Clinica Chimica Acta 480 (2018) 1–8

Contents lists available at ScienceDirect

Clinica Chimica Acta

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

# An endoglycosidase-assisted LC-MS/MS-based strategy for the analysis of site-specific core-fucosylation of low-concentrated glycoproteins in human serum using prostate-specific antigen (PSA) as example

Robert Lang[a,*], Andreas Leinenbach[a], Johann Karl[a], Magdalena Swiatek-de Lange[a], Uwe Kobold[a], Michael Vogeser[b]

[a] Roche Diagnostics GmbH, Nonnenwald 2, 82377 Penzberg, Germany
[b] University Hospital, LMU Munich, Marchioninistraße 15, 81377 Munich, Germany

ARTICLE INFO

*Keywords:*
Prostate-specific antigen
LC-MS/MS quantification
Core-fucosylation
Glycoprotein
Human serum
Endo F3

ABSTRACT

Recently, site-specific fucosylation of glycoproteins has attracted attention as it can be associated with several types of cancers including prostate cancer. However, individual glycoproteins, which might serve as potential cancer markers, often are very low-concentrated in complex serum matrices and distinct glycan structures are hard to detect by immunoassays. Here, we present a mass spectrometry-based strategy for the simultaneous analysis of core-fucosylated and total prostate-specific antigen (PSA) in human serum in the low ng/ml concentration range. Sample preparation comprised an immunoaffinity capture step to enrich total PSA from human serum using anti-PSA antibody coated magnetic beads followed by consecutive two-step on-bead partial deglycosylation with endoglycosidase F3 and tryptic digestion prior to LC-MS/MS analysis. The method was shown to be linear from 0.5 to 60 ng/ml total PSA concentrations and allows the simultaneous quantification of core-fucosylated PSA down to 1 ng/ml and total PSA lower than 0.5 ng/ml. The imprecision of the method over two days ranged from 9.7–23.2% for core-fucosylated PSA and 10.3–18.3% for total PSA depending on the PSA level. The feasibility of the method in native sera was shown using three human specimens. To our knowledge, this is the first MS-based method for quantification of core-fucosylated PSA in the low ng/ml concentration range in human serum. This method could be used in large patient cohorts as core-fucosylated PSA may be a diagnostic biomarker for the differentiation of prostate cancer and other prostatic diseases, such as benign prostatic hyperplasia (BPH). Furthermore, the described strategy could be used to monitor potential changes in site-specific core-fucosylation of other low-concentrated glycoproteins, which could serve as more specific markers ("marker refinement") in cancer research.

## 1. Introduction

Among protein post-translational modifications (PTMs), glycosylation is a frequently occurring and functionally important one involved in many physiological processes including cell adhesion, receptor activation, protein folding and immune response [1–4]. Estimates suggest that approximately half of all mammalian proteins are glycosylated [5]. Protein glycosylation is not a template-driven process such as DNA, RNA or protein synthesis, but is rather controlled by complex enzymatic pathways during protein passage through the endoplasmic reticulum and Golgi compartments. The activity of those enzymes depends on factors including their quantity, localization and substrate availability, which can largely vary based on differences in tissue, cell type and disease state [6–8]. Even under non-malignant conditions this greatly increases the complexity of protein glycosylation, resulting in extensive molecular micro- and macroheterogeneity of glycoproteins. The disturbance of this equilibrium in disease often leads to altered glycosylation of individual glycoproteins expanding the degree of heterogeneity beyond their natural forms [9]. This offers the potential for glycoproteins to serve as markers for a variety of biological processes. Fucose (Fuc), galactose (Gal), glucose (Glc), mannose (Man), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNac) and sialic acid (Sia) are the primary building blocks of a diverse set of human N- and O-glycan structures. Core-fucosylation, consisting of an $\alpha$-1,6 fucose modification on the innermost GlcNAc residue of the N-glycan core structure, has attracted attention, because it can be linked to various types of cancers such as hepatocellular carcinoma, pancreatic cancer, lung cancer and prostate cancer (PCa) [10–13].

* Corresponding author.
*E-mail address:* robert.lang.rl1@roche.com (R. Lang).

https://doi.org/10.1016/j.cca.2018.01.040
Received 10 December 2017; Received in revised form 22 January 2018; Accepted 22 January 2018

Although glycoprotein research has been improved by advancements in mass spectrometry (MS), core-fucosylation analysis in complex matrices remains challenging as concentrations of individual glycoproteins are usually very low due to glycan microheterogeneity at multiple glycosylation sites. Furthermore, representative glycopeptides have decreasing ionization efficiencies with glycan branching and sialylation and notably suffer from ion suppression from co-eluting non-glycopeptides during electrospray ionization (ESI) [14]. Several methods have been applied to overcome these challenges using large scale glycoprotein or glycopeptide enrichment steps, for example, lectin affinity chromatography, peroxidase oxidation prior to hydrazide coupling or hydrophilic interaction chromatography [10,15]. In contrast, specific enrichment of individual glycoproteins using IgG depletion followed by immunoprecipitation has been performed on high-abundance glycoproteins such as ceruloplasmin and α-2-macroglobulin [11,16]. Enriched and purified glycoproteins or glycopeptides are commonly enzymatically treated by endoglycosidases for complete or partial cleavage of their glycans and are analyzed by tandem MS with or without previous labeling [17,18]. Core-fucosylation analysis at the glycan level is usually achieved by using peptide-N-glycosidase (PNGase F) cleaving N-glycans from purified proteins or from biological mixtures [19]. This enzymatic release additionally allows for indirect identification of N-glycosylation sites of glycoproteins by deamidation of asparagine to aspartic acid providing a mass shift of +0.98 Da. However, information linking multiple glycosylation sites to its respective glycan structures is lost. Other enzyme-based strategies using galactosidases, sialidases or *endo-β-N*-acetylglucosaminidases retain site-specific information as these enzymes do not remove the complete glycan [20]. In addition, glycan microheterogeneity of partially truncated glycopeptides is simplified and ionization efficiency is increased compared to intact glycopeptides, making partial deglycosylation a useful tool in core-fucosylation analysis.

All of the examples mentioned above have been limited to the core-fucosylation analysis of high-abundance and highly purified glycoproteins or applied large scale screenings of core-fucosylated serum proteomes. By applying glycoproteome-wide studies, disease-specific modifications of a single, low-concentrated glycoprotein cannot be captured. The analysis of individual modifications may be a more effective biomarker refinement strategy as the discovery of the core-fucosylated α-fetoprotein (AFP-L3) approved for the early diagnosis of hepatocellular carcinoma by the Food and Drug Administration (FDA) recently showed [21]. Alternative, non-MS-based strategies for core-fucosylation analysis use enzyme-linked lectin assays (ELLA). These types of assays are based on a similar principle to the common enzyme-linked immunosorbent assay (ELISA) technique, in which the capture or detection antibody or both are replaced by a lectin. Approaches involving antibody-based capture of low-concentrated glycoproteins and subsequent detection of their fucosylation by lectins have been employed [13,22]. Major challenges with ELLA are the inherent glycosylation of the capture/detection antibody or non-specific binding by high-abundance glycoproteins from human matrices, which can cause a non-specific background signal by lectin detection, obscuring the analyte signal of interest. Furthermore, simultaneous measurement of total glycoprotein levels and corresponding core-fucosylated subpopulations is not feasible. Outside the biological context, lectins have low affinity for their target glycans as multivalent interactions are missing [23]. This makes glycosylation analysis in the low concentration range more difficult.

Prostate-specific antigen (PSA), also known as Kallikrein-3 (KLK3), is a 28–32 kDa glycoprotein composed of 237 amino acids. According to the NXT/S motif for N-glycosylation (if X is not proline), PSA possesses a single N-glycosylation site at Asn-69. In blood, PSA circulates in two predominant forms, either as free PSA or complexed to *α1*-antichymotrypsin and *α2*-macroglobulin [22]. PSA is almost exclusively secreted by epithelial cells of the prostate gland, which makes PSA a highly organ-specific biomarker [24]. PSA blood levels of 10 ng/ml or higher indicate the risk of cancer, and prostate biopsy is usually recommended [25]. However, PSA is not a cancer-specific biomarker because PSA blood levels do not efficiently distinguish between PCa and other prostatic diseases, such as benign prostatic hyperplasia (BPH) and prostatitis, especially in the so called “grey area” ranging from 4 to 10 ng/ml [26]. In addition, PSA serum levels cannot differentiate between indolent and aggressive PCa [27]. As a consequence, PSA screening resulted in tremendous over-diagnosis and over-treatment during the last decades [28]. Several groups proposed that altered glycosylation might increase the diagnostic potential of PSA [29]. Defined changes in the fucosylation degree of PSA in cancer samples have been described using different lectin-based approaches. For example, Fukushima et al. showed in a 40-sample cohort that α-1,2-fucosylated total PSA levels were higher in sera of PCa patients than in sera of BPH patients with > 95% probability [30]. The α-1,2-fucosylated form of free PSA was shown by Dwek et al. to be increased in sera of cancer patients with 92% specificity and 69% sensitivity for PCa over BPH [22]. In contrast, a significant decrease with 90% sensitivity and 95% specificity in α-1,6-core-fucosylated total PSA was found by Llop et al. in high-risk PCa that differentiated BPH and low-risk PCa from high-risk PCa patients in a 40-sample cohort in which total PSA concentrations ranged from 4.14 to 109.7 ng/ml [13].

Here, we present a hybrid immunoaffinity mass spectrometry (IA-MS) based approach for the simultaneous quantification of total PSA levels and its core-fucosylated subpopulation in the low ng/ml concentration range in human serum. Challenges in lectin-based approaches including weak affinity for low-concentrated targets could be tackled by combining the sensitivity of immunoassays with the specificity of mass spectrometric detection. In this work, the glycoprotein PSA served as an ideal example as its native serum concentrations usually are very low. The heart of the method was an immunoaffinity enrichment step followed by consecutive partial deglycosylation and proteolytic digestion while PSA was still captured by the immunoaffinity complex. Following, surrogate peptides of total PSA and core-fucosylated PSA were analyzed by LC-MS/MS. Based on calibration curves of total and core-fucosylated PSA, we evaluated the linear range, lower limit of detection (LLOD), lower limit of quantification (LLOQ) and imprecision expressed as coefficient of variation (CV). Altered core-fucosylation of serum glycoproteins might be the long sought diagnostic biomarkers associated with malignancies, such as cancer. However, specific and sensitive methods for the simultaneous quantification of low-concentrated glycoproteins and its core-fucosylated subpopulations are rare. To fill this gap, we suggest that this endoglycosidase-assisted MS-based strategy could be used for site-specific core-fucosylation analysis of other low-concentrated glycoproteins in human serum to evaluate their potential as biomarkers.

## 2. Experimental section

### 2.1. Reagents and materials

Streptavidin-coated magnetic particles (Ref. 11,641,786,001), biotinylated monoclonal antibody PSA36 against total PSA (=free and complexed PSA) binding to epitope 6b [4], total PSA CalSet II (Ref. 04485220190) and universal diluent (Ref. 11732277122) were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Phosphate buffered saline (PBS), trypsin from porcine pancreas, tween 20 and ammonium bicarbonate (ABC) were purchased from Sigma-Aldrich (St. Louis, USA). Endoglycosidase F3 (Endo F3) from *Elizabethkingia meningosepticum* was obtained from Ludger Ltd. (Oxfordshire, UK). Anhydrous sodium acetate and glacial acetic acid were from Merck (Darmstadt, Germany). Acetonitrile (ACN) and formic acid (FA) both ULC/MS grade were purchased from Biosolve (Dieuze, France). PSA purified from human seminal fluid was purchased from Scripps Laboratories (San Diego, USA). Peptides LSEPAELTDAVK (single-letter amino acid code) and a mixture of glycopeptides N(GlcNAc)K and N