

**Investigation of Post-translational Modifications of Recombinant
Polygalacturonase III from *Aspergillus niger* Using Mass Spectrometry**

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Overview

- ◆ **Determination of the Post-translational modifications occurring on recombinant Polygalacturonase III (PGC), including glycosylation and phosphorylation;**
- ◆ **Employment of multiple techniques : MALDI-MS, HPLC, ESI-MS/MS and LC-MS;**
- ◆ **First investigation of the post-translational modifications of PGC.**

Introduction

Aspergillus niger produces several polygalacturonases that, with other enzymes, are involved in the degradation of the pectic polysaccharides of plant cell walls. This specific property causes these enzymes to have a variety of industrial uses, such as clarification of fruit juices. It is known that post-translational modifications (PTMs), such as glycosylation and phosphorylation, alter the properties of some of these enzymes. To date, no work has been done to investigate the PTMs of PGC. The objective of this work is to identify the glycosylations and phosphorylation occurring on PGC by means of combining different MS, HPLC and proteolytic methods.

Methods

- ◆ **Are there any Post-translational modifications on PGC?**

Lectin blotting and ELISA performed on immunoassay plates using DIG Glycan Differentiation Kit for **glycosylation screening** and alkaline phosphatase conjugates (Rabbit IgG and Mouse IgG) for screening **phosphorylation**.

- ◆ **Determine the type of Glycosylation (figure 1)**

Obtain the molecular weight of intact PGC by **HP-MALDI-TOF MS**
PGC then treated with Endo-H directly on MALDI sample stage for 30 min to produce N- deglycosylated protein suitable for screening of high mannose N-linked glycans.

- ◆ **Determine the glycopeptide attaching site and glycan structure**

Generate tryptic peptides by standard protocols and analyze by MALDI-TOF MS for heterogeneity due to glycosylation (figure 2).

Analyze tryptic PGC fragments by LC-ESI-MS (Q-TOF) using **stepped orifice voltages technique** (figure 3).

- ◆ **Identify glycopeptide**

Identified using **carbohydrate marker ions** at m/z ratios of 163, 204 and 366 (figure 4,5).

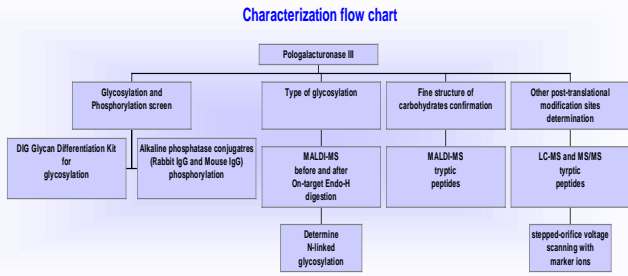
- ◆ **Further peptide mapping**

Tryptic PGC fragments then further analyzed by **LC-ESI- MS/MS** (figure 6).

Compare the experimental peptide amino acid sequences with published sequences from database to locate additional other post-translational modification sites.

Instrumentation

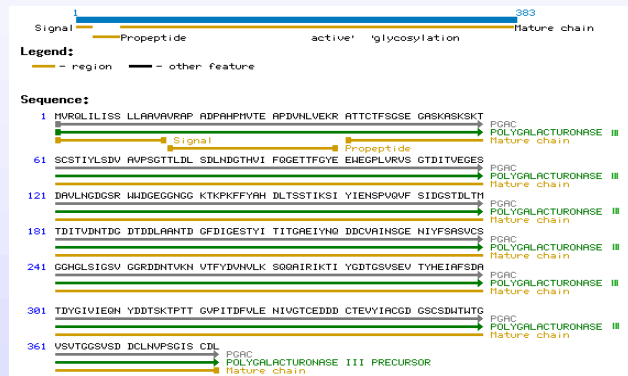
MALDI-MS:	Hewlett Packard G2025A time-of-flight mass spectrometer
Q-TOF setting:	Micromass Q-TOF 2; Electrospray ionization in positive ion mode MS 1st acquisition by cone voltage set to 60 from 50-250 m/z MS 2nd acquisition by cone voltage set to 30 from 400-2000 m/z



Results

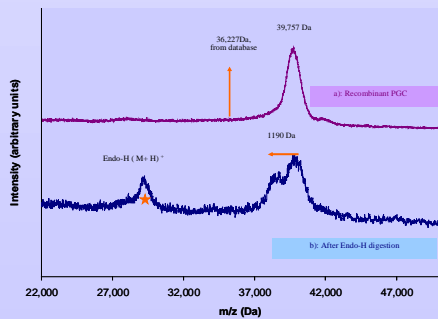
Lectin blotting and ELISA results indicate that PGC is both a glycosylated and phosphorylated protein.

SEQUENCE OF PGC FROM NCBI nr DATABASE



Molecular mass: 36,227 Da, pt: 3.9

Asn 260 is a potential N-linked glycosylation site: **KNVTFYDVLNVLK** (T12, MW=1,310.6 Da)



(a) The molecular mass of PGC is approximately 3530 Da larger than the mass calculated from the amino acid sequence. This indicated the protein experienced PTMs

(b) Endo-H digestion results a new peak with approximately 1190 Da mass less than the mass intact PGC, which indicated N-linked glycosylation exists. The relatively short digestion time results in incomplete digestion therefore intact PGC is still observed.

Figure 1. MALDI mass spectra (a) of the recombinant PGC from *A.niger* and (b) after PGC had been digested by Endo-H on-target for 30min. The peak marked with an asterisk represents ion from Endo-H.

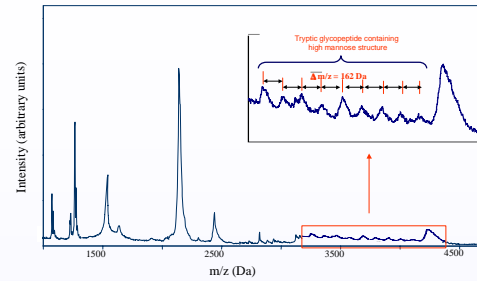


Figure 2. MALDI-MS of tryptic PGC serial peaks with average differences of 162 Da confirms that this peptide contains high mannose structure

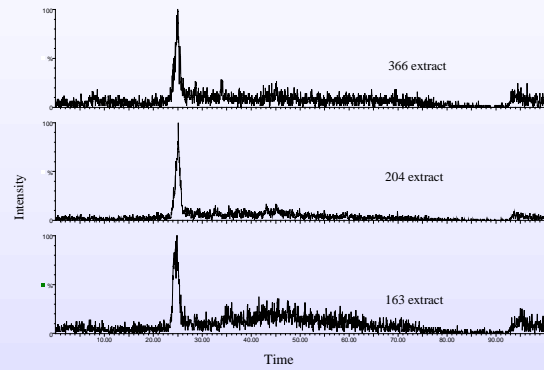


Figure 4. (a) ion chromatograms of three carbohydrate fragment masses extracted from TIC under high cone voltage acquisition; and (b) averaged spectrum from high cone voltage acquisition

(Glycans are fragmented under high cone voltage acquisition, and high cone voltage TIC is averaged and screened for carbohydrate ions; ions produced by Hexose (163 m/z), HexNAc(204 m/z) and Hex-HexNAc(366 m/z) were observed. The ion chromatography of three ions show the same elution time. This means only one glycopeptide appears in PGC.

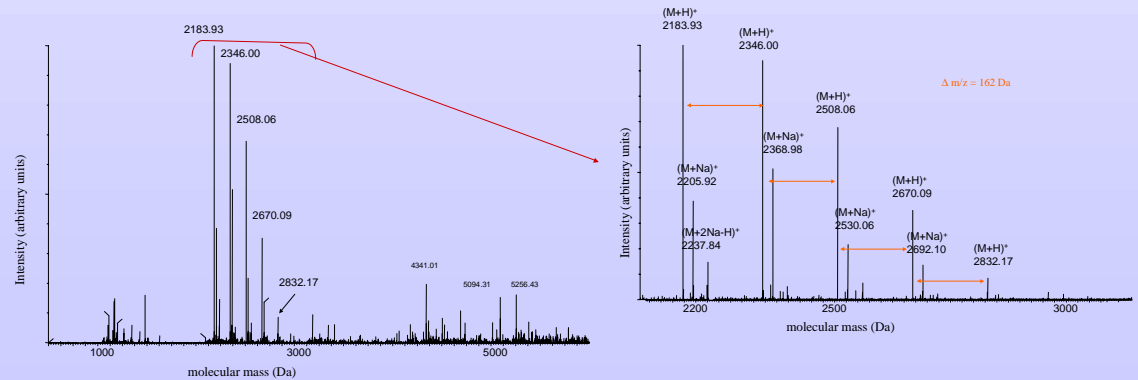


Figure 5. Spectra for the 25min peaks of the low cone voltage scan (Averaged and then converted into singly charged) Low cone voltage TIC is used to reveal the source of the carbohydrate fragment ions. The corresponding glycopeptide shows a high mannose series at mass region 2183.9–2832.2Da.

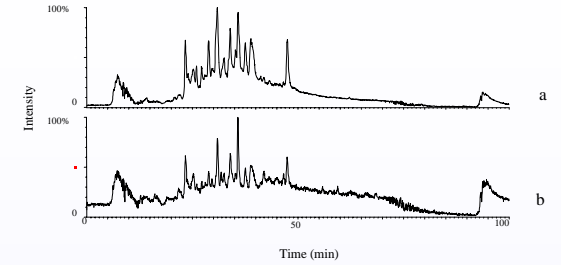


Figure 3. Total ion chromatograms (TIC) of MS acquisitions of tryptic PGC obtained under (a) high cone voltage and (b) low cone voltage. Spectra from (A) are averaged and reviewed for diagnostic fragment ions. If fragment ions are found, the masses of the source glycopeptide can be determined by averaging the spectra for that peak using the TIC from (B)

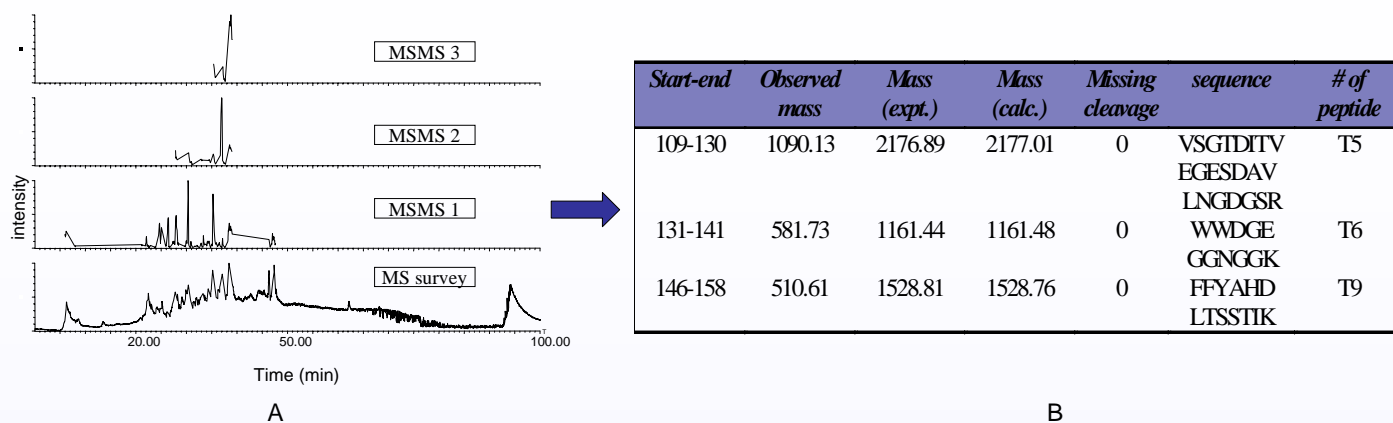


Figure 6. (A) MS/MS survey of tryptic PGC peptides and (B) the database searching result

The peptide mapping is performed by ESI-MS/MS survey with three different conditions. The spectra are treated with MassLynx software, and then sent to Mascot database for searching. Three peptides, T5, T6 and T9 are matching with database record, which suggests no post-translational modification occurred in those three peptides.

Conclusion

- ◆ PGC is both glycosylated and phosphorylated.
- ◆ The mass difference between the intact PGC and deglycosylated PGC demonstrates that a **N-linked glycosylation of high mannose structure is located at Asn 260**.
- ◆ The peptide containing this N-linked glycan eluted from LC around 24-25min and was found to have a mass at **2183.93-2832.17 Da** region.
- ◆ From Mascot searching results of MS/MS data, we can see three peptides: T5, T6 and T9 are perfectly matching with database records. This means the post-translational modifications won't occur on those three peptides.
- ◆ There is still an additional 2336 Da mass discrepancy which cannot be explained. Phosphorylation would be partially accounted for the mass difference that consists with ELISA analysis. Yet other post-translational modifications such as O-linked glycosylation cannot be excluded.

Future work

- ◆ Locate the sites of phosphorylations.
Either from MS/MS data or by the aid of labeling technique.
- ◆ Locate the sites and size of O-linked glycans.
Using enzymatic digestion, affinity chromatography and ESI-MS/MS.

References

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Acknowledgement

This research is gratefully supported by grants from National Institute of Health (NIHP41RRR05351), the North Atlantic Treaty Organization (NATO CRG973086), the Department of Energy (DOE DE-FG02-93ER20097 and DE-FG02-96ER20221) and the National Science Foundation (MCB-0115132).