Instability of Peptide and Possible Causes of Degradation

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Peptides are different from proteins. Although both are composed of amino acids, peptides are smaller molecules comprised of two or more amino acids linked by peptide bonds, while proteins are long chains of amino acids that may have a much larger number of amino acids. Peptide stability in aqueous solutions is critical when developing parenteral formulations, as the potency of a peptide is often compromised due to chemical or physical degradation pathways.

Keywords: therapeutic peptides ; stabilization formulations ; aqueous solutions

1. Introduction

Peptides can control various physiological processes, functioning as growth factors, neurotransmitters, and endocrine or paracrine signals at other sites of action. In diverse disease areas, such as endocrinology, oncology, hematology, and urology, peptides are used as therapeutic agents [1]. Several antibiotics, antitumor agents, hormones, and neurotransmitters are peptides.

Peptides are different from proteins. Although both are composed of amino acids, peptides are smaller molecules comprised of two or more amino acids linked by peptide bonds, while proteins are long chains of amino acids that may have a much larger number of amino acids. Unlike proteins with a defined tertiary and quaternary structure [2], peptides generally do not have a defined three-dimensional structure. Although peptides are mostly linear and usually do not have as much complexity in their structure as proteins, some can have a defined three-dimensional structure due to the presence of multiple disulfide bridges, hydrogen bonds, and hydrophobic interactions [3][4]. The hydrophobic sides of amino acids in peptides are buried inside their structure and tend to form aggregates. This is because hydrophobic, non-covalent interactions between non-polar or slightly polar molecules cause these side chains to avoid contact with water and interact instead. This tendency to aggregate can also be increased by changes in pH, temperature, ionic strength, and the presence of surfactants or other excipients [5]. Furthermore, their functionality in living organisms is different. While proteins usually act as structural and regulatory molecules [6], peptides regulate a broad spectrum of biological effects, including proteins [7][8]. Making a clear distinction between peptides and proteins based on the number of amino acids is challenging, and several definitions exist. First, the United States Food and Drug Administration defines peptides as short chains that contain less than 40 amino acid residues [9]. Malavolta [10] provides a similar definition, defining molecules containing 50 amino acid residues or more as proteins. Between them is a category called polypeptides that have 40–49 residues. Furthermore, Forbes [11] defines peptides as a short string of 2 to 50 amino acids, where oligopeptides contain between 10 and 20, and polypeptides contain more than 20 amino acids.

2. Instability of Peptide and the Possible Causes of Degradation

Peptides may be able to undergo several degradation pathways. Peptide degradation can occur through chemical and physical mechanisms. Chemical instability involves processes that alter the peptide by creating or breaking covalent bonds, leading to the formation of new chemical entities [12]. Oxidation, hydrolysis, β-elimination, deamidation, racemization, isomerization, and disulfide exchange are examples of chemical instability pathways [13][14]. Physical instability refers to structural changes in non-covalent interactions of the peptides and includes changes in secondary structure, adsorption, aggregation, and precipitation [15].

2.1. Hydrolytic Pathways

2.1.1. Chain Cleavage of the Peptide Backbone

Hydrolysis represents one of the main degradation pathways of peptides. Generally, hydrolysis is catalyzed by Bronsted acids and bases [16] and strongly depends on the pH. This pH dependency has been extensively investigated for the peptides gonadorelin and triptorelin. These peptides undergo acid-catalyzed hydrolysis at pH 1–3 through deamidation of the C-terminal amide. At pH 5–6, however, the peptide backbone can undergo hydrolysis at the N-terminal side of the serine (Ser) residue. This process is likely facilitated by the hydroxyl group on the Ser side chain, which acts as a nucleophile by attacking the adjacent amide bond. As a result of this reaction, a cyclic intermediate is formed, which ultimately leads to the fragmentation of the peptide [17][18]. At pH > 7, the primary degradation pathway of gonadorelin and triptorelin are base-catalyzed epimerization. The epimerization reaction most likely involves Ser via a carbanion intermediate. Gonadorelin and triptorelin have the capability to create hydrogen bridges in a relatively stable six-membered intermediate, which elucidates the reason for the Ser residue's relatively high rate of racemization in comparison to other amino acids. Apart from epimerization, the hydrolysis of gonadorelin and triptorelin under base-catalyzed conditions has also been detected [17][19][20]. Recombinant Glucagon-like Peptide-1 (r-GLP-1) has also been reported to undergo base-catalyzed racemization because of extreme pH exposure during purification that can impact its impurity profile and yield of bulk rGLP-1 [21]. The primary degradation route of recombinant human parathyroid hormone (rhPTH) occurs via cleavage at the aspartate (Asp) residue under acidic conditions. Conversely, when the pH is above 5, asparagine (Asn) deamidation is the primary degradation route [22]. The cholecystokinin peptide tends to undergo C-terminal and N-terminal cleavage as the primary degradation pathways when it is subjected to non-isothermal conditions [23].

Somatostatin and its analog octastatin have also been observed to undergo acid/base-catalyzed hydrolysis in aqueous formulations, with the rate of hydrolysis being influenced by the buffer species [24][25]. Octastatin, for example, experiences a higher degradation rate in a phosphate buffer than in a glutamate buffer solution, likely due to a catalytic effect of phosphate ions [24]. It appears that increasing phosphate concentration results in much faster degradation of octastatin. Conversely, increasing the concentration of glutamate in a buffer solution enhances the stability of the solution, as evidenced by hydrophobic and ionic interactions between glutamate and octastatin [22]. These findings underscore the significance of selecting appropriate buffer species and their concentrations when formulating peptides.

2.1.2. Deamidation of Asn and Gln Residues

Peptides containing glutamine (Gln) and Asn residues are susceptible to deamidation, leading to the formation of Glu and Asp, respectively, under physiological conditions. When the pH is lower than 3, Asn residues deamidation occurs primarily through the direct hydrolysis of the Asn amide side chain to generate Asp. Likewise, Gln residues undergo acid-catalyzed direct hydrolysis to form Glu [12]. Asn deamidation mostly transpires via a cyclic imide intermediate that forms through an intramolecular reaction where the amino acid residue's nitrogen next to Asn attacks the carbonyl carbon on the side chain of the Asn residue. Thus, the rate of deamidation through this pathway depends on the carboxyl-side amino acid residue's nature [26][27][28]. Under similar conditions, the deamidation of Gln residues proceeds much slower than the deamidation of Asn, because the cyclization of Asn residues into a five-membered ring is kinetically more favorable than the formation of a six-membered ring intermediate in Gln deamidation [12].

Peptide chain flexibility strongly favors a high rate of Asn deamidation [29]. The amino acid sequence in the peptides can also affect the rate of deamidation [30]. Amino acid residues following Asn, such as threonine (Thr), Ser, and Asp, may substantially increase the reaction rate since they are very susceptible to dehydration, forming a cyclic imide intermediate [5].

At alkaline and neutral pH, adrenocorticotropic hormone (ACTH), was shown to degrade via deamidation of its single Asn residue [26][31]. Asn or Gln deamidation was also observed for salmon calcitonin (sCT) under acidic conditions [32]. Oxytocin provides another instance of a peptide that can be subjected to Asn [33] and Gln [34] side chain amides deamidation through hydrolysis. Additionally, oxytocin's C-terminal glycine (Gly)-NH has been reported to undergo deamidation at pH 2 [35].

2.1.3. Isomerization of Asp Residues

The Asp transformation into isoAsp follows the equivalent succinimide ring intermediate as reported for Asn deamidation [36][37]. Moreover, racemization of L-succinimide into D-succinimide can produce D-Asp and D-isoAsp enantiomers [27][38]. The rate-limiting step for the isomerization of Asp and Asn deamidation reactions at physiological pH is the formation of the succinimide intermediate [39]. Isomerization of the Asp-hexapeptide into the isoAsp-hexapeptide through cyclic imide intermediate was also reported to be pH dependent [40].

2.2. Oxidative Pathways

Peptide oxidation is a reaction that increases the electronegative atom content in a peptide molecule [41], where oxygen or halogens are typically the electronegative heteroatoms [42]. Sulfur-containing residues such as Met and Cys are particularly susceptible to oxidation because sulfur atoms are highly reactive and can easily lose electrons, forming sulfur radicals when exposed to reactive oxygen species (ROS). Aromatic residues such as His, Trp, and Tyr are also prone to oxidation because the aromatic rings in these residues contain multiple carbon-carbon double bonds that are easily oxidized by various ROS [43].

Oxidation can be induced by contaminating oxidants, trace amounts of catalytic redox-active metals, and light exposure. Moreover, peptide oxidation may be affected by pH, temperature, and buffer composition [5]. Deprotonation of the mercapto group of Cys [44] and the phenoxy group of Tyr accelerates oxidation of these residues [45]. Deprotonation of the imidazole side chain of His favors metal binding and, potentially, oxidation [46].

2.2.1. Autoxidation

Frequently, the oxidative degradation of pharmaceuticals is referred to as “autoxidation”. However, the term “autoxidation” denotes “the spontaneous oxidation in an air of a substance not requiring catalysis” [47]. Hence, if peptides were to autoxidize, this would require the reaction of amino acids with molecular oxygen. In general, the reaction of “closed-shell” (i.e., non-radical) organic substances with oxygen is relatively slow [48], and it is unlikely that autoxidation contributes significantly to peptide oxidation except, perhaps, to the oxidation of the mercapto group of Cys under the condition that chain oxidation is possible. Conditions for the chain oxidation reaction of dithiols (i.e., dithiothreitol) have been defined by radiation chemical techniques [49].

2.2.2. Metal Induced Oxidation

Metal ion-catalyzed oxidation for peptides refers to the process by which metal ions can promote the oxidation of specific amino acid residues in peptides. This process usually requires the presence of a redox-active transition metal such as Fe^{2+} and Cu^{2+} that can undergo redox cycling reactions and produce ROS. In metal ion-catalyzed oxidation, metal ions act as catalysts, accelerating the conversion of hydrogen peroxide, superoxide anion radical, and hydroxyl radical. These hydroxyl radicals can then react with amino acid residues in peptides, causing degradation. Specifically, metal ion-catalyzed oxidation can cause oxidative damage to amino acid residues such as histidine (His), cysteine (Cys), and methionine (Met) [50]. Metal ion-catalyzed oxidation frequently implies a site-specific reaction catalyzed by transition metals complexed by metal-binding sites. Hence, metal ion-catalyzed oxidation frequently does not target the most solvent-accessible amino acids, but rather amino acids which are part of or are located close to metal ion-binding sites [51]. It was reported that the oxidation of hPTH (1–34) by ferrous ethylenediaminetetraacetic acid (EDTA)/H_2O_2, found that this system can cause oxidation of the methionine residue at position 8 (Met 8) and the histidine residue at position 9 (His 9) (1–34). The study found that the oxidation of Met 8 and His 9 in hPTH (1–34) resulted in the formation of sulfoxide and imidazole-5-aldehyde products, respectively. The oxidation of Met 8 was found to be highly selective, as this residue was oxidized much more rapidly than other methionine residues in the peptide. The oxidation of His 9 was also found to be highly selective, as other histidine residues in the peptide were not oxidized under these conditions. The study suggested that the oxidation of Met 8 and His 9 in hPTH (1–34) by ferrous EDTA/H_2O_2 may be relevant to the physiological and pathological roles of this peptide. For example, the oxidation of Met 8 may affect the biological activity of hPTH (1–34), as this residue is important for binding to the PTH receptor. The oxidation of His 9 may also affect the conformation of the peptide, as this residue is located near the N-terminus of the peptide and plays a role in stabilizing the peptide structure [52].

2.2.3. Light-Induced Oxidation

Light-induced oxidation usually affects peptides that contain aromatic amino acid residues such as Trp, Tyr, and Phe, or a disulfide bond [53]. The mechanisms of light-induced oxidation are complex and not completely understood. While much emphasis has been placed on the primary photophysics and photochemistry of Trp, Tyr, Phe, and cystine, secondary reactions can induce the formation of a large variety of products [54]. The photo-irradiation of Trp can lead to photoionization as well as the formation of singlet oxygen. Photoionization is associated with the release of an electron, which can react with suitable electron acceptors such as oxygen (to yield superoxide) or disulfides (to yield thiolate and thiyl radical) [55]. Similar mechanisms have been reported for Tyr and Phe, though photoionization may be a biphotonic rather than monophotonic process. The biphotonic process is initiated by two-photon absorption, whereas the monophotonic process involves a single photon. Oxytocin was reported to be sensitive to U.V. light at pH 4.0–5.0 and 7.0–8.0 [56]. Recently, a series of papers have focused on near U.V. and visible light-induced photo-oxidation of peptides

promoted by ligand-to-charge-transfer (LMCT) pathways of iron-buffer complexes [57][58][59]. These processes yield multiple reactive species and peptide oxidation products at relatively low light doses.

2.2.4. Peroxide Oxidation

Peroxide can cause the oxidation of amino acid residues including Met [60], Cys [61], and His [62], as well as the formation of hydroperoxides on amino acids and polypeptides during oxidative stress, which can potentially lead to biological damage. Accidentally, peroxide may be present in formulations due to the inclusion of surfactants or other excipients. For example, some surfactants, such as polysorbate 20 and polysorbate 80, can produce peroxide [63]. Therefore, surfactants or co-solvents such as polyethylene glycol usually have certain specifications related to the levels of peroxides.

2.3. β-Elimination

A disulfide bond of a peptide can undergo β-elimination leading to C-S cleavage, resulting in perthiolate/perthiol and dehydroalanine. It is frequently observed when materials are subjected to high temperatures in conjunction with a high pH environment. Cys and Ser-containing peptides undergo β-elimination at alkaline pH [64][65]. Even at neutral pH, when cystine-containing peptides are heated at 100 °C, they initially form perthiol and then convert to free thiols [66]. sCT degrades through β-elimination at the disulfide bridge between the Cys residues at positions 1 and 7. It has also been reported that the insertion of an additional sulfur forms a trisulfide and tetrasulfide bridge because of a β-elimination reaction [67]. It has also been observed in oxytocin after exposure to heat stress at an alkaline pH [35]

2.4. Disulfide Exchange

Disulfide exchange reactions can occur in peptides, leading to disulfide scrambling and contributing to forming dimers and larger aggregates. An investigation on the degradation of sCT recognized dimeric products generated through disulfide exchange reactions. However, dimers linked to disulfides can go through further disulfide reactions, ultimately regenerating monomers of sCT [67]. In an acidic aqueous solution, disulfide interchange can continue through the formation of sulfonium ions [68]. When disulfide bonds are subjected to hydrolysis, sulfenic acid intermediates are formed, which can further react with other cysteine residues or with water to produce sulfonium ions. These ions can then undergo disulfide interchange reactions, leading to the formation of new disulfide bonds between cysteine residues. There have been several studies conducted on disulfide exchange reactions and the significance of disulfide bridges in maintaining peptide stability. Several investigations have highlighted the importance of disulfide bonds for peptide stability and the impact of disulfide exchange reactions on peptide conformation and function. By developing strategies to stabilize disulfide bonds and prevent disulfide exchange reactions, researchers can improve the stability and bioactivity of peptides for use as therapeutic agents [64][67][68][69][70][71].

2.5. Dimerization, Aggregation, and Precipitation

Apart from intermolecular disulfide bond formation, peptides can dimerize/oligomerize via a series of oxidative reactions [15][72][73]. Some of these processes may even lead to larger aggregates. In addition, stress conditions, such as freezing, heating, or agitation, may induce aggregation. Aggregates can form through covalent bonds; such as dityrosine, ester, disulfide, or amide linkages; or electrostatic interactions or non-covalent bonds that occur through hydrophobic interactions. However, during sample preparation, relatively weak non-covalent bonds may be disrupted again, leading to incorrect results [74].

The formation of aggregates on peptides is not limited to a single pathway [12]. Instead, multiple mechanisms can occur concurrently, leading to the formation of both soluble and insoluble aggregates [75]. Aggregation occurs when peptides interact with each other to form larger, multi-molecular species, which can have altered conformation, solubility, and biological activity. At higher concentrations, peptides are more likely to interact with each other due to increased intermolecular forces, resulting in faster aggregation. As aggregation proceeds, the peptides can become more insoluble and eventually precipitate out of solution [76]. In addition to precipitation, higher concentrations of peptides have been reported to form gel-like aggregates. Calcitonin, deterelix, leuprolide, and β-amyloid peptide are examples of peptides that are capable of forming gel-like aggregates under certain conditions [77]. Gel-like aggregates form because the structure shifts from an α-helix or β-turn structure to a β-sheet structure. As a result, they have strength, elasticity, and plasticity that can maintain their shape.

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