Application Note: 335

Characterization of Protein Glycosylation Using ESI Chip™ Static Nanospray Ion Trap MS^n Mass Spectrometry

Dirk Chelius[1] and Sheng Zhang[2]; [1]Thermo Fisher Scientific, San Jose, CA; [2]Advion BioSciences, Inc., Ithaca, NY

Key Words

- LTQ™
- Advion NanoMate™
- MS^n
- Nanospray
- Protein Glycosylation

Introduction

The accurate identification of glycopeptides is challenging because of their complex structures, labile nature, and microheterogeneity. A variety of mass spectrometry based techniques for determining the structure of glycopeptides have been used, including Time of Flight (TOF), triple quadrupole, and ion trap, coupled with a variety of ionization techniques including fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI), and electrospray ionization (ESI). This application report describes the use of a static nanospray ionization technique in combination with a novel linear ion trap (Thermo Scientific LTQ) for identification and characterization of glycosylation in complex mixtures.

Goal

Determine in a single experiment the complete structure of glycosylation group(s) attached to the protein, the amino acid sequence of the glycosylated peptide(s), and the exact site(s) of the glycosylation linkage using MS^n techniques.

Experimental Conditions

Reduction, alkylation, and proteolytic digestion

One milligram of ribonuclease B (Sigma, St. Louis, MO) was reconstituted in 100 µL of solution containing 20 mM Tris-HCl pH 7.8, 6 M guanidine-HCl and 10 mM DTT. The mixture was incubated for 30 minutes at 50°C, and 50 µL of 0.2 M iodoacetamide and 50 µL of 0.2 M ammonium bicarbonate pH 7.8 were added. The mixture was incubated at room temperature in the dark for two hours. The alkylated solution was dialyzed against 20 mM ammonium bicarbonate pH 7.8 overnight at 4°C using a Slide-A-Lyzer® MINI Dialysis Unit. The dialyzed protein was digested by adding a 1:60 ratio (enzyme:sample) of a proteolytic enzyme (Promega, Madison, WI) at a concentration of 0.52 mg/mL in 50 mM ammonium bicarbonate pH 7.8 and incubated overnight at 37°C.

Sample Analysis

Samples were introduced into the LTQ using the NanoMate™ 100 mounted in front of the LTQ and 5 µL samples (at 1 pmol/µL in 50% methanol; 0.1% formic acid) were infused at a flow rate of 100 nL/min.

MS Spectrometry

Mass Spectrometer: LTQ run in tune mode
Ionization Mode: Nano-electrospray
Ion Polarity: Positive
Spray voltage: 1.55 kV
Spray pressure: 0.2 psi
Capillary temperature: 150°C
Normalized Collision Energy™: 20–25% for MS^n experiments
Maximum scan time: 50 ms
Number of micro scans summed for each scan: 2–3

Results and Discussion

The proteolytic digest of bovine pancreatic ribonuclease B (1 pmol/µL) was analyzed using chip-based static nanospray mass spectrometry as described in the experimental section. Tandem mass spectrometry was performed on all of the major peaks, and additional fragmentation (MS^n) was recorded manually. The full-scan mass spectrum (Figure 1) shows the complex peptide mass fingerprint of ribonuclease B. Several peptides were identified using fragmentation information from MS/MS spectra using the Thermo Scientific BioWorks™ 3.1 software package. The software correlates theoretical MS/MS data from a database with actual observed spectra for identification of proteins (Eng J.K, McCormack A.L, and Yates J.R.I. *J. Am. Soc. Mass Spectrom.* 1994). The identified peptides and the protein coverage are shown in Table 1. Nine out of fourteen possible proteolytic peptides were detected, resulting in over 87% coverage. The software did not identify several major peaks, although the fragmentation pattern of those peaks appeared to be of high quality. The unidentified peaks show a typical pattern for high mannose-type glycopeptides with mass shifts of 81 Da for doubly charged ions. Collision-induced fragmentation of the unidentified peaks (Figures 2 a-e) confirms the presence of high mannose-type glycosylation by generating doubly charged fragment ions that differ by 81 Da. Additionally, the presence of N-acetyl-D-glucosamine can be easily detected in the tandem mass spectra based on the marker ion at 204 Da. Assuming complete proteolytic digestion, the only possible site for N-linked glycosolation in ribonuclease B is the peptide NLTK. The molecular weight information of the unidentified peaks, together with the molecular weight of the amino acid sequence and

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the fragmentation information, confirms the presence of five different high mannose type glycopeptides in ribonuclease B. As shown in Figures 3 a-c, the MS^n capability of the LTQ was used to characterize the nature and extent of the glycosylation. Sequential MS/MS experiments allow the sequential linkage of the oligosaccharide units to be determined. The large ion populations trapped by the LTQ facilitate acquisition of high quality tandem mass spectra of up to MS^5 and enables complete characterization of the carbohydrate structure.

Since glycopeptides tend to fragment first within the saccharide portion of the glycopeptide, it is often difficult to determine the peptide sequence and the site of oligosaccharide attachment. The need for high sensitivity MS^n is clearly shown in Figure 4, where an MS^4 experiment enables the peptide sequence to be determined.

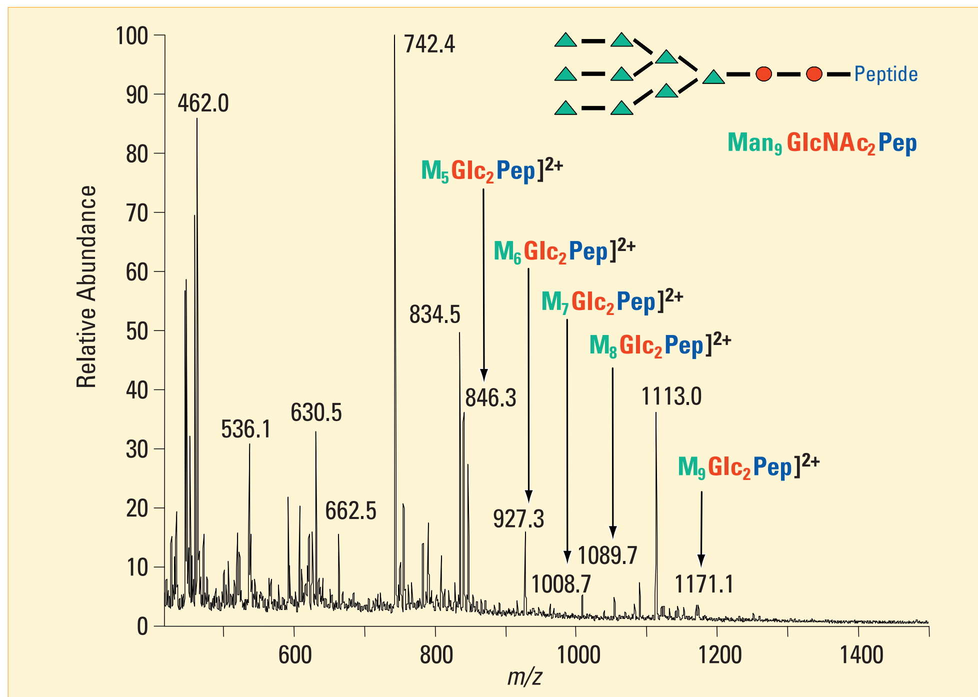

Figure 1: Full scan mass spectrum of the proteolytic digest of pancreatic ribonuclease B at a concentration of 1 pmol/mL as described in the experimental section

ETAAAK FER **QHMDSSTSAASSSNYCNQMMK** SR **NLTK**

DR CK **PVNTFVHESLADVQAVCSQK** **NVACK**

NGQTNCYQSYSTMSITDCR **ETGSSK** **YPNCAYK** **TTQANK**

HIIVACEGNPYVPVHFDASV

Table 1: Amino acid sequence and the predicted peptides from a proteolytic enzyme digestion of bovine ribonuclease B. Identified peptides are shown in **bold**; N-glycosylation site is shown in **red**

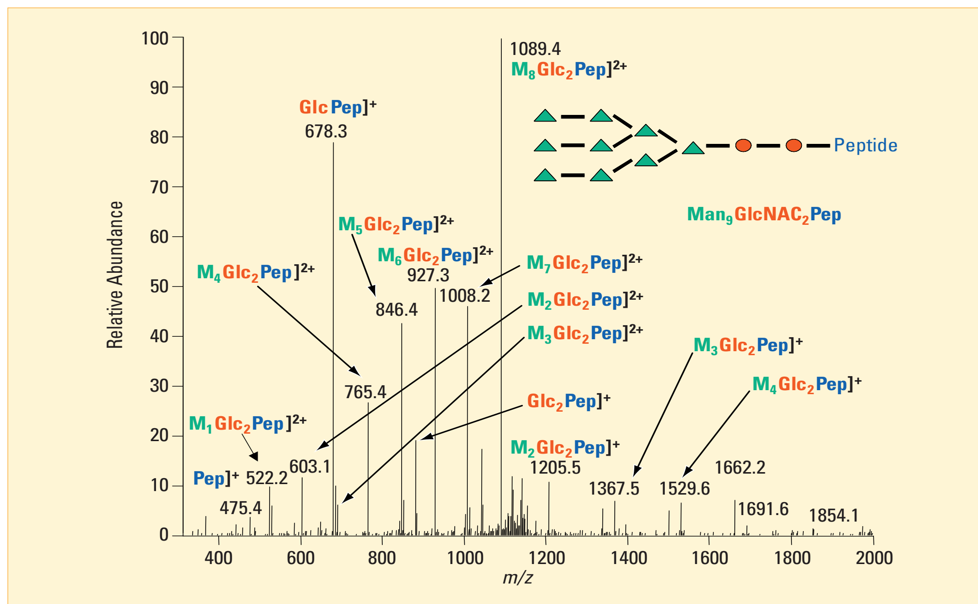

Figure 2a: Tandem mass spectra derived by collision-induced dissociation of the $(M+2H)^{2+}$ precursor ion of the ribonuclease glycopeptides, m/z = 1171.1

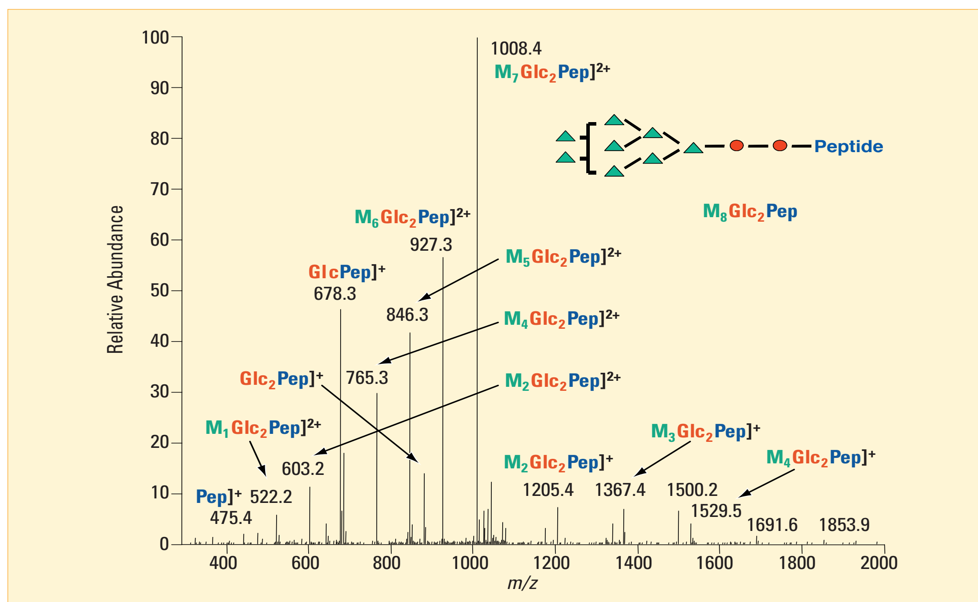

Figure 2b: Tandem mass spectra derived by collision-induced dissociation of the $(M+2H)^{2+}$ precursor ion of the ribonuclease glycopeptides, m/z = 1090.8

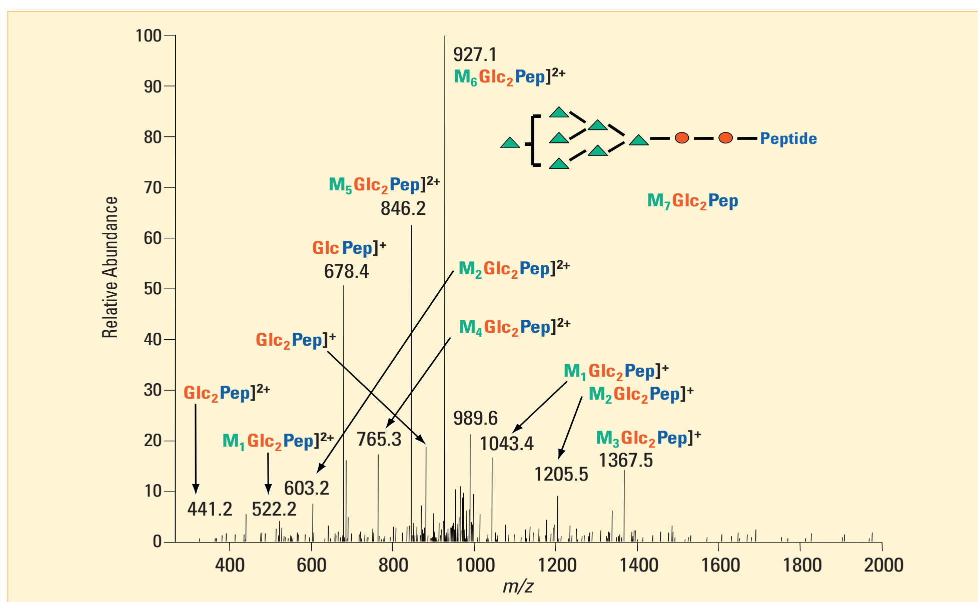

Figure 2c: Tandem mass spectra derived by collision-induced dissociation of the $(M+2H)^{2+}$ precursor ion of the ribonuclease glycopeptides, m/z = 1008.4

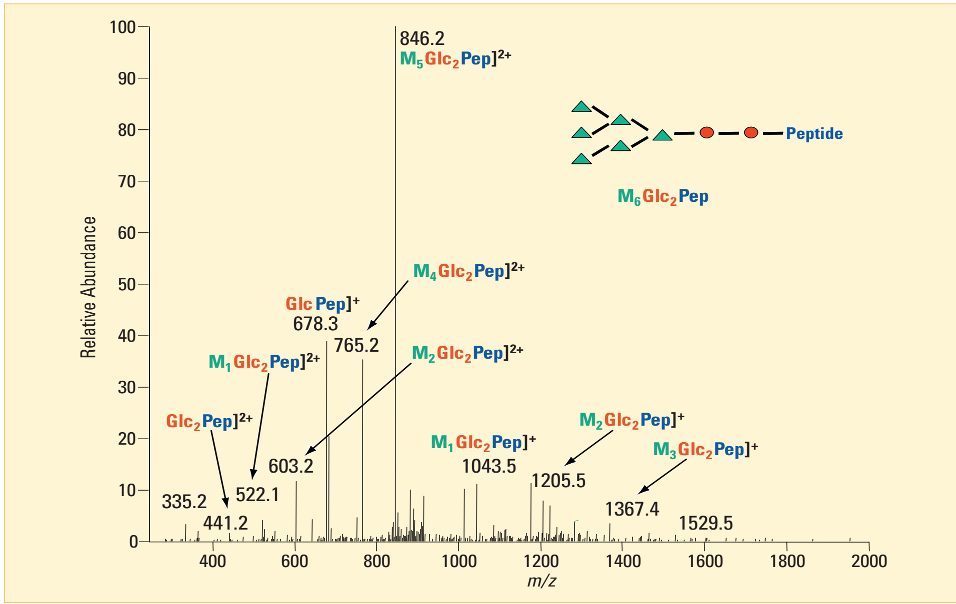

Figure 2d: Tandem mass spectra derived by collision-induced dissociation of the $(M+2H)^{2+}$ precursor ion of the ribonuclease glycopeptides, m/z = 927.3

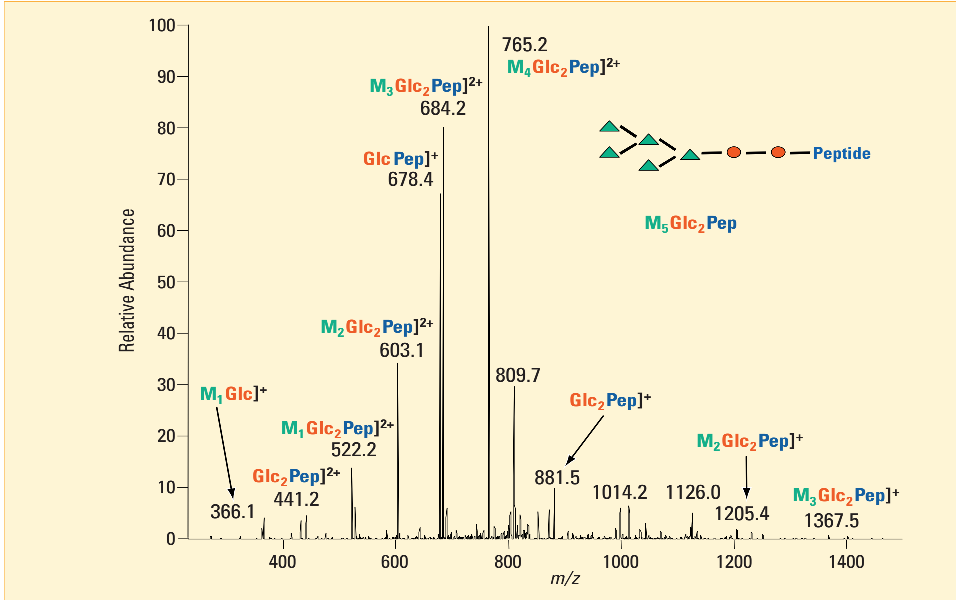

Figure 2e: Tandem mass spectra derived by collision-induced dissociation of the $(M+2H)^{2+}$ precursor ion of the ribonuclease glycopeptides, m/z = 846.0

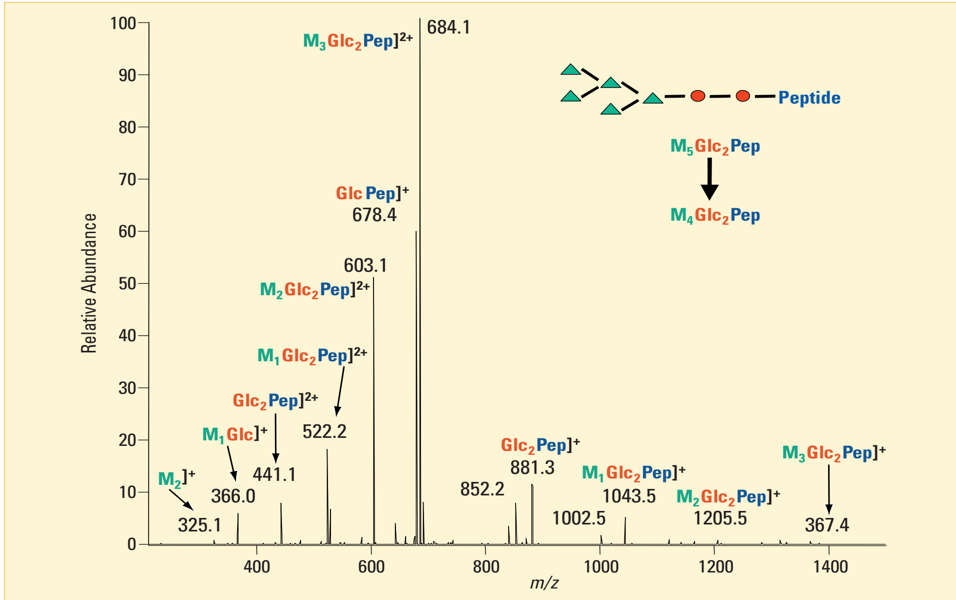

Figure 3a: MS^{3-5} mass spectra derived by collision-induced fragmentation of $(M+2H)^{2+}$, m/z = 864,0→765.0, for the stepwise removal of terminal mannose residues

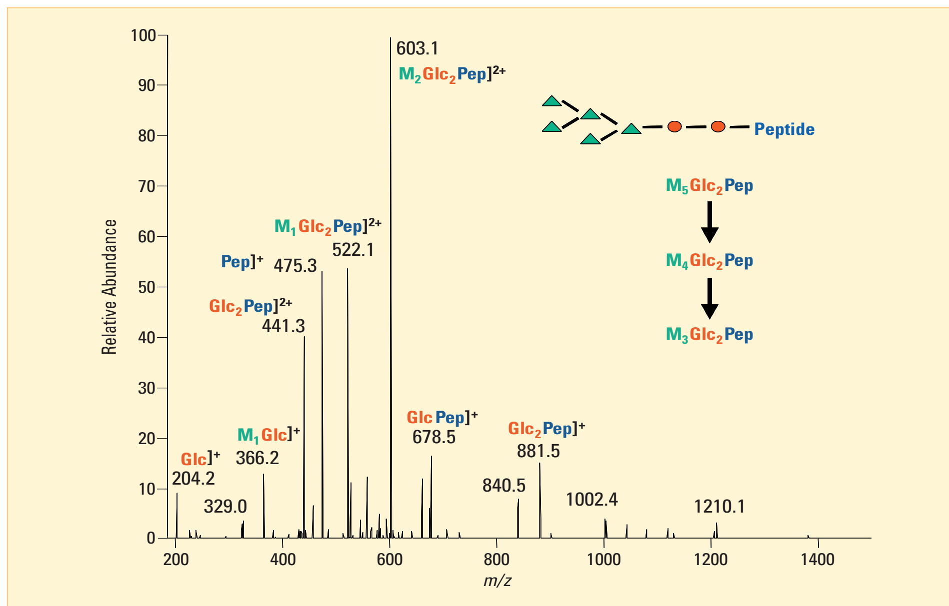

Figure 3b: MS3-5 mass spectra derived by collision-induced fragmentation of $(M+2H)^{2+}$, *m/z* = 864,0→765.0→684.0, for the stepwise removal of terminal mannose residues

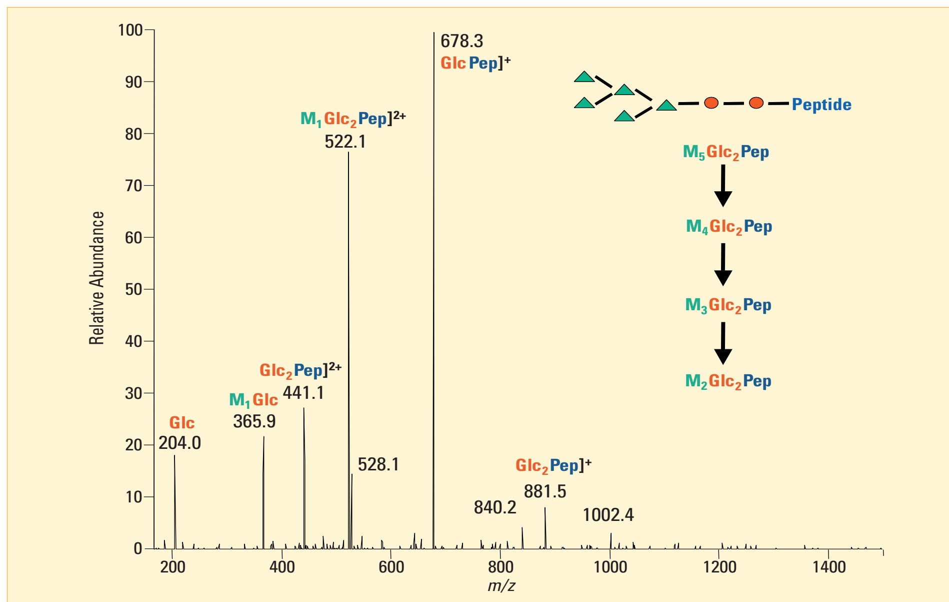

Figure 3c: MS3-5 mass spectra derived by collision-induced fragmentation of $(M+2H)^{2+}$, *m/z* = 864,0→765.0→684.0→603.0 for the stepwise removal of terminal mannose residues

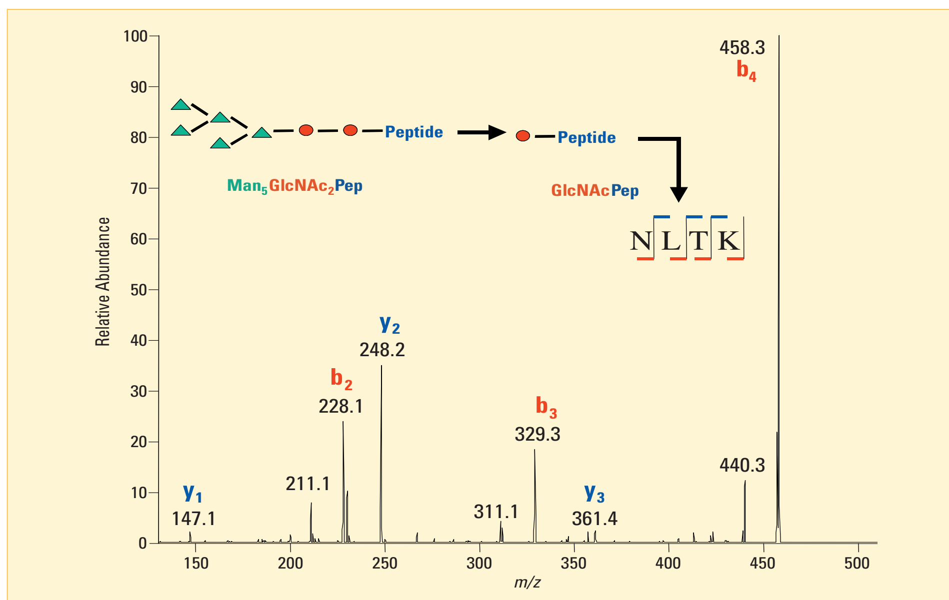

Figure 4: MS4 mass spectra derived by collision-induced fragmentation of $(M+2H)^{2+}$, *m/z* = 864,0→765.0→475 for the identification of the amino acid sequence of the ribonuclease glycopeptide

Conclusion

The LTQ ion trap mass spectrometer has the sensitivity and scan functionality for the analysis of glycoproteins. Using the unique MS/MS and MS^n functions, combined with BioWorks protein identification software, the glycosylated peptide was identified and the sugar structure was determined. The MS^4 spectra provided the additional information of the site of sugar attachment at the peptide backbone. The Advion NanoMate allows full automation of the nanospray process, resulting in excellent reproducibility while minimizing sample consumption and eliminating cross-contamination.

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