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Structural analysis of *N*- and *O*-glycans using ZIC-HILIC/dialysis coupled to NMR detection

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ABSTRACT

Protein glycosylation, an important and complex post-translational modification (PTM), is involved in various biological processes, including the receptor–ligand and cell–cell interaction, and plays a crucial role in many biological functions. However, little is known about the glycan structures of important biological complex samples, and the conventional glycan enrichment strategy (i.e., size-exclusion column [SEC] separation) prior to nuclear magnetic resonance (NMR) detection is time-consuming and tedious. In this study, we developed a glycan enrichment strategy that couples Zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) with dialysis to enrich the glycans from the pronase E digests of RNase B, followed by NMR analysis of the glycoconjugate. Our results suggest that the ZIC-HILIC enrichment coupled with dialysis is a simple, fast, and efficient sample preparation approach. The approach was thus applied to analysis of a biological complex sample, the pronase E digest of the secreted proteins from the fungus *Aspergillus niger*. The NMR spectra revealed that the secreted proteins from *A. niger* contain both *N*-linked glycans with a high-mannose core similar to the structure of the glycan from RNase B, and *O*-linked glycans bearing mannose and glucose with 1→3 and 1→6 linkages. In all, our study provides compelling evidence that ZIC-HILIC separation coupled with dialysis is very effective and accessible in preparing glycans for the downstream NMR analysis, which could greatly facilitate the future NMR-based glycoproteomics research.

1. Introduction

Post-translational modifications play essential roles in controlling physiological cellular processes by affecting protein folding, conformation, interaction, and activities (Kamath et al., 2011). Protein glycosylation, an important and complex post-translational modification, is involved in various biological processes, including receptor–ligand and cell–cell interaction, and plays a crucial role in many biological functions (Mann and Jensen, 2003). In fungi, a significant number of secreted biomass-degrading enzymes are known to be glycosylated, and a variety of studies have assessed the functional effects of both *N*- and *O*-linked glycosylation (Beckham et al., 2012). Two types of glycosylation linkages exist: *N*-type and *O*-type. The *N*-glycans are linked to the amide nitrogen of asparagine (Asn) with the motif sequence of Asn-X-serine (Ser)/threonine (Thr) (X is any amino acid except proline [Pro] or aspartic acid [Asp]). The attached sugar residue is *N*-acetylglucosamine (GlcNAc). A common pentasaccharide core among *N*-linked oligosaccharides containing two *N*-acetylglucosamines and three mannoses exists (Kornfeld and Kornfeld, 1976). *O*-glycans can be significantly diverse, most of which are linked to the hydroxyl group of Ser/Thr (Kornfeld and Kornfeld, 1976). *N*-glycans can be removed from the proteins with an amidase such as PNGase F (Marino et al., 2010), while *O*-glycans are cleaved primarily using chemical approaches (Carlson, 1966; Merry et al., 2002).

Structural and functional characterization of glycoproteins presents significant challenges and is a growing research area, mainly because glycan analysis is involved in various disciplines (e.g., medical research and drug discovery) and is vital for many emerging areas including glycoproteomics. The investigation of glycan composition and structures can not only explain the discrepancies in protein molecular weight and/or chromatographic retention

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time between the experimental data and the *in silico* analysis of peptide sequence (Marino et al., 2010), but also provide critical clues to the understanding of the physiological functions (Freeze and Aebi, 2005) and enzymatic activities (Wittwer et al., 1989) of glycoproteins. Therefore, the development of easy and fast methodologies for the analysis of glycan structures is important to advance glycoproteomics research.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for studying the molecular structure, molecular interaction, molecular kinetics—or dynamics—and composition of biological mixtures (Keeler, 2010). The analyte size within the capacity of NMR detection ranges from a small organic molecule or metabolite, to a mid-sized peptide or a natural product, up to proteins of several tens of kDa in molecular weight. In addition, two-dimensional NMR spectroscopy can be employed to provide the information complementary to one-dimensional NMR analysis, which is particularly useful in simplifying the determination of the molecular structures from a chemical mixture even without further chromatography separation. NMR-based methods have been extensively used in characterization of glycan structures for glycoproteins (Duus et al., 2000). For medium-sized polysaccharide molecules, NMR-based glycan analyses show significant advantage over chromatography and mass spectrometry (MS) analysis because NMR is highly quantitative and reproducible, and provides more detailed molecular structure information, such as the ring chain linkage and the conformation (Davis et al., 1994; Keeler, 2010).

The natural low abundance of glycoproteins in biological samples requires efficient strategy to enrich glycans for subsequent NMR measurement. Size-exclusion separation (SEC), based on the differences of the molecular sizes of analytes, was used almost extensively for the fractionation of oligosaccharides prior to NMR analysis (Gonzalez et al., 1998; Young et al., 2002). However, the sample preparation is tedious and time-consuming (e.g., a single SEC running time is ~5 h, and purification of glycans normally requires more than one type of SEC fractionation approach) (Harvey, 1997), and the peptides could be co-eluted with the glycans of the same size. In addition, the glycan analysis typically has special requirements, such as radiolabeling, fluorescent labeling, or mass spectrometry, which are not straightforward to couple online with SEC fractionation for localizing the glycan-containing fractions. Therefore, the lack of an easy approach to enrich glycans hinders the characterization of glycans by NMR.

On the other hand, ZIC-HILIC separation has recently been widely used in MS-based glycoproteomics research (Di Palma et al., 2011; Intoh et al., 2009; Takegawa et al., 2006). The stationary phase of the ZIC-HILIC column contains positively charged quaternary ammonium groups and negatively charged sulfonic acid groups on the surface (Di Palma et al., 2011; Intoh et al., 2009), forming a hydrophilic layer that serves as the basis for the interaction between the stationary phase and analytes. Samples are dissolved in the high-concentration organic solvent and eluted by increasing the water gradient (Di Palma et al., 2011). Therefore, the ZIC-HILIC column is suitable for separating glycans from a peptide mixture because of the difference of hydrophobicity. To our knowledge, the ZIC-HILIC enrichment has not been used for NMR analysis of glycan structures.

Herein, we developed the strategy that coupled ZIC-HILIC separation with dialysis to enrich *N*- and/or *O*-linked glycans prepared by pronase E digestion of the pure glycoprotein or biological complex sample. For the proof-of-principle experiment, the known glycosylated protein RNase B (Fu et al., 1994) was digested with pronase E followed by separation through the ZIC-HILIC column coupled with or without dialysis, and the enriched glycans were analyzed by NMR. The results demonstrate that the glycan enrichment using ZIC-HILIC coupled with dialysis to remove non-specifically bound small molecules is ideal for the glycan preparation for the downstream NMR analysis. Therefore, this enrichment strategy was further applied to the enrichment of glycans from the *Aspergillus niger* secretome. *A. niger* is a well-known fungal species that has been used extensively for production of citric acid and polysaccharide hydrolases. The secreted glycoproteins contain the majority of the enzymes responsible for hydrolyzing biomass into fermentable sugars. *N*-glycosylation sites for *A. niger* secreted proteins have been determined (Wang et al., 2011a), and some genetic analysis of the pathway has been performed (Dai et al., 2013), but little is known about the glycan structures. Therefore, we took advantage of the ZIC-HILIC coupled with dialysis strategy to enrich the glycans derived from pronase E cleavage of the secretome, followed by NMR analysis of the glycan structure. We were able to simultaneously characterize both *N*- and *O*-linked glycan structures from the *A. niger* secretome: the *N*-linked glycans contain high-mannose core structure similar to RNase B, and the *O*-linked glycans contain mannose and glucose with 1→3 and 1→6 linkages. In all, our study demonstrates that ZIC-HILIC separation coupled with dialysis is ideal for the glycan enrichment because of its rapidity, ease, and effectiveness, and thus greatly facilitates the future glycoproteomics research.

2. Materials and methods

2.1. Chemicals

RNase B, dithiothreitol, iodoacetamide, pronase E from *Streptomyces griseus*, methoxyamine hydrochloride, 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt (TSP), and *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide were from Sigma–Aldrich (Milwaukee, Wisconsin, USA). Deuterium oxide was from VWR (Radnor, Pennsylvania, USA).

2.2. Growth of A. niger and preparation of secretome from A. niger

A. niger (ATCC11414) was maintained on complete agar medium, which is widely used for fungal growth (Bennette and Lasure, 1991). Conidia of spore inocula were grown on complete medium at 30 °C and harvested after 4 days. 1×10^6 spores/ml were inoculated into 2 × 200 ml of modified minimal medium as described previously (Wang et al., 2011b). The cultivation was performed at 30 °C in a 1-L baffled flask in the incubator shaker (New Brunswick Scientific) at 200 rpm. After 24 h of growth, the supernatant was collected by filtering the culture through two layers of sterile miracloth and then centrifuging it at 15,000*g* at 4 °C for 10 min to remove cell debris. About 400 ml of supernatant was concentrated to 20 ml in a stirred cell (Millipore, Billerica, Massachusetts, USA) with an ultrafiltration membrane (NMWL 3 kDa, Millipore, Billerica, Massachusetts, USA) overnight at 4 °C with continuous pressure of 40 psi N_2. Cold (−20 °C) acetone was mixed with the protein solution at a volume ratio of 5:1, and incubated at −20 °C for 1 h. The precipitated proteins (~30 mg) were harvested by centrifugation at 18, 000*g* for 15 min at 4 °C.

2.3. Protein digestion with pronase E

Twelve mg of RNase B or 30.6 mg of *A. niger* secretome dissolved in NH_4HCO_3 buffer (100 mM, pH 8) were reduced with 10 mM dithiothreitol at 60 °C for 30 min and then alkylated with 40 mM iodoacetamide in the dark at 37 °C for 60 min. With the addition of 1 mM $CaCl_2$, the chemically treated proteins were digested with pronase E from *S. griseus* (mass of protein substrate/mass of pronase E = 1/1) at 37 °C for 6 h and further digested with newly added fresh pronase E at 37 °C for 18 h. The