ApplicationNOTE

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THE USE OF Q-Tof™ FOR THE CHARACTERIZATION OF AN N-LINKED GLYCOSYLATION SITE IN NATIVE CAULIFLOWER XYLOGLUCAN ENDOTRANS GLYCOSYLASE

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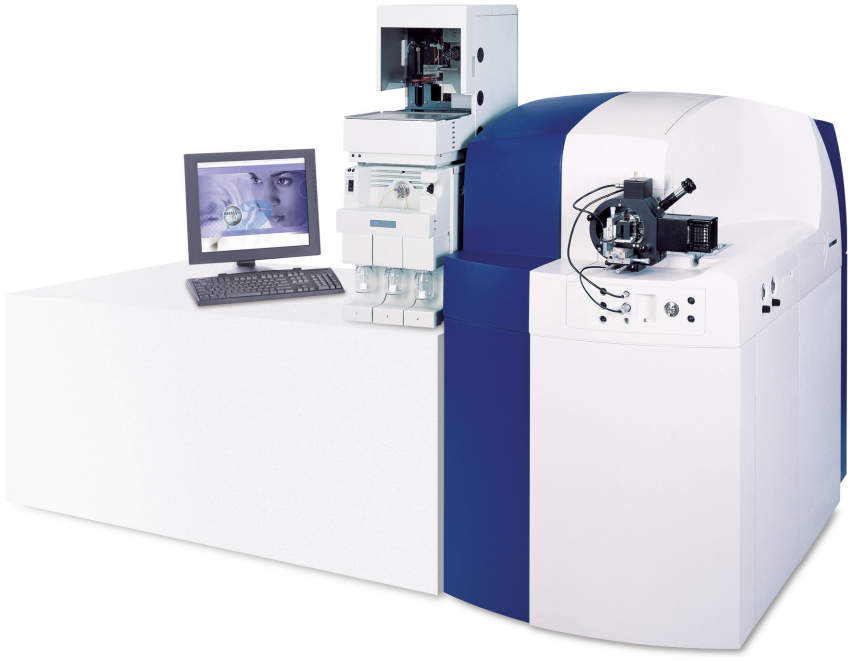
OVERVIEW

- Here we demonstrate use of relative molecular protein mass determination on a Waters Micromass® Q-Tof™ mass spectrometer to detect the presence of a post-translational modification (PTM).
- Key "marker ions" specific to oligosaccharides have been used to detect a post-translationally modified peptide.
- LC/MS/MS analysis of tryptically-digested peptides with the Waters CapLC® Q-Tof™ system enabled sequencing of post-translationally modified peptides and location of the PTM.

INTRODUCTION

Proteins frequently possess post-translational modifications, thus resulting in an increase in protein mass, which often cannot be predicted from the gene sequence. Common post-translational modifications are phosphorylation, sulfation, acetylation and glycosylation. The combination of capillary liquid chromatography, electrospray ionisation and Time-of-Flight (Tof) mass spectrometry provides a powerful approach for the analysis of these structural modifications[1].

Xyloglucan endotransglycosylases (XETs) are a class of enzymes closely related to the well-studied glycosylhydrolases (GH), which have been implicated in playing a role in plant cell wall expansion during growth and development. XETs catalyse the cleavage and re-ligation of high molecular weight xyloglucan, which acts as a "glue" to hold cellulose microfibrils together in a composite matrix thorough hydrogen bonding interactions[2]. Early studies have indicated that protein glycosylation may be important for the catalytic function of heterologously expressed *Arabidopsis thaliana* XET[3]. Interestingly, a conserved N-glycosylation site is found in Family 16 XETs proximal to the active site glutamic acid residues (Figure 1).



Waters Micromass protein characterization system.

ArabidopsisTCH4	SAGTVTTLYLKSPGTTWD	EIDF EFLG NSS	GEPYTLHTNVYTQGKGDKEQQFKLWFDPTANFH	140
Kiwi	SAGTVTAFYLSSQNSEHD	EIDF EFLG NRT	GQPYILQTNVFTGGKGDREQRIYLWFDPTKDYH	142
Tomato	SAGVVTAFYLSSNNAEHD	EIDF EFLG NRT	GQPYILQTNVFTGGKGNREQRIYLWFDPTKGYH	150
Tobacco	SAGVVTAFYLSSNNAEHD	EIDF EFLG NRT	GQPYILQTNVFTGGKGDREQRIYLWFDPTKGYH	149
Soybean	SAGTVTAFYLSSQNAEHD	EIDF EFLG NRT	GQPYILQTNVFTGGKGDREQRIYLWFDPTKEYH	147
MS/MS seq.		YLSSTNNEHD	EIDF EFLG DRT	GQPVLLQTNVFTGGK	

Figure 1. Sequence alignments of some GH family 16 XETs, showing the conserved catalytic residues and potential N-glycosylation site. The sequence obtained from the MS/MS analysis of two tryptic peptides (m/z 1201.0 and m/z 830.5) are also shown.

EXPERIMENTAL

- Purified cauliflower XET protein was digested with trypsin and then separated using the Waters CapLC system.
- The CapLC system was configured with a trapping column (320 µm x 5 mm, C18) where samples were first loaded via the autosampler and desalted. A 10-port valve was switched after ca. 3 mins and a gradient was run to elute the tryptic peptides from the trap onto the analytical column (LC Packings, 15 cm x 75 µm, C18 PepMap). A precolumn split gave a resultant flow though the column of 250 nL/min, with the pump delivering a flow of 2.5 µL/min. The peptides were eluted over 45 mins, using an acetonitrile/water gradient.
- The intact and digested protein samples were infused separately in to the mass spectrometer via borosilicate nanovials. A voltage of 850 V was applied to the tip of the needle to generate electrostatic nebulization. The proteins were dissolved to a final concentration of 1 pmol/µL in an aqueous solution containing 0.5% formic acid and 50% acetonitrile.

Mass Spectrometry

- Mass spectrometric analysis was carried out on a Q-Tof 2 mass spectrometer fitted with a NanoFlow ZSpray™ source. The instrument was calibrated over the mass range m/z 50-2500, using a sodium iodide solution.
- For intact protein analysis, the Q-Tof mass spectrometer was used to acquire MS data over the mass range m/z 400-2500.
- Data Directed Analysis (DDA™) was used to automatically acquire MS/MS data from the tryptically digested samples. An initial MS survey scan was acquired over the m/z range 350-1600.
- The instrument was programmed to switch into MS/MS on the four most intense, multiply-charged ions present in each MS survey scan.
- In each MS/MS experiment, the collision energy was tuned to optimize fragmentation of the selected ions based on their individual m/z and charge state. Data for MS/MS was acquired over the m/z range 50-2000.
- Switch back into MS survey mode was triggered after a time period of 4 seconds.

RESULTS AND DISCUSSION

Mass determination of the post-translational modification by intact protein analysis

- The raw MS data (figure 2) from the intact proteins was combined and background subtracted. The data was then deconvoluted using Maximum Entropy 1 (MaxEnt™ 1) to obtain a relative molecular protein mass.

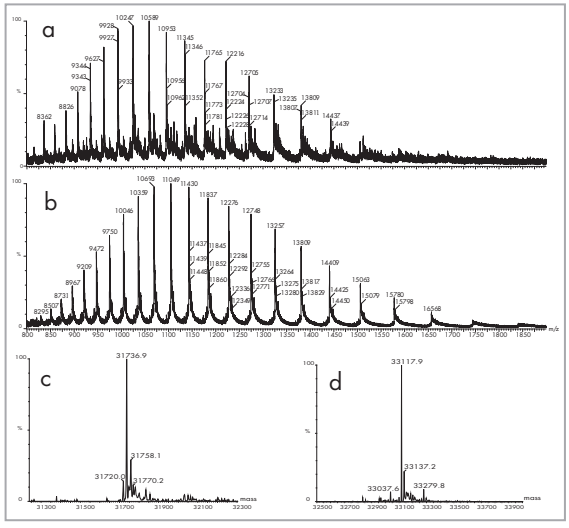


Figure 2. TOF-MS spectra of N-glycosidase F-treated (a) and native (b) cauliflower xyloglucan endotransglycosylase. The deconvoluted (MaxEnt 1) spectra are also shown, c (F-treated) and d (native).

- The experimentally determined relative molecular masses for the N-glycosidase F-treated XET (Figure 2 c) and native XET (Figure 2 d) were 31736.9 Da and 33117.9 Da respectively.
- The mass difference between the native XET and the Nglycosidase F-treated XET was 1381.0 Da, which is consistent with the mass of an N-linked $GlcNAc_2Hex_6$ oligosaccharide.

Detection and characterization of the post-translationally modified peptide

- During LC/MS/MS analysis of glycopeptides, diagnostic carbohydrate marker ions can be produced from the decomposition of the oligosaccharide moieties in the collision cell of the Q-Tof mass spectrometer. Therefore, detection of the marker ions at m/z 163.1 (protonated hexose residue [Hex]), m/z 204.1 (protonated N-acetylhexosamine residue [Hex Nac]) and m/z 366.1 (protonated N-acetylhexosamine residue [Hex Hex Nac]) can easily be used to identify the presence of a glycopeptide.

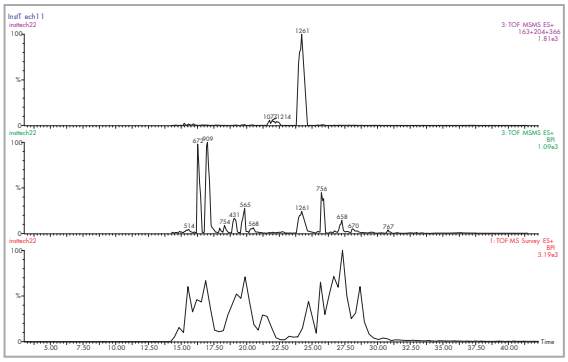


Figure 3. Data from the LC/MS/MS analysis of a tryptic digest of native cauliflower xloglucan endotransglycosylase. The lower base peak intensity chromatogram represents the eluting peptides in MS survey data. The middle base peak intensity chromatogram represents one of the MS/MS components and the peptides selected for MS/MS. The upper diagram is the reconstructed ion chromatogram for the diagnostic ions at m/z 163.1, 204.1 and 366.1.

- Figure 3 shows the data from the LC/MS/MS (DDA) analysis of tryptically-digested native XET protein. A reconstructed ion chromatogram was produced for m/z 163.1, 204.1 and 366.1 (upper trace) from the MS/MS data (middle trace).
- As can be observed from this reconstructed ion chromatogram in Figure 3 (upper trace) there is a large peak in the MS/MS data at m/z 1260.5, indicating the presence of an oligosaccharide component.
- The raw MS/MS data was deconvoluted into a simplified form for interpretation using Maximum Entropy 3 (MaxEnt 3) software. MaxEnt 3 data is shown here for m/z 1260.5 (Figure 4).

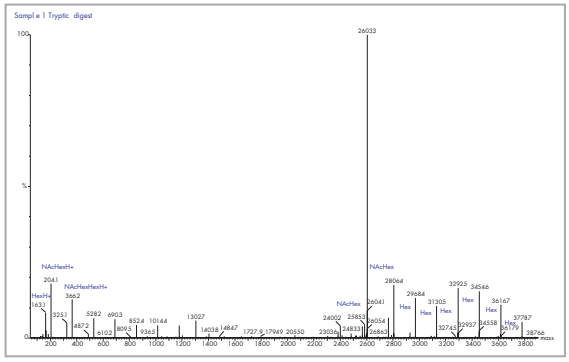


Figure 4. Maximum Entropy 3 (MaxEnt 3) deconvoluted spectrum obtained from MS/MS of the ion m/z 1260.5. The progressive neutral loss of hexose and N-acetyl hexoseamine have been annotated as Hex and NAcHex respectively

- Multiple neutral losses of 162.0 amu and 203.0 amu at the upper end of the spectrum can be observed corresponding to the loss of hexosamine and N-acetylhexosamine, respectively, from the peptide bound oligosaccharide chain.
- At the lower end of the spectra the ions of /mz 163.1, 204.1 and 366.2 correspond to protonated hexose, protonated N-acetyhexosamine and a protonated hexose N-acetylhexosamine respectively.
- It can be clearly observed that there are six neutral losses of 162.0 amu and two neutral losses of 203.0 amu, corresponding with the postulated composition of an N-linked $GlcNAc_2Hex_6$ oligosaccharide.This is consistent with the mass difference between the N-glycosidase F-treated and untreated cauliflower xyloglucan endotransglycosylase.

Determining the site of post-translational modification

- The tryptic digest of N-glycosidase F-treated (deglycosylated) XET was further analyzed by ESI/MS/MS. The sample was first desalted, in an aqueous solution containing 0.5% formic acid and 50% acetonitrile, using a C18 Ziptip. It was then infused into the mass spectrometer via a borosilicate nanovial which enabled extended MS/MS analysis on ion of interest.
- MS analysis of the peptide mixture enabled detection of a doubly charged ion at m/z 1201.0 which corresponded to one of the deglycosylated peptides present in the F-treated XET sample.
- MS/MS data was subsequently acquired from the ion at 1201.0 m/z and processed using MaxEnt 3. The peptide sequence was then automatically derived with the de novo sequencing algorithm, MassSeq™ (Figure 5a). Full sequence coverage was obtained for the deglycosylated peptide.

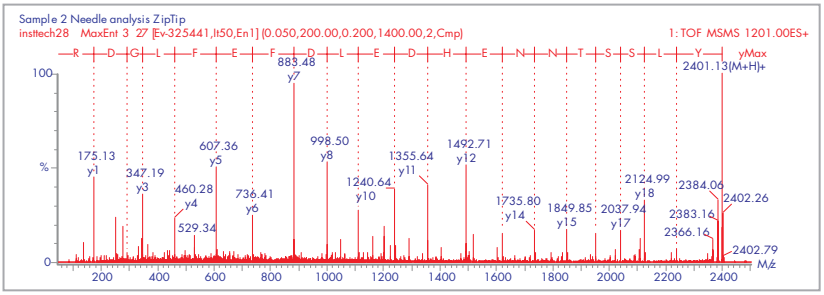


Figure 5a. Full sequence coverage obtained from MS/MS of the doubly charged precursor ion m/z 1201.0

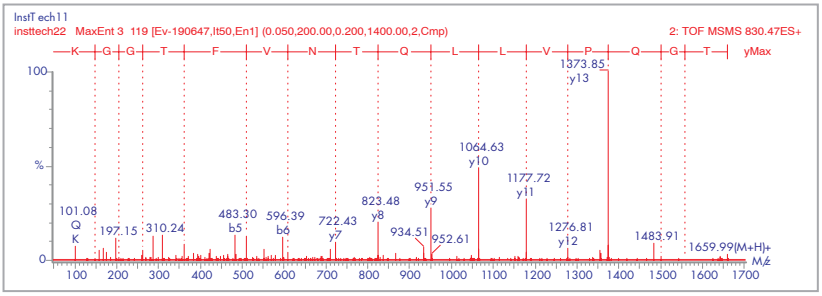


Figure 5b. Full sequence coverage obtained from MS/MS of the doubly charged precursor ion m/z 830.5

- MS/MS and de novo sequence analysis was carried out in an identical manner for the ion at m/z 830.5. This corresponds to the adjacent C-terminal peptide within the XET sequence. Again, full sequence coverage was obtained using MassSeq (Figure 5b).
- The C-terminal residues of peptide m/z 1201.0 are aspartic acid and arginine (D & R), and the N-terminal residue of peptide m/z 830.5 is threonine (T). This corresponds to an internal concensus sequence DRT, whereas the conserved glycosylation site within the xyloglucan endotransglycosylases is actually NRT (asparagine-argininethreonine) (Figure 1).
- This observation can be explained since the removal of the oligosaccharide by N-glycosidase F- treatment results in the hydrolysis of the asparagine (N) residue to aspartate (D, Figure 6)

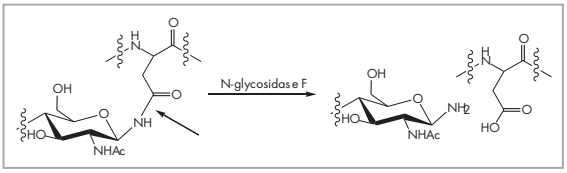


Figure 6. Schematic representation of N-glycosidase F hydrolysis of an N-linked oligosaccharide. Prior to hydrolysis the oligosaccahride is attached to an asparagine residue. Upon release of the oligosaccharide the asparagine residue is hydrolysed to an aspartic acid residue.

- The one mass unit shift (asparagine to aspartic acid) was identified by a combination of MS/MS analysis and de novo sequencing with MassSeq. This enabled confirmation of the conserved glycosylation site within cauliflower xyloglucan endotransglycosylase.

CONCLUSION

- MS analysis of intact cauliflower XET protein using nanoscale infusion on a Waters Micromass Q-Tof system, followed by subsequent analysis of the MaxEnt 1 deconveluted data, produced evidence of a post-translational modification consistent with an N-linked $GlcNAc_2Hex_6$ oligosaccharide.
- A combination of LC/MS/MS and nanoscale infusion MS/MS allowed the identification of the post-translational modification and full sequence characterization of the oligosaccharide moiety, confirming the $GlcNAc_2Hex_6$ sequence.
- The conserved consensus sequence for the bound oligosaccharide was mapped and confirmed to be NRT.

REFERENCES

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3. P. Campbell and J. Braam (1998) *Plant Journal*, 15, 553-561

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