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Elucidating heterogeneity of IgA1 hinge-region O-glycosylation by use of MALDI-TOF/TOF mass spectrometry: Role of cysteine alkylation during sample processing☆

Vojtěch Franc[a], Pavel Řehulka[b,*], Martin Raus[c], Jiří Stulík[b], Jan Novak[d,**], Matthew B. Renfrow[e], Marek Šebela[a,c,***]

[a]Department of Protein Biochemistry and Proteomics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic
[b]Institute of Molecular Pathology, Faculty of Military Health Sciences, University of Defence, Třebešská 1575, CZ-500 01 Hradec Králové, Czech Republic
[c]Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic
[d]Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, USA
[e]Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, USA

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ABSTRACT

Determining disease-associated changes in protein glycosylation provides a better understanding of pathogenesis. This work focuses on human immunoglobulin A1 (IgA1), where aberrant O-glycosylation plays a key role in the pathogenesis of IgA nephropathy (IgAN). Normal IgA1 hinge region carries 3 to 6 O-glycans consisting of N-acetylgalactosamine (GalNAc) and galactose (Gal); both sugars may be sialylated. In IgAN patients, some O-glycans on a fraction of IgA1 molecules are Gal-deficient. Here we describe a sample preparation protocol with optimized cysteine alkylation of a Gal-deficient polymeric IgA1 myeloma protein prior to in-gel digestion and analysis of the digest by MALDI-TOF/TOF mass spectrometry (MS). Following a novel strategy, IgA1 hinge-region O-glycopeptides were fractionated by reversed-phase liquid chromatography using a microgradient device and identified by MALDI-TOF/TOF tandem MS (MS/MS). The acquired MS/MS spectra were interpreted manually and by means of our own software. This allowed assigning up to six O-glycosylation sites and demonstration, for the first time, of the distribution of isomeric O-glycoforms having the same molecular mass, but a different glycosylation pattern. The most abundant Gal-deficient O-glycoforms were $GalNAc_4Gal_3$ and $GalNAc_5Gal_4$ with one

Abbreviations: ACN, acetonitrile; CAM, carbamidomethylation; CHCA, α-cyano-4-hydroxycinnamic acid; DTT, dithiothreitol; ECD, electron capture dissociation; ETD, electron transfer dissociation; FEP, fluorinated ethylene propylene; FT-ICR, Fourier transform ion cyclotron resonance; IAM, iodoacetamide; IgA, immunoglobulin A; IgAN, IgA nephropathy; HR, hinge region; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PSD, post-source decay; PAM, propionamidation; RPLC, reversed-phase liquid chromatography; TFA, trifluoroacetic acid.

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* *Correspondence to:* P. Řehulka, Institute of Molecular Pathology, Faculty of Military Health Sciences, University of Defence, Třebešská 1575, CZ- 500 01 Hradec Králové, Czech Republic. Tel.: +420 973255199.

** *Correspondence to:* J. Novak, Department of Microbiology,University of Alabama at Birmingham, 845 19th Street South, BBRB 761A, Birmingham, AL 35294, USA. Tel.: +1 205 934 4480.

*** *Correspondence to:* M. Šebela, Department of Protein Biochemistry and Proteomics, CRH, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic. Tel.: +420 585634927; fax: +420 585634933.

E-mail addresses: rehulka@pmfhk.cz (P. Řehulka), jannovak@uab.edu (J. Novak), marek.sebela@upol.cz (M. Šebela).

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Gal-deficient site and $GalNAc_5Gal_3$ and $GalNAc_4Gal_2$ with two Gal-deficient sites. The most frequent Gal-deficient sites were at Ser230 and/or Thr236.

Biological significance
In this work, we studied the *O*-glycosylation in the hinge region of human immunoglobulin A1 (IgA1). Aberrant glycosylation of the protein plays a key role in the pathogenesis of IgA nephropathy. Thus identification of the *O*-glycan composition of IgA1 is important for a deeper understanding of the disease mechanism, biomarker discovery and validation, and implementation and monitoring of disease-specific therapies. We developed a new procedure for elucidating the heterogeneity of IgA1 *O*-glycosylation. After running a polyacrylamide gel electrophoresis under denaturing conditions, the heavy chain of IgA1 was subjected to in-gel digestion by trypsin. *O*-glycopeptides were separated from the digest on capillary columns using a microgradient chromatographic device (replacing commonly used liquid chromatographs) and subjected to MALDI-TOF/TOF mass spectrometry (MS) and tandem mass spectrometry (MS/MS) involving post-source decay fragmentation. We show that the complete modification of cysteines by iodoacetamide prior to electrophoresis is critical for successful MS/MS analyses on the way to deciphering the microheterogeneity of *O*-glycosylation in IgA1. Similarly, the removal of the excess of the reagent is equally important. The acquired MS/MS allowed assigning up to six *O*-glycosylation sites and identification of isomeric *O*-glycoforms. We show that our simplified approach is efficient and has a high potential to provide a method for the rapid assessment of IgA1 heterogeneity that is a less expensive and yet corroborating alternative to LC-(high-resolution)-MS protocols. The novelty and biological significance reside in the demonstration, for the first time, of the distribution of the most abundant isoforms of HR *O*-glycopeptides of IgA1. As another new feature, we introduce a software solution for the interpretation of MS/MS data of *O*-glycopeptide isoforms, which provides the possibility of fast and easier data processing.
This article is part of a Special Issue entitled: Posttranslational Protein modifications in biology and Medicine.

1. Introduction

Immunoglobulin A (IgA) is the most abundantly produced antibody with an important role in mucosal immunity. It occurs in two structurally and functionally distinctive subclasses IgA1 and IgA2 [1]. In contrast to IgA2, the hinge region (HR) of IgA1 (Fig. 1) contains two octapeptide repeats with multiple Pro, Ser, and Thr residues and 3 to 6 *O*-glycan chains [2–4]. IgA1 *O*-glycans consist of *N*-acetylgalactosamine (GalNAc) that may carry galactose (Gal) and/or sialic acid (Neu5Ac) giving rise to several different IgA1 *O*-glycoforms (Fig. 1) [5–9]. An abnormal glycosylation of serum immunoglobulins and other glycoproteins has been observed in several human diseases [10–15]. In 1968, IgA nephropathy (IgAN) was described as a clinical entity [16] and has been since recognized as the most common primary glomerulonephritis and an important cause of end-stage renal disease [17–19]. Although the precise mechanism of IgAN pathogenesis is still being elucidated, it clearly involves formation of immune complexes that contain IgA1 with Gal-deficient *O*-glycans [10,20]. IgAN is diagnosed based on the evaluation of renal biopsy; no alternative noninvasive diagnostic method is currently available [21–23]. Patients with IgAN have elevated levels of circulatory IgA1 with Gal-deficient *O*-glycans; these molecules are bound in pathogenic immune complexes (for review see: [22,23]). Therefore, identification of the *O*-glycan composition of IgA1 is important for a deeper understanding of the disease mechanism, biomarker discovery and validation, and implementation and monitoring of disease-specific therapies [24].

O-glycosylated isoforms from a single biological source show a distinct distribution of heterogeneity with respect to the number and structure of glycan chains [25]. Many methodological approaches have been employed for the analysis of aberrant *O*-glycosylation in IgA1 [26]. The Human Disease Glycomics/Proteome Initiative associated with the Human Proteome Organisation recently coordinated a multi-institutional study that evaluated methodologies with a wide use for defining *N*-glycan content in glycoproteins [27]. Such activities have also been extended to *O*-glycans [28]. These studies have shown that mass spectrometry (MS) is the most powerful tool for both identification and quantification of *N*- and *O*-glycans. The possibility of a precise assessment of mucin-type *O*-glycans has been successfully demonstrated using tandem mass spectrometry (MS/MS) with electron capture dissociation (ECD) or electron transfer dissociation (ETD) [6,25,29–31].

So far, two main strategies have been adopted to assess the heterogeneity of *O*-glycans in HR of IgA1: lectin binding assays ideally combined with monosaccharide compositional analysis [32–34] and MS analysis. Notably, only a few studies have shown a direct assignment of multiple sites of *O*-glycan attachment [3,6,25,29]. There have been several reports based on matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [7,35,36], but there is always a limitation in resolving power and mass accuracy. Every single *O*-glycoform of IgA1 represents a unique glycoprotein species with different abundance and possibly different biological role. Microscale solid-phase extraction methods have been used in