University of Basrah
College of Pharmacy

Review about challenges of the stability of protein type drugs

A project submitted to the department of pharmaceutics for graduation in College of Pharmacy

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(5th stage)

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Abstract

Proteins are the most versatile macromolecules in living systems and serve crucial functions in essentially all biological processes. They function as catalysts, they transport and store other molecules such as oxygen, they provide mechanical support and immune protection, they generate movement, they transmit nerve impulses, and they control growth and differentiation.

Protein stability is a topic of major interest for the biotechnology, pharmaceutical and food industries, in addition to being a daily consideration for academic researchers studying proteins. An understanding of protein stability is essential for optimizing the expression, purification, formulation, storage and structural studies of proteins. In this review, discussion will focus on the main physical and chemical instabilities that are related to the protein structures (primary, secondary, tertiary and quaternary) in addition to the most important factors affecting protein stability.

Acknowledgement

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1. Introduction

Maintaining protein-based therapeutics is a challenging task in part due to the difficulties in maintaining protein solutions safe and efficacious throughout the drug product development process, storage, transportation and patient administration. Bulk drug substance goes through a series of formulation, fill and finish operations to provide the final dosage form in the desired formulation and container or delivery device. Different process parameters during each of these operations can affect the purity, activity and efficacy of the final product. Common protein degradation pathways and the various physical and chemical factors that can induce such reactions have been extensively studied for years. This review presents an overview of the various formulation-fill-finish operations with a focus on processing steps and conditions that can impact product quality. Various manufacturing operations including bulk freeze-thaw, formulation, filtration, filling, lyophilization, inspection, labeling, packaging, storage, transport and delivery have been reviewed. The article highlights our present day understanding of protein instability issues during biopharmaceutical manufacturing and provides guidance on process considerations that can help alleviate these concerns.

However, the aim of this research is to provide a basic overview of the subject in order to equip the reader with knowledge of various factors affecting stability of proteins and protein drugs. (1)

2. Protein structures and classifications

Proteins are macromolecules consisting of one or more polypeptides (Table 1). Each polypeptide consists of a chain of amino acids linked together by peptide (amide) bonds. The exact amino acid sequence is determined by the gene coding for that specific polypeptide. When synthesized, a polypeptide chain folds up, assuming a specific three-dimensional shape (i.e. a specific conformation) that is unique to it.

The conformation adopted is dependent upon the polypeptide's amino acid sequence, and this conformation is largely stabilized by multiple, weak non-covalent interactions. Any influence (e.g. certain chemicals and heat) that disrupts such weak interactions results in disruption of the polypeptide's native conformation, a process termed denaturation.

Denaturation usually results in loss of functional activity, clearly demonstrating the dependence of protein function upon protein structure. A protein's structure currently cannot be predicted solely from its amino acid sequence. Its conformation can, however, be determined by techniques such as X -ray diffraction and nuclear magnetic resonance (NMR) spectroscopy.

Proteins are sometimes classified as 'simple' or 'conjugated'. Simple proteins consist exclusively of polypeptide chain(s) with no additional chemical components present or being required for biological activity. Conjugated proteins, in addition to their polypeptide components(s), contain one or more non-polypeptide constituents known as prosthetic group(s). The most common prosthetic groups found in association with proteins include carbohydrates (glycoproteins), phosphate groups (phosphoproteins), vitamin derivatives (e.g. flavoproteins) and metal ions (metalloproteins) (2).

Table (1): Examples of proteins The number of polypeptide chains and amino acid residues constituting the protein are listed, along with its molecular mass and biological function (2).

Protein	No. polypeptide chains	Total no. amino acids	Molecular mass (Da)	Biological function
Insulin (human)	2	51	5 800	Complex, includes regulation of blood glucose levels
Lysozyme (egg)	1	129	13 900	Enzyme capable of degrading peptidoglycan in bacterial cell walls
IL-2 (human)	1	133	15 400	T-lymphocyte-derived polypeptide that regulates many aspects of immunity
EPO (human)	1	165	36 000	Hormone that stimulates red blood cell production
Chymotrypsin (bovine)	3	241	21 600	Digestive proteolytic enzyme
Subtilisin (*Bacillus amyloliquefaciens*)	1	274	27 500	Bacterial proteolytic enzyme
Tumour necrosis factor (human TNF-α)	3	471	52 000	Mediator of inflammation and immunity
Haemoglobin (human)	4	574	64 500	Gas transport
Hexokinase (yeast)	2	800	102 000	Enzyme capable of phosphorylating selected monosaccharides
Glutamate dehydrogenase (bovine)	~40	~8 300	~1 000 000	Enzyme interconverts glutamate and α-ketoglutarate and NH_4^+

Table (2): The 20 commonly occurring amino acids (2).

R group classification	Amino acid	Abbreviation		Molecular mass	Occurrence in 'average' protein (%)
		3 letters	1 letter		
Nonpolar, aliphatic	Glycine	Gly	G	75	7.2
	Alanine	Ala	A	89	8.3
	Valine	Val	V	117	6.6
	Leucine	Leu	L	131	9
	Isoleucine	Ile	I	131	5.2
	Proline	Pro	P	115	5.1
Aromatic	Tyrosine	Tyr	Y	181	3.2
	Phenylalanine	Phe	F	165	3.9
	Tryptophan	Trp	W	204	1.3
Polar but uncharged	Cysteine	Cys	C	121	1.7
	Serine	Ser	S	105	6
	Methionine	Met	M	149	2.4
	Threonine	Thr	T	119	5.8
	Asparagine	Asn	N	132	4.4
	Glutamine	Gln	Q	146	4
Positively charged	Arginine	Arg	R	174	5.7
	Lysine	Lys	K	146	5.7
	Histidine	His	H	155	2.2
Negatively charged	Aspartic acid	Asp	D	133	5.3
	Glutamic acid	Glu	E	147	6.2

2.1 The primary structure

The primary structure of a polypeptide refers to its exact amino acid sequence, along with the exact positioning of any disulfide bonds present. Nineteen of these amino acids contain a central (α) carbon atom, to which is attached a hydrogen atom (H), an amino group (NH2) a carboxyl group (COOH), and an additional side chain (R) group – which differs from amino acid to amino acid. The amino acid proline is unusual in that its R group forms a direct covalent bond with the nitrogen atom of what is the free amino group in other amino acids.

Amino acids are joined together during protein synthesis via a 'peptide' (i.e. amide bond) as explained in (figure 1). This is a condensation reaction, as a water molecule is eliminated during bond formation. Each amino acid in the resultant polypeptide is termed a 'residue', and the polypeptide chain will display a free amino (NH2) group at one end and a free carboxyl (COOH) group at the other end. These are termed the amino and carboxyl termini respectively. (2)

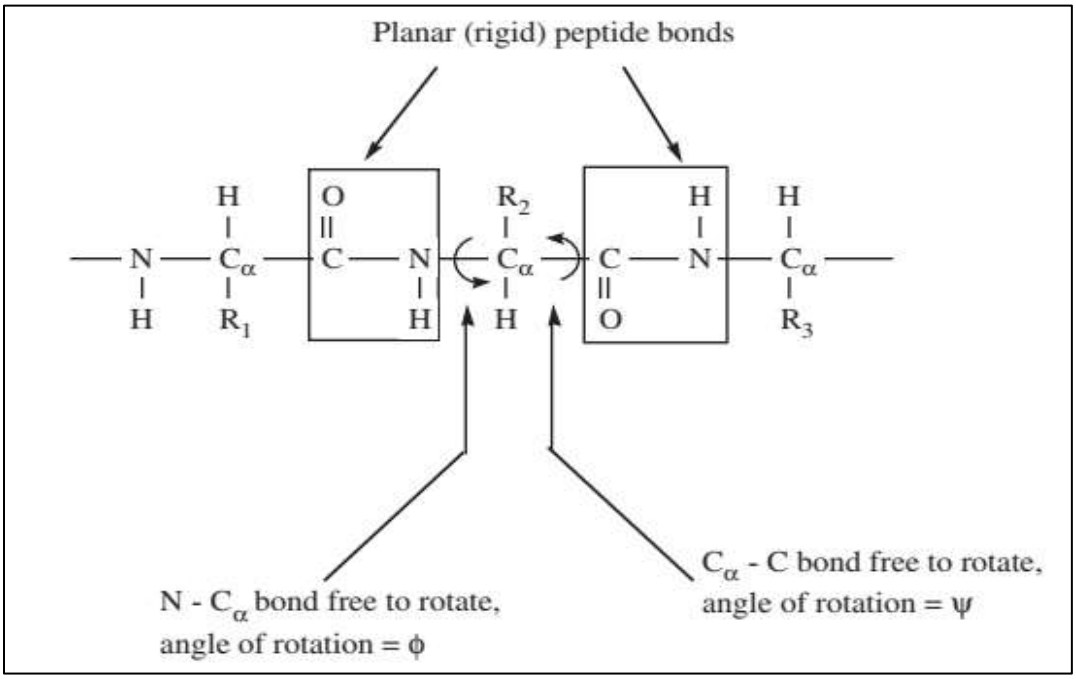

Figure (1): Fragment of polypeptide chain backbone illustrating rigid peptide bonds and the intervening (N-Cα) and (Cα-C) backbone linkages, which are free to rotate2. (2)

2.2 secondary structure:

By studying the backbone of most proteins, stretches of amino acids that adopt a regular, recurring shape usually become evident. The most commonly observed secondary structural elements are termed the α-helix and β-strands, which are usually separated by stretches largely devoid of regular, recurring conformation. The α-helix and β-sheets are commonly formed because they maximize formation of stabilizing intramolecular hydrogen bonds and minimize steric repulsion between adjacent side chain groups, while also being compatible with the rigid planar nature of the peptide bonds as shown in (figure 2) (2) .

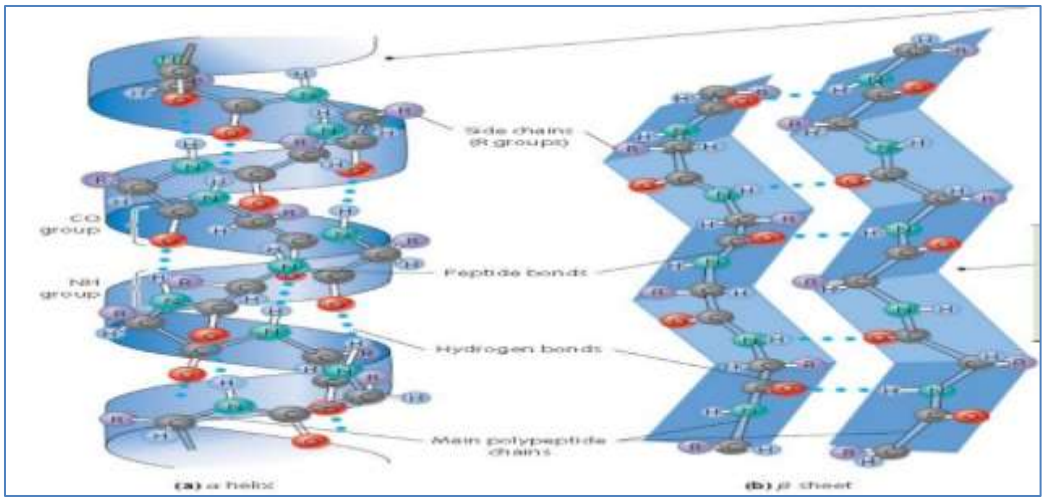

Figure (2): Secondary structure of proteins (3).

2.3 Tertiary structure:

A polypeptide's tertiary structure refers to its exact three-dimensional structure, relating the relative positioning in space of all the polypeptide's constituent atoms to each other. The tertiary structure of small polypeptides usually forms a single discrete structural unit (see figure 3).

However, when the three-dimensional structure of many larger polypeptides is examined, the presence of two or more structural subunits within the polypeptide becomes apparent. These are termed domains. Domains, therefore, are (usually) tightly folded sub regions of a single polypeptide, connected to each other by more flexible or extended regions. As well as being structurally distinct, domains often serve as independent units of function. Cell surface receptors, for example, usually contain one or more extracellular domains (some or all of which participates in ligand binding), a transmembrane domain (hydrophobic in nature and serving to stabilize the protein in the membrane) and one or more intracellular domains that play an effector function (e.g. generation of second messengers). Many therapeutic proteins also display several domains. Tissue plasminogen activator (tPA) (2).

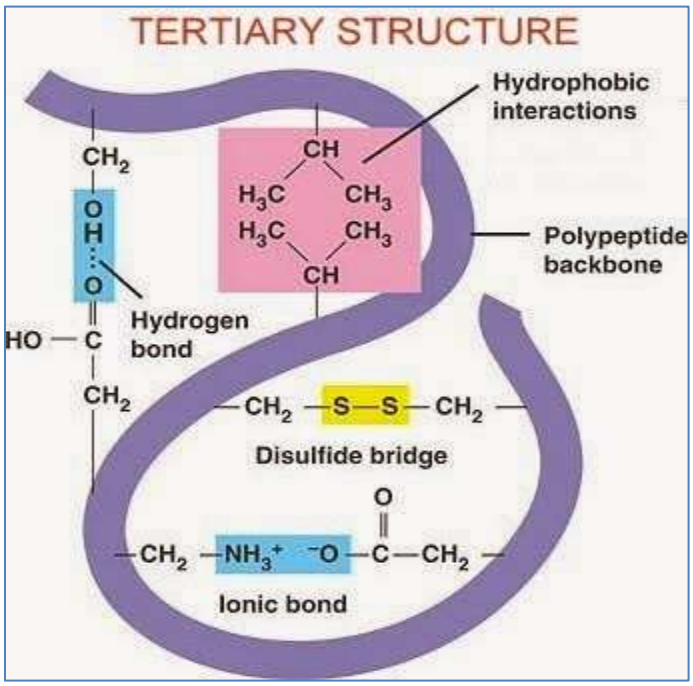

Figure (3): Tertiary structure (4)

2.4 Quaternary Structure

Quaternary structure is the interaction of two or more folded polypeptides as explained in figure (4). Many proteins require the assembly of several polypeptide subunits before they become active. If the final protein is made of two subunits, the protein is said to be a dimer. If three subunits must come together, the protein is said to be a trimer, four subunits make up a tetramer, etc. If the subunits are identical, the prefix "homo" is used, as in "homodimer." If the subunits are different, we use "hetero," as in "heterodimer."

Hemoglobin is the protein responsible for carrying oxygen in the blood. It is made up of four polypeptides: two α- and two β-subunits. One α-subunit and one β-subunit will come together to form a heterodimer, and two of these heterodimers will interact to form one hemoglobin molecule. Hemoglobin can therefore be thought of as a dimer of dimers, which come together to give the final protein its quaternary structure (5).

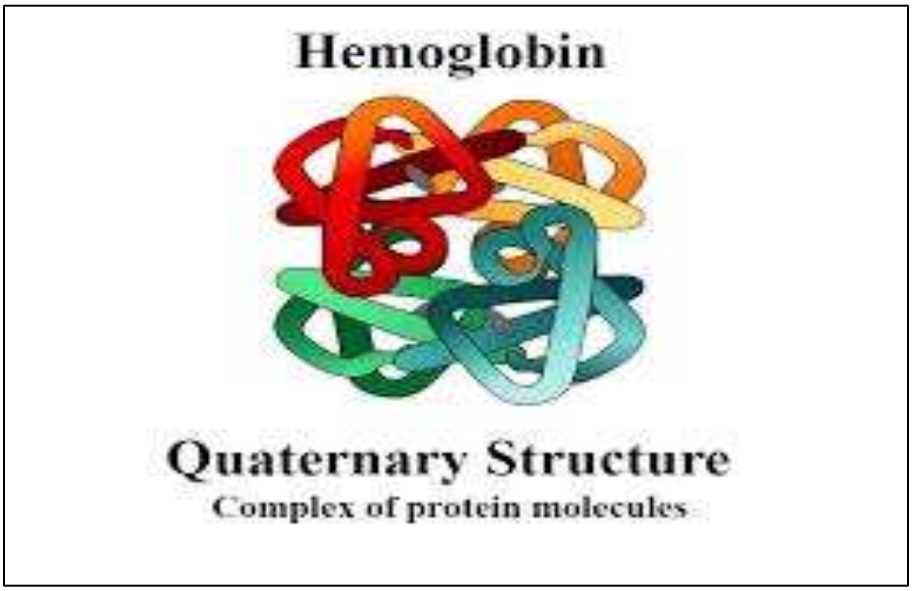

Figure (4): Quaternary structure (6)

3. Instabilities of protein and peptide type drugs

3.1 Physical instabilities

3.1.1 Denaturation:

Denaturation refers to an alteration of the global fold of a biopharmaceutical with higher order structures without an accompanying change in the primary structure. For proteins, these higher order structures refer to the secondary, tertiary, and quaternary structures with different levels of complexity. Although nucleic acids are structurally less complex than proteins, more elaborate structures such as double helix and supercoiling exist in DNAs, and many RNAs also have well defined tertiary structures. Any conformational changes in these structures can lead to denaturation of these biopharmaceuticals. (7)

The conformational integrity of native protein is maintained by a proper balance of weak nonbonding forces. Conceptually, an equilibrium exists between the native folded and the denatured-unfolded forms of the protein, with or without the involvement of unfolding intermediate(s), or molten globule(s). The native structure of the protein is readily disrupted by a change of environment (e.g., application of heat, change of pH, and addition of detergents or chaotropic agents) (8).

Such structural perturbation, which is either permanent or temporary, leads to a change of conformationally sensitive physical properties (e.g., optical rotation, viscosity, and UV absorption) and, most seriously, to a concomitant loss of biological activities. If the denaturation process is reversible, the loss of native structure can be recovered once the stress is removed. However, no such structure recovery is possible with irreversible denaturation involving covalent bond formation such as disulfide exchange or cross-linking.(7)

Irreversible denaturation also leads to aggregation and precipitation with accompanying changes in the secondary and/or tertiary structures of the proteins.

The soluble aggregates derived from denatured-unfolded protein and unfolding protein intermediates are prone to aggregation and precipitation in pharmaceutical formulation, resulting in a severe activity loss, as illustrated by recombinant human granulocyte colony stimulating factor (8).

Addition of sucrose to this protein leads to decrease in aggregation and an increase in conformational stability. Almost all water-soluble proteins are denatured by heat treatment. In an actual situation, once the temperature is raised and a significant fraction of the protein ensemble is unfolded, aggregation and/or precipitation can proceed rapidly, resulting in irreversible denaturation. In addition to heat denaturation, cold denaturation of proteins can occur during freeze-drying. In general, freeze-drying causes considerable conformational changes and aggregation in protein molecules, thereby promoting the formation of the β-sheet structure at the expense of the α-helix and random structures (9).

Protein stability is highly sensitive to pH changes and decreases sharply at acidic or basic pH. Because of the differences in pKa of certain amino acid groups between the native and unfolded proteins, protons released in response to pH changes can play an important role in the unfolding process (10) it has been shown that protein conformational fluctuations generally agree with the predictions based on the pKa values of the titrating groups in the protein (11). Aside from pH manipulation, protein unfolding can be achieved with chaotropic agents (e.g., urea and guanidine hydrochloride). However, in contrast to the less-than-complete protein unfolding induced by thermal denaturation, the denatured state produced by such chaotropes is virtually devoid of structure, resembling a random coil conformation (12).

3.1.2 Protein aggregation and precipitation

A major event of physical instability may be occurred under certain conditions (or simply with time) in which the secondary, tertiary, and quaternary structure of a protein may change and lead to protein unfolding and/or aggregation,. Protein aggregates may have no or reduced activity, reduced solubility, and altered immunogenicity. Presence of any insoluble aggregates in a protein pharmaceutical is generally not acceptable for product release. (13)

Protein aggregation (see figure 5) in many cases results from intermolecular association of partially denatured protein chains (14) Recent evidence suggests that aggregation may occur by specific interaction of certain conformations of protein intermediates rather than by nonspecific co-aggregation (15)The aggregation process can be roughly divided into three steps: initiation, propagation, and termination (16). Proteins aggregate to minimize thermodynamically unfavorable interactions between solvent and exposed hydrophobic residues of proteins.

Hydrophobic interaction, the reluctance of nonpolar groups to be exposed to water, is considered to be the major driving force for both protein folding and aggregation. Both protein aggregation and folding represent a balance of exposed and Buried hydrophobic surface areas (17) The balance is so delicate that a change of one amino acid in a protein may substantially change its aggregation behavior (18).

Protein aggregation may be induced by a variety of physical factors, such as temperature, ionic strength, vortexing, surface adsorption, etc. These factors can increase the hydrophobic surface area of proteins causing aggregation.(19)

Protein aggregation may result from chemical degradations or modifications and subsequent exposure of the hydrophobic surface(s). Proteins can directly form covalent aggregates such as insulin (20) or aggregate indirectly such as human relaxin after oxidation of His and Met residues.

Physical and chemical aggregations may occur simultaneously. For example, bFGF (basic fibroblastic growth factor) in citrate buffer (pH 5) forms intact and truncated dimers and trimers after storage at 25°C for 7 weeks (21).

Protein aggregation may start from a single protein molecule (unimolecular: intramolecular process) or more than one protein molecule (multimolecular: intermolecular process). Unimolecular processes include b-elimination and intrachain disulfide scrambling or formations such as IL-1ra in aqueous solution (22). Examples of multimolecular aggregation include thiol disulfide interchange for bovine serum albumin, thiol-catalyzed disulfide exchange for insulin, other covalent aggregation such as ribonuclease A, and non-covalent aggregation such as tetanus toxoid (23) If the percentage of precipitates: aggregates increases with increasing protein concentrations, multimolecular aggregation: precipitation processes may be involved such as aggregation of hIGF-I (24).

Aggregation can lower protein potency simply by decreasing the effective protein concentration. Moreover, it can increase the immunogenicity of proteins, as observed for insulin, human growth hormone, and recombinant human interferon-α2a (25).

Various low-molecular-weight excipients have been investigated for their potential to minimize protein aggregation or stabilize protein conformation (31) Simple sugars, polyhydric alcohols, and amino acids have all been shown to effectively prevent freeze/thaw-induced aggregation in chimeric L6, a mouse human monoclonal antibody (26) Sucrose is known to inhibit the aggregation of recombinant human granulocyte colony stimulating factor (27) Sorbitol has also been demonstrated to reduce moisture-induced aggregation in tetanus and diphtheria toxoids (28) β-Cyclodextrins have also been found to inhibit the aggregation of recombinant human growth hormone (29).

However, there are also excipients that can adversely impact the protein aggregation and precipitation. For example, benzyl alcohol can induce precipitation of human insulin-like growth factor I from solution (30) as well as aggregation of human interleukin-1 receptor antagonist (a predominantly β-sheet protein) in aqueous solution (31) and reconstituted lyophilized formulation (32).

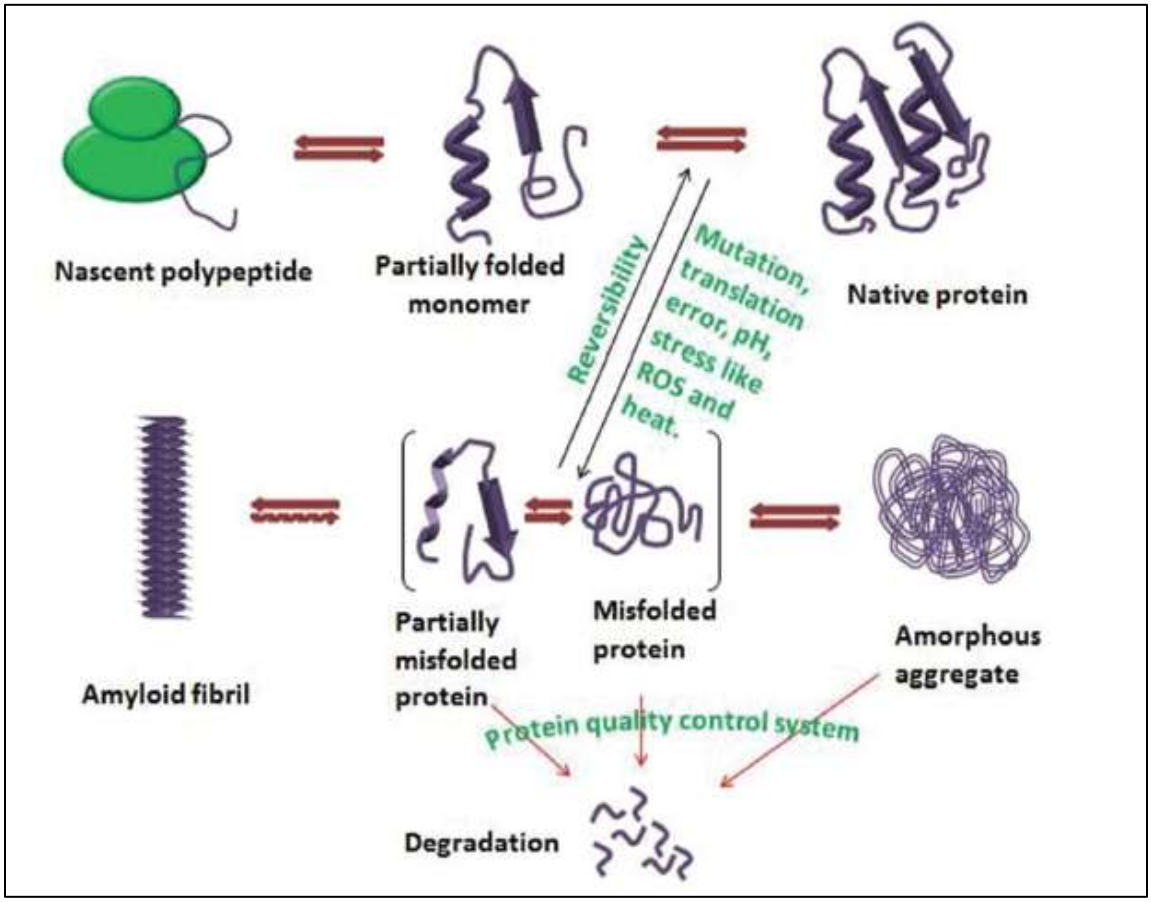

Figure (5): Protein aggregation (33)

3.1.3 Adsorption

Interfaces between two separate phases, such as air/water, oil/water, and solid/ water, are potential adsorption sites of biopharmaceuticals. Adsorption often involves simple diffusion of surface-active solute molecules in the bulk to the interface, and hence the rate of adsorption is generally dependent on the solute concentration. At saturation, a close packed monolayer of protein molecules corresponding to 0.1 to 0.5μg/cm2 is normally formed at the interface (34) and this adsorption behavior is of particular concern for high-potency therapeutic proteins.

However, certain proteins do not conform to such saturation-limited adsorption behavior and tend to show increased adsorption with increasing protein concentration, attaining a local protein concentration at the interface 1000 times higher than the initial concentration in the bulk solution (35) The surface activity resulting from different amino acid compositions of polypeptides has an important bearing on the extent of surface adsorption. As amino acids can be hydrophobic (e.g., tryptophan, phenylalanine, and isoleucine), hydrophilic (e.g., serine and threonine), negatively charged (e.g., aspartic acid and glutamic acid), or positively charged (e.g., histidine), the proteins involved can potentially adsorb on to solids such as plastics and glasses with different affinities. Owing to their intrinsic polyelectrolyte nature, the adsorption of proteins on solids is highly pH dependent, reaching a maximum at their respective isoelectric points (p*I*s) (33).

It has been demonstrated that recombinant human interleukin 11, which is monomeric and highly basic (p*I* > 10.5), displays a nonspecific loss to container in alkaline solution (36) Surface adsorption alone can cause more than 40% activity reduction of interleukin 11 in solution after 3 hours of storage at room temperature (37).

3.2 Chemical instabilities

3.2.1 Hydrolytic Reactions

3.2.1.1 Deamidation:

It appears to be the most common degradation in protein pharmaceuticals. In many cases, it is a major degradation pathway in proteins, such as recombinant human deoxy ribonuclease (rhDNase)(44) and rhVEGF at pH 5–6 (Goolcharran). The deamidated rhDNase has only about 40–50% of the original activity.

Several deamidation mechanisms have been reported and discussed (38). Asn and Gln are the two amino acids susceptible to deamidation in proteins and Asn is much more labile (39) Deamidation of Asn in proteins and peptides in an aqueous solution can proceed at a much higher rate than hydrolysis of a peptide bond (40).

The rate, mechanism, and location of deamidation in peptides or proteins are pH-dependent. Deamidation of Asn-X appears favored mostly at neutral or alkaline conditions (40). Maximum stability of Asn residues within peptides is found between pH 2-5. Between pH 5 and 12, the reaction proceeds rapidly and entirely through a cyclic imide (succinimide) intermediate, while slow deamidation at pH 1–2 seems to bypass the succinimide intermediate (40).

In insulin, however, deamidation originates from a rate-limiting intramolecular nucleophilic attack at the C-terminal AsnA-21 with a cyclic anhydride intermediate to form desamido insulin (41) since the unionized carboxyl group at the C terminal is the catalyzing group; increasing solution pH inhibits deamidation of AsnA-21. Therefore, the reaction is favored at low pH (<5). In neutral solutions, deamidation in insulin takes place predominantly at AsnB-3 residue (42).

3.2.1.2 Peptide Bond Cleavage at Aspartic Acid Residues*:*

The breakdown of proteins and peptides may occur by cleavage at the amide bonds. Although all amide bonds are theoretically susceptible to hydrolysis, they normally exhibit different hydrolytic rates. For instance, the rates of acid-catalyzed hydrolysis in polypeptides containing the aspartic acid residues (see figure 6) are at least 100 times higher than those of the other peptide bonds (39/40/41).

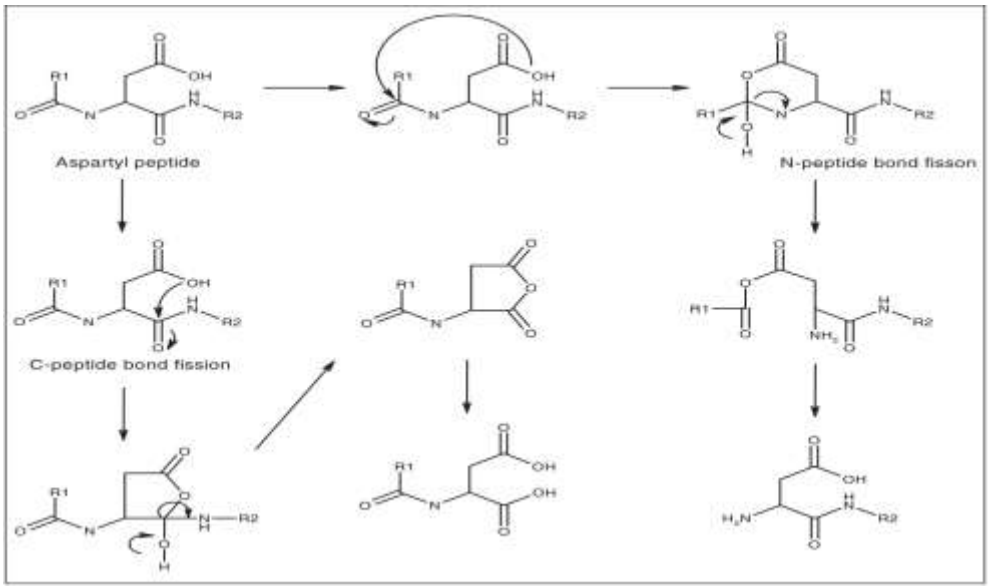

Figure (6): Peptide Bond Cleavage at Aspartic Acid Residues (7)

It is well established that aspartic acid-proline peptide bonds are particularly labile and undergo hydrolysis under conditions where other aspartic acid peptide bonds are stable, e.g., at low pHs (43).

The hydrolysis proceeds via intramolecular catalysis by carboxylate anion displacement of the protonated nitrogen of the peptide bond (44) This mechanism has been demonstrated for recombinant human interleukin 11, which undergoes peptide cleavage specifically at aspartic acid133- proline134 residues in acidic solution (45).

3.2.1.3 N-terminal Degradation

If the proteins and peptides possess a penultimate proline residue in an N-terminal sequence, the first amino acid in the primary sequence may be lysed from the polypeptide skeleton via the formation of diketopiperazine (DKP). This reaction is commonly termed nonenzymatic aminolysis, which results from the intramolecular nucleophilic attack of the N-terminal nitrogen on the carbonyl carbon of the peptide bond between the second and the third amino acid residues (Figure 7)) (46).

Pharmaceutical materials known to follow this degradation pathway include substance P recombine (47) ant human growth hormone (48) as well as aspartame and aspartyl phenylalanine in the solid state (49).

The rate of DKP formation generally increases with increasing temperature, as shown for RMP-7 Avoidance of general base catalysis or decreasing the buffer concentration at high pHs can minimize this cyclization reaction (50/51).

The degree of ionization of the N-terminal group, which is governed by the pKa of the amino group and the pH of the medium, also affects the rate of DKP formation because the first protonated amino acid in the sequence will not be available to effect base-catalyzed reaction.

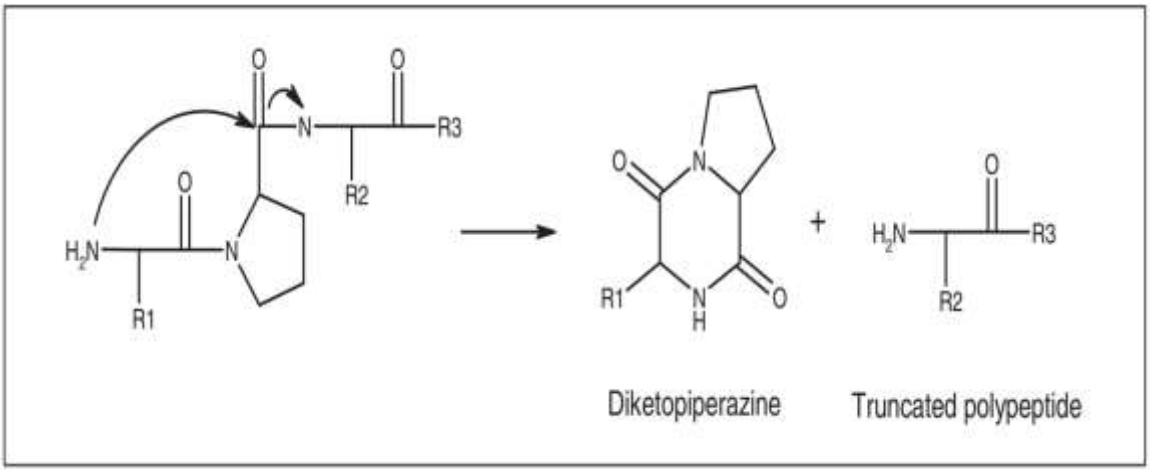

Figure (7): N-terminal Degradation (7)

3.2.2 Oxidation

Oxidation is one major chemical degradation pathway for biopharmaceuticals. The reaction involves reactive oxygen species such as hydroxyl radical (OH), hydrogen peroxide (H_2O_2), superoxide (O_2), and singlet oxygen ($1O_2$), which can be generated by autoxidation, photo-activation, and metal catalyzed oxidation. Autoxidation refers to the oxidation in the sheer absence of oxidants. True autoxidation, being characterized by extremely slow reaction rate, is normally not regarded as an important degradation pathway for proteins (52/53).

Oxidation can be catalyzed by various external factors, such as the presence of transition metal ions and ultraviolet (UV) irradiation (52/53) among them, Fenton-type metal catalyzed oxidation is the most relevant generating source of reactive oxygen species in pharmaceutical formulations where contaminating peroxides may react with traces of redox-active transition metals such as iron or copper. (52/53)

3.2.2.1 Metal Catalyzed Oxidation

It is known to adversely influence the stability of proteins, peptides, plasmid DNAs, and nucleic acid-derived drugs by inducing conformational and functional changes in these biopharmaceuticals (53/54/55/56) In proteins and peptides where metal catalyzed oxidation is extremely common, the presence of trace metal ions can catalyze oxidation by either directly reacting with the side chains of certain amino acid residues to produce free radicals or complexing with oxygen to produce various reactive oxygen species. Due to site-specificity, a faster oxidation rate is commonly observed for amino acid residue having closer proximity to the metal-binding sites (55). However, site-specific oxidation can be greatly reduced by replacement of the existing amino acid residues with their larger, bulkier, or charged counterparts, as in the case of condon-222 mutant subtilisin (57).

3.2.2.2 Specific Oxidation Reactions in Proteins and Peptides

In general, the amino acid residues exposed on the surface of proteins are more susceptible to oxidation than those buried within the hydrophobic core. For instance, different methionine residues in proteins can exhibit substantial differences in oxidation rate, as observed for human growth hormone (58) granulocyte colony-stimulating factor (59) and human parathyroid hormone (60) Such differences in methionine reactivity are consistent with the location and solvent accessibility of the methionine residues. Oxidation in proteins and peptides are highly amino acid specific. The amino acid residues that are susceptible to oxidation generally fall into two groups, those containing a Sulphur atom (methionine, cysteine) and those with an aromatic side chain (histidine, tryptophan and tyrosine) (61).

3.2.3 Maillard Reaction

It is responsible for the nonenzymatic browning of materials, and has been extensively investigated with food materials, but less so with pharmaceutical substances. The reaction consists of an initial reaction of reducing sugars with amino or free amine groups, followed by Schiff base conversion and the formation of brown pigments via Amadori and Heyns rearrangement

For proteins and peptides, the amino acid residue involved in the reaction is usually lysine because of its free ε-amino group, but other bases such as arginine, asparagine, and glutamine can also react with the sugars (62/63).

It can give rise to significant changes/losses in physico-chemical properties and pharmacological activities of therapeutic protein in addition the presence of particular concern in this regard (the modification of protein antigenicity), which is associated with the formation of advanced glycation end product (AGE) (64).

Glucose, a monosaccharide with reducing property, is particularly reactive toward the amino acid residues in proteins and peptides (65-67) whereas other sugars such

as trehalose and mannitol have very little effect, as attested by the findings of the formulation studies with these sugars on glucagon (68) and relaxin (65).

Sucrose can also cause glycation when it is hydrolyzed to glucose and fructose by heat treatment, as observed for β-lactoglobulin variant A, lysozyme, and rHSA during antiviral heat bioprocessing (65-67).

3.2.4 Covalent Dimerization and Polymerization in Proteins:

The formation of precipitates may be due to covalent polymerization and/or noncovalent aggregation. In covalent polymerization, dimer formation usually precedes the precipitation, and the phenomenon is best illustrated by insulin, which has been extensively reported to form covalent dimers. Covalent dimer is the main degradation product of insulin upon storage.

Covalent trimer, tetramer, oligo-, and polymer formation is also possible with insulin when stored at ambient or higher temperatures. In general, the rate of insulin polymerization is virtually independent of the protein source (e.g., bovine, porcine, and human), but it varies with the composition and formulation of the protein and, for the isophane preparation, with the strength of preparation.

The rate of polymerization is one order of magnitude slower than insulin hydrolysis except for the NPH preparations (68).

The covalent aggregation of insulin can be inhibited by self-association at high insulin concentration (69).

Both deamidation and covalent dimer formation in human insulin solution at low pH seem to originate from a common cyclic anhydride intermediate (70).

Besides Insulin, other biopharmaceuticals known to exhibit covalent polymerization include recombinant tumor necrosis factor-alpha, which forms nonreducible dimers and oligomers (71) and human insulin-like growth factor I, which is prone to covalent aggregation (72).

4. The most important factors that must be controlled in storage of protein products

4.1 Temperature

The stability of biopharmaceuticals is highly temperature dependent. Multistep reaction pathways are particularly common in biopharmaceuticals because of the existence of higher order structures. For instance, with recombinant bovine granulocyte colony stimulating factor, a reversible equilibrium between the native protein and an intermediate state is established, followed by irreversible aggregation Although individual denaturation steps seem to obey the Arrhenius law, the overall kinetic behavior for product formation may not (73/74).

Thus, for protein pharmaceuticals, extrapolated shelf-life prediction from accelerated stability test must be viewed with caution and real-time stability monitoring should also be conducted whenever feasible. As many therapeutic proteins are formulated in aqueous solution and stored at 2–8°C, they are liable to cold denaturation at such low temperatures and possibly to freezing/thawing inactivation arising from temperature fluctuation during storage or handling. When a protein solution freezes, water crystallizes out as solid ice and the solution becomes more concentrated in the protein and salt components present, causing dramatic changes in pH and ionic strength and hence protein inactivation (76) For instance, during freezing of sodium phosphate solutions, crystallization of the disodium salt can reduce the pH by as much as 3 units (77).

The effects of freezing and thawing rates on the stability of model proteins have been investigated in the absence of cryoprotectants, and higher activity recovery at a slower freezing rate and a faster thawing rate has been observed.

During fast freezing, small ice crystals are preferentially formed and the associated increase in total surface area of the ice–liquid interface will enhance the exposure of protein molecules to the interface and increase the protein damage.

Upon thawing, additional damage to proteins may be caused by recrystallization (78).

Many biopharmaceuticals, including recombinant human interferon-γ (77) and tetanus toxoid conjugate vaccines (78) show a reduction in activity upon freezing/thawing. To circumvent the aforementioned protein inactivation problems, various excipients have been used in biopharmaceutical formulations. For example, Tween 80, a polysorbate surfactant, has proved useful for reducing surface-induced protein denaturation during freezing (79) and glycine can also stabilize proteins through the preferential exclusion mechanism (76).

4.2 Moisture

It is well documented to adversely affect the chemical stability of solid biopharmaceuticals. The presence of water enhances the mobility and flexibility of the bioactive macromolecules in solid excipient matrix, thereby facilitating their rearrangement and increasing their susceptibility to chemical degradation. For instance, the deamidation rate of an asparagine containing hexapeptide in lyophilized poly(vinyl alcohol) (PVA) and poly (vinyl pyrrolidone) (PVP) increases with increasing moisture uptake and water activity of the system . The asparagine deamidation seems to correlate closely with the extent of water-induced plasticization of PVA and PVP matrices (as determined by the respective glass transition temperature *Tg*), suggesting that the chemical stability of the peptide may be predicted by the physical state of the formulation (80).

The presence of moisture can also reduce physical stability. The mechanisms of moisture-induced aggregation are well documented and have been exemplified by a good number of solid biopharmaceuticals, including salmon calcitonin spray dried powders for inhalation (81) and recombinant human albumin (82).

To prevent such protein aggregation, excipients are often employed in protein formulation. The excipients, which comprise mostly sugars such as mannitol,

lactose and trehalose which prevents protein aggregation by occupying the water-binding sites of protein in the dried state (83).

However, these sugar excipients may also present formulation problems, notably sugar crystallization at high excipient-to-protein ratios (83) and phase separation due to prevalent sugar–sugar interactions (84).

The conventional wisdom of moisture content control for biopharmaceuticals is "the drier, the better." As has been demonstrated with a lyophilized humanized monoclonal antibody formulation, moist cakes tend to have higher aggregation rates than drier samples if stored above their *Tg* (85) However, excessive moisture removal can destabilize the protein, as observed with bovine immunoglobulin (86).

4.3 Light and Ionizing Radiation

Radiations of different wavelengths, i.e., UV-visible light and x-ray, are known to induce degradation in proteins.

Radiation, which is commonly employed for sterilization purpose, can generate free radicals and lead to peptide chain cleavage and aggregation in solid protein pharmaceuticals.

In aqueous solution, the protein may also degrade through the destruction of the amino acid residues by the hydroxyl radicals and electrons produced from water molecules (87).

Although amber glass containers can be used for shielding against UV light, their utility in light protection is often limited by the glass thickness. Light-induced degradation or photo degradation can be minimized by formulation into solid dosage forms. By formulating into a lyophilized form, recombinant human factor VIII showed no significant loss of activity after accelerated photostability testing, whereas the reconstituted preparation displayed partial activity loss after similar light exposure, and the observed photodegradation could be effectively prevented by packaging the protein in tinfoil wrap (88).

4.4 Physical Stress

Biopharmaceuticals may be inactivated by physical stress associated with vial filling, shipping, storage, and handling. Insulin is a well-known example of agitation-induced instability, and patients are advised to avoid vigorous shaking of the insulin preparation. Shaking is known to accelerate the degradation of insulin by way of covalent dimerization (89).

Apart from insulin, monomeric recombinant human growth hormone has been shown to aggregate rapidly within 10 hours of shaking (90) The presence of Tween 20, a nonionic surfactant, at an excipient: protein molar ratio larger than 4 effectively inhibited this aggregation. Similar aggregation was observed with IgG1- antibody upon the application of physical stress. However, differences in aggregation mechanism exist among different mechanical stress methods (e.g., shaking and stirring) (91).

Moreover, the aggregation kinetics is dependent on the shear rate applied, and trimer formation is particularly evident at a high shear rate, as observed for human serum albumin (92) even simple vortexing for just 1 minute can cause substantial aggregation and precipitation, as shown for recombinant human growth hormone (93).

4.5 pH

At extreme pH values, far away from isoelectric point of proteins, electrostatic repulsions between like charges in proteins increase, resulting in a tendency to unfold (94) The process of unfolding leads to a reduction of charge density, thus lowering electrostatic free energy (95).

A minor factor contributing to the unfolding tendency is the decreased capability of salt bridge formation at extreme pH values. In addition, the pKa values of charged groups in the folded state are different from those in the unfolded state. Changes in pH lead to titration of groups only in the unfolded form, which causes destabilization of overall native protein structure (96).

pH-dependent unfolding has been found to be due only to a small number of groups with anomalous pKa's (97) These ionizable groups with anomalous pKa's may stabilize or destabilize a protein in different pH ranges, as those in hen egg-white lysozyme (98).

Proteins are often stable in a narrow pH range such as pH 6.5–7.0 for recombinant factor VIII SQ (FVIII SQ) (99) 6–7 for low molecular weight urokinase (LMW-UK, 33 kD) (109) and 4.5–5 for relaxin (100).

Protein refolding to a wrong conformation is often seen at a pH close to its isoelectric point (101) pH-induced denaturation of proteins can be reversible, such as porcine pancreatic elastase, which denatures on lowering the pH to less than 5.0, but renatures completely by immediately adjusting the pH back to 5.0 (102).

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