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Review on characterization of deamidation in therapeutic proteins by mass spectrometric methods

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Abstract

Deamidation is a critical post-translational modification that influences the stability, structure, and function of therapeutic proteins, impacting their efficacy in clinical applications. This spontaneous process, primarily affecting asparagine (Asn) and glutamine (Gln) residues, leads to the formation of aspartic acid (Asp) and glutamic acid (Glu), respectively, and is influenced by environmental factors such as pH, temperature, and ionic strength. Understanding deamidation is essential, particularly in monoclonal antibody (mAb)-based therapies, where structural integrity determines therapeutic performance. Mass spectrometry (MS) has emerged as a powerful tool for deamidation analysis due to its sensitivity, accuracy, and ability to characterize complex protein modifications. Various MS approaches, including top-down, middle-down, and bottom-up methods, facilitate the identification and quantification of deamidation events. Additionally, reversed-phase liquid chromatography (RPLC), often integrated with MS, supports therapeutic protein analysis but faces challenges such as peak broadening, secondary interactions, and protein denaturation. Recent advancements in proteomics and MS techniques continue to refine our understanding of deamidation, offering new insights into protein stability, aging-related modifications, and disease mechanisms. This review aims to provide a comprehensive perspective on deamidation studies, emphasizing its implications for biopharmaceuticals and therapeutic protein development.

Keywords: Deamidation, therapeutic proteins, monoclonal antibodies, protein stability, mass spectrometry (MS), proteomics, post-translational modifications (PTMs), reversed-phase liquid chromatography (RPLC), top-down MS, bottom-up MS, middle-down MS.

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Introduction

Studies related to therapeutic proteins and deamidation have been significant for years. Therapeutic proteins are used in diverse clinical settings including cancer, inflammatory diseases, kidney transplantation, cardiovascular medicine, and infectious diseases[1]. Deamidation is the most common chemical reaction that has an effect on the charge of protein changing the stability, the structure, or the natural function. In this reaction, an amide group is removed leading to degradation of the protein because it damages the amide-containing side chains of the amino acids. Also, it can change the hydrophobicity, leading to aggregation of a drug product at low pH[1]. It happens spontaneously on asparagine (Asn) in acidic conditions to produce aspartic acid (Asp) and Glutamine (Gln) residues to produce glutamic acid (Glu) in alkaline conditions [2]Figure 1. This process is faster on Asn than Gln and during the reaction, the covalent amide functional groups are converted to carboxylic acid[3,4]. High temperature and ionic strength anticipate the deamidation rate[5]. The characterization by mass spectrometric techniques has been used to understand the biological role of therapeutic protein deamination in different diseases [6]. MS is important for all deamidation studies of proteome due to its high sensitivity, speed, and specificity [7]. New advances in preparation methods, and MS techniques, have enabled the characterization of protein modifications. This fact depends on the process development of how deamidation occurs in all the proteomes of human cells and tissues[8,9]. Therefore, this knowledge will enable a better understanding of how deamidation contributes to the pathology of biological aging and major degenerative diseases. Due to the increasing of proteomics tools and structural MS approaches, we hope that this review will be helpful in providing a wide perspective on deamidation studies[10,11,12].

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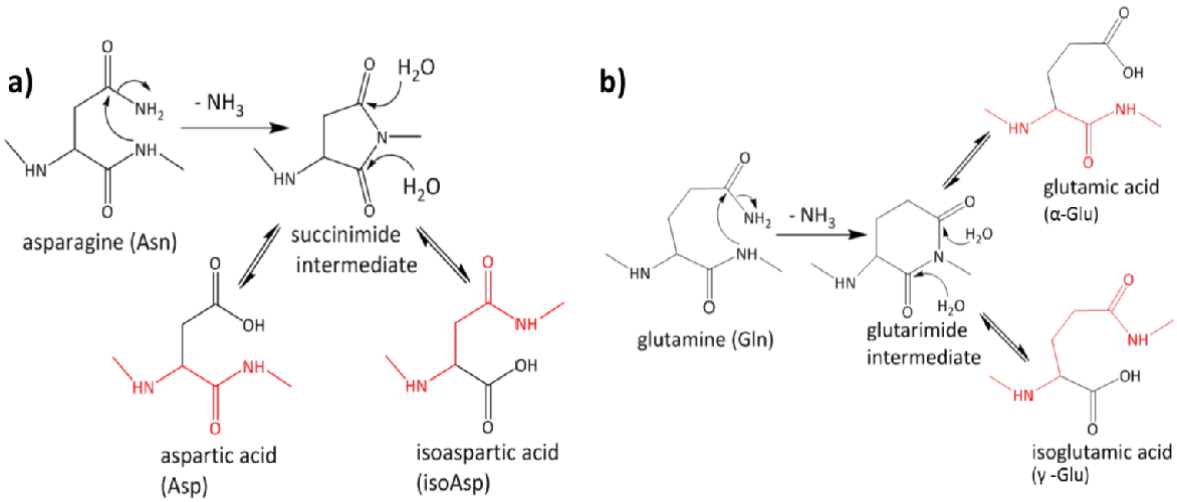

Figure 1 a) deamidation of Asn and isomerization of Asp to isoAsp via hydrolisis of the succinimide intermediate and b) deamidation of Gln [2]

Theoretical Part

The deamidations in peptides and proteins have different stages and prediction of deamidation remains challenging[4]. We could say that the biological effects of protein deamidation could not be totally negative, because this process is known as a mechanism for regulating biomolecule longevity and duration of principal processes[12]. To have a better understanding of the biological roles of protein deamidation in aging and diseases, MS has been used in the identification of deamidated protein species, and deamidation states of protein including therapeutical monoclonal antibodies (mAbs)[12]. mAbs are immunoglobulins (IgG) attached to a specific protein of the pathogens or abnormal cells and inactivate their ability to bind or invade new cells. Many of these mAbs are used to treat cancer and infectious viral and bacterial diseases. This treatment is a targeted therapy with fewer side effects and more specificity[14]. Before MS in deamidation studies takes place, it is better to continue the identification of deamidated species by electrophoresis or chromatography based on band or peak shifts between deamidated and non-deamidated species. According to recent publications, three main MS approaches exist, top-down, middle-down, and bottom-up. All methods can study protein deamidation with high sensitivity, throughput, and accuracy. The advances in top-down and middle-down MS methods help to find new strategies for protein deamidation analysis and lead to new knowledge for the biological significance of protein deamidation during diseases[6]. The recent science field that provides the study of proteins and their reactions and interactions in

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organisms is called Proteomics, in which the mass gives information on the protein identity, its chemical modifications, and its structure[15] Figure 2.

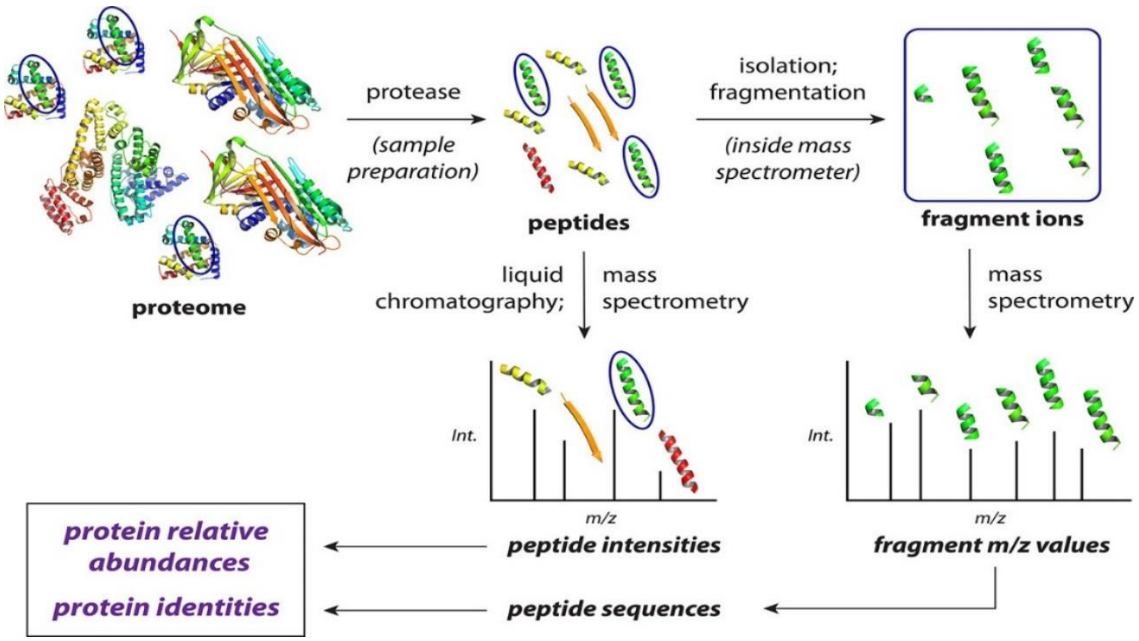

Figure 2 Scheme of proteomic analysation[15]

So, according to the recent literature, top-down analysis is generally used to study the deamidation of purified, intact proteins with low molecular weights, bottom-up analysis can additionally be used to characterize deamidation in complex protein mixtures (short proteolytic peptides) and middle-down analyzing polypeptides between 2–20 kDa with rapid limited digestion (long proteolytic peptides)[15,16]Figure 3. Using the bottom-up technique acquires data for the identification and sequence determination of proteins. Bottom-up analysis requests the proteins of interest were digested with an enzyme called trypsin, to form peptides. In that way, hydrolyzed proteins were then ionizable by electrospray ionization (ESI) or matrix-assisted laser desorption (MALDI)[17]. Middle-down MS is a hybrid approach that takes the advantages of both bottom-up and top-down MS. Limited protease digestion of large proteins is employed in middle-down MS to produce polypeptides at a size more amenable for MS or MS/MS analysis than top-down MS.

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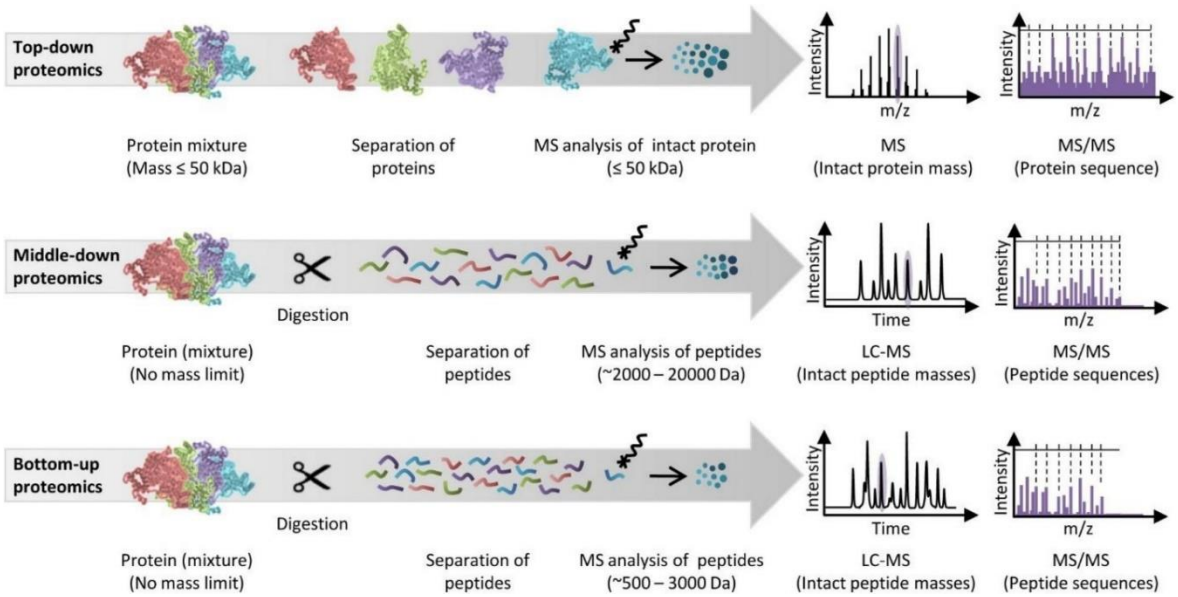

Figure 3 Simple scheme of three different approaches for protein analysis[6]

For MS, protein ions have to be analyzed in the gas phase. In tandem with MS (MS/MS), a current ion corresponding to a peptide, is selected in MS^1 for further fragmentation in MS^2. All MS contains three pieces:

- Ionsource (e.g., MALDI, Electrospray Ionization (ESI))
- mass analyzer (e.g., Quadrupoles (MS^n), time-of-flight (TOF), Fourier transform ion cyclotron resonance (FT-ICR))
- detector (e.g., electron multiplier)

The ion source produces peptide ions, the analyzer separates these ions due to their mass-to-charge (*m/z*) and the detector detects the charged particles and amplifies the response to give a signal. The new development of MS confirmed that deamidation was identified based on the mass increase in peptides and product ions during MS/MS analysis[8]. MS/MS spectrometry could contain multiple quadrupoles (MS^n), coupled to other analyzers. The mass analyzer coupled to MALDI determines the type of MS/MS analysis, that can be carried out. During the development of ESI, the combination of ESI with MS/MS analysis became common in the characterization of protein deamidation. According to these experiments, these precursor ions are fragmented and their (*m/z)* values of the resulting product ions, are used to evaluate the sequence of the peptide or protein[16,17].

It is well known that therapeutic proteins include a large- heterogeneous variety of products showing different pharmacologic activity and molecular types[18]. Between them, we can mention, antibody-based drugs that are the largest and fastest-growing class including monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), bi-specific antibodies

(bsAbs), peptibodies, and Fc-fusion proteins. Reversed-phase liquid chromatography (RPLC) in combination with MS is one of the most widely used chromatographic approaches in this field because it is able to answer several issues due to therapeutic proteins product development, such as assessment of identity, purity, verification of disulfide bonds isoformsformation; confirmation of molecular weight, primary structure; and identification of post-translational modifications (PTMs)[18,19].

In comparison to other chromatographic modes, RPLC provides higher kinetic efficiency and is compatible with MS detection[20]. Experimentally, it is noticed that this method has some limitations, one of which is related to the phenomena of broadening band as the result of the slow diffusivity and mass transfer resistance of big protein molecules. Related to this fact, multiple isoforms of therapeutic proteins can contribute to the peak broadening[19]. Also, there is another limitation, due to the adsorption of mAbs onto the stationary phase. Actually, this process happens by secondary electrostatic interactions, especially when it happens at the intact level. However, this seems to be a less problematic limitation when the analyses are performed at the middle- or bottom-up level. The problems of the peak tailing are directly connected to ionic secondary interactions. The next point is related to the denaturation of therapeutic protein during RPLC at 60^0-90^0C by applying a water/organic solvent gradient. These facts prove that RPLC analyses suffer from limited selectivity when large proteins have been analyzed[20].

Results

This review is focused mainly on the new scientific developments in the last five years, related to the new advanced technique of mass spectrometric methods on the deamidation reaction on the therapeutic proteins. Therefore, a lot of articles use as their major spectrometric method the three techniques called “top-down”, “bottom-up” and “middle-down” MS analyzing peptides and proteins. Referring to the biological point of view, deamidation essentially intersects with the concept of protein stability. Converting Asn and Gln into Asp/IsoAsp and Glu/IsoGlu changes the composition and the structure of proteins compared to their time of synthesis[18]. The improvement of mass accuracy and power increases with the development of mass analyzers such as the time-of-flight (TOF), Fourier transform ion cyclotron resonance (FT-ICR), and Orbitrap[20]. Also, the methods of new ions activation or dissociation such as electron capture/transfer dissociation (ECD, ETD), ultraviolet photo-dissociation (UVPD), and more efficient matrix-assisted laser desorption

ionization (MALDI), drastically increase the sequence and the information that can be obtained in Top-Down MS experiments[21,22,23,24,25]. The development of these new technological methods has been sustained by the corresponding Top-Down/Middle-Down-specific advances in data analysis tools, such as ion distributions and product ion annotation approaches[22]. Otherwise, in the Bottom-Up approaches, top-down-MS provides sequence coverage and proteoform mapping in only one single experiment and sustains sample handling protocols minimizing the presence of artifactual modifications (e.g., oxidation and deamidation)[26,27,28]. We can say that proteoform mapping secures valuable information on the entirety of a mAb, giving information about the structural integrity of all mAb in solution, which is actually lost in the Bottom-Up approach[23].

Also, deamidation confuses sometimes MS analyses of proteins, especially when the reaction occurs during the actual sample preparation steps. In this case, is necessary to differentiate these deamidation artifacts from those really present in the original sample using 18 O labeling[24]. The biopharmaceutical manufacturers have noticed that the rates of asparaginyl deamidation are quite slow for the typical conditions used. So, this is related to the place where deamidation was found during the biopharmaceutical production chain. Deamidation does not take place during processing but during long-term product storage[24]. In the beginning, MS analysis was firstly only established on simple samples (peptides or protein digests) to detect the increase between the monoisotopic mass of Asn (114.0429) and Asp/IsoAsp (115.0269) which indicates the typical 0.984 Da difference[29]. Before a long time ago, different experiments reported that was impossible to detect fragmented ions due to the progressive degradation of proteins over time. Later, it was successfully reported the differentiation between Asp and IsoAsp residues in intact β2-microglobulin using top-down MS[7].

According to the past year's studies, the first triple-quadrupole MS analysis of deamidation sites has been used in the proteolytic peptides of a monoclonal antibody (mAb). During recent studies, ESI-TOF-MS methods together with top-down and bottom-up approaches have been used to identify the specific site of deamidations and other modifications of mAb[24]. While, during the experimental work of mAb modification and degeneration in vivo, has been developed a method of assessing the extent of Asn deamidation and the ratio of Asp to isoAsp residues over time. So, according to this fact, the monoclonal antibody was for the first time immobilized by antigen (or goat anti-human IgG Fc gamma antibody) and then was subjected to the sequential digestion in trypsin and endoproteinase Glu-C. The

analyses were followed by RPLC-MS/MS on an ESI-Q-TOF mass spectrometer, known as a fast technique, highly sensitive, and adaptable only to the study of the deamidation of pharmaceutical protein/peptide in a complex in vivo setting[25].

Deamidated forms of hemoglobin were found to have a lower affinity for oxygen compared with non-deamidated variants[24]. Another approach to understanding the impact of deamidation is to produce purified, recombinant protein carrying one or more acidic substitutions, and analyze these mutants for impact upon structure or function. Levels of deamidation in biopharmaceutical products should be relatively constant during pre-clinical and clinical development, and finally manufacture and storage. In addition to keeping amounts of these species within defined limits, it is important that biopharmaceutical product developers take necessary steps to understand the effect that deamidation has on the final product[24,25,26].

In 2019, another innovation due to the application of RPLC in the field of therapeutic protein characterization is the development of native RPLC (nRPLC), an MS-compatible alternative to HIC (hydrophobic interaction chromatography) that has been recently reported in the literature[29]. This is explained by the increasing of the organic component which allows the reverse-phase separation and low MS-compatible salt concentration that could be able to preserve the native form of proteins to keep secure their retention on bonded phases having limited hydrophobicity. Actually, the changes in solvent strength and temperature can affect strongly the gradient separation of protein therapeutics in RPLC mode[30]. The new scientific developments are important for the reduction of RPLC problems during protein analysis, referring to band-broadening, peak tailing, strong adsorption, and possible protein denaturation/degradation.

Discussion and Outlook

It is well known in all scietific studies, that deamidation is a widely studied post-translational modification (PTM) in proteins, especially in therapeutic mAbs, but it is rather difficult to clarify all of the variants that may occur in such a large protein due to deamidation, let alone in combination with other possible PTMs[4]. This review has highlighted recent advances in protein deamidation and their characterization by mass spectrometric methods. To study site-specific deamidation in purified proteins is necessary top-down and bottom-up mass spectrometry-based proteomic approaches and ECD/ETD that contribute to the reliable identification of isoAsp peptides based on the detection of diagnostic ions. Top-down MS is

the most ideal and powerful method to study proteoforms including protein deamidation[7]. Direct analysis of large intact proteins using top-down MS-based proteomics is more technically challenging than analyzing small tryptic peptides at the current proteomics technology; therefore, bottom-up MS is the more commonly used method for the proteome-wide study of protein deamidation in complex samples, and many of the challenges associated with analyzing primary biological specimens have now been addressed. However, special attention must be paid to sample preparation and data analysis strategies in order to mitigate against the false-positive identification of deamidated and/or isoAsp peptides[8,9,10]. The development of specific enrichment methods, such as immunoprecipitation and affinity purification, could potentially enhance the analysis of isoAsp-containing peptides in the near future. The recent development of these methodologies for studying protein deamidation in complex samples will greatly advance current understanding of its biological role in human aging and disease conditions[9].

According to recent studies and laboratory applications, is interesting the combination of MS methods for mAb analysis afforded by a conventional bottom-up CID/HCD-based approach, integrated with intact mAb measurements and middle-up MS of mAb subunits produced by IdeS digestion and disulfide bond reduction. The MALDI-based ISD fragmentation approach performed on either TOF or FT-ICR MS nowadays, has demonstrated a particularly attractive efficiency for mAb sequencing[20]. These advances may establish the basis for the use of MALDI-based methods for mAb structural analysis and lead to the wider acceptance of Top-Down and Middle-Down methods in the industrial environment. This will promote the success of another MALDI-based technology, where MALDI TOF MS is normally used for microorganism identification via intact mass measurements in many hospitals and healthcare organizations[30,31].

“Middle-down” analysis of antibodies is showing greater promise than “top-down”. “Middle-down” LC-MS/MS is carried out in the same way as “top-down” except the antibody is first cleaved into large fragments (usually Fc/2, Fd and light chain, or Fab and Fc) which are analyzed separately by LC-MS/MS)[6]. Since the cleaved fragments are significantly smaller than the full-sized antibody, the MS/MS spectra are simpler, and the fragment ion signal is improved. However, the fragmentation data is still complex, and complete amino acid sequence coverage continues to be an issue. It remains to be seen whether the technical challenges associated with “top-down” and “middle-down” MS will be

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surmounted in the coming years and the use of these promising technologies becomes more widespread[32].

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