Identification of Degradation Products of Synthetic Peptides with Nano-LC/MS on an Orbitrap Mass Spectrometer

P1605

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Overview

Purpose: Synthetic peptides are widely used as therapeutic drugs. In order to guarantee their biological activity, they must be characterized thoroughly. Also, during long-term storage, degradation of the product may occur.

Methods: The peptides calcitonin and glucagon were stored long-term under different conditions and subsequently analyzed using an Orbitrap mass spectrometer coupled to a nano-LC system.

Results: Degradation of synthetic peptides can result in complex mixtures. Even low amounts of degradation products can be identified and quantified using nano-LC-coupled mass spectrometry (MS).

Introduction

Calcitonin is a 32-amino acid polypeptide hormone that regulates the calcium level in the body. Glucagon is a 29-amino acid polypeptide hormone that regulates the blood sugar level in the body.

Both peptides were stored at accelerated conditions including high temperature and high humidity. Characterization of the degradation products is important in understanding how the quality of the peptide varies due to storage conditions, and to validate the analytical procedures used.

Degradation products can occur as a modification of an amino acid in the sequence or as a hydrolysis of the peptide backbone. Both events can be monitored using MS.

As shown in Figure 1, calcitonin contains a well-characterized N-terminal acetylation site. It also contains a possible O-Acetylation site on the Ser^{13}. Acetylation of an amino acid results in a mass shift of +42.011 (N-terminal) or +42.0367 (Serine) for the precursor mass and the fragment ion series.

The glucagon sequence contains many asparagines and glutamines that are prone to deamidate during long- term storage. Deamidation results in a mass shift of +0.984, and the amino acid asparagine is converted to aspartic acid and the amino acid glutamine is converted to glutamic acid. The relatively small mass shift can only be detected when using a mass spectrometer with high mass accuracy.

FIGURE 1. Calcitonin and glugacon sequences.

Calcitonin

H-Cys[1]-Ser[2]-Asn[3]-Leu[4]-Ser[5]-Thr[6]-Cys[7]-Val[8]-Leu[9]-Gly[10]-Lys[11]-Leu[12]-Ser[13]-Gln[14]-Glu[15]-Leu[16]-His[17]-Lys[18]-Leu[19]-Gln[20]-Thr[21]-Tyr[22]-Pro[22]-Arg[23]-Thr[24]-Asn[25]-Thr[26]-Gly[27]-Ser[28]-Gly[29]-Thr[31]-Pro[32]-NH_2

Glucagon

H-His[1]-Ser[2]-Gln[3]-Gly[4]-Thr[5]-Phe[6]-Thr[7]-Ser[8]-Asp[9]-Tyr[10]-Ser[11]-Lys[12]-Tyr[13]-Leu[14]-Asp[15]-Ser[16]-Arg[17]-Arg[18]-Ala[19]-Gln[20]-Asp[21]-Phe[22]-Val[23]-Gln[24]-Trp[25]-Leu[26]-Met[27]-Asn[28]- Thr[29]-OH

Acetylation, Oxidation, Deamidation

Methods

Sample Preparation

Calcitonin (powder) was stored at -20 °C (control) or at 5 °C for 3 years (accelerated). The intrachain disulfide bridges were reduced and alkylated prior to LC-MS analysis. Approximately 0.7 fmol of each of the control and accelerated calcitonin were analyzed using LC-MS.

The stability sample of the glucagon (powder) was stored at-20 °C (control stability sample) or at 25 °C/60% (relative humidity accelerated stability sample) both for 6 months.

The glucagon stress test sample (powder) was stored at 20 °C or at 40 °C/75% relative humidity or at 60 °C (relative humidity not controlled). Each stress test sample was stored for 11 days at the above condition and afterwards was placed in a -20 °C freezer for 8 years.

Additionally, one aliquot of glucagon was dissolved in water and stored for 3 hours at 75 °C. This sample was used as a system suitability test (SST). Approximately 0.7 fmol of each glucagon sample preparation was analyzed by LC-MS.

Liquid Chromatography

Nanoscale liquid chromatography was performed on an Thermo Scientific EASY-nLC II nano liquid chromatograph. The flow rate was 200 nL/min. The column used was a "Magic C18" column from Spectronex (particle size 5 µm, length 100 mm, ID 100 µm). The column also served as an emitter for the nanospray. Column equilibration and loading was done with 5% acetonitrile (ACN) in 1% acetic acid. The peptides were eluted with a 70 min gradient up to 40% acetonitrile. Column washing was done using 90% ACN.

Mass Spectrometry

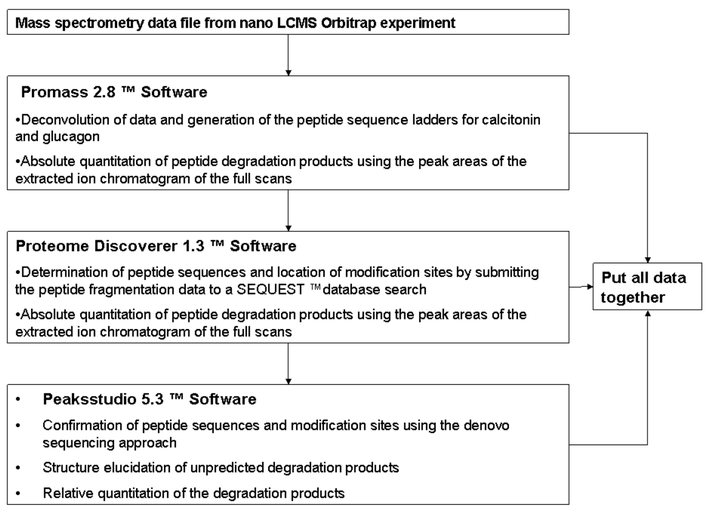
The experiment was performed on the Thermo Scientific LTQ Orbitrap Velos hybrid mass spectrometer. Both full and fragmentation scans were measured in the Orbitrap™ mass analyzer using 30000 and 7500 resolution, respectively. Peptides were fragmented using collision induced dissociation (CID) and higher-energy collisional dissociation (HCD). The 10 highest peaks for each full scan were subjected to MS/MS. A dynamic exclusion time of 60 sec was used to fragment the low abundance compounds.

Data Analysis

Large biomolecules such as peptides are predominantly multiply-charged when ionized by nanospray. In positive ion mode, peptides are protonated at multiple sites due to the basic amino acids and the amino terminus. These complex spectra must be "deconvoluted" to determine the uncharged mass of the peptide. Thermo Scientific ProMass deconvolution software was used to screen the MS survey scans for target peptide masses, impurities and post-translational modifications in the samples. ProMass™ deconvolution software cannot locate the site of the modification, nor is it able to find modifications that show small mass shifts such as a deamidation. It is also not able to confirm the sequence of a peptide. Therefore Thermo Scientific Proteome Discoverer software version 1.3 was used. The peptide fragmentation data was submitted to a SEQUEST® database search. The search contained the original sequence of the peptides and the sequence of some known impurities, and was performed using the "non-cleavage option." For calcitonin carbamidomethylation of the cysteine and amidation of the C-terminus was used as a fixed modification. Acetylation was used as variable modification for the N-terminus and for the serines.

Database search algorithms like SEQUEST only work if the original target amino acid sequence is known and present in the database. It fails if the sequence is unknown or when unexpected modifications and degradation products are present in the sample. PEAKS™ Studio 5.3 *de novo* sequencing software (Bioinformatics Solutions, Inc.) was used to screen for unexpected degradation products and modifications, and to confirm the findings of the SEQUEST search. Figure 2 summarizes of the data analysis steps used.

FIGURE 2. Data analysis steps.



Results

Calcitonin

Using the ProMass software's ladder sequencing and peak extraction functions, the results (shown in Figure 3) were obtained. Carbamidomethylated calcitonin was the base peak. Acetylation is the most common modification and its amount does not change during storage. The same applies to the side product Des-Tyr^{22} calcitonin.

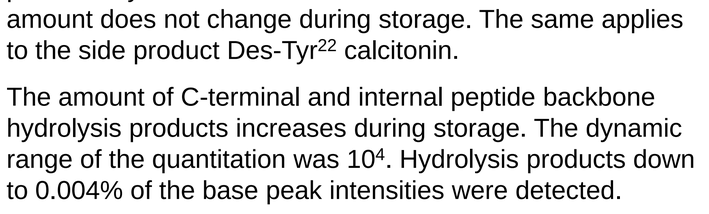
The amount of C-terminal and internal peptide backbone hydrolysis products increases during storage. The dynamic range of the quantitation was 10^4. Hydrolysis products down to 0.004% of the base peak intensities were detected.

FIGURE 3. Results for calcitonin.

Retention time [min]	MH+ (Monoisotopic mass)	Identity	% of Base peak intensity in Control Sample	% of Base peak intensity in Accelerated Sample
24.3	3547	Carbamidomethylated Calcitonin	Base peak	Base peak
32.8	3589	Acetylated Calcitonin	50.4	53
56.4	3384	Des-Tyr22 Calcitonin	0.77	0.4
24.9	3349	Calcitonin Cys1-Gly30 hydrolysis product	4	6
25.6	3204	Calcitonin Cys1-Gly28 hydrolysis product	1.65	4
26.7	3046	Calcitonin Cys1-N26 hydrolysis product	1.5	6.8
14.7	3186	Calcitonin Leu4-P32 hydrolysis product	not determined	3.6

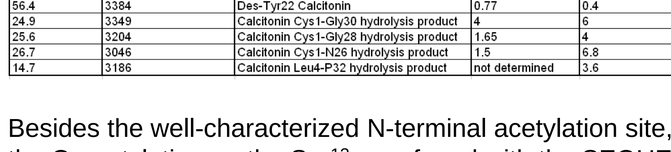
Besides the well-characterized N-terminal acetylation site, the O-acetylation on the Ser^{13} was found with the SEQUEST database search in both calcitonin samples (Figure 4).

FIGURE 4. O-acetylation on the Ser^{13}.



Identities of the hydrolysis products were confirmed using PEAKS Studio software. The calcitonin stored at accelerated conditions showed a higher number of C-terminal hydrolyzed degradation products (Figure 5). N-terminal hydrolysis products were hardly detected, probably because the N-terminal acetylation suppresses the signal of the b-ions.

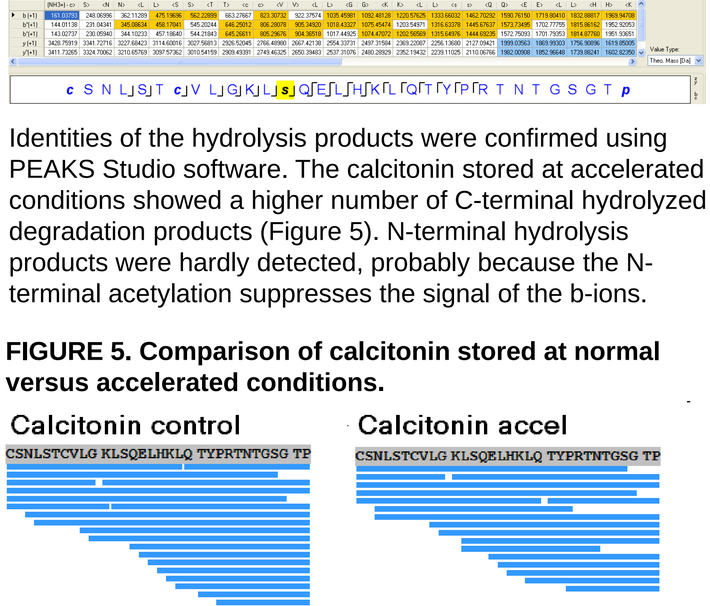
FIGURE 5. Comparison of calcitonin stored at normal versus accelerated conditions.



Glucagon

Using ProMass ladder sequencing and peak extraction functions, the results shown in Figure 6 were obtained. Oxidized glucagon was the base peak. Oxidation is the most common modification and its amount increases if the peptide is stored at higher temperature. The side product Des-Thr5 glucagon was found in low amounts in the sample that was stored at 40°C and 60°C.

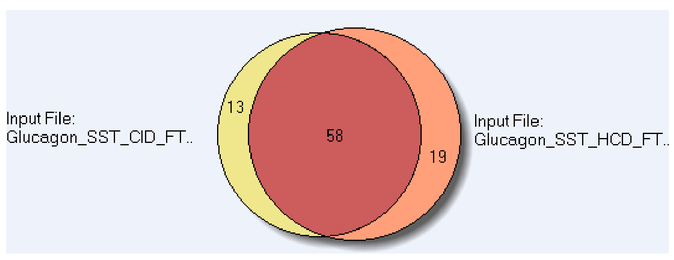
FIGURE 6. Results for glucagon.



In addition, several N- and C-terminal and internal peptide backbone hydrolysis products were detected. Except for the glucagon S2-L26 hydrolysis product, the detected amounts were very low (below 1% base peak intensity).

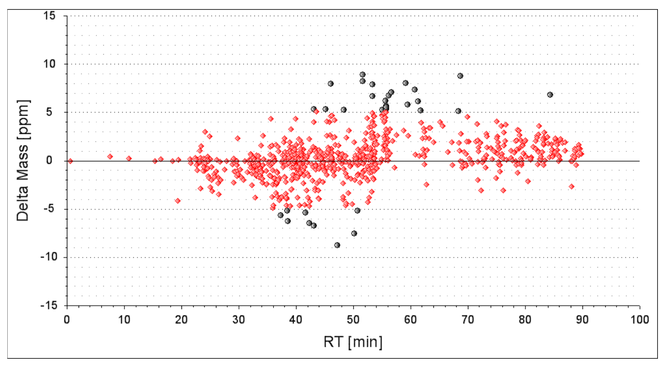
The SEQUEST database search found a deamidation product on the Gln20 in all glucagon samples. The deamidation product on the Asn 28 was also present in all samples but was only identified if the peptide precursor was fragmented using HCD. This was likely because HCD does not have a low mass cut off in the MS2 scans. As shown Figure 7, more than 19 unique peptides were identified using HCD as compared to 13 unique peptides using CID. Both CID and HCD fragmentation scans were measured in the Orbitrap mass analyzer.

FIGURE 7. Sample stored in solution (SST).



Throughout the LC-MS analysis the Orbitrap mass spectrometer had a mass error of less then 5 ppm for most of the measured precursor masses (Figure 8.)

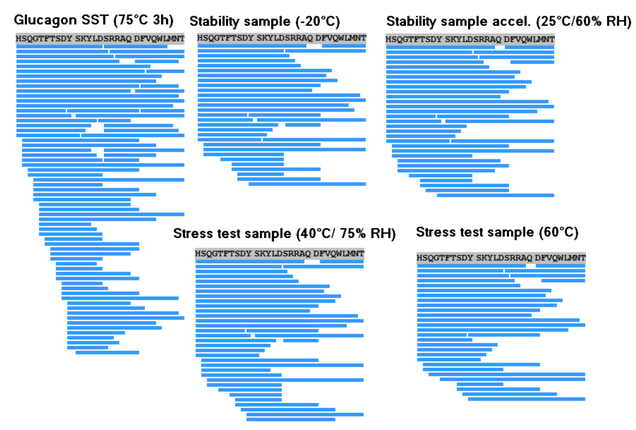
FIGURE 8. Mass error.



Although deamidation products were identified in all samples, in most they were below the limit of quantitation (signal to noise ratio<2). Deamidation product could only be quantified in the sample stored in solution (SST) for 3 h at 75 °C. About 45% of the total peak area was deamidated glucagon.

The identity of the peptide backbone hydrolysis products were confirmed using PEAKS Studio 5.3 *de novo* sequencing software. As shown in Figure 9, the amount of hydrolysis products increased if the peptide was stored at high temperature and high humidity. If glucagon was stored as a powder we observed mostly N-terminal and C-terminal peptide hydrolysis. When stored in solution, internal peptide hydrolysis products were detected.

FIGURE 9. Amount of hydrolysis.



Conclusions

1. Degradation of synthetic peptides at accelerated conditions can result in complex mixtures. The degraded peptides are a result of a modification on the original peptide sequence and/or hydrolysis of the peptide backbone.
2. Even modifications that cause only a small shift in the masses of the precursor ion and the fragment ions can be detected using the Orbitrap mass analyzer.
3. Using nano-LC-coupled MS, even low amounts of degradation products can be identified and quantified.
4. In order to identify both expected and unexpected peptide degradation products, a database search algorithm must be used in conjunction with a *de novo* sequencing approach.

References

1. Ma B., et. al. PEAKS: powerful software for peptide *de novo* sequencing by tandem mass spectrometry. *Rapid Commun Mass Spectrom*. **2003**;17(20):2337- 42.
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3. John R. Yates, et.al. An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino Acid Sequences in a Protein Database. *American Society for Mass Spectrometry*. **1994**,1044-0305

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