Analytica Chimica Acta 787 (2013) 140–147

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Dephosphorylation of intact glycoprotein to greatly improve digestion efficiency coupled with matrix-assisted laser desorption/ionization–Fourier transform ion cyclotron resonance mass spectrometric analysis

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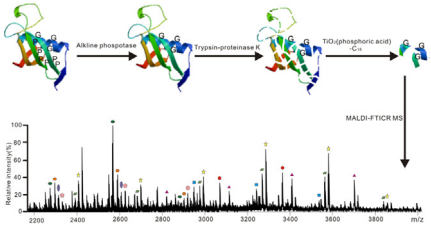
HIGHLIGHTS

- Dephosphorylation of intact glycoprotein to greatly improve digestion efficiency.
- The enrichment efficiency for multisialylated glycopeptides were enhanced using phosphoric acid solution as elution buffer.
- Validation was made by analyzing human serum α2-macroglobulin and transferrin.

GRAPHICAL ABSTRACT



The stereostructure of bovine fetuin based on Benkert,P., *et al.* Bioinformatics 2011, 27. 343-50.
G: glycan; P: phosphoric acid.

ARTICLE INFO

Article history:
Received 13 February 2013
Received in revised form 20 May 2013
Accepted 23 May 2013
Available online 2 June 2013

Keywords:
Dephosphorylation
Intact glycoprotein
Titanium dioxide
Matrix-assisted laser desorption/ionization–Fourier transform ion cyclotron resonance mass spectrometry
Sialylated glycopeptide

ABSTRACT

Sialylation is essential for a variety of cellular functions. Herein, we used bovine fetuin with three potential N-linked glycosylation sites containing complex-type glycan structures, four potential O-linked glycosylation sites and six potential phosphorylation sites as a model compound to develop a highly-efficient digestion strategy for sialylated glycoproteins and efficient enrichment strategy for sialylated glycopeptides using titanium dioxide. The former according to the process of alkaline phosphatase digestion followed by tryptic digestion and then proteinase K digestion could greatly improve the enzymatic efficiency on fetuin, and the latter could obviously enhance the enrichment efficiency for multisialylated glycopeptides using phosphoric acid solution as elution buffer. The mass spectra of the enriched glycopeptides derived from fetuin reveal that several series of the ion clusters with mass difference of 291 Da correspond to the presence of multisialylated glycopeptides. In addition, the approach was applied to characterize the sialylated status of α2-macroglobulin and transferrin, respectively, from the sera of healthy subjects and sex- and age-matched patients with thyroid cancer, and their spectra indicate that the change in the amount of the glycoforms containing different number of sialic acid (SA) residues from one glycosylation site may be used to differentiate between healthy subjects and cancer cases.

Abbreviations: PTM(s), post translational modification(s); DHB, 2,5-dihydroxylbenzoic acid; ACN, acetonitrile; TFA, trifluoroacetic acid; MALDI-FTICR MS, matrix-assisted laser desorption/ionization–Fourier transform ion cyclotron resonance mass spectrometry; DTT, dithiothreitol; IAA, iodoacetamide; CBB, coomassie brilliant blue; SA, sialic acid; TP, tryptic digestion followed by proteinase K digestion; TPA, tryptic digestion followed by proteinase K digestion and then alkaline phosphatase digestion; ATP, alkaline phosphatase digestion followed by tryptic digestion and then proteinase K digestion; IG/I(G-H_2O), an intensity ratio of sialylated glycopeptide to its dehydrated counterpart, where G represents sialylated glycopeptide; IG/I(G-SA), an intensity ratio of sialylated glycopeptide to its less sialylated counterpart; CDG, carbohydrate-deficient glycoproteins.

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http://dx.doi.org/10.1016/j.aca.2013.05.044

1. Introduction

Sialylated glycoproteins are a major subclass of glycoproteins, glycan moieties of which can profoundly affect myriad biological processes, such as protein folding and stability, modulation of protein function, and receptor–ligand interaction [1–3]. Sialic acid (SA) belongs to the family of nine-carbon backbone sugars, which is an obvious exception of the common carbohydrate chains composed generally of five- or six-carbon sugars, and is typically found attached to the distal ends of glycans, bridging interactions with environmental agents skillfully [4]. Previous studies have reported that different physiological conditions generated different glycans and glycan diversification at one glycosylation site of one protein may show distinct functions [5,6]. Previous studies have also shown that aberrant sialylations of proteins are significantly associated with diseases [5,7–11].

Traditional methods for the characterization of the sialylated glycans involve proteolysis of glycoproteins with several enzymes. Trypsin is the most commonly employed enzyme by virtue of its high enzymatic specificity and activity, however, suffering steric hindrance and resulting in large mass peptides [12–15]. These peptides would likely contain more than one potential glycosylation sites, which severely decrease ionization efficiency and complicate site-specific glycosylation analysis. Nonspecific proteases, such as pronase and proteinase K, are alternative choices to hydrolyze glycoproteins and to result in much shorter peptides. Actually, these approaches do not overcome the effect of inherent steric hindrances due to post-translational modifications (PTMs), such as phosphorylation and glycosylation. Glycans attached to proteins or peptides are well known steric hindrance [12–15], and phosphorylation is another important and common PTM [16]. Thus we hypothesize that phosphoric acid group attached to the proteins may breed considerable steric hindrance both electronically and spatially due to its negative charge and size, which prohibits nonspecific enzyme capable of cleaving any peptide bond, resulting in the glycopeptides with multiple glycosylation sites or larger peptides. So the identification of glycosylation sites and the characterization of site-specific glycan structure are challenging task.

The remarkable microheterogeneity of glycan structure with regard to the branching patterns and the sequence of each antenna also challenges sialylated glycoprotein analysis. Furthermore, non-sialylated glycopeptides are prone to suppress sialylated glycopeptide signals [17–19], especially sialylated glycopeptides harboring more polar residuals [20]. As a consequence, the separation of the sialylated glycopeptides from complex digestion mixture is critical to the identification of the sialylated glycopeptides. Currently, the separation methods have been utilized for sialylated glycopeptide analysis, such as titanium dioxide (TiO_2) [21–23], and the mixture of graphite carbon to activated charcoal [24]. Among them, TiO_2 chromatography is a preferred choice for the sialylated glycopeptide analysis [21]. TiO_2 has extremely high specific affinity toward SA residuals, whereas the enrichment efficiency remained to be enhanced due to the sporadic distribution of the sialylated glycopeptides in mass spectra [21,22].

Herein, we used bovine fetuin with three potential N-linked glycosylation sites, four potential O-linked glycosylation sites and six potential phosphorylation sites [25] and human thyroglobulin with up to seventeen potential N-linked glycosylation sites and one potential O-linked glycosylation site [26] as model compounds to develop a highly-efficient digestion strategy for sialylated glycoproteins and an efficient enrichment strategy for the sialylated glycopeptides coupled with matrix-assisted laser desorption/ionization (MALDI)–Fourier transform ion cyclotron resonance (FTICR) mass spectrometric (MS) analysis.

2. Material and methods

2.1. In-solution digestion of fetuin and thyroglobulin

One $\mu g\,\mu L^{-1}$ of bovine fetuin and thyroglobulin (Sigma–Aldrich, St. Louis, MO, USA) was prepared in water, respectively. The solutions were treated as previously described with slight modifications [22]. Briefly, 10 μL of the protein solution was denatured using boiling water for 6 min. 1.25 μL of 500 mM dithiothreitol (DTT) in 25 mM NH_4HCO_3 was added into the solution for the protein reduction at 37 °C for 2 h. After the reduced sample was allowed to cool to room temperature, 2.5 μL of 500 mM iodoacetamide (IAA) was added, and the mixture was incubated for 45 min at room temperature in the dark. Subsequently, 1.25 μL of 500 mM DTT was added to quench the reaction. Three enzymatic digestion strategies were employed as follows: (1) The reduced and alkylated glycoprotein was digested with 2% (w/w) sequencing grade modified trypsin (Roche diagnostics, Mannheim, Germany) at 37 °C for 20 h, and then the tryptic digest was further hydrolyzed by 1% (w/w) proteinase K (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C for 20 h. This digestion strategy is termed as TP. (2) The digest derived from the TP was further dephosphorized with 0.75 units of alkaline phosphatase (Promega Corporation, Madison, WI, USA) at 37 °C for 4 h. This digestion strategy is termed as TPA. (3) The reduced and alkylated glycoprotein was first dephosphorized with 0.75 units of alkaline phosphatase at 37 °C for 4 h followed by the TP. This digestion strategy is termed as ATP (Fig. 1). All of the digested samples were dried by SpeedVac vacuum concentrator at −45 °C and stored at −80 °C until analysis.

2.2. Isolation of α2-macroglobulin and transferrin from human serum

The separation of α2-macroglobulin and transferrin from human serum of healthy blood donors in our laboratory and the patients with thyroid cancer with written informed consent was performed via a well-established approach as reported previously [27,28]. Briefly, 10 μL of sera pooled from three patients or three healthy controls was subjected to native-thin-layer-IEF as the first dimensional electrophoresis, followed by native-PAGE as

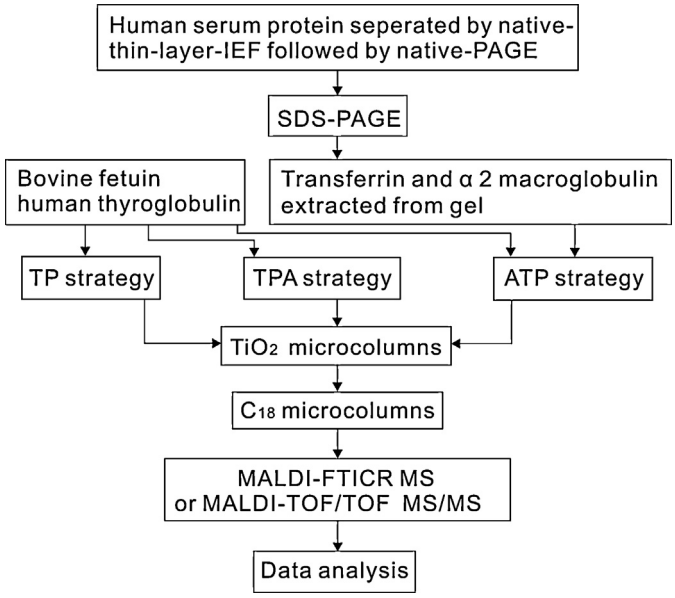

Fig. 1. Schematic to investigate the effect of phosphoric acid group attached to the intact glycoproteins on the digestion efficiency coupled with MALDI-FTICR MS analysis.