Macedonian pharmaceutical bulletin, **51**, (1,2) 3-8 (2005)
ISSN 1409 - 8695
UDC: 577.112

Review

Instabilities of proteins: theoretical aspects, degradation products and methods for their detection

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Received September 2005, accepted December 2005

Abstract

Recombinant DNA technology has led to a significant increase in the number of peptide and protein based pharmaceuticals, giving a new approach to combat poorly controlled diseases. This particular development has been reached in the last two decades. However, proteins are highly susceptible of physical and chemical degradation resulting in a decrease or complete loss of biological activities. Reasons for their physical and chemical instabilities and the methods for their examination, become a challenge for the pharmaceutical scientists for successful development of stabile protein - based pharmaceuticals.

The stability of protein - based pharmaceuticals is significant in terms of their pharmaceutical quality and biological activity. In addition, a right choice of suitable analytical methods is needed in order to detect an early formation of degradation products or modified forms.

Key words: proteins, physical instability, chemical instability, methods

Inroduction

During the past two decades, peptides and proteins have become an important class of potent therapeutic drugs. They have gained significant importance in the treatment of several severe diseases including autoimmune diseases, memory impairment, hormonal disorders, organ transplantations and different cancers. Major advantages of protein drugs are both their extremely specific activity and their high tolerability (1).

While recombinant DNA technology, which is providing exciting opportunities for new pharmaceutical development and new approaches to the diagnosis, treatment and prevention of diseases, has led to a significant increase in the production of peptides and proteins for pharmaceutical purposes, this has not been matched by the number of peptide and protein based drugs available on the market. The reasons for that are multiple: proteins highly susceptibility to chemical and physical degradation, which is associated with relevant difficulties in purification, storage and delivery, multifaceted metabolic properties, variable tissue penetration and toxicity related to the stimulation of the immune or allergic reaction (2, 3, 4).

The structure and function of proteins is determined by their amino acid sequence which defines the peptide backbone, *the primary structure*. In spite of the enormous number of naturally occurring proteins, a mere of 20 amino acids construct proteins. The vast difference in the three-dimensional structure and, therefore, also in protein function originates solely from a different amino acid sequence, i.e. the unique structure of a protein is determined by the chemical and physical properties of the amino acids aligned within the protein sequence. *The secondary structure* describes the folding or the shape of the polypeptide chains into regular, ordered structures like α-helices and β-sheets. Furthermore, areas with increased flexibility – the so-called turns or loops – are to be subsumed in this level of protein structure. The domains of the secondary structure and all noncovalent interactions such as hydrogen bonds and hydrophobic, electrostatic, or van der Waals interactions generate the intrinsic, three-dimensional arrangement of a protein, *the tertiary structure*. Some proteins consist of several

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polypeptide chains. *The quaternary structure* characterises the non-covalent interactions binding these chains into a single protein molecule (5). For example, haemoglobin consists of four polypeptide chains, which are associated by one Fe^{2+} ion. The retention of the tertiary structure is deemed the primary requirement for the biological activity of protein molecules. However, the biochemical and structural complexity of these molecules is the reason for proteins to react sensitive to even marginal changes in their natural environment (6).

In the context of protein structure, the term stability can be defined as the tendency to maintain a native (biologically active) conformation.

As proteins and peptides continue to enter the pharmaceutical market, their stability becomes a pressing issue for the pharmaceutical scientists. Native proteins are only marginally stable and highly susceptible to degradation, both chemical and physical (7-9).

Chemical instability refers to the formation or destruction of covalent bonds, within a protein molecule, i.e. some amino acid side chains are chemically reactive, whereas others are chemically inert. It has been demonstrated that „labile" amino acid residues are susceptible to covalent modifications via bond formation or cleavage through nonenzymatic reactions, including hydrolysis, deamidation, oxidation, racemisation, β-elimination and cystine destruction/disulfide exchange (10, 11). These changes alter the primary structure of the protein, and impact higher level of its structure.

Physical instabilities include aggregation and precipitation, and adsorption to surface (7- 9, 12,). Chemical instabilities such as deamidation and disulphide bond cleavage, may also lead to physical instabilities, and vice versa (6, 11).

The primary focus of this minireview is to present and discuss the main reasons for the most common physical and chemical instabilities and the methods for their detection. It is important to remember that every protein is unique, both physically and chemically, and therefore exhibits unique stability behaviour.

Denaturation

The loss of tertiary structure, and frequently also of secondary structure is generally referred to as denaturation of the protein. Perturbation of secondary or tertiary structure can lead to exposure of previously buried amino acid, facilitating its chemical reactivity; thereby leading to loss of its native or original characteristics. Denaturation can be caused by destabilizing agents such as excipients (reducing sugars, antioxidants, surfactants, metal ions), heat, hydrolysis by strong acid or alkali, enzymatic action, exposure to urea or other substances, or exposure to ultra violet light. Excipients like reducing sugars can react with protein amino groups to form schiff's bases (Maillard reaction) which can be shown by LC/MS (13). Antioxidants may contain reducing agents that will destroy disulphide bonds. Several surfactants like Tween 20 and Tween 80 can cause oxidation of aminoacid due to residual peroxides present in these materials, which can be detected by RP-HPLC (14). Many chemical reactions involving polypeptides and proteins are catalyzed by metal ions such as Zn^{2+}, Cd^{2+}, Pb^{2+}. At high temperature (80-100°), aspargine and glutamine are susceptible to deamidation, Aspartate-Xaa peptide bonds are susceptible to hydrolysis, disulphide bonds rupture, and Xaa-Prolin peptide bonds undergo cis-trans isomerisation (where Xaa is any amino acid). High temperature can result in physical degradation due to irreversible denaturation. Residual moisture can be responsible for protein instability in the solid state. pH has also strong influence on denaturation and aggregation rate (15, 16).

The folded state of proteins is connected to conformational stability, which is expressed as the free energy change ΔG during the unfolding/denaturation reaction under physiological conditions (17). The higher the ΔG value, the greater the stability of the protein. However, the reported ΔG values for proteins of 45 +/- 15 kJ/mol indicate that the folding state is only marginally more stable than the denatured state (18). As a matter of fact, the conformational stability of a protein in aqueous solution tallies with only a few hydrogen bonds or ion pairs (2). Simplified, protein denaturation can be described as:

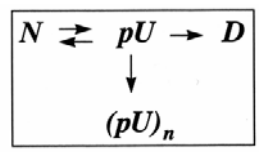

Figure 1: Equation of the native and denatured state of a protein

The native state N exists in an equilibrium with a partially unfolded state pU. This unfolding of the native protein can be reversible, e.g. an increase of the temperature causes unfolding, which can be reversed by a subsequent temperature decrease (7). Generally, the loss of the tertiary structure implies an increase in the protein molecule's reactivity, which results in a decrease in their stability. Hydrophobic regions, which were accumulated in the core of the folded protein, are then exposed to surrounding solvents. As a consequence, side reactions can now lead to an irreversible, denatured state D. Alternatively, partially unfolded proteins may encounter irreversible aggregation (pU)n. Constantly elevated temperatures, extreme pHs, the formation of interfaces during shaking, shearing, adsorption to hydrophobic surfaces, high pressure, and denatu-

rants such as urea and guanidine hydrochloride foster irreversible transitions (2,19-23).

Physical instability

Physical instability of proteins denotes that these super structured molecules can undergo changes independent of any chemical modification, i.e. includes the reactions that do not involve the formation or cleavage of covalent bonds.

Aggregation and precipitation

Protein aggregation is arguably the most common and troubling manifestation of protein instability, encountered in almost all stages of protein drug development, during refolding, purification, sterilization, shipping and storage processes (24).

The aggregation of protein molecules into non-native assemblies in vivo can have profound pathological implications, as in the aggregation of β-amyloid proteins in Alzheimer's disease and the aggregation of prion protein in numerous neurodegenerative diseases (24-26). The presence of aggregates in therapeutic protein pharmaceuticals can cause adverse effects within patients, ranging from immune response to anaphylactic shock (27, 28).

Protein aggregation is defined as the association of at least two denatured protein molecules. With increasing numbers of molecules the solubility of these species will decrease, eventually resulting in the precipitation of the protein. Aggregation can occur even under conditions where the proteins native conformation is favored thermodynamically compared to the unfolded state and at concentrations well below the proteins solubility limit (24, 29, 30). To effectively inhibit aggregation, both in vivo and in vitro, a more complete understanding of the mechanisms by which proteins aggregate is needed (31).

Mechanisms of protein aggregation are still not fully understood. One plausible mechanism is that aggregation is catalyzed by the presence of a small amount of a contaminant. That contaminant could be a damaged form of the protein product itself, host cell proteins, or even non-protein materials such as silica particles (32). Damaged forms of a protein product can arise from chemical modification (such as oxidation or deamidation) and from conformationally damaged forms arising from thermal stress, shear, or surface-induced denaturation. A second mechanism begins with partial unfolding of the native protein during storage. Protein conformation is not rigid – the structure fluctuates around the time-averaged native structure to different extents depending on environmental conditions. Some partially or fully unfolded protein molecules are always present at equilibrium in all protein solutions, but most such molecules simply refold to their native structure. However, those unfolded proteins may instead aggregate with other such molecules or may be incorporated into an existing aggregate nucleus, eventually to form larger aggregates as described above. Factors such as elevated temperature, shaking (shear and air-liquid interface stress), surface adsorption, and other physical or chemical stresses may facilitate partial unfolding. A third aggregation mechanism is reversible self-association of the native protein to form oligomers. The tendency of different proteins to reversibly associate is highly variable, and the strength of that association typically varies significantly with solvent conditions such as pH and ionic strength. Such reversible oligomers often eventually become irreversible (they are a first step along a pathway to irreversible aggregation). Detection of reversible aggregates can be especially challenging. One of the reasons is that such aggregates can dissociate from dilution during the measurement process (33-36).

Regardless of the mechanism of aggregation, preventing aggregation problems requires sensitive and reliable technologies for quantitative determination of aggregate content and aggregate characteristics (37). Size-exclusion chromatography (SEC) has been a workhorse for detecting and quantifying protein aggregation (2, 38-39). Native gel electrophoresis and SDS-PAGE have also been used to observe protein aggregation. Column-free techniques such as analytical ultracentrifugation (AUC), field-flow fractionation (FFF), and dynamic light scattering (DLS) now find increasing application in aggregation analysis (36-37, 40). As it can be concluded, there are various techniques for assessing protein aggregation, which will enable detection of dimmer and aggregate formation at the very beginning. All that is important for pharmaceutical quality and biological activity of protein - based drugs.

Chemical instability

Chemical instability of proteins can be potentiated or induced by temperature, pH, light and composition of the formulation buffer. Such chemical modifications may result in alteration of structure, loss of function, acquired immunogenicity and altered pharmacokinetics.

Oxidation

Methionine, cystine, tryptophan and tyrosine residues are susceptible to oxidation. Air, residual peroxide content, or intense fluorescent light, can convert thioether to sulfoxide, and then sulfone (41-46). The polysorbate-80

and polysorbate-20, which are usually used in the protein formulations like surfactants, have a tendency to produce peroxides, which in turn, can lead to oxidation of the methionine residues. This peroxide contamination appears to have a greater effect on protein oxidation than the presence of atmospheric oxygen in the vial headspace or the effects of product foaming in the fill lines (14). Oxidative modification can be variable (47). Peptide maps are convenient for detecting methionine oxidation, and RP-HPLC is used to separate the oxidized forms. Oxidation causes a protein to become more hydrophilic and oxidized proteins elute before the native form in RP-HPLC (48).

Oxidation occurs in a number of proteins favored by factors like temperature, pH etc. Most proteins lose biological activity when oxidized. Thereby, formulation approaches include addition of anti oxidants (sodium thiosulphate, catalase, or platinum) and adjustment of environmental conditions (pH or temperature) to prevent oxidation (49).

Deamidation

For many proteins deamidation is one of the most often observed stability problems and occurs more rapidly than any of the other degradation routes (14). Deamidation of asparginine residues (glutamine residues to lesser extent) to aspartate or isoaspartate via succinimide intermediates (positive to negative charge) is a major cause of spontaneous degradation and loss of aminoacid sequence homogeneity. Deamidation can make protein prone to proteases and denaturation. This can affect the *in vivo* half-life, activity, and conformation of protein, and also increase the immunogenicity of certain protein. Deamidation often results in the loss of biological activity (50, 51).

The deamidation process is mainly dependent on the storage temperature, on the pH of the formulation buffer and on the sequence and conformation. Deamidation can be detected by isoelectric focussing and quantified by densitometric scanning of the gel and by RP-HPLC (50, 52, 53). A common means of determining deamidation is to digest the protein with tripsin and to look for new peptide fragments eluting slightly later than fragments containing asparagine in RP-HPLC, because under acidic conditions aspartic acid is slightly more hydrophobic than asparagines (50). At neutral pH, fragments containing asparagines elute after the aspartic acid deamidation products because they are less hydrophobic under these conditions (53).

Conclusions

Proteins are only marginally stable and highly susceptible to physical and chemical degradation. Physical and chemical instabilities of proteins are the major road barriers, hindering rapid commercialization of potential protein drug candidates. Thereby, the successful development of stabile protein-based pharmaceuticals is highly dependent on a thorough understanding of their physico-chemical and biological characteristics and of the mechanisms by which proteins stabilizes.

Due to the complexity and heterogeneity of protein structures, multiple analytical methods for stability testing must be employed. Stability indicating test methods must be validated to be suitable to detect potential degradation products or modified forms. Detection of degradation products or modified forms at the very beginning signalling the stability changes important for pharmaceutical quality and biological activity of protein - based pharmaceuticals.

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Резиме

Нестабилност на протеини: Теоретски аспекти, деградациони продукти и методи за нивно детектирање

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Клучни зборови: протеини, физичка стабилност, хемиска стабилност, методи

Рекомбинантната ДНК технологија доведе до значителен пораст на бројот на пептидни и протеински фармацевтски препарати, давајќи нов пристап во третманот на оние заболувања, за кои досега применуваните фармацевтски препарати не даваат задоволителни резултати. Особено брз развој на ова поле е постигнат во последните две децении. Меѓутоа, како проблем се јавува нестабилноста на протеинските молекули. Имено, познато е дека протеините се подложни на физичка и хемиска деградација, што резултира со намалување или целосно губење на нивната биолошка активност. Токму поради тоа, причините што доведуваат до нивна физичка и хемиска нестабилност, како и методите за нивно испитување, претставуваат предизвик за фармацевтите за развој на стабилни протеински фармацевтски препарати.

Стабилноста на протеинските фармацевтски препарати е многу значајна во однос на нивниот фармацевтски квалитет и биолошка активност. Поради тоа, потребен е правилен избор на соодветни аналитички методи кои ќе овозможат детектирање во почетниот стадиум на формирањето на дегдрадационите продукти или модифицирани форми.