatives. It is found that the order of stabilization is **SM2** > **SM4** > **SM3** > **SM1** > **3**. This result supports the Th-T data given above.

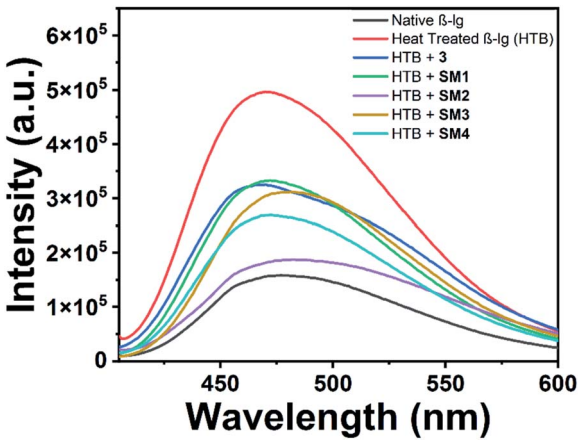

Fig. 5 ANS-fluorescence emission spectra of native β-lg and β-lg incubated separately at 78 °C for 1 h in the absence and presence of different coumarin derivatives (1 : 1) at pH = 7.4. β-lg concentrations throughout all emission experiments were kept at 0.25 mg mL^{-1}. Results were the mean of three different experiments.

Rayleigh light scattering (RLS) study

Protein aggregation can also be investigated with the help of RLS measurements. The scattering of light is increased in the presence of colloidal particles in the medium. Therefore, higher protein aggregates can scatter light efficiently over the smaller aggregates. In this study, RLS data were collected after incubation of β-lg solutions at 78 °C for 1 h in absence and presence of coumarin derivatives. Our result as shown in Fig. 6 indicates that maximum scattering intensity was observed with heat-treated β-lg in absence any coumarin derivative. On thermal denaturation, the structure of a protein is lost and it forms maximum aggregates. Decreased RLS intensities were noted in the presence of different coumarin derivatives indicating the formation of smaller β-lg aggregates. Thus it is worth to point out that coumarin derivatives maintain the structural integrity of the protein during the thermal denaturation. It was found that **SM2** is superior to other coumarin derivatives in the inhibition of protein–protein interactions during thermal exposure as the minimum RLS intensity value (closer to that of native β-lg) was obtained (Fig. 6). Lowering in RLS intensities confirms the smaller aggregate formation during thermal incubation. In our present study the order of inhibition of thermal aggregation of β-lg by the coumarin derivative is the same as that obtained with other experiments and it follows **SM2** > **SM4** > **SM3** > **SM1** > **3**.

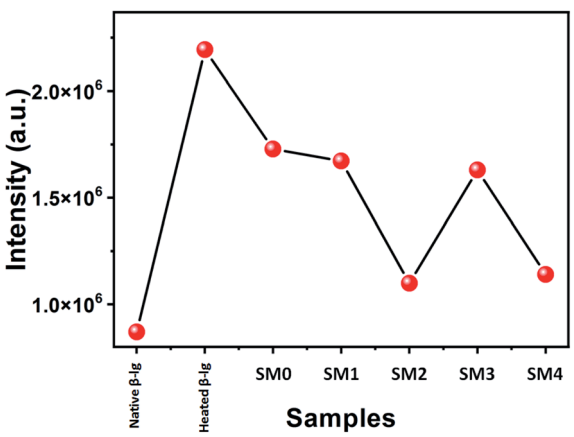

Fig. 6 Rayleigh light scattering data of β-lg at native form and β-lg incubated at 78 °C for 1 h in the absence and presence of different coumarin derivatives (1 : 1) at pH = 7.4.

Dynamic light scattering (DLS) measurements

Dynamic light scattering (DLS) was unambiguously used to identify the heat-induced oligomers of β-lg and also to measure their hydrodynamic size. The size distribution profile of the native β-lg, its heat-treated form without and with the different coumarin derivatives were investigated and have been shown in Fig. 7. The size distribution curves of heated (78 °C, 1 h) β-lg with and without coumarin compounds showed the formation of different sized protein aggregates. The hydrodynamic radius of native β-lg was ranging from 12 nm to 50 nm and its size was enhanced to 1250–3200 nm range when the protein was incubated at 78 °C for 1 h, clearly indicating the formation of large β-lg aggregates having greater light scattering effect. In presence of different coumarin derivatives the scattering intensity of β-lg solution decreased. The sizes of protein aggregates were decreased in the presence of **3** and it was in the region 1500–2750 nm. Minimum hydrodynamic radius was noted during incubation with **SM2**. The size of the aggregates decreased minimally in the range of 100–450 nm and aggregates with smaller diameter became prominent during thermal incubation with the other coumarin molecules. The hydrodynamic radii of protein aggregates in presence of coumarin derivatives have been found to follow the order **3** > **SM1** > **SM3** > **SM4** > **SM2**. Therefore, it is interesting to note that all these coumarin compounds are potent inhibitors of β-lg fibrillation and the order of the ability of inhibition by the coumarin derivatives is **SM2** > **SM4** > **SM3** > **SM1** > **3** (Fig. 7). This result also corroborated with AFM imaging of different β-lg aggregates.

Monitoring the changes in secondary structure of β-lg during thermal incubations with circular dichroism spectroscopy

Far-UV CD technique has been employed to investigate the potential effect of different coumarinoid compounds used in this study on the secondary structural transformation of β-lg. The β-lg solutions were incubated with or without different coumarin derivatives at 78 °C for 1 h and the different secondary structures were determined by CD spectroscopy. CD measurements were carried out by scanning the spectra in the region 190–250 nm and represented in Fig. 8. Native β-lg showed two negative bands at 208 nm and 215 nm. These two bands of β-lg represented the existence of ordered secondary structure that contained α-helix and β-sheet.[26] The oligomeric structure of incubated β-lg at 78 °C is found to have greater amount of beta structural contents (~61%) with lesser alpha helical structure (~9.0%) than the native (Table S1, see ESI†). This data provided the information regarding the formation of greater β-sheet structures linked with the thermal aggregation of the protein. The CD spectrum of β-lg also showed a significant shift in the band positions. Then we analysed the change of secondary structure of heat-treated β-lg in presence of different coumarin compounds. With **3**, a small change of peak position with negative ellipticity value differing from heated β-lg was observed. CD spectra of β-lg in presence of **SM1** showed lower MRE values at 215 and 207 nm. In both the cases lesser β-structures has been formed compared heat-treated β-lg alone indicating structural transitions leading to the disaggregation in the presence of **3** and **SM1**. It is interesting to mention that the shape of CD signal of β-lg in presence of the coumarin derivative **SM2** is similar to that of the native though decreased negative ellipticities are observed in the vicinity of 208 nm and 215 nm. The difference in MRE values could be related to the structure of the derivatives. The amount of different β-structures decreased most significantly compared to heat-stressed β-lg alone (Table S1†) showing maximum inhibitory power **SM2** against the thermal aggregation of β-lg. Other two coumarin derivatives **SM3** and **SM4** displayed almost similar CD spectra in this region but CD results revealed than **SM4** is more potent than **SM3** in the suppression of fibrillation of β-lg. Hence all the coumarin compounds used in this study showed their effectiveness in the suppression of thermal aggregation of β-lg and the order of their inhibition is **SM2** > **SM4** > **SM3** > **SM1** > **3**. The calculation of secondary structural changes of β-lg in the

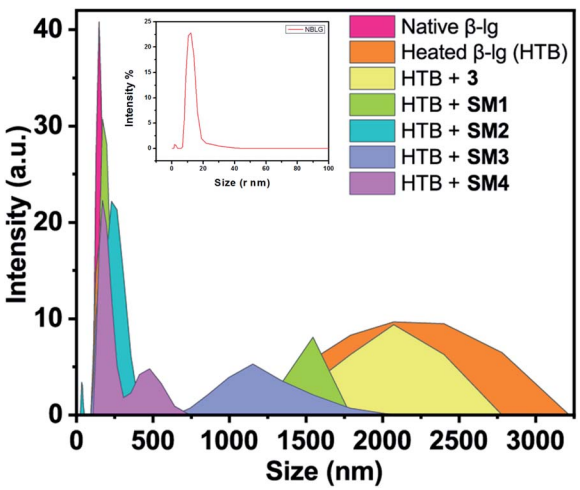

Fig. 7 Number particle size distribution spectra in DLS studies of β-lg at native form and β-lg incubated at 75 °C for 1 h in the absence and presence of different coumarin derivatives (1 : 1) at pH = 7.4.

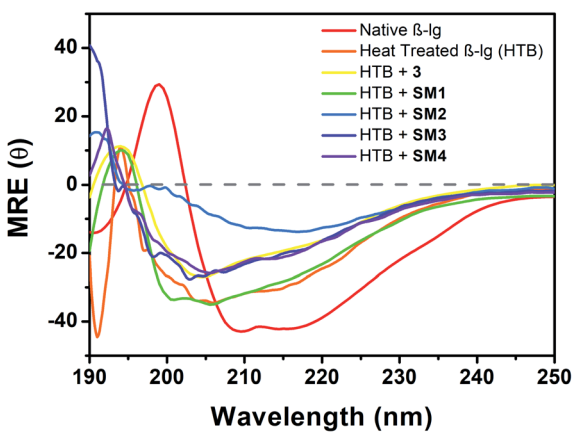

Fig. 8 Far-UV CD spectra (190–250 nm) of native β-lg, heat treated β-lg (78 °C, 1 h), and β-lg incubated (78 °C, 1 h) in the presence of different coumarin derivatives (1 : 1) at pH = 7.4 showing secondary structural changes during thermal aggregation.

absence and presence of different coumarin compound was done by CDNN 2.1 software and shown in Table S1.†

Morphological studies with atomic force microscope (AFM)

To gain more insight, atomic force microscopy was also employed; it has been proved to be a powerful tool in the study of fibril formation.[27] AFM analysis was performed to visualize the extent of disruption of β-lg samples aggregated alone or with coumarin derivatives at molar concentration ratios of 1 : 1.

The morphology of the β-lg aggregates in different conditions is shown in Fig. 9. It is revealed that the three-dimensional AFM images of the aggregates are globular, not fibrillary. The AFM image of β-lg shows that it is smaller globular particles (Fig. S3†). Large globular aggregates of β-lg formed after incubation of the β-lg solution alone at 78 °C for 1 h (Fig. 9a). During thermal incubation the β-lg monomers are swelled owing to change of its conformation, forming the aggregates.

To investigate the effect of different coumarin derivatives on the aggregation mechanism, the β-lg sample was co-incubated at 78 °C for 1 h in the presence of coumarin derivatives at a molar concentration ratio 1 : 1. The compound **3** was found to be inefficient in the inhibition of protein aggregation because the nature and size of aggregates are very similar to the β-lg aggregates obtained after heat treatment (Fig. 9b). A similar type of results were obtained in the case of **SM1**, large globular type aggregate (Fig. 9c). But the AFM image analysis clearly demonstrates that the larger and numerous globular aggregates of β-lg were disappeared when it was thermally incubated with the coumarin compound **SM2** (Fig. 9d). Here, the density of the aggregates was very low. Thus it was found to be most efficient to inhibit and arrest the aggregation of β-lg. However, **SM3** is less efficient than that of both **SM2** and **SM4** but better than other compounds (Fig. 9e). The size of the aggregate particles formed with **SM3** is sufficiently smaller than with heat-treated β-lg (Fig. 9a) or with heat-treated β-lg in presence of **3** (Fig. 9b). Thus **SM3** is also efficient to suppress the formation larger globular aggregates of β-lg. The AFM studies also support the order of inhibition efficiency of coumarin derivatives obtained in all the previous experiments and the order is **SM2** > **SM4** > **SM3** > **SM1** > **3**.

Docking studies

The small molecules can interact with the protein and can stabilise the native protein conformation and it may be attributed to the anti-protein aggregation effect of these small molecules. To understand the effect of coumarin derivatives on the native structure of β-lg, docking studies were performed. It is found that the binding energies (ΔG°) of **3**, **SM1**, **SM2**, **SM3** and **SM4** are −5.6, −5.8, −7.6, −6.3, and −6.9 kcal mol^{-1} respectively. The result indicates that the order of stabilization by these molecules is **SM2** > **SM4** > **SM3** > **SM1** > **3**. This order of stabilization of β-lg structure with these molecules is corroborated with their protein-aggregation inhibition order. Therefore, the coumarin molecules can stabilize the β-lg conformation and locked a protein structure to inhibit the protein–protein interactions, playing the key role in the protein aggregation pathway.

Analysis of the docking results can provide an insight of the molecular interactions required for the freezing a confirmation of protein. The **SM2** binds at the narrow end of the barrel of the β-lg (Fig. 10a). The cavity in this region encapsulates the molecule (Fig. 10b). It was observed that the methoxy oxygen

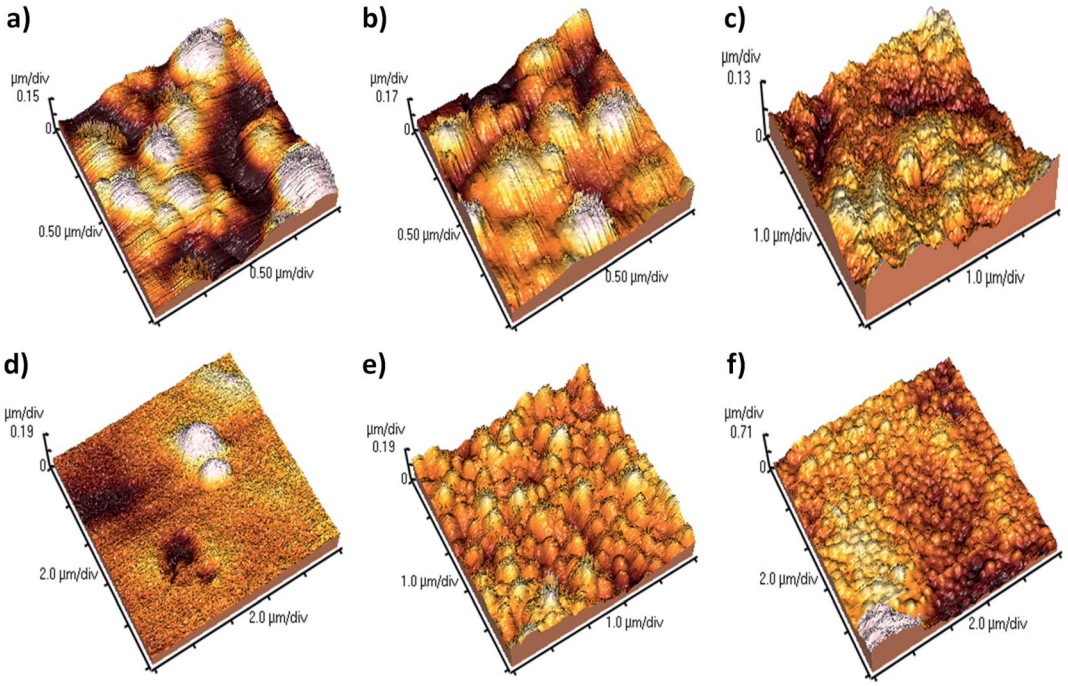

Fig. 9 AFM images representing (a) the aggregate morphologies of heat treated β-lg and (b–f) heat treated β-lg in the presence of coumarin derivatives: (b) with **3**, (c) with **SM1**, (d) with **SM2**, (e) with **SM3**, (f) with **SM4**.

atoms and the hydroxyl group are involved in the hydrogen bonding with N88, N98 and S116 amino acid residues. The non-conventional hydrogen bonding was also observed between ring C=O of coumarin and S116. The methyl of methoxy group found to be interacted with L39 through hydrophobic interactions (Fig. 10c). Docking studies reveals that the positions of OH groups play an important role in their protein binding. The OH groups at C4 and C7 are unable to involve hydrogen bonding with amino acid residues. However, hydroxyl groups at C4, and C5 can interact with the amino acid residues in their corresponding active site (Fig. S4 and S5†). Therefore, hydrogen bonding stabilization of protein–SM complexes can influence the protein aggregation.

The protein can exist as dimer in native state. In this quaternary form of the protein have one additional binding pocket (Fig. S6 and S7a†). It is possible that coumarin derivative able to bind this site to show aforesaid effect. The molecular docking was performed to find such binding possibility. In this case, energy minimized dimeric protein is used for docking. It is found that the binding energies (ΔG°) of **3**, **SM1**, **SM2**, **SM3** and **SM4** are −3.2, −3.9, −5.2, −4.3, and −4.7 kcal mol^{-1}, respectively. These binding energies are quite lower than that of the values obtained from monomeric form of the protein. All the docking poses are shown in the Fig. S7(b)–(f).†

Experimental

Synthesis of coumarin derivatives[20]

To a round bottomed flask a mixture of resorcinol (110 mg, 1.0 mmol) and dimethyl acetylenedicarboxylate (170 mg, 1.2 mmol) were taken in 1.3 mL dry toluene and stirred magnetically in a 10 mL round bottom flask. Then 4 mg CuO (0.05 mmol) catalyst was added to the mixture and allowed the stirring for 3 h at 110 °C. The degree of conversion into the products was noticed by thin layer chromatography. The solvent of the reaction mixture was removed under low pressure, and the pure product was obtained after column chromatography with the help of silica gel (100–200 mesh). The pure compounds were characterized using ^{1}H NMR and ^{13}C NMR spectroscopy. All the data are given in the ESI.†

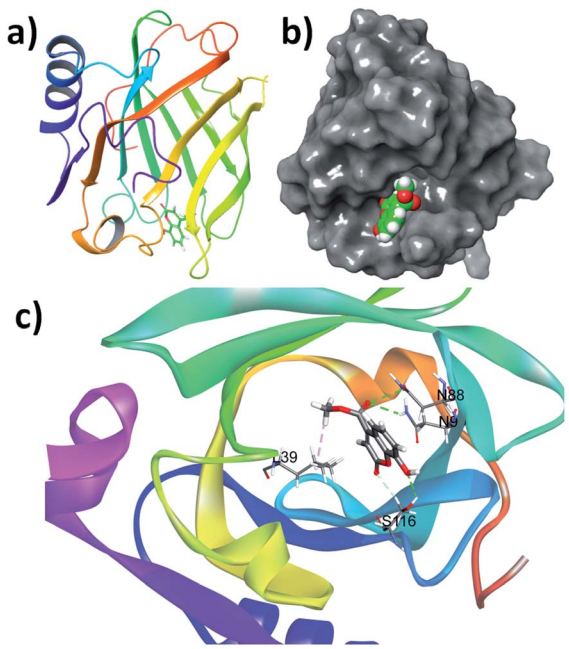

Fig. 10 (a) The structures of the most stable β-lg–coumarin **SM2**, bind at the terminal of the barrel, (b) binding of **SM2** at the hydrophobic pocket, (c) different non-covalent interactions in the binding site of β-lg with **SM2**.

Preparation and purification of beta-lactoglobulin[13]

Bovine β-lg was isolated and purified from cow milk as described by Aschaffenburg and Drewry.[13] The final product was lyophilized and stored at 4 °C. For spectroscopic sample preparation, β-lg was weighed and dissolved in 0.01 M Na–phosphate buffer pH 7.4 solution containing 2% ethanol. Protein stock solutions were prepared using phosphate buffer pH-7.4. Since the extinction coefficient of β-lg (0.96 mg^{-1} mL^{-1} cm^{-1} at 280 nm) is known, different concentrations of protein samples were prepared by dissolving β-lg samples in Milli-Q-water and then measuring the O.D. at 280 nm.

UV-visible spectroscopy

Utilizing UV-visible JASCO V700 spectrophotometer (Model no. V-730, Serial no. B184461798 and JASCO Spectra Manager software), absorption spectra were recorded to check the binding affinity and binding constant at room temperature (25 °C). To perform this experiment two PerkinElmer quartz cell of path length of 1 cm were used for both reference and samples. The intensity *vs.* wavelength spectra for the absorbance measurement was recorded over the wavelength range 200–600 nm. For reference cell 10 mm phosphate buffer of pH 7.4 were taken. Concentration of the sample solutions were 13 μm.

Intrinsic fluorescence study

Fluorescence measurements were carried out using Horriba Fluorometer (Model: FLUOROMAX-4C, Serial no. 1734D-4018-FM). Stock solution of β-lg and others solution of 13 μm were taken in a fluorescence quartz cell of path length of 1 cm and excited at 295 nm. Emission spectra were recorded in the range of 300 to 550 nm. Excitation and emission slit were set at 5 nm. Data were recorded at a scan rate of 100 nm s^{-1}.

Thioflavin T (Th-T) fluorescence

Th-T binds with the aggregates of β-lg and other amyloid fibrils. After binding it shows enhanced fluorescence at 480 nm. For this experiment stock β-lg samples of 54.3 mM were mixed with 3.13 mM Th-T solution. The assay solution was excited at 450 nm (ref. 28) and the emissions were measured over the range 460 to 600 nm using Horriba Fluorometer (Model: FLUOROMAX-4C, Serial no. 1734D-4018-FM) and Fluoromax Software. Slit widths for both excitation and emission were kept at 5 nm.

ANS-fluorescence study to monitor the hydrophobicity change

Hydrophobicity of protein molecules were measured using fluorescent probe which binds with the hydrophobic packets of protein surface.[25] A well-known polarity sensitive prove is 1-anilinonapthalene-8-sulfonate (ANS). To measure the hydrophobicity a stock solution of ANS is prepared and added to the each samples (2 mL volume). The final concentration of ANS in each sample is maintained 30 μM. Fluorescence spectra were measured at 390 to 550 nm after excitation at 380 nm and emission spectra were recorded using Shimadzu spectrofluorometer (Shimadzu 5301 PC). Path length was 1 cm. Excitation slit was 5 nm and emission slit was also 5 nm.

Raleigh light scattering (RLS)

The formation or change in the presence of turbidity or aggregates after the incubation of β-lg in absence and presence of different coumarin samples (**3–SM4**) with respect to the native β-lg were quantitatively measured using Raleigh light scattering study. For this study samples were excited at 350 nm and then emission intensity were recorded at 350 nm using Horriba Fluorometer (Model: FLUOROMAX-4C, Serial no. 1734D-4018-FM) and Fluoromax Software. Samples were prepared using 10 mM phosphate buffer (pH – 7.4). 2 mL 13 μm solution of each samples were taken in a quartz cell of 1 cm path length for this experiment and excitation and emission slit were 5 nm.

Analysis of secondary structures by CD spectroscopy

Using Jasco spectropolarimeter (J-815) (Jasco, Tokyo, Japan) CD spectra of β-lg, heat treated β-lg and heat treated β-lg in presence of different coumarin compounds were recorded with the help of Jasco Spectra Manager Software. For this experiment in the far UV region (190–260 nm), each sample containing 13.6 μM β-lg was taken in a PerkinElmer quartz cell of path length of 0.2 cm and then data was recorded in the inner nitrogen atmosphere of the instrument at 190 nm to 260 nm. Scan speed was 100 nm min^{-1} and temperature was 30 °C. The results were expressed as mean residual ellipticity (MRE) in deg cm^2 $dmol^{-1}$ which is defined as:

$$\mathrm{MRE} = \theta_{\mathrm{obs}}\,(\mathrm{mdeg})/10 \times n \times C_{\mathrm{p}} \times l$$

where θ_{obs} is the CD in millidegree, n is the number of amino acid residues in one subunit (162 for β-lg), l is the path length of the cell in centimeters and C_{p} is the molar fraction of proteins.

Spectra were recorded using Jasco Spectra Analysis tool. And secondary structural elements were calculated from CDNN 2.1 software.

Dynamic light scattering (DLS)

Dynamic light scattering (DLS) study is used to determine the size of proteins, nucleic acids, and complexes or to monitor the binding of ligands. Also, diffusion of tiny particle of nano sized range alters the intensity of the scattered light. DLS studies are also used to determine the presence of different molecules and supramolecules as it is very sensitive to particle size.[29] Using Zetasizer Nanos (Malvern Instrument, U.K.) equipped with 633 nm laser and using 2 mL rectangular helma cuvette of 1 cm path length DLS Measurements were done at 20 °C taking 250 μL of samples (β-lg, heat treated β-lg, and heat treated β-lg in presence of different coumarin compounds) in 1.75 mL 3 mM glycine–KOH buffers of pH 7.4. The time-dependent auto correlation function was acquired with twelve acquisitions for each run.

Morphological study with AFM

For this experiment AFM grid slides is prepared. For grid preparation drop casted solution of different samples (each containing 5 mg mL^{-1} β-lg) in the surface of a glass slide and then sample is spread all over the slide and then dried overnight for the observation. AFM microscope image of β-lg, heat treated β-lg and β-lg incubated at 78 °C in the presence of different coumarin compounds were developed using VEECO DICP II autoprobe (Model AP 0100).

Molecular docking study

The AutoDock 4.2.0 based docking studies of coumarin derivatives molecule with β-lg (1BSY) were carried out. The structure of coumarin derivatives used in docking after minimized its energy by DFT optimization using Gaussian 09W. Lamarckian genetic algorithm (LGA) was utilized for molecular docking. In this calculation, 126 × 126 × 126 grid box was used.

Conclusions

In summary, the work presents application of some coumarin derivatives for the inhibition of β-lg aggregation. Protein aggregation can occur due to protein–protein interaction of a particular conformation of a protein. The synthesized coumarin derivative can stabilize a protein conformer so that it unable to reach the conformation essential for protein–protein interaction. We have tested five coumarin derivatives to prevent the protein aggregation. The results showed that the coumarin derivatives have inhibition ability in the order **SM2** > **SM4** > **SM3** > **SM1** > **3**. In the molecule **SM2**, one hydrogen bond donor (OH) and two hydrogen bond acceptor (ring O and C=O) groups are in a row. This structural feature of the molecule may help to fit the molecule in the barrel of the protein through hydrogen bonds. This makes **SM2** the best inhibitor. We utilised UV-visible, fluorescence, and CD spectroscopy, Raleigh light scattering, dynamic light scattering (DLS) and AFM techniques to determine the order and to understand the mechanism of inhibition of protein aggregation. Therefore, the work showed the protein aggregation through protein–protein interactions can be inhibited through locking of a protein at its native-like conformation using protein–small molecule interactions.

Author contributions

HP performed most of the experiments. SB and RD were involved in the preparation and purification of protein. SP, BM, and SS helped HP in the analysis of the results. GM synthesized

and characterized all the coumarin derivatives. NS and UCH were performed theoretical work and prepared the manuscript with the help of other authors. UCH designed and supervised the work.

Conflicts of interest

The authors declare no competing financial interest.

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New insight into the alcohol induced conformational change and aggregation of the alkaline unfolded state of bovine β-lactoglobulin†

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Accumulation of ordered protein aggregates (or amyloids) is responsible for several neurodegenerative diseases. β-Lactoglobulin (β-lg) an important globular milk protein, self-assembles to form amyloid-like fibrils on heating at low pH. But here we report for first time the self-assembly of β-lg from its alkaline unfolded state. The present work describes the folding and self-assembly of β-lg from a reversible unfolded state at pH 10.5 in the presence of methanol, 2-propanol, *t*-butanol and 2,2,2-trifluoroethanol (TFE). The extent of secondary and tertiary structure formation is in the order methanol < 2-propanol < *t*-butanol < TFE. Exposure of the hydrophobic core of the protein molecules in an apolar environment of TFE seems to promote intermolecular cluster formation. Methanol and TFE induce aggregation through the α-helical structure whereas isopropanol and *t*-butanol favour the formation of the β-structure leading to aggregation at higher concentrations. *In vitro* aggregation generates various nanometer structures such as nanofibrils, nanovesicles and nanotubes depending on the nature and concentration of the alcohols.

1 Introduction

During the last few decades the stabilization of proteins has become one of the big concerns of scientists. Due to the high complexity of protein structures, stabilization of protein molecules plays an important role in controlling the protein aggregation. Protein molecules are stabilized through a balance between the intramolecular interactions of functional groups and their interactions with the solvent environment.[1–4] Protein stability depends on electrostatic interactions, steric interactions, hydrogen bonding and hydrophobic interactions which are disturbed by the addition of osmolytes or co-solvents.[5–8] The instability of the protein structure leads to the formation of pre-molten globule states, molten globules (MGs), partially folded intermediates, and aggregates of native proteins. These misfolded, aggregated amyloid protein states are either a cause or a major evidential symptom for a variety of current neurodegenerative disease states which includes Alzheimer's disease, Huntington's disease, Parkinson's disease and diabetic-related disorders.[9,10] Protein aggregates are used to develop novel biomaterials for a wide range of application in biomedicine and biotechnology, also these protein aggregates are utilised in dairy industry to recover the whey proteins.[11a,b]

Alcohols also play an important role to destabilize the native structure of proteins although some of them are used as protein precipitant during the purification of human serum albumin from human plasma. Alcohols such as methanol, ethanol, or 2,2,2-trifluoroethanol (TFE) when used as co-solvent, denature the tertiary structures of proteins while enhancing their helicity.[12–15] Several alcohols weaken non-local hydrophobic interactions while promoting local polar interactions (*e.g.*, hydrogen bonds), stabilizing the extended helical rods in which hydrophobic side chains are exposed while polar amide groups are shielded from the solvent.[16–18] In comparison with non-fluorinated alcohols, TFE is often preferred for such studies because of its high potential to stabilize α-helical structures.[19,20] The effectiveness of TFE in inducing secondary structures appears to be related to its three fluorine atoms.[21] Methanol, ethanol, or 2,2,2-trifluoroethanol (TFE) not only stabilize the native-like secondary structure but also transform other structural elements of the protein leading to the formation of non-native structures. The secondary structures stabilized by TFE are believed to reflect the conformations existing in early stages of protein folding.[20,22,23] Molten globule (MG), a compact collapsed form of a protein with a pronounced secondary structure but lacking rigid tertiary structure, is such an intermediate conformation.[24,25] However, in several instances, the MG has been shown to possess well-defined tertiary contacts, *i.e.*, a pre-molten globule state.[26,27] Study of these structural

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Facile synthesis and characterization of beta lactoglobulin–copper nanocomposites having antibacterial applications†

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The synthesis of Cu^0 nanoparticles and Cu–protein nanocomposites is a great challenge. Here we describe a simple and convenient method for the synthesis of Cu–β-lactoglobulin nanocomposites using very cheap $CuSO_4 \cdot 5H_2O$ and the retinol binding model protein bovine β-lactoglobulin (β-lg) at pH 10.0 in ammoniacal medium. Then addition of hydrazine hydrates in the reaction mixture and heating the solution at 55 °C for 2 h resulted in the formation of hexagonal Cu–β-lg nanocomposite (average size 0.5 μm) containing the embedded Cu-nanoparticles as revealed from SEM and TEM analysis. The important feature of this method is that the highly stable Cu-nanoparticle present in the composites were synthesized without employing any inert atmosphere; decomposition of hydrazine hydrate generated the nitrogen *in situ* which produced the inert atmosphere for this reaction. Synthesis of this nanocomposite is justified by a docking study. The synthesized nanocomposite exhibits potential antibacterial activity against both Gram positive and Gram negative bacterial strains. Thus it can be employed in different medical applications and also in the preparation of various nanomedicines.

Introduction

The synthesis and development of metallic nanoparticles (NP) have received a great deal of attention during the last few years. NPs not only act as carriers for various drugs, but they themselves can be effectively used as sources for biosensing, bioimaging, luminescence tagging, immunoassay and inhibiting the formation of amyloid aggregates of protein.[1–5] NPs of certain inorganic materials also possess antimicrobial and healing properties. Particularly Cu-NPs have drawn significant attention because of their unique properties such as electrical conductivity, catalytic activity, antimicrobial activity, drug delivery, biosensing and chemical stability.[6–10] In the last few years huge efforts have been made to invent new synthetic routes to produce Cu-NPs, such as a radiation method,[11] thermal decomposition,[12] and microemulsion.[13,14]

However, since copper is redox-active, it is usually difficult to prepare metallic copper *via* reduction of simple copper salts in aqueous solution in contrast with noble metals, such as Au, Ag, Pt. The reason being, even though zero valence copper is initially formed, it gets easily oxidized in the solvents with high dipolar moments under ambient conditions.[15] The stabilization as well as the control of size and growth rate of nanoparticles can be achieved in polymeric matrices resulting in the formation of nanocomposite, an advanced functional material composed of nanoparticles dispersed inside the polymeric matrix and coated by polymer.[16–18] The resulting nanocomposites material combines the suitable properties of both partners thus have enhanced functions as compared to individual components. Thus increasing attention has been paid to the fabrication of bifunctional nanostructures consisting of discrete domains of two materials.[19] Various methods are available for the preparation of copper nanocomposites in various media and with a variety of agents.[20–22] Cu nanocomposite is now used to remove nitrate from ground water where it shows both catalytic reduction and chemical reduction.[23,24] Cu nanocomposite has good antibacterial activity such as on *Staphylococcus aureus* and *Escherichia coli* as pathogen microorganisms and it exhibited good antibacterial potential against both Gram positive and Gram negative bacterial strains.[25] Graphene–copper nanocomposite can be used as antifriction additive.[26] In our present work we have synthesized the Cu–β-lactoglobulin nanocomposite (a Cu–protein nanocomposite) having good antibacterial property by a simple chemical reduction method where metallic Cu-NPs were formed without the using of any inert environment. β-lg is a most widely studied whey protein and a member of the lipocalin family. This protein contains 162 amino acids with a molecular mass of ~18 400 Da, featuring an eight-stranded β-barrel (strands A-H)

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Curcumin inhibits the Al(III) and Zn(II) induced amyloid fibrillation of β-lactoglobulin *in vitro*†

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Accumulation of ordered protein aggregates (or amyloids) is responsible for several neurodegenerative diseases. The behaviour of amyloidal fibril formation of β-lactoglobulin (β-lg) during heat treatment depends on the environmental conditions. In this study the Al(III) and Zn(II) induced amyloid fibrillations of β-lg, in the absence and presence of curcumin, were evaluated using fluorescence, Thioflavin T, Congo red, Rayleigh scattering, dynamic light scattering analysis, FT-IR, CD spectroscopy and transmission electron microscopy. Curcumin, a natural phenolic antioxidant, is capable of binding with Al^{3+}, Zn^{2+} and β-lg. Our experimental findings demonstrate that the metal–curcumin mixture can inhibit the transition from less structured oligomers to β-sheet rich protofibrils which act as seeding factors for further fibrillization. The Al(III)–curcumin mixture has greater inhibition capability than the Zn(II)–curcumin mixture of heat treated metal induced aggregation of β-lg.

1 Introduction

Amyloid plaques containing amyloid β (Aβ) peptides are conceived as pathological hallmarks of Alzheimer's disease (AD). The progressive accumulation of Aβ aggregates *via* a fibrillation process is widely believed to be fundamental to initialize the neurodegenerative pathology and trigger a cascade of events which include neurotoxicity, oxidative stress and inflammation during the progression of AD.[1–3] Other peptides and proteins generate morphologically similar or different amyloid fibrils under carefully chosen conditions through dimer and oligomer formation. Their growth into the protofibrils and fibrils is really a complicated nucleation process.[4] Recently, the leading role of the soluble aggregates in the neurodegenerative disorders has been widely accepted.[5]

Several attempts have been made in searching the therapeutic agents which can inhibit the formation of such toxic oligomers or disintegrate the preformed fibrils. A number of experimental parameters (protein concentration, pH, temperature, ionic strength, presence of co-solvents, additives, *etc.*) can be varied to modulate the protein aggregation process and diversify the morphology, such as worm-like or rigid fibrils and amorphous aggregates.[6–8] Interactions with metal ions can deeply affect protein aggregation, causing rapid precipitation, increased fibrillogenesis, and morphology alterations.[9–11] It has been shown that the senile plaques, typical of the Alzheimer's disease, contain a great amount of transition metals ions such as Cu(III), Fe(III) and Zn(II). So far, their role is not very well clarified. The formation of amyloid fibrils from the β-amyloid peptide, main constituent of amyloid plaques in the brain of Alzheimer's disease/patients, became faster in the presence of copper and zinc.[12,13] A similar behaviour has been noticed in α-synuclein, a protein involved in Parkinson's disease. The generally accepted argument on the function of divalent metals in protein aggregation is based on their ability to act as bridges, as well as to provide an electrostatic screening between the negatively charged groups of the neighbouring protein molecules.[14] In fact, aggregation of protein is generally promoted by the electrostatic screening due to the action of monovalent and/or divalent metal ions.[15]

In our experiment we have selected the β-lg because of its two important interest – (i) it is a model β-protein in the aggregation process and (ii) used as a thermal marker in the industrial processes involved in preparation of milk.[16] In general, β-lg is the major protein in whey of ruminant milk. The globular molecule has molecular weight of approximately 18.3 kDa and is made of 162 amino acid residues. β-lg is composed of nine β-strands and one α-helix, in which the hydrophobic sequences are mostly buried.[17,18] In addition, β-lg possesses two intramolecular disulfide bonds (Cys66–Cys160, Cys106–Cys119) and one free thiol group (Cys121) which is buried between the β-barrel and the major α-helix.[19] Furthermore, it consists of 162 amino acids and capable of binding and transporting small hydrophobic molecules. This carrier property makes it an attractive candidate to serve as a transporter for delivering important hydrophobic nutrients to improve their bioavailability. β-lg exists in the form of a dimmer at room temperature and neutral pH. However, it

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Multispectroscopic analysis and molecular modeling to investigate the binding of beta lactoglobulin with curcumin derivatives†

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Bovine beta lactoglobulin (β-lg), the major whey protein, has a great affinity for a wide range of organic compounds like fatty acids, retinol *etc.* Curcumin, a polyphenolic antioxidant present in turmeric and its isoxazole (IOC) and pyrazole (PY) derivatives have been elicited worldwide for their therapeutic activities. However, the nature of interaction of β-lg with these derivatives remains unexplored. Fluorescence quenching studies suggest a static quenching mechanism for both the compounds. The average distances of 7.28 nm and 7.33 nm have been determined for IOC and PY respectively for energy transfer based on FRET which have application in many biological and biophysical fields. Circular dichroism spectra (CD) and Fourier transform infrared spectroscopy (FTIR) have been utilized to analyze the influence on the secondary structure of the protein. Docking simulation reveals a possible mechanism for different quenching behaviours and modes of binding preferred by the two compounds. Our findings will be helpful in the design of the drugs and other biologically active molecules that bind more strongly to β-lg and have the ability to show FRET.

1 Introduction

Curcumin, a yellow lipid-soluble phenolic β-diketone dietary spice of turmeric, is found in the powdered root of *Curcuma longa* and is well known as a food coloring agent.[1] The pharmacological attributes of curcumin, such as antimalarial,[2] antioxidative,[3] anti-inflammatory,[4] anti-angiogenic,[5] anti-amyloid,[6] anti-cancer,[7] anti-microbial,[8] wound healing,[9] and protective activities have been undergoing thorough research during last few decades due to its nontoxicity and bio-compatibility.[10] This non-steroidal phytochemical has a medicinal value for liver diseases (jaundice), indigestion, urinary track diseases, rheumatoid arthritis & insect bites.[11] The β-diketone moiety of curcumin is responsible for keto–enol tautomeric behaviour, exerted enolic –OH (hydroxyl) group, and its instability *in vitro* as well as *in vivo*.[12] Anand *et al.* reported that the presence of β diketone moiety may play the crucial role in bioactivities but recent studies also show that the bioactivity of curcumin derivatives without β-diketones also revolved.[13]

The pyrazole derivatives of curcumin has been deployed to study lipoxygenase inhibitory activity,[14] endothermal cell proliferation and cytotoxicity.[12] Pyrazole and isoxazole derivatives also maintain the binding to the sub domain of PKCs, protein kinase c6.[15] Chakraborti *et al.* showed that both these derivatives were equally competent of binding to tubulin and resisting the tubulin self-assembly formation.[16] The proposal for using these derivatives as an anti-cancer drug also had been under consideration due to their enhanced stability and free radical scavenging property.[17] Nonetheless, pyrazole (PY) & isoxazole (IOC) derivatives are used as a potent ligand of fibrillar Aβ-42 aggregates.[18] Various attempts have been made to increase the bioavailability and solubility of curcumin and its analogs either by nano capping or by nano encapsulation to various biological macromolecules.[19] Bovine β-lg, a protein with hydrophobic core which consist with eight antiparallel β-strands called β-barrel or calyx, is one of the mostly used accepted carrier protein for hydrophobic ligands, having pH dependant opening, encapsulating property and a unique acidic pH resistivity.[20] In this way, bovine beta lactoglobulin (β-lg) performs the well-controlled drug delivery mechanism indeed.[21] This encourages us to choose β-lg as a model carrier protein. Moreover, R. Narlawar *et al.* reported that curcumin derived isoxazoles and pyrazoles inhibit or modulate APP metabolism by interfering with γ-secretase activity.[18]

In order to go to mechanistic insight, our present work aims to study the interaction between these derivatives and the model protein β-lg utilizing different spectroscopic techniques (Scheme 1). These synthesized IOC and PY derivatives (shown in Scheme 1) exhibit excellent efficiency of energy transfer (FRET) from the Trp moiety (W_{19}) of β-lg in bound condition which will

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Inhibition of amyloid fibril formation of β-lactoglobulin by natural and synthetic curcuminoids†

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The aggregation of proteins has been associated with several aspects of daily life, including food processing, blood coagulation and many neurodegenerative infections. However, the actual mechanisms responsible for amyloidosis, the irreversible fibril formation of various proteins, which is linked to disorders such as Alzheimer's disease, Creutzfeldt–Jakob disease and Huntington's disease, have not yet been fully elucidated. Curcumin, a potent anti-oxidant, exhibits anti-amyloid activity; however, its activity is limited due to its instability. Therefore, chemical modifications of curcumin have been performed to obtain molecules with enhanced stability and superior anti-amyloid activity. Herein, the main objective of this study is related to the inhibitory effects of three stable analogs of curcumin against bovine β-lactoglobulin (β-lg) fibrillization. We inferred that a pyrazole derivative of curcumin showed remarkable potency in arresting the fibrillization of β-lg, as revealed by biophysical techniques. Molecular docking demonstrated that pyrazole-mediated inhibition of β-lg fibrillogenesis may be initiated by interacting with aggregation-prone regions of the protein and preventing interactions between monomers, leading to suppression of the overall aggregation process. This work alludes to a possible broader scope for discovery of other small molecules that may exert similar effects against amyloid formation and its associated neurodegenerative diseases.

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Introduction

The anomalous self-assembly and accumulation of misfolded proteins is known to have common cellular and molecular mechanisms, including protein aggregation, which is the known leading causative agent of a number of conformational diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and prion disease.[1,2] The aggregates consist of fibers with cross β-sheet structures, termed harmful 'amyloids', and their morphological features are not associated with behaviors of specific proteins. Although the proper aetiology of AD remains controversial, diverse factors appear to play vital roles in the pathophysiology of the disease; these include abnormal β-amyloid (Aβ) deposits in extracellular amyloid plaques, which lead to progressive neuronal death, tau protein hyperphosphorylation, metal ion dyshomeostasis, oxidative stress, and neurotransmitter system dysfunction.[3–7] Different peptides and proteins generate morphologically similar or different amyloid fibrils through dimer and oligomer formation; this has been hypothesized to occur in a stepwise fashion, with a slow phase of nucleation of the precursors of the amyloid fibrils followed by a relatively fast elongation phase.[8] Moreover, in the formation of amyloid fibrils, the incentive to assemble arises from favorable solvation energies and side-chain interactions accompanying the formation of β-sheet structures.[9] Currently, numerous clinical and experimental studies are revealing that soluble oligomeric and protofibrillar forms of proteins are potentially neurotoxic.[10,11]

Recently, the most challenging research task has focused on the inhibition of fibril formation[12–14] by the employment of small molecules. These potent modulators are believed to stabilize monomers by blocking the formation of toxic oligomers and to divert the monomeric proteins to off-pathway non-toxic intermediates. Small molecules are being developed to inhibit aggregation of Aβ,[15] α-synuclein[16] and prions.[17]

Much evidence has shown that polyphenols, which have structural constraints, are effective in the inhibition of amyloid fibrillation.[18,19] Curcumin, a classical active yellow lipid-soluble β-diketone dietary polyphenolic component, has been

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Silver nanoparticle modulates the aggregation of beta-lactoglobulin and induces to form rod-like aggregates

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ABSTRACT

Silver nanoparticles (SNPs) have been increasingly used in medicines and biomaterials as a drug carriers and diagnostic or therapeutic material due to their smaller size, large surface area and cell penetration ability. Here we report the preparation of SNPs of diameter 10 ± 3 nm by using silver nitrate and sodium borohydride and the interaction of synthesized SNPs with our model protein β-lactoglobulin (β-lg) in 10 mM phosphate buffer at pH 7.5 after thermal exposure at 75 °C. Heat exposed β-lg forms amyloidal fibrillar aggregates whereas this protein aggregates adopt rod-like shape instead of fibrillar structure in presence of SNP under the same conditions. Size of the synthesized SNPs is confirmed by UV–Visible spectroscopy, SEM and TEM. Interactions and subsequent formation of molecular assembly of heat stressed β-lg with SNP were investigated using Th-T assay and ANS binding assay, DLS, RLS, CD, FT-IR, SEM, TEM. Docking study parallely also support the experimental findings.

1. Introduction

Silver nanoparticles (SNPs) play a significant role in catalytic reaction, wound dressing, optical property, electrical property, antimicrobial activity, purification of ground water, removal of some chemical hazards, medical implants, prevention in infection, drug delivery, cancer treatment, antifungal activity, molecular linkers, pharmacological application, etc. [1–4]. Silver nanocrystals encapsulated in mesoporous silica nanoparticles displayed antimicrobial activity against both Gram-positive and Gram-negative bacteria [5]. Some researchers applied silver nanoparticles in purification of ground water in a convenient way. This nanoparticle is suitably used to detect some chemical hazards like Hg^{2+}, Cu^{2+} and S^{2-} [6–7]. It is also increasingly used in cosmetics and it has also been proposed in medical implants and instruments for the prevention of infection [8–10]. The silver nanocrystals encapsulated with silica nanoparticles are now used for imaging and drug delivery purpose [11]. Very low concentrations of silver nanoparticles are effective in induction of apoptosis in cancer cells [12]. Recent study demonstrates the antitumor activity of green-synthesized SNPs against lung cancer in vitro and in vivo [13]. The interactions between SNPs and various DNA bases (adenine, guanine, cytosine, and thymine) are also used as molecular linkers because of their biological significance [14]. The interaction of silver nanoparticles with proteins like SNPs-BSA and SNPs-BSA-emodin, interactions over the protein structure as well in the protein-drug binding show that silver nanoparticles may play a good role in biomedical and pharmacological applications [15].

Several diseases are known to occur due to the misfolding and aggregation of the proteins that are naturally present for normal the functioning of our body. Protein misfolding and aggregation are associated with many neurodegenerative diseases like Alzheimer's (AD), Parkinson's (PD), amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), diabetes type II and Huntington's diseases are the notorious examples for this kind [16–20]. The pathologic characteristics of AD are the formation of amyloid-beta (Aβ) fibrils enriched with cross β-sheet structures caused by the misfolding of Aβ peptide [21,22]. The mechanism of the formation of the fibrils is formed based on the nucleation and growth mechanism [23]. In the progression, the monomers are transformed into oligomers and the oligomers act as nuclei for the formation of fibril followed by the elongation of the fibrils through the addition of the monomers [24]. In our previous study, we have shown that gold nanoparticles (GNP) inhibit the amyloid fibril formation of β-lg [25]. β-Lg has been extensively utilized in the study of protein folding and aggregation.

β-Lg is a predominantly β-sheet protein having 162 amino acid residues and molecular weight 18.4 kDa. Basically it is a well-known globular whey protein of pI 5.2. In its β-barrel structure there are 8 strands (A to H) which are succeeded by three turn α-helix and a final β-strand (strand I) [26]. At pH 7.0 the protein exists as a reversible dimer and extent of dimerization depends on pH, temperature, protein

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REGULAR ARTICLE

Antioxidant ferulic acid prevents the aggregation of bovine β-lactoglobulin *in vitro*

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Abstract. Amyloids, a well-ordered β-sheet-enriched structural network, can be broadly defined as insoluble protein aggregates that are linked to a wide variety of diseases including systemic amyloidosis and some neurodegenerative disorders. Ferulic acid (FA), a phenolic acid, abundant in antioxidant and efficient pharmaceutical has beneficial effects against several ailments. Based on this, we have investigated the protective role of FA on amyloid formation of bovine β-lactoglobulin (β-lg), a model globular protein. Using a set of *in vitro* biophysical methods, such as UV-Vis spectroscopy, fluorescence, circular dichroism, transmission electron microscopy, etc., our research group has concluded that FA significantly inhibits the heat-induced amyloid formation of β-lg and this inhibitory effect is dose-dependent. Exposed surface hydrophobicity of β-lg amyloid fibrils decreased significantly in the presence of FA. Docking study revealed that ionic and hydrogen bonding interactions between FA and β-lg prevented protein conformational changes leading to fibrillation. We anticipate that our finding would give an insight into the protein aggregation inhibited by the antioxidant compound, FA and pave the way for finding and developing other new small molecules (protein misfolding inhibitors) that give similar result against amyloid fibril formation and its allied neurodegenerative disorders.

Keywords. Antioxidant; ferulic acid; β-lactoglobulin; aggregation.

1. Introduction

In the present area of research, a very interesting topic is the alteration of native (often soluble) proteins into non-native folded fibrillar structures; these are often not soluble in various solvents as well as in water. These protein fibrils are usually called amyloid fibrils and are the hallmark for numerous ailments, including Alzheimer's, Huntington's, type II diabetes, Parkinson's, prion-associated encephalopathy diseases and others.[1–6] Amyloid fibrils are highly organised polypeptide aggregates and are rich in β-sheet secondary conformation.[7] These fibrils are stable against temperature,[8] hydrolytic pressure,[9] proteolytic enzymes[10] and denaturants.[11] Several proteins and peptides, e.g. amyloid β-peptide (Aβ), β-lactoglobulin (β-lg) islet amyloid polypeptide (IAPP), insulin, α-synuclein, and transthyretin have been identified as amyloidogenics,[12–16] but it has been observed that there is no similarity in primary structure among them.[17] The aggregation pattern of such peptides and proteins differs owing to their differential forms.[18–20]

In recent research work, a number of endeavours have been applied to find or design the compounds which can prevent the formation of these toxic oligomeric species or break up the pre-formed fibrils. Several working parameters, e.g. concentration of protein, pH of experimental solution, ionic strength of the reaction medium, reaction temperature, existence of co-solvents, etc., can be altered to modulate the aggregation process of β-lg into the oligomers or fibrils.[21–23]

Small organic molecules (either from natural origin or synthetically derived) play a significant role in

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Modulation of amyloid fibrillation of bovine β-lactoglobulin by selective methionine oxidation†

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Deposition of oxidation-modified proteins during normal aging and oxidative stress are directly associated with systemic amyloidoses. Methionine (Met) is believed to be one of the most readily oxidisable amino acid residues of protein. Bovine beta-lactoglobulin (β-lg), a model globular whey protein, has been presented as a subsequent paradigm for studies on protein aggregation and amyloid formation. Herein, we investigated the effect of *t*-butyl hydroperoxide (*t*BHP)-induced oxidation on structure, compactness and fibrillation propensity of β-lg at physiological pH. Notably, whey protein modification, specifically Met residues, plays an important role in the dairy industry during milk processing and lowering nutritional value and ultimately affecting their technological properties. Several bio-physical studies revealed enhanced structural flexibility and aggregation propensity of oxidised β-lg in a temperature dependent manner. A molecular docking study is used to predict possible interactions with *t*BHP and infers selective oxidation of methionine residues at 7, 24 and 107 positions. From our studies, it can be corroborated that specific orientations of Met residues directs the formation of a partially unfolded state susceptible to fibrillation with possible different cytotoxic effects. Our studies have greater implications in deciphering the underlying mechanism of different whey proteins encountering oxidative stress. Our findings are also important to elucidate the understanding of oxidation induced amyloid fibrillation of protein which may constitute a new route to pave the way for a modulatory role of oxidatively stressed proteins in neurological disorders.

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Introduction

Oxidative damage is considered as a likely cause of age-related brain dysfunction because the brain is believed to be particularly vulnerable to oxidative stress due to a relatively high rate of oxygen free radical generation without commensurate levels of anti-oxidative defenses.[1] In particular, one category of disease in which oxidative damage is found extensively is neurodegenerative diseases including Alzheimer's (AD), Huntington's (HD), Parkinson's diseases (PD), and prion diseases.[2–6] In particular, post-mortem analysis of the AD brain has shown elevated levels of protein oxidation, lipid peroxidation, and oxidative damage to mitochondria.[7,8] Specifically, protein oxidation involved covalent cross-linkages, fragmentation of covalent bonds, and modification of amino acids including methionine, cysteine, histidine, tryptophan, and tyrosine.[9] Methionine (Met) is one of the most oxidation-prone amino acid and is converted to methionine sulfoxide or sulfone derivatives by different mechanisms, such as hydrogen peroxide treatment, metal catalyzed reactions, and UV exposure.[10]

Recently, most research work has been focused on the inhibition of fibril formation by the employment of small molecules. These potent modulators are believed to stabilize the monomer by blocking the formation of toxic oligomers and divert the monomeric protein to off-pathway non-toxic intermediates. Small molecules agents are being developed to inhibit aggregation of Aβ,[12] α-synuclein[13] and prions.[14]

It has been demonstrated that oxidation of the susceptible Met residues of proteins has been shown to result in structural changes,[11,12] decreased stability,[13,14] increased propensity to aggregation[15] and loss of biological functions. It is worth mentioning that even subtle modifications at single residue level in protein structures may reorganize specific inter-protein and protein-solvent interactions. In addition, methionine oxidation serves as a common modulator of fibril formation and has been shown to suppress the aggregation of β-amyloid peptide, prion protein (PrP), transthyretin, apolipoprotein C-II α-synuclein and human serum albumin.[16–21] Interestingly, in

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