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Evaluation of Degradation Products of Insulin using High Resolution Mass Spectrometry with Proteome Discoverer and BioPharma Finder

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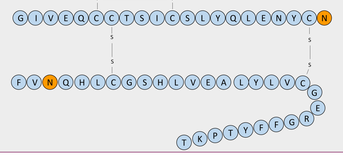
PURPOSE

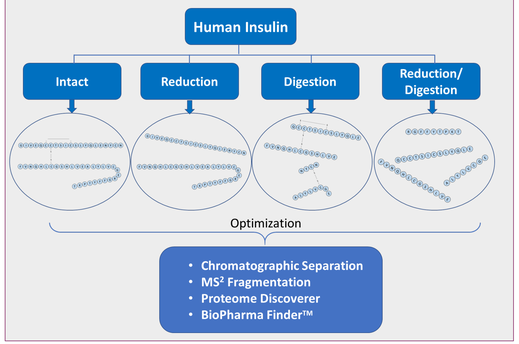
Human insulin

The development of Insulin and its analogs has played an important role in diabetes treatment as alterations in sequence and formulation have been exploited to develop short and long acting therapy options. To facilitate further design of analogs with greater stability, a thorough evaluation of insulin degradation products is required. Insulin can undergo both physical degradation, such as aggregation and fibrillation, as well as chemical degradation including deamidation of AsnA21 and AsnB3. Additionally, oligomers can form from transpeptidation between the A and B chain, and disulfide interchange reactions can be initiated by cleavage of the CysA7-CysB7 bridge. This poster presents a thorough approach to characterizing insulin degradation utilizing multiple strategies; intact, A/B chain, and Glu-C digested analysis. The procedure has been optimized for chromatography, MS, and data mining parameters. In addition, the entire data set can be analyzed in one batch utilizing a single HPLC column and mobile phase.



METHOD

- Human Insulin was forced degraded in 0.1% formic at 60° C for 10 days
- Degradation products were evaluated by high resolution mass spectrometry (Q Exactive Plus)
- Insulin was reduced and alkylated using triethylphosphine and iodoethanol, and digested with Glu-C
- Data was mined using Proteome Discoverer and BioPharma Finder



RESULTS

Intact Analysis

Intact insulin and its degradation products were separated on a Waters BioResolve RP mAb Polyphenyl (2.1 X 150 mm) HPLC column with a gradient from 20-35% acetonitrile in 0.1% formic acid. Figure 1 represents the separation between insulin and its deamidated products achieved in 13 minutes. Precursor mass search and spectral deconvolution performed in BioPharma Finder (Xtract algorithm) identified two separate 1X deamidated products eluting at approximately 9.8 minutes. Peaks representing 2X deamidations eluted at 10.2 and 10.8 minutes, and dimers of insulin eluted between 11-12 minutes. A summary of detected precursor masses shown in table 1 indicate that the main degradation product was 1 X deamidation with fractional abundance of 43 percent. In addition to deamidated products, peaks correlating to possible ammonia loss (-17) and + 39 conjugation were detected.

Figure 1. Chromatography of Insulin Degradation Products

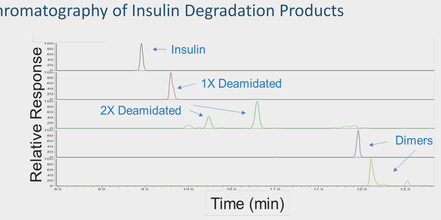


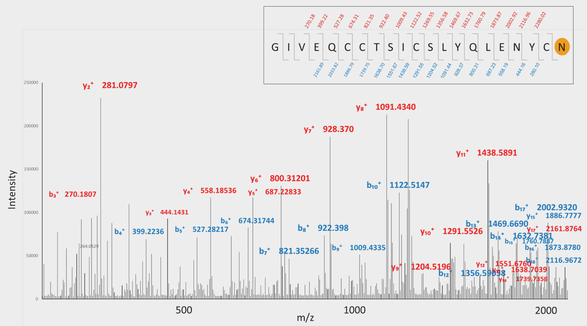
Table 1. Intact Degradation Products

Modifications	Delta Mass	Fractional Abundance	Retention Time
Insulin	0	17.5	9.2
Deamidation 1X	0.9886	42.8	9.6
NH3 loss	-17.0075	2.3	9.4
Deamidation 1X +38/39	38.9176	2.5	9.6
Deamidation 2X	1.9860	1.7	10.8
Deamidation 1X , NH3 loss	-16.0147	1.4	9.6
Deamidation 2X, NH3 loss	-15.0222	0.4	9.7
*Additional Fractional abundance included Dimer, Trimer corresponding products			

A and B Chains

Intact insulin was reduced using triethylphosphine at 60 °C (pH 10) for 1 hour to form the A and B chain. Reduced A and B chains eluted at 3.2 and 4.4 minutes respectively on the BioResolve HPLC column utilizing a gradient from 25-30% acetonitrile. Independent optimization of collision energy was required for the A/B chain analysis to obtain representative MS2 spectrum for proper identification of modified peptides. A representative product ion spectrum indicating deamidation on AsnA21 is shown in figure 2. Deamidations were identified on AsnA18, AsnA21, AsnB3 and GlnB4 which are summarized in Table 2.

Figure 2. Product MS Spectrum for deamidated A Chain I



Glu-C Digestion

Digestion of insulin with Glu-C for 1 hour at pH 7.0 resulted in cleavage of 2 disulfide peptides (Table 2, Glu-C A & B). The MS2 spectrum for Glu-C Peptide A shown in figure 3 verified that deamidation was at AsnA21. PSMs for Glu-C digested Peptide B identified both 1X and 2X deaminated products. However, the MS2 fragmentation pattern of this peptide did not allow confirmation of the deamidation sites for the B chain. Reduction and digestion of insulin resulted in Glu-C cleavage peptides (Reduced 1-4) and one mis-cleavage product (Reduced 5). Reduced 2 did confirm deamidation on both AsnB3 and GlnB4. The missed-cleavage product from this same digestion (Reduced 5) confirmed deamidation on AsnA18.

Figure 3. Product MS Spectrum Glu-C A

* Spectrum in Red = deamidation

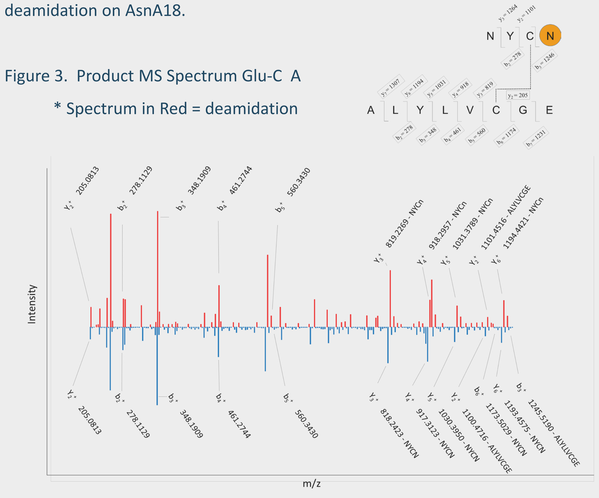


Table 2. Sequence Summary

A Chain	GIVEQCCTSICSLYQLENYCN
B Chain	FVNQHLCGSHLVEALYLVCGERGFFYTPKT
Reduced 1	QCCTSICSLYQLE
2	FVNQHLCGSHLVE
3	ALYLVCGE
4	RGFFYTPKT
5	QCCTSICSLYQLEN
Glu-C	A: NYCN, ALYLVCGE; B: QCCTSICSLYQLE, FVNQHLCGSHLVE

CONCLUSIONS

Degradation of human insulin was investigated using a multi-pronged HRMS approach. All evaluations for intact, reduced, and digested peptides were conducted on a single BioResolve RP mAb Polyphenyl column (Waters) accomplishing separations for degradation products in a single batch. Degradation products identified included deamidations on AsnA18, AsnA21, AsnB3 and GlnB4. These were detected in the A and B chain analysis and verified using digestion with and without reduction. The major degradation product in acid/heat degraded insulin solutions was a 1X deamidation, 43% of the fractional abundance of intact samples. Additional peaks correlated to possible ammonia loss (-17), and addition of 39 Da consistent with deamidation and aldimine modification at PheB1 and LysB29. Future work includes optimization of fragmentation and software parameters to further characterize degradation products.

References

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Weiss, Michael A. Design of ultra-stable insulin analogues for the developing world. Journal of Health Specialties. 1, 59-70 (2013)

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