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Development and application of mass spectrometric methods for the analysis of progranulin *N*-glycosylation

Kriangsak Songsrirote[a], *Zhi Li*[b], *David Ashford*[c], *Andrew Bateman*[b], *Jane Thomas-Oates*[a,*]

[a]*Department of Chemistry, and Centre of Excellence in Mass Spectrometry, University of York, York, UK*
[b]*Endocrine Research Laboratories, Department of Medicine, Royal Victoria Hospital, McGill University, Montreal, Quebec, Canada*
[c]*Department of Biology, and Centre of Excellence in Mass Spectrometry, University of York, York, UK*

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ABSTRACT

PGRN is a modular protein with 7 1/2 repeats of the granulin domain separated by short spacer sequences. Elevated expression of PGRN is associated with cancer growth, while mutations of PGRN cause frontotemporal lobar degeneration (FTLD), an early onset form of dementia. PGRN is a glycoprotein, containing five *N*-glycosylation consensus sequons, three of which fall within granulin domains. A method tailored to enable detailed analysis of the PGRN oligosaccharides and glycopeptides has been developed. The approach involves in-gel deglycosylation using peptide-*N*-glycosidase F (PNGase F) followed by permethylation of the released oligosaccharides. Permethylation was applied for rapid sample clean-up and to improve sensitivity of MS detection and mass spectrometric fragmentation. Reversed-phase monolithic LC–ESI–MS/MS was used for analysis of permethylated oligosaccharides, enabling structural characterization of released *N*-linked glycans in one chromatographic run. In-gel tryptic digestion was further applied to the gel pieces containing deglycosylated protein, for *N*-glycosylation site determination. In addition, glycopeptides were produced using in-solution pronase digestion to identify species of *N*-glycan attached at particular sites. The method developed was applied to progranulin (PGRN) to characterize the structures of the released glycans and to identify the sites of glycosylation. Glycosylation of four out of five potential PGRN *N*-glycosylation consensus sites was demonstrated (the final one remains undetermined), with one of the four observed to be partially occupied. Two of the observed glycosylation sites occur within granulin domains, which may have important implications for understanding the structural basis of PGRN action.

1. Introduction

Progranulin (PGRN) is a glycoprotein found in all vertebrates and most invertebrates; in mammals it plays a role in a range of functions including embryo development [1,2], wound healing [2] and inflammation [3,4]. High expression of PGRN is also associated with tumorigenesis in many cancers [reviewed in 5]. Monoclonal antibodies against PGRN inhibit the growth of liver cancers in murine models, suggesting that PGRN may be a suitable target for the development of anti-cancer therapies [6]. Moreover, mutations in the gene for PGRN cause an early onset dementia called frontotemporal lobar degeneration [7,8]. This is often due to haploinsufficiency of the PGRN gene [7,8] but mutants in which translocation and abnormal glycosylational processing have been reported [9], and cause frontotemporal dementia. PGRN is a modular protein with 7 1/2 repeats of the 12-cysteine granulin–epithelin motif [10,11] separated by short intervening sequences. While the 3-dimensional conformation of the granulin–epithelin motif has been demonstrated as a stack of beta-turns pinned by an axial rod of 6-disulfide bridges,

* *Corresponding author*. Tel.: +44 1904 434459; fax: +44 1904 432516.
E-mail address: jeto1@york.ac.uk (J. Thomas-Oates).

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[12,13] the overall conformation of the full-length protein is unknown. Defining the glycosylation of PGRN is an essential step towards better understanding the molecular structure of this protein.

Carbohydrate attachment to proteins can modify the intrinsic properties of the proteins. Because of the importance of protein glycosylation in mediating a wide range of biological processes, characterization of glycan structures is necessary in understanding the structure function relationship. The wide range of glycan structures, with several possible isoforms makes their full structure analysis very demanding. Moreover, the small amount of available glycan material increases further the demands of the analysis. Consequently, a range of sample handing, derivatization and separation approaches which are compatible with proteomic and glycoproteomic analyses have been employed in combination with analytical systems, to improve analysis scale and sensitivity.

N-glycan chains are attached to the amide nitrogen on the asparagine's side chain in the consensus sequence (Asn-X-(Ser or Thr)), where X can be any amino acid except proline. The enzyme PNGase F releases *N*-linked glycans converting the glycosylated asparagine to aspartic acid. Permethylation of the released glycans helps to improve ionization efficiency resulting in increasing sensitivity of detection [14–18] and makes glycans amenable to RP-LC/MS, using solvents that are compatible with mass spectrometry.

Although the use of PNGase F to deglycosylate glycoproteins can be exploited to locate glycosylation sites by virtue of the conversion of N to D, the information on which N bears a particular glycan species is lost in glycoproteins that contain more than one glycosylation site. The analysis of glycopeptides which consist of both peptide backbone and oligosaccharide resolves this problem. To produce glycopeptides of manageable size for mass spectrometry, a non-specific protease, pronase, has been exploited [19–21].

In this work, the released *N*-linked oligosaccharides from progranulin have been characterized using a modification of the method of in-gel deglycosylation presented by Küster et al. [22], followed by permethylation. The permethylated glycans were analysed by MALDI-MS/MS and ESI-LC–MS/MS; a reversed-phase monolithic column which shows several advantages over the traditional particle-supported RP chromatography [23–25] was employed for the first time for permethylated glycan analysis. Moreover, the deglycosylated polypeptide remaining in the gel was then digested with trypsin and the released deglycosylated peptides subjected to RP-LC–ESI/MS for glycosylation site determination. Pronase digestion of progranulin was performed to produce glycopeptides that are amenable to analysis by MS. Prior to MS analysis, the released glycopeptides were isolated using a mixed bed microcolumn. To characterize the sites of glycosylation of each glycan, CID MS/MS was exploited.

2. Materials and methods

2.1. Chemicals

All solvents (methanol, ethanol, acetonitrile, DMSO, HPLC-grade water) used were from Fisher Scientific. Ovalbumin and bovine pancreatic ribonuclease B were purchased from Sigma. NuPAGE® Novex 4–12% Bis–Tris gels, and LDS sample buffer were from Invitrogen. PNGase F from Sigma was dissolved in HPLC-grade water to a final concentration of 1000 units/mL. Modified porcine trypsin (sequencing grade) was purchased from Promega. Pronase (mixture of proteases from *Streptococcus griseus*) was from Sigma-Aldrich. 2-mercaptoethanol was purchased from Sigma. Dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma-Aldrich. Sodium dodecyl sulfate (SDS) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Fluka. Dowex AG-50 X8 (H^+) and Dowex AG-3 X4A (OH^-) ion-exchange resins were from Sigma-Aldrich. C18 resin was taken from a disposable SPE Strata C18-E 70 Å cartridge. The PGRN was prepared by transient transfection in COS7 cells as previously described [36]. After transfection the cells were cultured for 48 h in serum free medium, the medium collected, centrifuged to remove debris and then concentrated using 15 mL Amicon Ultra-15 Centrifugal Filters with a 30 kDa cut-off (Millipore). The concentrate was then purified by reversed-phase high pressure liquid chromatography as described [26]. The PGRN content of HPLC fractions was determined by Western blot analysis.

2.2. Gel electrophoresis

Approximately 10 μg each of ovalbumin and RNaseB, and 15 μg of PGRN were mixed with LDS sample buffer and DTT, and heated at 70 °C for 10 min, before loading onto a 1D SDS-PAGE NuPAGE® mini gel. The gel was run in the Xcell Surelock™ mini cell (Invitrogen) with NuPAGE® MES buffer (Invitrogen) at a constant 200 V for 45 min. The glycoproteins were detected with 0.1% Coomassie blue staining. The protein bands were excised from the gel, and destained with 40% methanol and 10% acetic acid overnight to remove background staining.

2.3. In-solution de-N-glycosylation

Approximately 15 μg of PGRN was dissolved in 45 μL of 20 mM sodium bicarbonate ($NaHCO_3$). Then 5 μL of denaturation solution (0.2% SDS with 100 mM 2-mercaptoethanol) was added. The solution was heated at 100 °C for 10 min. After the solution was cooled down to room temperature, 2 μL of 1000 U/mL PNGase F was added. The reaction mixture was incubated at 37 °C for 16 h.

2.4. In-gel de-N-glycosylation

The gel bands containing glycoprotein were washed twice with 20 mM $NaHCO_3$ for 30 min each. The wash was removed and replaced with 300 μL of fresh 20 mM $NaHCO_3$. 20 μL of 45 mM dithiothreitol was added to the solution to reduce the protein. The solution was heated at 60 °C for 30 min, and then left at room temperature (RT) to cool. 20 μL of 100 mM iodoacetamide was added to alkylate the protein, and kept for 30 min in the dark at RT. All of the solution was removed, and the protein band was washed by incubation in 50% acetonitrile in 20 mM $NaHCO_3$ for 30 min twice. The gel band was cut into small pieces, and dried in a vacuum centrifuge.

30 μL of 100 U/mL PNGase F solution was added to the dried gel pieces. After the gel absorbed PNGase F solution for 30 min at RT, an additional 20 mM $NaHCO_3$, 100 μL, was added to cover the pieces. The digest was incubated at 37 °C overnight.