Rapid and sensitive methods for the analysis and identification of O-glycans from glycoproteins

Kozak, R.P.

Citation

Kozak, R. P. (2017, January 24). *Rapid and sensitive methods for the analysis and identification of O-glycans from glycoproteins*. Retrieved from https://hdl.handle.net/1887/45434

Cover Page

Author: Kozak, R.P.
Title: Rapid and sensitive methods for the analysis and identification of O-glycans from glycoproteins
Issue Date: 2017-01-24

Chapter 4

Improved nonreductive O-glycan release by hydrazinolysis with ethylenediaminetetraacetic acid addition

Radoslaw P. Kozak[1], Louise Royle[1], Richard A. Gardner[1], Albert Bondt[2,3], Daryl L. Fernandes[1] and Manfred Wuhrer [2,4]

[1]Ludger Ltd., Culham Science Centre, Oxfordshire, OX14 3EB, United Kingdom

[2]Center for Proteomics and Metabolomics, Leiden University Medical Center, The Netherlands

[3]Department of Rheumatology, Erasmus University Medical Center, 3000 CA Rotterdam, The Netherlands

[4]Division of BioAnalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, VU University Amsterdam, The Netherlands

Abstract

The study of protein O-glycosylation is receiving increasing attention in biological, medical and biopharmaceutical research. Improved techniques are required to allow reproducible and quantitative analysis of O-glycans. An established approach for O-glycan analysis relies on their chemical release in high yield by hydrazinolysis, followed by fluorescent labelling at the reducing terminus and HPLC profiling. However, an unwanted degradation known as "peeling" often compromises hydrazinolysis for O-glycan analysis. Here we address this problem using low-molarity solutions of ethylenediaminetetraacetic acid (EDTA) in hydrazine for O-glycan release. O-linked glycans from a range of different glycoproteins were analysed including bovine fetuin, bovine submaxillary gland mucin and serum IgA. The data for the O-glycans released by hydrazine with anhydrous EDTA or disodium salt dihydrate EDTA show high yields of the native O-glycans compared to the peeled product, resulting in a markedly increased robustness of the O-glycan profiling method. The presented method for O-glycan release demonstrates significant reduction in peeling and reduces the number of sample handling steps prior to release.

Introduction

Protein glycosylation is one of the most common post-translational modifications (PTMs) that influences many protein functions.[1,2] Detailed knowledge of O-linked glycan (serine- or threonine-linked oligosaccharides) structures is invaluable for better understanding their biological functions. O-glycans have a number of different functions including, but not limited to, the protection of the underlying protein as well as epithelial cell surfaces, maintaining protein conformations and controlling epitopes and antigenicity.[3] They have been reported to determine the cell surface expression and function of cell surface receptors and may be involved in growth regulation.[3] They are also known to be involved in blood clotting, embryogenesis, development and cell death [3-5] and they are associated with cancer [6,7] and other human diseases and disorders.[8-10]

Monitoring and controlling O-glycosylation is important in biopharmaceutical development and quality control (QC) in the context of existing and potential drugs that are O-glycosylated (e.g. human erythropoietin (EPO)).[11,12] The monitoring of any changes in drug glycosylation during manufacturing processes is tightly regulated by the health authorities around the world (e.g. US Food and Drug Administration or European Medicines Agency). Biopharmaceutical manufacturers must demonstrate that any process changes do not alter the clinical safety or efficacy of the biopharmaceutical.[13,14]

A number of techniques for the release, recovery and analysis of O-glycans are available.[15] Almost all of these techniques use chemical release methods, as there is no enzyme available for universal O-glycan release from proteins. Two endo-α-*N*-acetylgalactosaminidases have been described that have a high specificity but are limited to the release of the neutral core 1 disaccharide, Galβ1-3GalNAcα1.[16-18]

The most common method for the chemical liberation of O-glycans is reductive β-elimination.[19-21] This method of release leads to O-glycans that are present in their reduced forms (alditols) which means they are not amenable to labelling with a fluorescent or UV tag. Therefore, the range of analytical methods available for the characterization of O-glycans released by reductive β-elimination is restricted, with high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [22], mass spectrometry and NMR being the predominantly applied methods.[23-26]

Several chemical methods for obtaining O-glycans in their non-reduced form have been described, using ethylamine [27], ammonia [28] or lithium hydroxide in an automatic setup [29] but the yields of released O-glycans were low and variable. It has been reported that the best method for universal removal of O-glycans in their non-reduced form in high yields is hydrazinolysis.[15,30-32] Release of glycans by non-reductive methods is compatible with reducing-end labelling using reagents such as 2-aminobenzamide (2-AB) or 2-aminobenzoic acid (2-AA) allowing HPLC with fluorescent or colorimetric (UV) detection.[33]

Analysis of O-glycosylation by HPLC based methods is preferred for a number of reasons. For example, the HPLC or UPLC-profiling of 2-AB labelled O-glycans is quantitative, highly sensitive, has a reasonably high throughput and has the ability to provide detailed information about both monosaccharide sequence and the types of glycan linkage.[34,35]

Unfortunately, all of the chemical O-glycan release methods (reductive and non-reductive) show stepwise degradation of the polysaccharides. The degradation is observed as the removal of one sugar residue at a time from the reducing end. This unwanted side reaction is known as "peeling". [15,32]

Peeling is a general problem when performing the release of O-glycans and often results in poor repeatability with variable amounts of the small peeled glycans. This is a major problem for comparability studies or quality control. Many researchers have investigated this peeling phenomenon and it has been reported that the degree of peeling can be minimized by removal of water prior to hydrazinolysis.[32] We have recently shown that the degree of peeling can be greatly reduced by the removal of calcium or other cations prior to hydrazinolysis by employing several washes of the glycoprotein samples with a solution of 0.1% trifluoroacetic acid (TFA) or low-molarity solutions of ethylenediaminetetraacetic acid (EDTA).[35]

Although these "washing" steps are effective, they are also time consuming and can lead to loss of sample. Here we present an improved hydrazinolysis protocol that allows efficient suppression of peeling, but does not require the series of washing steps prior to the release reaction.[35] The method relies on including EDTA – either as its disodium dihydrate salt or in its anhydrous form - as part of the hydrazinolysis reaction mixture. Analysis of the O-glycans by both HPLC with fluorescence detection and LC-MS, demonstrates the efficient suppression of peeling reactions, allowing the acquisition of O-glycan profiles in a reproducible manner. The method is shown to work on commercially available standards, but also proves to be successful when analysing IgA purified by affinity enrichment from human plasma.

Materials and Methods

Materials

Anhydrous hydrazine (99.9%) and all other reagents for hydrazinolysis were from Ludger Ltd (Oxford, UK). Ethylenediaminetetraacetic acid disodium dihydrate salt (EDTA salt) and anhydrous EDTA were obtained from Sigma-Aldrich (Dorset, UK). Acetonitrile (Romil; 190 SpS for UV/ gradient quality) was obtained from Charlton Scientific (Charlton, Oxon, UK). Bovine fetuin was from Ludger Ltd and bovine submaxillary gland mucin (Type I-S) (BSM) as well as secretory IgA were obtained from Sigma-Aldrich.

Purification of serum IgA

The immunoglobulin A (IgA) was captured from 10 μL human plasma of a presumably healthy volunteer using CaptureSelectTM IgA Affinity Matrix (Life Technologies/Invitrogen) in a 96-well plate format. Captured IgAs were washed with PBS buffer and water (3 x 200 μL) and eluted with 100 mM formic acid (100 μL; Fluka, Steinheim, Germany). Eluates from 4 wells were pooled and dried by vacuum centrifugation. An estimated total of 20 μg IgA was captured per well.

Release of O-glycans

The O-glycans were released from fetuin glycoprotein by the addition of a range of solutions: anhydrous hydrazine; 50 mM, 20 mM and 10 mM EDTA disodium dihydrate salt (EDTA salt) in hydrazine or 50 mM, 20 mM, 10 mM anhydrous EDTA in hydrazine. Samples were incubated at 60°C for 6 h.[32-34] Excess hydrazine was removed by centrifugal evaporation. The samples were placed on ice for 20 min (0°C) and were re-*N*-acetylated by the addition of a 0.1 M sodium bicarbonate

solution (200 µL) and acetic anhydride (21 µL). Samples were cleaned up by passing them through LudgerClean CEX cartridges (Ludger Ltd). The glycans were eluted off the cartridges using water (3 x 0.5 mL). Eluates were dried by vacuum centrifugation prior to fluorescent labelling.

The O-glycans from BSM and serum IgA samples were released as for fetuin using the following solutions: anhydrous hydrazine, 50 mM EDTA salt in hydrazine or 20 mM EDTA anhydrous in hydrazine.

2-AB labelling and purification

Released O-glycans were fluorescently labelled with 2-aminobenzamide (2-AB) by reductive amination according to the method of Bigge *et al.* [36] using a Ludger 2-AB Glycan Labelling Kit (Ludger Ltd). The released glycans were incubated with labelling reagents for 3 h at 65°C. The 2-AB labelled glycans were cleaned up using LudgerClean T1 Cartridges (Ludger Ltd). 2-AB labelled glycans were eluted from the LudgerClean T1 Cartridges with water (1 mL). The samples were dried by vacuum centrifugation and re-suspended in water (100 µL) for further analysis.

Sialidase treatments

Sialidase digestion was performed on the released 2-AB labelled glycans in 10 µL solution containing enzyme at standard concentrations in 50 mM sodium acetate for 16 h at 37°C according to Royle *et al.*[37] The enzyme used was: sialidase from *Arthobacter ureafaciens* (a368S) which is specific for α2-3, α2-6, α2-8 and α2-9 sialic acids (E-S001; QABio, Palm Desert, CA). After digestion, samples were separated from the sialidase by binding onto a LudgerClean Post-Exoglycosidase clean-up plate (Ludger Ltd.) for 60 min followed by elution of the glycans from the plate with water. The samples were analysed by HILIC-HPLC.

HPLC analysis

HILIC-HPLC of 2-AB labelled glycans was performed using the 4.6 x 150 mm LudgerSep-N2 column (Ludger Ltd) on a 2795 HPLC with a 2475 fluorescence detector, controlled by Empower software (Waters, Manchester, UK). The flow rate was 1 mL/min. Solvent A was 50 mM ammonium formate pH 4.4, solvent B was acetonitrile. Samples were injected in 20% water / 80% acetonitrile; injection volume 25 μL. Gradient conditions were 20% - 38% solvent A at flow rate of 1.0 mL/min over 36 min, followed by 2 min at 100% solvent A, then finishing with 20% solvent A, giving a total run time of 45 min.

Waters GPC software with a cubic spline fit was used to allocate GU values to peaks and 2-AB labelled glucose homopolymer (CAB-GHP-30, Ludger Ltd) was used as a system suitability standard as well as an external calibration standard for GU allocation.[14]

LC-ion trap-MS/MS analysis

Nano liquid chromatography (LC) was performed using an Ultimate 3000 LC system (Thermo Scientific; Breda, The Netherlands). Samples were applied to a C18 trapping column (C18 Acclaim PepMap100, 5 μm 0.1 mm × 20 mm; Thermo Scientific) and washed with 100% solvent A (0.1% formic acid in water) at a flow rate of 15 μL/min for 2 min prior to separation on a C18-RP analytical column (C18 Acclaim PepMapRSLC 100 Å, 2 μm, 75 μm x 150mm; Thermo Scientific) at a flow rate of 500 nL/min and elution was monitored by UV absorption (215 nm). Separation was achieved with 5 min 0% of solvent B (95% ACN, 5% water) as starting condition and a gradient to 25% in 15 min and in further 5 min to 70% of solvent B.

Afterwards, the column was washed for 5 min with 70% solvent B. The LC system was coupled via an online ESI-nanosprayer source to an Esquire AmaZonSpeed

ETD ESI-ion trap-MS (Bruker Daltonics). Capillary tubing with an inner diameter of 20 µm was used for nano-electrospray (ca 1200 V) and the solvent was evaporated at 250°C employing a nitrogen stream of 10 L/min. Spectra were acquired in positive-ion mode over a *m/z* range from 300 to 1800.
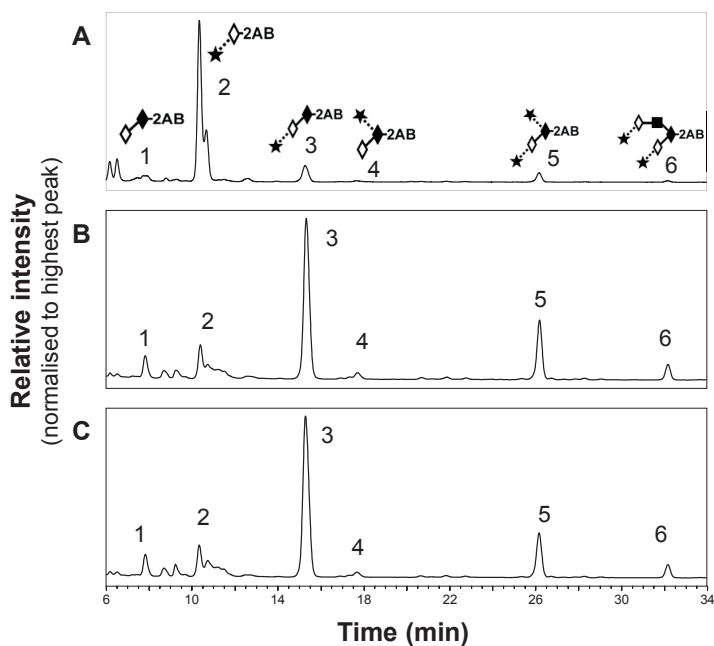

Figure 1. Comparison of HPLC profiles of 2-AB labelled fetuin O-glycans. The O-glycans were released with (A) hydrazine; (B) 50 mM EDTA salt in hydrazine; (C) 20 mM anhydrous EDTA in hydrazine. The following symbols are used to depict glycan structures[38]: ◇, galactose; ◆, *N*-acetylgalactosamine; ◈, fucose; ■, *N*-acetylglucosamine; ★, *N*-acetylneuraminic acid; ☆, *N*-glycolylneuraminic acid; dashed line, α-linkage; solid line, β-linkage.

Structure		Fetuin samples released with:								
		Hydrazine (n=6)	Hydrazine		50 mM EDTA salt dihydrate in hydrazine (n=12)	50 mM EDTA anhydrous in hydrazine (n=9)	20 mM EDTA salt dihydrate in hydrazine (n=9)	20 mM EDTA anhydrous in hydrazine (n=12)	10 mM EDTA salt dihydrate in hydrazine (n=6)	10 mM EDTA anhydrous in hydrazine (n=6)
			washed with 0.1% TFA prior to release (n=18)	washed with 100 mM EDTA prior to release (n=12)						
◆-2AB	Average % area	3.8	9.2	7.1	7.1	7.7	6.1	7.9	5.4	5.8
	STDEV	2.4	2.2	1.2	1.7	1.8	1.3	1.9	0.6	1.0
	p-value	-	0.36	0.35	0.09	0.06	0.03	0.09	0.07	0.13
◇★-2AB	Average % area	71.3	19.1	17.4	24.5	20.7	28.3	21.8	26.2	23.6
	STDEV	23.3	7.3	3.0	4.6	3.1	3.3	2.9	2.3	1.2
	p-value	-	0.0	0.01	0.03	0.01	0.00	0.02	0.05	0.03
★-2AB	Average % area	16.1	49.8	51.8	48.3	51.7	46.6	53.1	49.0	53.7
	STDEV	15.5	4.3	2.9	4.0	3.0	3.0	4.8	1.4	1.3
	p-value	-	0.01	0.01	0.03	0.01	0.00	0.01	0.04	0.01
★◆-2AB	Average % area	1.1	2.6	2.7	2.3	2.7	2.6	2.4	2.7	2.4
	STDEV	0.7	1.0	0.4	0.7	0.5	0.6	0.6	0.2	0.2
	p-value	-	0.06	0.05	0.18	0.02	0.00	0.10	0.00	0.02
★-2AB	Average % area	6.5	14.1	16.8	14.2	13.5	13.1	11.6	13.4	11.3
	STDEV	3.8	2.6	1.7	2.4	1.0	1.2	1.0	1.3	1.6
	p-value	-	0.00	0.01	0.05	0.03	0.03	0.09	0.14	0.25
★■-2AB	Average % area	1.2	3.6	4.2	3.5	3.6	3.2	3.1	3.4	3.1
	STDEV	1.1	1.0	0.6	0.7	0.4	0.4	0.3	0.4	0.4
	p-value	-	0.01	0.01	0.05	0.02	0.02	0.04	0.09	0.13

Table 1. Evaluation of EDTA addition during hydrazinolysis for O-glycosylation analysis of fetuin. Fetuin O-glycans were released by hydrazinolysis after pre-treatments (TFA wash or EDTA wash) or with addition of EDTA. O-glycan profiles were analysed by HILIC-UPLC of 2-AB labelled glycans. Relative abundances and standard deviations are given. Relative abundances of samples with TFA or EDTA treatment were compared to non-treated samples by t-test, and the resulting p-values are listed. Changes were significant for the p-value ≤ 0.05. Data for O-glycans released from fetuin that had been buffer exchanged with 0.1% TFA or 100 mM EDTA were taken from[35].

Results and discussion

HPLC analysis of 2-AB labelled O-glycans

We used bovine fetuin as a model to optimize the release and minimize the formation of peeling product. The O-glycans from dried bovine fetuin were released using a range of solutions: anhydrous hydrazine; 50 mM EDTA salt in hydrazine; 50 mM anhydrous EDTA in hydrazine; 20 mM EDTA salt in hydrazine; 20 mM anhydrous EDTA in hydrazine; 10 mM EDTA salt in hydrazine and 10 mM anhydrous EDTA in hydrazine. Released O-glycans were 2-AB labelled and analysed by HILIC-HPLC. For comparison two fetuin glycoprotein samples were buffer exchanged with 0.1% TFA or 100 mM EDTA prior to release with anhydrous hydrazine.[35] The HPLC data for this experiment is shown in Figure. 1 and Supplemental Figure. 1. The O-glycan structures from fetuin were consistent with published data.[36] The fetuin profile contains: core 1 Galα1-3GalNAc (peak 1), mono-sialylated and di-sialylated core 1 O-

glycans: Neu5Acα2-3Galβ1-3GalNAc (peak 3); Neu5Acα2-6(Galβ1-3)GalNAc (peak 4); di-sialylated core 1 O-glycan Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc (peak 5); di-sialylated core 2 structure Neu5Acα2-3Galβ1-3(Neu5Acα2-3Galβ1-4GlcNAcβ1-6)GalNAc (peak 6) as well as the peeled product Neu5Acα2-3Gal (peak 2) (Supplemental Table 1).

The highest amount of the peeled product (71%) was observed for the fetuin sample that was incubated with anhydrous hydrazine only, without prior washes (peak 2, Figure. 1A). For the samples washed with 0.1%TFA or 100 mM EDTA, the degree of peeling was consistent with published results [35] with an average relative abundance of 19% and 17%, respectively (peak 2, Supplemental Figure. 1B, C, Table 1.). The amount of peeling for fetuin samples that were released with a range of low-molarity solutions of EDTA salt or anhydrous EDTA in hydrazine was between 20% and 28% (Table 1.). The most efficient reduction of peeling was observed for the samples released with 50 mM and 20 mM EDTA anhydrous in hydrazine (22% and 21% relative intensity for peak 2; Supplemental Figure. 1E, G; Table 1.).
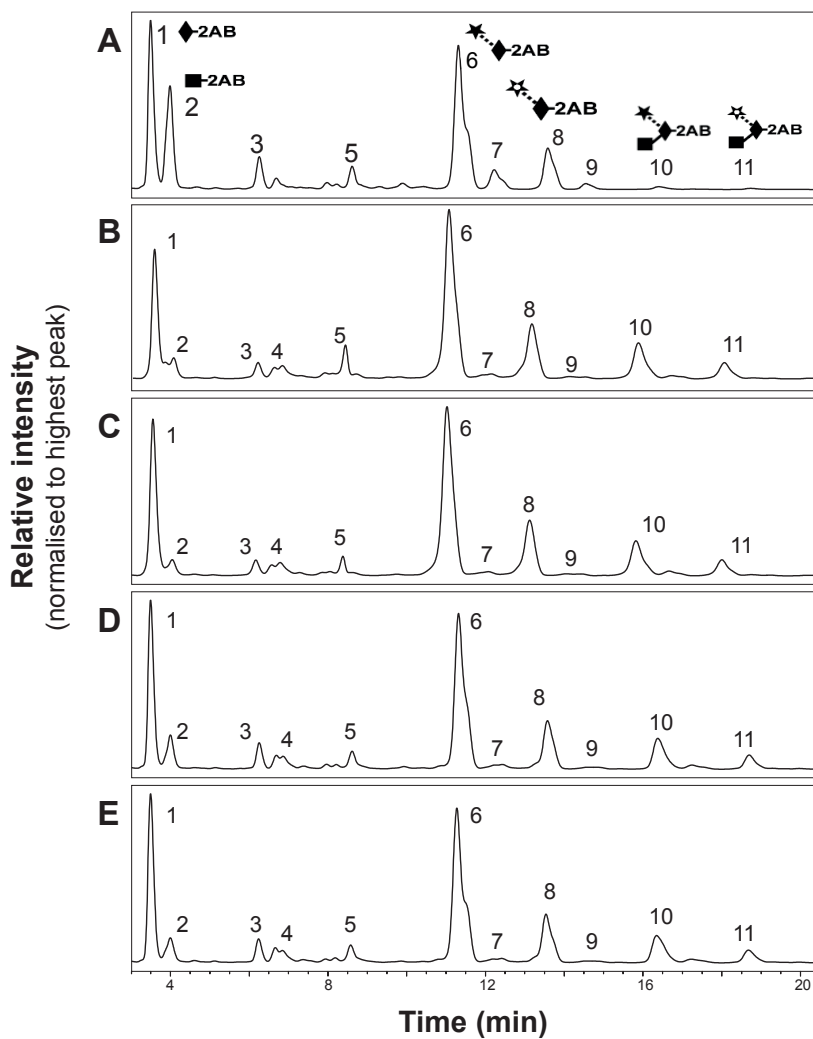
Figure 2. Comparison of HPLC profiles of 2-AB labelled BSM O-glycans. The O-glycans were released with (A) hydrazine; (B) sample was buffer exchange with 0.1% TFA prior to hydrazinolysis; (C) sample was buffer exchange with 100 mM EDTA prior to hydrazinolysis; hydrazinolysis was performed in (D) 50 mM EDTA salt in hydrazine or (E) 20 mM anhydrous EDTA in hydrazine.

Structure		Hydrazine (n=6)	Hydrazine washed with 0.1% TFA prior to release (n=9)	Hydrazine washed with 100 mM EDTA prior to release (n=9)	50 mM EDTA salt dihydrate in hydrazine (n=6)	20 mM EDTA anhydrous in hydrazine (n=6)
◆-2AB	Average % area	22.3	18.2	18.8	22.0	24.0
	STDEV	-	1.4	2.2	0.7	2.4
	p-value	-	0.84	0.51	0.50	0.35
■-2AB	Average % area	16.6	4.8	4.2	5.7	5.7
	STDEV	0.5	1.3	0.9	0.5	1.8
	p-value	-	0.01	0.00	0.00	0.01
fucosylated structure	Average % area	4.7	0.2	3.5	3.7	4.0
	STDEV	-	0.2	0.6	0.0	0.8
	p-value	-	0.05	0.07	0.00	0.26
◣◆-2AB	Average % area	-	2.4	2.3	2.1	1.5
	STDEV	-	0.7	0.2	0.1	0.8
	p-value	-	0.00	0.00	0.00	0.08
sialylated structure	Average % area	4.0	4.3	2.9	3.4	3.4
	STDEV	-	0.5	0.3	0.1	0.4
	p-value	-	0.38	0.01	0.01	0.08
✦◆-2AB	Average % area	34.8	39.5	41.0	36.9	36.5
	STDEV	-	0.7	1.2	0.5	2.0
	p-value	-	0.00	0.00	0.02	0.27
sialylated structure	Average % area	5.0	1.5	1.6	2.0	1.9
	STDEV	-	0.5	0.4	0.3	0.1
	p-value	-	0.00	0.00	0.00	0.00
✦◆-2AB	Average % area	9.9	13.1	13.3	11.8	11.6
	STDEV	-	0.3	0.4	0.2	0.9
	p-value	-	0.00	0.00	0.00	0.08
sialylated structure	Average % area	1.4	0.8	0.7	0.9	0.8
	STDEV	-	0.3	0.2	0.0	0.1
	p-value	-	0.01	0.01	0.00	0.00
✦◣◆-2AB	Average % area	0.8	8.3	8.3	8.0	7.4
	STDEV	-	0.9	0.9	0.5	1.0
	p-value	-	0.00	0.00	0.00	0.01
✦◣◆-2AB	Average % area	0.3	3.5	3.5	3.4	3.2
	STDEV	0.0	0.4	0.4	0.2	0.5
	p-value	-	0.00	0.00	0.00	0.01

Table 2. Evaluation of EDTA addition during hydrazinolysis for O-glycosylation analysis of bovine submaxillary mucin (BSM). BSM O-glycans were released by hydrazinolysis after pre-treatments (TFA wash or EDTA wash) or with addition of EDTA. O-glycan profiles were analysed by HILIC-UPLC of 2-AB labelled glycans. Relative abundances and standard deviations are given. Relative abundances of samples with TFA or EDTA treatment were compared to non-treated samples by t-test, and the resulting p-values are listed. Changes were significant for the p-value ≤ 0.05. Data for O-glycans released from BSM exchanged with 0.1% TFA or 100 mM EDTA were taken from[35].

Based on the results for fetuin we analysed the O-glycosylation of a second model glycoprotein, BSM.[39] Mucin O-glycans were released with anhydrous hydrazine, 50 mM EDTA salt and 20 mM anhydrous EDTA solution in hydrazine, 2-AB labelled and analysed by HILIC-HPLC.

The HPLC results were compared with previously published data for the BSM samples cleaned up prior to hydrazinolysis with 0.1% TFA and 100 mM EDTA [35]. The identities of the observed peaks were confirmed by comparison with previously reported data.[36,40,41] Six different glycan species were identified: GalNAc (peak 1), GlcNAcβ1-3GalNAc (peak 3), Neu5Acα2-6GalNAc (peak 6); Neu5Gcα2-6GalNAc (peak 8); GlcNAcβ1-3(Neu5Acα2-6)GalNAc (peak 10) and GlcNAcβ1-3(Neu5Gcα2-6)GalNAc (peak 11) along with peeled product GlcNAc (peak 2) (Supplemental Table 1). Moreover, BSM was also found to contain additional sialylated species (peaks 5, 7 and 9) and a fucosylated structure (peak 3) which were not fully structurally characterised.

The highest relative abundance of unwanted peeled product was detected for the sample that was released with anhydrous hydrazine (17%, peak 2, Figure. 2A; Table 2). For the samples released with solutions of 50 mM EDTA salt and 20 mM anhydrous EDTA in hydrazine the degree of degradation product was much lower (6%, peak 2, Figure. 2D, E; Table 2) and consistent with published results.[35]
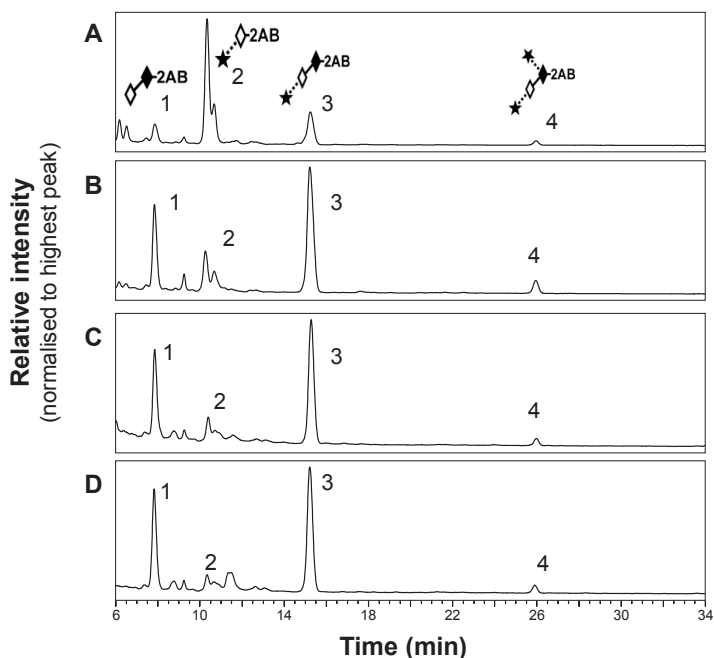
Figure 3. Comparison of HPLC profiles of 2-AB labelled serum IgA O-glycans. The O-glycans were released with (A) hydrazine; (B) sample was buffer exchange with 0.1% TFA prior to hydrazinolysis; release was performed with (C) 50 mM EDTA salt in hydrazine and (D) 20 mM anhydrous EDTA in hydrazine.

To further evaluate the improved hydrazinolysis protocol, more experiments were performed using IgA purified from human plasma. The O-glycans were released from IgA samples using the following conditions: (i) sample without any clean-up was released with anhydrous hydrazine; (ii) sample was cleaned by centrifugal filtration with 0.1% TFA before release with anhydrous hydrazine according to previously described procedure [35]; (iii) sample without any clean-up was released with 50 mM EDTA salt in hydrazine; (iv) sample without any clean-up was released with 20 mM anhydrous EDTA in hydrazine. The HPLC chromatograms obtained from these four IgA experiments are shown in Figure. 3. The IgA O-glycan profiles contain four major peaks corresponding to: core 1 Galα1-3GalNAc (peak 1), mono-sialylated and di-sialylated core 1 O-glycans: Neu5Acα2-3Galβ1-3GalNAc (peak 3); di-sialylated core

1 O-glycan Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc (peak 4), and the sialylated peeled product Neu5Acα2-3Gal (peak 2, Figure. 3.) (Supplemental Table 1). The mono-sialylated and di-sialylated core 1 (peaks 3 & 4, Figure. 4) digested to the neutral core 1 (peak 1, Figure. 4) and the sialylated peeled product peak was digested to galactose (peak 2, Figure. 4A) following treatment with sialidase. These structures are in agreement with previously published structures.[37,42]
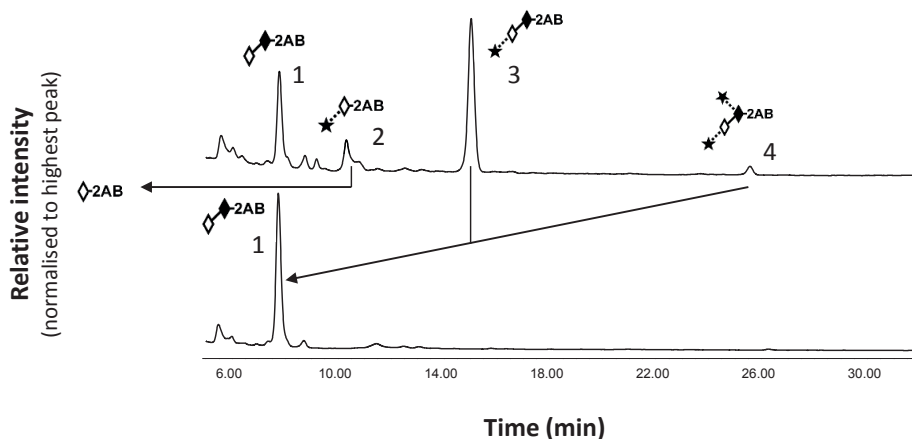

Figure 4. Glycan sequencing of IgA O-glycans before and after treatment with sialidase. The O-glycans were released with 20 mM anhydrous EDTA in hydrazine and 2-AB labelled. Aliquots of the total O-glycan pool were incubated with sialidase. O-glycans were analyzed by HILIC-HPLC before (A) and after sialidase treatment. Arrows indicate digestion pathways.

A significant difference in the relative abundance of peeling products was apparent between the different reagents used for the release. The highest occurrence of peeling was observed for the samples released with anhydrous hydrazine that did not have any EDTA added (68%, peek 2, Figure. 3A, Table 3).

Structure		Hydrazine (n=3)	Hydrazine washed with 0.1% TFA prior to release (n=3)	50 mM EDTA salt dihydrate in hydrazine (n=6)	20 mM EDTA anhydrous in hydrazine (n=6)
			IgA samples released with:		
◇◆-2AB	Average % area	8.2	21.5	29.8	31.5
	STDEV	-	2.4	0.9	0.3
	p-value	-	0.08	0.00	0.01
★◇-2AB	Average % area	68.1	25.5	14.1	12.2
	STDEV	-	5.9	0.7	2.4
	p-value	-	0.06	0.00	0.02
★◇◆-2AB	Average % area	21.5	48.9	53.0	53.4
	STDEV	-	2.8	0.3	2.0
	p-value	-	0.05	0.00	0.03
★◇◆★-2AB	Average % area	2.3	4.2	3.1	3.0
	STDEV	-	0.7	0.1	0.1
	p-value	-	0.16	0.01	0.09

Table 3. Evaluation of EDTA addition during hydrazinolysis for O-glycosylation analysis of serum IgA. IgA O-glycans were released by hydrazinolysis after TFA wash or with addition of EDTA. O-glycan profiles were analysed by HILIC-UPLC of 2-AB labelled glycans. Relative abundances and standard deviations are given. Relative abundances of samples with TFA or EDTA treatment were compared to non-treated samples by t-test, and the resulting p-values are listed. Changes were significant for the p-value ≤ 0.05.

Structure	Composition			Mass calculated $[M+H]^+$	Mass registered $[M+H]^+$		
	Hex	HexNAc	Neu5Ac		anhydrous hydrazine	50mM EDTA (salt) in hydrazine	20mM EDTA (anhydrous) in hydrazine
					IgA samples released with :		
◇◆-2AB	1	1	0	504.22	nd	504.26	504.27
★◇-2AB	1	0	1	592.23	592.27	nd	nd
★◇◆-2AB	1	1	1	795.31	795.37	795.36	795.35

Table 4. Mass spectrometric data and corresponding structures of the IgA O-glycans peaks. nd, not detected.

LC-ion trap-MS/MS analysis of 2-AB labelled IgA samples.

To confirm the identities and the amounts of observed peaks (peaks 1, 2 and 3, Figure. 3) the 2-AB labelled O-glycans released from serum IgA were fractionated by HILIC-HPLC and characterized by LC-ion trap-MS/MS.
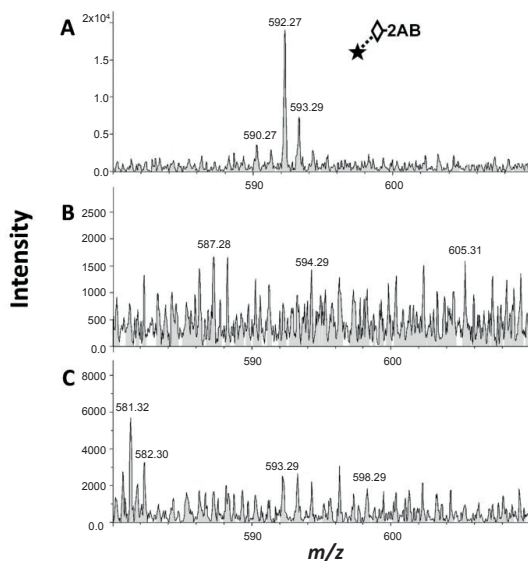
Figure 5. LC-ESI-MS mass spectra of collected peak 2 (peeled product) of the three IgA O-glycan samples generated with (A) hydrazine; (B) 50 mM EDTA salt in hydrazine; (C) 20 mM anhydrous EDTA in hydrazine (see Fig. 4). Spectra were summed over the entire elution range of the peeling product.

The observed molecular masses and the proposed structures based on previously reported data [37,42] [40-41] are summarized in Table 4. The results from LC-MS showed high level of peeled product for the samples released with anhydrous hydrazine (Figure 5A). For the IgA samples released with solutions of 50 mM EDTA salt and 20 mM anhydrous EDTA in hydrazine the peeled product was not detected (Figure 5B & C).

Conclusions

Here we present an improved hydrazinolysis protocol for the release of O-glycans (in their non-reduced form) from glycoproteins. We demonstrate that undesirable peeling can be efficiently reduced by adding EDTA directly to the hydrazine. These results are comparable with previously published data where the

samples are cleaned up with low-molarity EDTA solutions prior to hydrazine release [35] suggesting that the chelation of cations may be beneficial for suppressing O-glycan peeling.

We applied the current method to the analysis of O-glycans from bovine fetuin, bovine submaxillary gland mucin (BSM) and serum IgA and found that undesirable peeling was reduced from 71% for the fetuin O-glycans released with anhydrous hydrazine to 17% for the same sample released with solutions of 20 mM anhydrous EDTA or 50 mM EDTA salt in hydrazine. Significant reductions in the amount of peeled product were also observed for the BSM and human serum IgA samples. For the BSM, the peeled product decreased from 17% to 6% and for the IgA sample it decreased from 68% to 12%.

When compared to the previously published protocol [35] which relied on several consecutive washes prior to O-glycan release, this improved method has a reduced number of sample handling steps which make the process much quicker and helps prevent sample losses when working with low amounts of materials.

Acknowledgements

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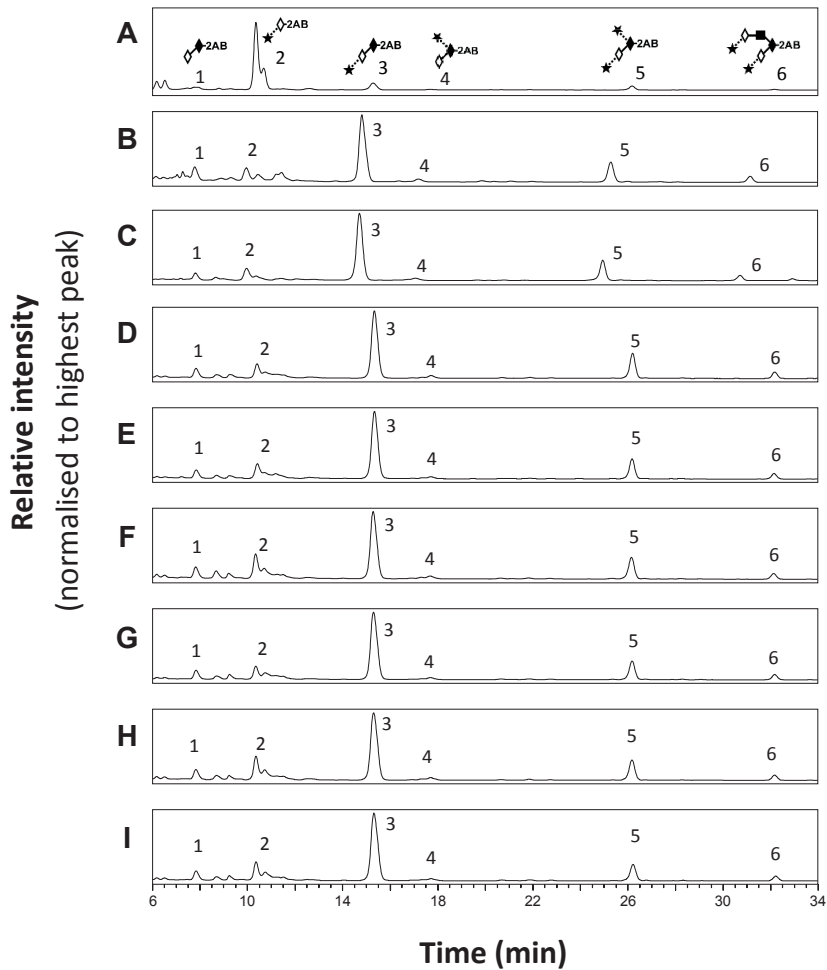
Supplementary Figure 1. Fetuin O-glycan profiling after hydrazinolysis. (A) Sample was subjected to conventional hydrazinolysis without pretreatment; (B) sample was buffer exchanged with 0.1% TFA prior to hydrazinolysis; (C) sample was buffer exchanged with 100mM EDTA prior to hydrazinolysis. In (D-I) the following additives were used with hydrolysis: (D) 50mM EDTA (salt) in hydrazine; (E) 50mM EDTA (anhydrous) in; (F) 20mM EDTA (salt) in hydrazine; (G) 20mM EDTA (anhydrous) in hydrazine; (H) 10mM EDTA (salt) in hydrazine; (I) 10mM EDTA (anhydrous) in hydrazine. The following symbols are used to depict glycan structures[37]: ◇, galactose; ◆, N-acetylgalactosamine; ◈, fucose; ■, N-acetylglucosamine; ★, N-acetylneuraminic acid; ☆, N-glycolylneuraminic acid; dashed line, α-linkage; solid line, β-linkage.