Separation and Characterization of Deamidated Isoforms in Insulin Analogue and its Underlying Mechanism

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Abstract
Deamidation of proteins has remained an elusive phenomenon largely down to the gap that exists in its understanding. One of the primary reasons, is that the experimentation involved is time consuming. However, it remains one of the most important phenomenon in proteomics both in-vitro as well as in-vivo. Deamidation results in the quick conversion of an asparagine residue to a mixture of isoaspartate and aspartate. In glutamine residues deamidation occurs at a much lower rate. Deamidation of asparagine residues is one of the most common post-translational modifications occurring in therapeutic proteins produced using recombinant DNA technology and is the major cause for degradation of bio- pharmaceuticals. Herein, we present the separation and characterization of deamidated Isoforms in a recombinantly produced Insulin analogue by ion exchange chromatography and enzymatic digestion. The deamidation site was confirmed by using state of the art technique: LC-ESI-MS-MS. Additionally secondary structural differences were observed between the unmodified and deamidated variant of the protein.

Keywords: Deamidated isoforms, Insulin analogue.

Introduction
Protein post-translational modification (PTM) increase the functional diversity of the proteome by the covalent addition of functional groups or proteins, proteolytic cleavage of regulatory subunits or degradation of entire proteins.(1) These protein modifications include misfolding - aggregation, oxidation of methionine, deamidation of asparagine and glutamine, variable glycosylation, and proteolysis.(2)

Such modifications not only pose challenges for accurate and consistent bioprocessing, but also may have consequences for the patient in that incorrect modifications or aggregation could lead to an immune response to the therapeutic protein.(2) Therefore, identifying and understanding PTMs is critical in the study of cell biology, disease treatment and prevention. The mechanism of formation of the de-amidated species from these amino acids are given in (Fig. 1). This process is annotated as a deamidation because the amide in the asparagine or glutamine side chain is replaced by a carboxylate group. The deamidation of one amino acid leads to an addition of 1 Da mass to the molecular weight of a protein. Liquid chromatography coupled with mass spectrometry is the most widely used technique for studying the nature and site of deamidation in proteins.

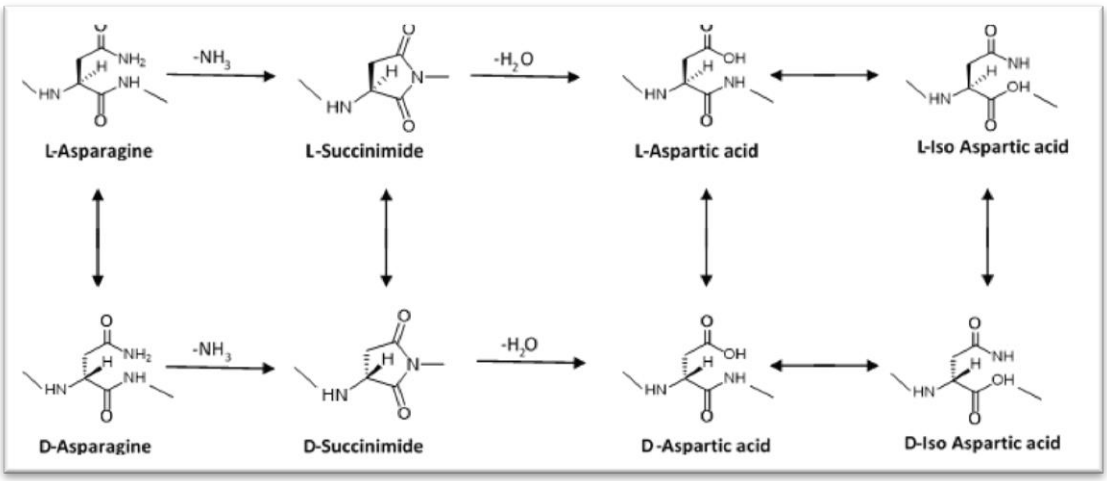

Fig. 1: Schematic representation for deamidation of asparagine to aspartic acid and iso-aspartic acid through succinimide intermediate

Recombinantly produced Insulin analogue used for this study consists of two chains (Chain-1 & Chain-2) which are linked by three disulphide bonds; two inter-chain disulphide bonds and one intra chain disulphide bond within chain I. Deamidation is a common post-translational modification associated with amino acids with free N- terminal viz., Asparagine and Glutamine. These amino acids are converted into a mixture of isoaspartate and aspartate or glutamine to glutamic acid or pyroglutamic acid. These reactions are important to the structure, stability. or function of the protein. The resulting mass is an increase of 1 Da. One of the possible explanations for the likely effect of deamidation is intracellular protein degradation.

Asparagine deamidation occurs more often. The non-enzymatic modification of asparagine and aspartate residues in-vivo, is a result of intramolecular rearrangement.[3] In the first step of the deamidation process the asparagine is converted to a five carbon cyclic intermediate ring referred to as a succinimide or cyclic imide. The intermediate is then hydrolysed at either the alpha or beta carbonyl group giving rise to either iso-aspartate (beta-aspartate) or aspartate. At low pH (<2), direct hydrolysis of the side chain amide generates only aspartate.

The difference between optical isomers of a protein can only be detected using complex methodology.[4] This is because it is extremely difficult to resolve isomeric forms. The difficulty is highlighted by the unequal probability of stereo inversion seen as result of protonation of succinimide intermediate. However, as shown in Figure1, racemization is inevitable in asparagine residues as it occurs following the formation of succinimide intermediate.

The reaction occurs as follows (a.) When Asparagine is in L- α form its carbonyl group is attacked by the nitrogen from the amide group of the succeeding residue resulting in the formation of L-succinimide due to cyclization; (b.) The conversion of L-succinimide to D-succinimide may occur because of a prochiral intermediate forming in the same plane; (c.) Hydrolysis of either racemic succinimide can occur at the carbonyl end yielding both Asp and Iso-Asp residues, respectively shown in Figure1.[5,6]

When amino acids with relatively short side chains for example, serine and threonine follow sequentially from an Asparagine residue in a peptide; the frequency of deamidation is neither high nor low. When an amino acid with a large bulky side chain (I, L,V,P) is immediately preceded by the sequence Asparagine-Alanine the rate of deamidation is as low as 1/6th the rate if the preceding residue is a glycine.[7] This may be due to steric hindrance as a result of the bulky side chain thereby reducing the rate of reactivity. Peptide bond deprotonation increases with increasing pH leading to a higher rate of succinimide formation.[7]

In addition, it has been purported that the rate of deamidation is increased by both high pH and ionic strength. Certain buffers have been identified as contributing to increased reaction rate at alkaline pH. However, there have been experiments suggesting otherwise.[7]

In order to highlight the importance of storage conditions for the proteins,[8] study was performed by creating stressed microenvironment around the protein. This was achieved by subjecting the Insulin analogue to pH of 4 for a period of 11 days at room temperature.

In our study deamidation of insulin analogue has been established and site characterised. Further isoforms were identified and elucidated. The deamidated isoforms were separated by using a well-known ion exchange chromatographic technique. IEX allows for the separation of different species that have the same molecular weight but only moderate variation in charge and the conformation of the molecule. This results in electrostatic interactions with molecules of opposite charges present on the stationary phase. One drawback of the method is that it can only be used for molecules containing ionisable groups. The retention time of the molecules to be separated is an indicator of the degree of charge based affinity of the molecule to the stationary phase. Different variables influence the interaction of charged molecules with the stationary phase. It is possible to separate different charge variants by modulating factors such as pH and concentration.[9] The variants separated by CIEX method were enriched. Desalting of proteins, which is necessary for mass and structural determination (LC-MS) was carried out using C18 resins. The desalted charge variants were analysed using LC/MS. LC-MS analysis overcomes the challenges associated with more conventional methods of analysis. This allowed for an increase in the diversity of organic molecules studied. The analyte molecules are ionised using various sources of ionisation such as ESI, APCI, TOF and chemical ionisation. The resultant ions are analysed using their different m/z ratios. Based on the results obtained from the LC-MS analysis two deamidated isoforms were identified. To further understand the deamidation site and elucidate the reasons for two deamidated isoforms the protein was subjected to PMF analysis. In order to ascertain the conformation of deamidated variants, the enriched deamidated samples were analysed using CD spectroscopy.[10,11] The variation was detected in the conformation of the individual variants. Using the information obtained it was possible to elucidate the presence of two deamidated variants with two distinct conformations.

Materials and Methods

Insulin analogue Hydrochloric acid (12N 37% Sigma Aldrich), Acetonitrile (ACN) (J.T Baker), Ammonium formate, Sodium chloride (NaCI) (Sigma Aldrich), Polysulphoethyl, C-18 Zip-Tips (Merck), Trifluoroacetic acid (Sigma Aldrich(TFA)) ,C18 column (ACE),C8-column (Waters), Dithiothreitol

(DTT), Glu-c (Endoproteinase, Roche), Asp-N (Endoproteinase, Roche), tris(hydroxymethyl)aminomethane (Sigma Aldrich (TRIS))

IEX: The protein was dissolved in 0.01N Hydrochloric acid so as to control the pH of the final solution. Acidic pH of the protein solution is expected to result in the formation of a higher concentration of deamidated isoforms. ACN, Ammonium formate, NaCl buffer combinations were used as mobile phase for separation of charge variants by CIEX. A column of Polysulphoethyl ATM (100 x 4.6 mm; particle & pore size: 3µ & 300 Å) was used for separation of variants.

The combination buffer with ammonium formate and ACN is used as Buffer A which is 100% in a gradient for 30min and gradually decreased to 20% till 25min and kept constant at 100% up to 40min. Buffer B contains a combination of NaCl with mill molar concentration of ammonium formate and ACN.

Desalting: As the buffers used in ion exchange chromatography method for separating deamidated isoforms are salt based, the enriched fragments (peaks) contain moderate amount of salt which is not recommended for further analysis in a mass spectrometer. In order to remove the salt, the enriched fragments were subjected to desalting process by commercially available C-18 Zip-Tips (Merck Ltd.) where the resin is C18. The protein interacts with the resin and salt is eluted. The bound protein is eluted by 0.01% TFA. The desalted samples were used for identification and characterization by LC-MS and for secondary structure estimation by using circular dichroism spectroscopy.

LC-MS: The variants were characterized and identified by state of art technique i.e. ESI LC-MS with a top down approach. In this approach, the variants are analysed using an established LC-MS method (ACE 5µ C18-300Å 250*4.6mm). The intact mass of variants was detected and +1 Da variants were observed. These +1Da variants were reduced with DTT in order to remove the di-sulphide bonds present between two chains. The primary structure was determined by injecting the standard sample and the +1Da variants in LCMS. The chromatographic separation of the samples was carried out using RP C-8 (waters 5µ C8 250*4.6mm) column which is simultaneously ionized to give mass to charge ratio (m/z) for particular species. The difference in the standard protein chain mass and variant chain masses was observed and the site of +1Da variant was identified. The variants were digested with glutamyl endopeptidase (commercially available as Glu-C from Roche). The pH of the sample was adjusted to 7.0 with 1M TRIS and endoproteinase Glu-C enzyme is added in the enzyme-protein ratio of 1:25. The mixture of reaction was incubated at 37°C for three hours. Further, the disulphide bonds were reduced by using 1M DTT with 1:10 protein to ratio DTT and incubated for an additional hour. The variants were digested and reduced to individual fragments which were identified using LC-MS.

In order to confirm the Deamidated iso variants samples were subjected to enzymatic digestion with ASP-N (Endoproteinase AspN is a zinc metalloendopeptidase which selectively cleaves peptide bonds N-terminal to aspartic acid residues).

Circular Dichroism: Circular Dichroism (CD) refers to the differential absorption of left and right circularly polarized light and the spectrum obtained due to this phenomenon is called CD spectrum in which the CD signal is represented in terms of Milli-degrees (mdeg). Wavelength scans, using a CD spectrometer, in the "far-UV" spectral region (190-260 nm) and the “near-UV" spectral region (260-350 nm) result in CD spectra that are respectively characteristic of the secondary and tertiary structure of a protein.

Secondary structure of a variants was determined by CD spectroscopy in the "far-UV" spectral region (190-260 nm). At these wavelengths the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment. Alpha-helix, beta-sheet, and random coil structures each give rise to a characteristic shape and magnitude of CD spectrum.

Results and Discussion

Insulin analogue and its deamidated isoforms were isolated using ion exchange chromatography (Fig. 2) followed by the identification of mass fragments based on m/z ratio.

The schematic representation of deamidation of aspargine to aspartic acid and its isomeric form is explained in Fig. 1. The process is detailed in the figure wherein, Aspargine losses its amide group leading to the formation of succinimide intermediate. The succinimide upon loss of water gets converted to aspartic acid. The variation in the deamidated product is observed because aspartic acid can exists in two isomeric forms known as aspartic and iso-aspartic acid. At acidic pH, the side chain of an asparagine is prone to attack from the amine group of the adjacent residue. This results in the formation of an energetically favourable succinimide intermediate which stabilizes to form either aspartic or isoaspartic acid. The succinimide intermediate formed during the deamidation of asparagine being more energetically favourable results in the deamidation of asparagine occurring at a rate ten times faster than glutamine.

The intact mass of the standard protein and that of the two deamidated isoforms are elucidated in Fig. 3. The difference in mass between the three proteins analysed were 1 one Da. Despite, the identical masses observed, there were two distinct species.

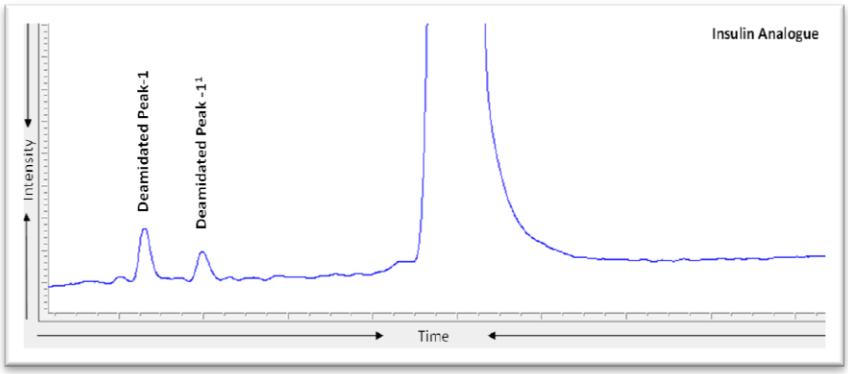

Fig. 2: IEX profile of insulin analogue showing the separation of deamidated isoforms

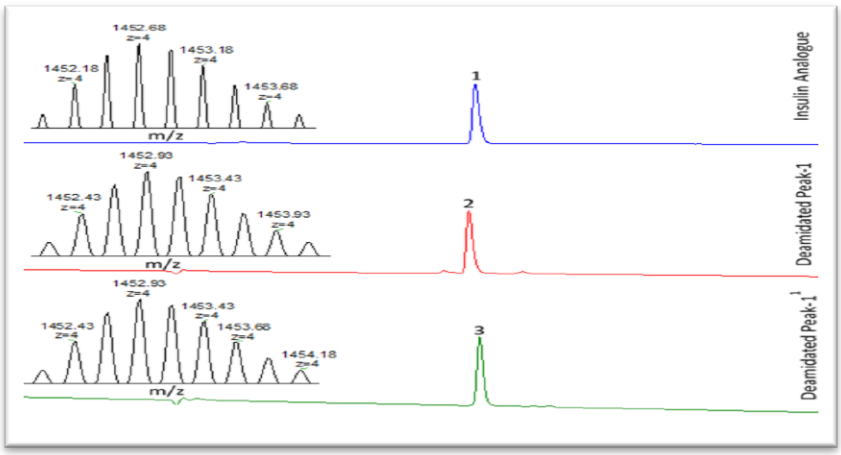

Fig. 3: LC-MS data for intact mass analysis of standard insulin and deamidated isoform 1 and 1[(1)]

In order to understand the variance in charge reduction with DTT was performed yielding two chains by breaking the intra di-sulphide bridges. From the mass spectrometric data obtained from reduction of protein and deamidated isoforms, it is confirmed that the mass difference i.e. increase in 1 Da of mass is in chain-1 for both the isoforms (Fig. 4*)* and the chain-2 is unmodified. Fig. 4 represents the two chains and the masses obtained by mass spectrometer with m/z is represented in the form of isotopes.

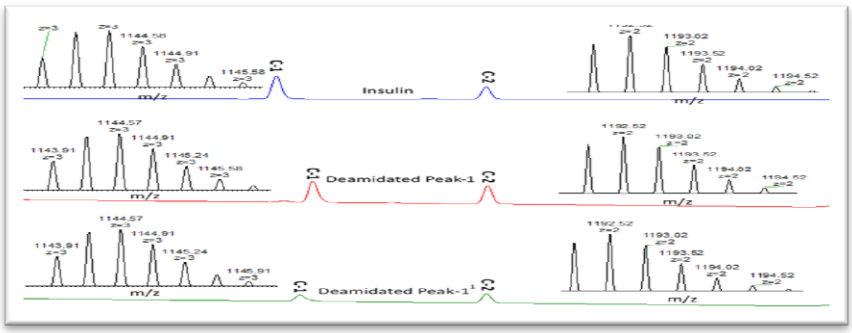

Fig. 4: LC- MS data for reduced mass analysis of deamidated isoforms comparing with standard insulin to confirm the site

Further proceeding for digestion in order to increase the rate of reactivity of enzyme, the deamidated isoforms samples was treated with 1M TRIS to maintain the pH at 7.0. The sequence of chain-1 is elucidated in Table 1. The samples were subjected to digestion with Glutamyl endopeptidase which cleaves the N-terminal of glutamine to yield the fragments.

Table 1: Theoretical sites of Glu-c digestion in sequence of insulin, Expected fragments of insulin on digestion with Glu-c, Expected fragments of deamidated insulin on digestion with Glu-c, Theoretical sites of Asp-N digestion in sequence of deamidated insulin, Theoretical fragments on Asp-N digestion of D Insulin, Theoretical fragments on Asp-N digestion of Iso-D insulin

Glu-C Cleavage Sites	**Asp-N Cleavage Sites**	
GIVE--QCCTSICSLYQLE—NYCN FVNQHLCGSHLVE--ALYLVCGE--RGFFYTPKT	**GIVEQCCTSICSLYQLENYCN FV--DQHLCGSHLVEALYLVCGERGFFYTPKT**	
Theoretical Fragments	Deamidated Insulin Fragments	Fragments obtained for de-amidated Insulin analogue (**B3-Aspartic acid**)
GIVE	GIVE	1. GIVEQCCTSICSLYQLENYCN
QCCTSICSLYQLE	QCCTSICSLYQLE	2. DQHLCGSHLVEALYLVCGERGFFYTPKT
NYCN	NYCN	
FVNQHLCGSHLVE	FVNQHLCGSHLVE	Fragments obtained for de-amidated Insulin analogue (**B3-Iso-Aspartic acid**)
ALYLVCGE	ALYLVCGE	**1**. GIVEQCCTSICSLYQLENYCN
RGFFYTPKT	RGFFYTPKT	**2**. **FV**DQHLCGSHLVEALYLVCGERGFFYTPKT

Fig. 5 shows that the first fragment from chain-1 of isoforms was deamidated. Sequence of fragment-1 in chain-1 as shown in Table 1 is susceptible to deamidation at only the 3rd position, which is Asparagine. The possible ways of deamidation are conversion of asparagine to aspartic acid and its isomer (Iso-D), and the other way is aspartic acid to isoaspartic acid. It was not possible to identify the inversion of D to iso-D aspartic acid in the sequence as the cleavage sites reults in fragments that are not affected by this inversion.

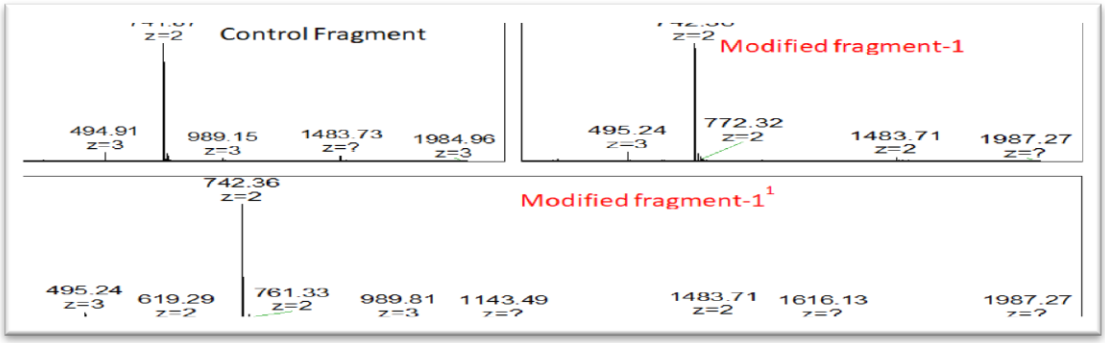

Fig. 5: Fragment of chain -1 for standard and deamidated isoforms obtained by Glu-c digestion followed by reduction

The fragment-1 of chain-1 for deamidated isoform-1, the asparagine present in the 3rd position is converted into aspartic acid and for deamidated isoform-2, asparagine in the 3rd position is converted to isoaspartic acid (Fig. 6).

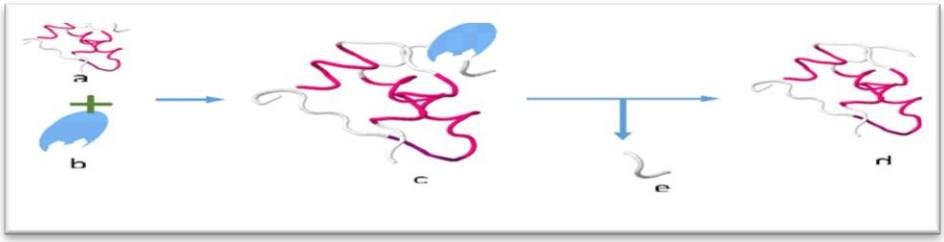

Fig. 6: **Peptide (a.) cleaved with proteolytic enzyme Asp-N (b.) which is specific for N-terminal of Aspartic acid (mechanism of action (c.)) resulting in desired fragmentation(d.&e.)**

These two variants are differentiated by ASP-N enzymatic digestion which cleaved specifically at the N-terminal of aspartic acid (Table 1) but not isoaspartic acid. The mass as confirmed after enzymatic digestion is elucidated in Table 2.

Table 2: Potential cleavage sites of chain-1 following ASP-N digestion

Asp N digestion	S.No	Peptides Location	Marker peptide	Sequence	Comments
	1	B1-30	3429.9	FVNQHLCGSHLVEALYLVCGERGFFYTKPT	C-1(Standard)
	2	B1-30	3430.9	FV**D**QHLCGSHLVEALYLVCGERGFFYTKPT	N→**iso-D**
	3	B3-30	3184.6	**D**QHLCGSHLVEALYLVCGERGFFYTKPT	N→**D**

As seen in Figure 7, the chain-1 standard and the variant consisting of aspartic acid in the 3rd position is susceptible to cleavage by Asp-N resulting in the scission of the **FV** fragment from chain-1. Whereas, the variant of chain-1 that has iso-aspartic acid at position 3 is not cleaved by Asp-N.

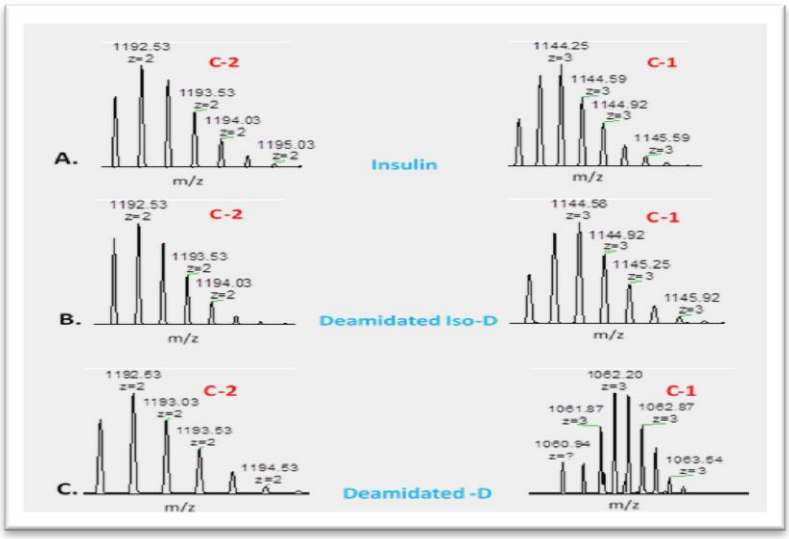

Fig. 7: MS-data for Asp-N digestion followed by reduction of protein A.) Standard protein showing the expected m/z of chain -1 and chain-2 B.) Expected m/z of chain-1 and chain-2 for N→iso-D C.) Expected m/z of chain-1 and chain-2 for N→D

In order to understand the effect of deamidation on secondary structure the standard protein as well as the deamidated variants were analyzed using CD spectroscopy. As seen from Fig. 8a there is an obvious difference between the secondary structures of the standard and deamidated variant-1 which is in terms of beta sheet (~ 220nm). There is also a notable difference in the tertiary structure of the standard and the deamidated variant as evident from Fig. 8b.

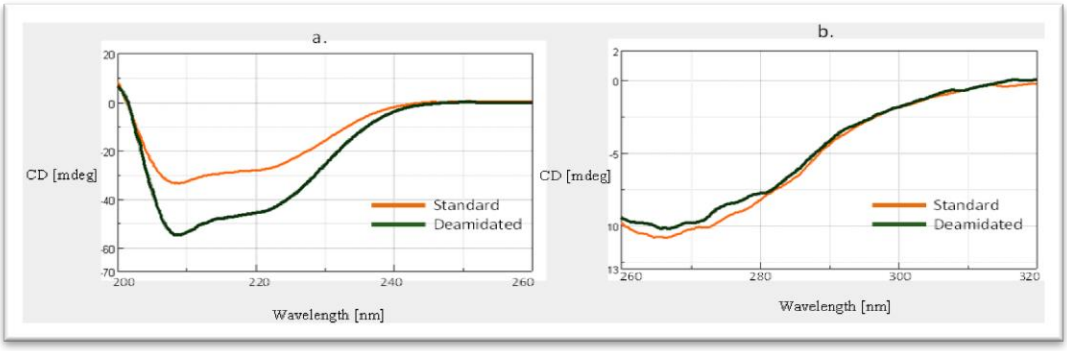

Fig. 8: Secondary and tertiary structure of standard and deamidated protein

This is further confirmed by the data shown in Table 3. One of the possible reasons for the notable difference is the effect of deamidation on the folding of protein.

Table 3: Secondary structure prediction by circular dichroism of standard and deamidated protein

Structure	Standard	Deamidated
Helix	22.7 %	21.1 %
Beta	18.1 %	33.8 %
Turn	25.6 %	18.1 %
Random	33.7 %	27.0 %

Conclusion

The mixture of deamidated and Iso-deamidated variants produced under acidic conditions have been elucidated and their mechanisms explained through the use of state of art LC-MS. It was possible to identify the change in mass of one Dalton and confirm the site of deamidation. The two variants of deamidation present as an isoform mixture were individually separated and completely characterized. The influence of isomeric form was most evident in the fragments obtained in post Asp-N digestion. In case of **N→D,** enzymatic cleavage by Asp-N results in the scission of the **FV** fragment from chain-1 which is not observed in case of **N→**Iso-**D.** In addition the effect of deamidation on secondary and tertiary structure was observed. Given the information obtained it is possible to generate deamidated variants which would help in study their effects on long term stability of proteins.

Acknowledgements

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Conflict Of Interest Statement

There is no conflict of interest.

Non-standard abbreviations: CIEX (Cation ion-exchange chromatography**), CD (**Circular dichroism**), LC-MS (**Liquid chromatography-Mass spectrometer**), ESI (**Electro spray ionisation**), APCI (**Atmospheric pressure chemical ionisation**), TOF (**Time of flight**), PMF (**Protein Mass Fingerprinting**)**

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