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

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Chapter 3

Suppression of peeling during the release of O-glycans by hydrazinolysis

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Abstract

The analysis of O-glycans is essential for better understanding their functions in biological processes. Although many techniques for O-glycan release have been developed, the hydrazinolysis release method is the best for producing O-glycans with free reducing termini in high yield. This release technique allows the glycans to be labelled with a fluorophore and analysed by fluorescence detection. Under the hydrazinolysis release conditions, a side reaction is observed which causes the loss of monosaccharides from the reducing terminus of the glycans (known as peeling). Using bovine fetuin (as it contains the sialylated O-glycans most commonly found on biopharmaceuticals) and bovine submaxillary glands mucin (BSM) we here demonstrate that peeling can be greatly reduced when the sample is buffer exchanged prior to hydrazinolysis with either solutions of 0.1% trifluoroacetic acid (TFA) or low molarity (100, 50, 20 and 5 mM) ethylenediaminetetraacetic acid (EDTA). Addition of calcium chloride to fetuin resulted in an increase in peeling, while subsequent washing with EDTA abolished this effect, suggesting a role of calcium and possibly other cations in causing peeling. The presented technique for sample preparation prior to hydrazinolysis greatly reduces the level of undesirable cleavage products in O-glycan analysis and increases the robustness of the method.

Introduction

Changes in glycosylation have been associated with many states of health and diseases providing diagnostic and prognostic information.[1-3] Analysis of glycosylation is not only important in clinical and biological research but also in biopharmaceutical research where glycosylation throughout the drug life cycle must be optimised and monitored.

There are two main types of protein glycosylation found in eukaryotes, N-linked glycosylation and O-linked glycosylation. While the methods for release and analysis of N-glycans (asparagine-linked) are well established, O-glycan (covalently linked to serine or threonine) release and analysis still remains very challenging. Various enzymatic and chemical techniques for the release and recovery of O-glycans have been developed. Each of these techniques has advantages and disadvantages. An enzymatic release method would be preferred as this would avoid degradation or chemical modification. Unfortunately, no enzyme has yet been ìsolated for the universal release of O-glycans from glycoproteins. There are two O-glycanases available [4-6] but their mode of action is restricted to the liberation of the neutral core 1 disaccharide Gal-GalNAc from serine or threonine. Therefore, chemical release is currently the only effective method for obtaining the full range of O-glycans. Several techniques for chemical release of O-glycans from glycoproteins have been reported. The most common one is reductive β-elimination, a method that combines the use of sodium hydroxide and sodium borohydride. [7,8] Glycan release is accomplished by sodium hydroxide whilst sodium borohydride reduces the terminal sugar converting it to an alditol, thus preventing the oligosaccharide from degrading during and after the release. This degradation, referred to as peeling, produces the

glycan structure NeuAcα2-3Gal from glycoproteins carrying sialylated T-antigens (Neu5Acα2-3Galβ1-3GalNAc) [9] and happens when 3-O-substituents of the reducing termini of the released O-glycans 'peel' off causing loss of the terminal monosaccharide(s). Peeling is a general problem found with chemical release methods for O-glycans and results in poor repeatability and stoichiometry for analytical glycan profiles. The mechanism for formation of this degradation product is not fully understood. Due to the use of the reducing agent to prevent peeling, the reductive β-elimination method results in glycans with reduced ends. This makes it impossible to introduce a fluorescent or colorimetric label such as 2-aminobenzamide (2-AB) or 2-aminobenzoic acid (2-AA) for fluorescence detection or 1-phenyl-3methyl-5pyrazolone (PMP) for UV detection.[10] Therefore alternative detection methods such as mass spectrometry [11] or HPAEC-PAD (high pH anion exchange chromatography with pulsed amperometric detection) [12] have to be used for the analysis of alditols.

To overcome this problem with alditols non-reductive β-elimination methods have been developed. These methods use various reagents such as ammonia [13], 70% (w/v) aqueous ethylamine [14] and lithium hydroxide [15] to release O-glycans without subsequent reduction. However, these methods have not found wide acceptance in the scientific community.

A combination of enzymatic digestion with chemical release using solid-phase permethylation of O-linked oligosaccharides has been described by Goetz *et al.* [16] Firstly, glycoproteins are incubated with pronase and then the whole reaction mixture is subjected to solid phase permethylation. This technique allows the release of O-glycans with free reducing termini in high yield without peeling. However, as the

glycans are released they become permethylated including methylation of the reducing-end anomeric hydroxyl group. Therefore, they cannot be labelled with a fluorophore of chromophore and mass spectrometric analysis is required.

Recently, Zauner et al.[17], Wang et al.[18] and Furukawa et al.[19] have described a combined method for O-glycan release and labelling using a β-elimination method with dimethylamine, [17] ammonia [18] or sodium hydroxide [19] for the release and concomitant labelling with 1-phenyl-3-methyl-5-pyrazolone (PMP). The technique is quick, provides good release and labelling and is reported to have a reduced or very low degree of peeling. However, PMP labelling only allows UV detection, which is not as sensitive and specific as fluorescence detection, or mass spectrometric analysis of the released PMP-labelled glycans.

Another widely used procedure for the release of O-glycans is the hydrazinolysis method.[9,20,21] Depending on the specific protocol, N- and/or O-glycans can be released. Whilst N-glycans are released from glycoproteins using more vigorous conditions (85°C to 100°C, 5-16 hours), [21] O-glycans are released and recovered under milder conditions (60°C, 6 hours).[9] Although hydrazinolysis of O-glycans produces intact glycans with free reducing termini in high yield, a major concern is the occurrence of undesirable peeling. Merry et al.[9] reported that removal of water from the sample before hydrazinolysis is essential to minimise peeling. The presence of water may lead to the alkaline conditions that favour β-elimination and produce higher amounts of peeling. Although removal of water does reduce peeling, it still remains a significant problem that has been proved difficult to control.

Despite significant amounts of work to develop improved methods for O-glycan release and recovery, currently there are no general, fast methods available for the removal of O-glycans with free reducing termini from glycoproteins (to allow

sample analysis by fluorescent detection) in high yield without partial degradation. In the course of our studies into this peeling phenomenon, we investigated a variety of ways to reduce or completely avoid peeling. In this article we examine the effect of removing buffer salts and other low-molecular weight materials from glycoprotein samples by a range of acid and ethylenediaminetetraacetic acid (EDTA) washes prior to hydrazinolysis.

Materials and Methods

Materials

Anhydrous hydrazine (99.9%) and all other reagents for hydrazinolysis were from Ludger Ltd (Oxford, UK). EDTA (99.0%) was obtained from VWR (Leicestershire, UK). Acetonitrile (Romil; 190 SpS for UV/ gradient quality) was obtained from Charlton Scientific (Charlton, Oxon, UK). Centrifugal filter devices with a molecular weight cut off membrane of 10 kDa were obtained from Fisher Scientific UK (Loughborough, Leicestershire, UK). Bovine fetuin was from Ludger Ltd (Oxford, UK) and bovine submaxillary gland mucin (Type I-S) (BSM) was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). All other reagents were obtained from Sigma-Aldrich (Dorset, UK).

Sample preparation for hydrazinolysis

Fetuin samples (375 µg) were dissolved in 150 µL of water or a range of solvents: 0.1 M PBS; 0.1% TFA; 0.1% HCl; 0.1% H_2SO_4; 0.1% HCOOH; 0.1% CH_3COOH; 100 mM, 50 mM, 20 mM, 5 mM EDTA. Each 150 µL solution of fetuin glycoprotein was then transferred to a separate centrifugal filter device (10 kDa,

molecular weight cut off membrane), which had been pre-washed with water (5 mL). Each centrifugal filter device was centrifuged at 4000 rpm for 10-12 min (until between 0.3 and 0.5 mL of solution remained).

The washing was then repeated a further five times with 5 mL of the appropriate washing solution for each sample. The remaining 0.3 to 0.5 mL of solution was divided and transferred into three pyrolysed 5 mL glass vials (for triplicate release) and dried down for 16 hours by vacuum centrifugation (without heating) prior to addition of hydrazine.

BSM samples (375 µg) were prepared as for fetuin with solvents: water; 0.1% TFA and 100 mM EDTA.

Release of O-glycans by hydrazinolysis

The O-glycans were released from fetuin or BSM glycoprotein by the addition of hydrazine and incubation at 60°C for 6 hours as previously described by Merry et al.,[9] Patel et al.[21] and Ashford et al.[20] 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 mixed and incubated at 0°C for 10 min. A further aliquot of acetic anhydride (21 µL) was added to each sample followed by vortexing and incubation at room temperature for 60 min. 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.

Fluorescent labelling

Released O-glycans were labelled with 2-aminobenzamide (2-AB) according to Bigge et al.[22] using a Ludger 2-AB Glycan Labelling Kit. The released glycans were incubated with labelling reagents for 3 hours at 65°C. The 2-AB labelled glycans were cleaned up using LudgerClean S Cartridges (Ludger Ltd). 2-AB labelled glycans were eluted from the LudgerClean S Cartridges with water (2 x 0.5 mL). The samples were evaporated to dryness under high vacuum using centrifugal evaporation and re-suspended in water (100 µL) for further analysis.

Exoglycosidase digestions

Exoglycosidase sequencing was performed according to Royle et al..[23] The released, 2-AB labelled glycans were incubated with exoglycosidases at standard concentrations in a final volume 10 µL in 50 mM sodium acetate for 16 hours at 37°C. The enzymes used were: Sialidase from *Arthobacter ureafaciens* (a368S): specific for α2-3, 6, 8, 9 sialic acids (E-S001; QABio, Palm Desert, CA); Beta galactosidase from *Streptococcus pneumoniae* (b4G): specific for β1-4 galactose (E-BG07; QABio, Palm Desert, CA); Bovine kidney α-fucosidase (a6F): specific for α1-6>2 fucose (EC 3.2.1.51; Sigma-Aldrich, UK). After digestion, samples were separated from the exoglycosidases by binding onto a protein-binding plate for 60 min followed by elution of the glycans from the plate with water. The samples were analysed by HILIC-HPLC.

HPLC analysis

2-AB labelled samples were analysed by HILIC-HLPC using 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). Solvent A was 50 mM ammonium formate pH 4.4, Solvent B was acetonitrile. Samples were injected in 20% aqueous/80% acetonitrile; injection volume 25 µL. Gradient conditions were 20% - 38% 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. 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. [23]

Acis used for washing fetuin sample prior to hydrazinolysis	Molarity (mM)	pH
0.1% HCl	3.6	1.4
0.1% H2SO4	10.2	1.7
0.1% HCOOH	21.7	2.7
0.1% CH3COOH	16.7	3.3
0.1% TFA	8.8	2.0

Table 1. Molarity and pH of acid solutions used for sample cleanup prior hydrazinolysis.

Results and Discussion

Release and recovery of O-glycans with free reducing termini is important for structural and functional analysis. Using fetuin as a model substance, we evaluated the effect of removing buffer salts and other low-molecular weight materials from glycoprotein samples before O-mode hydrazinolysis, to see if these measures could reduce peeling.

Bovine fetuin was buffer exchanged by washing with water and a range of inorganic and organic acid solutions: 0.1% TFA; 0.1% HCl; 0.1% H_2SO_4; 0.1% HCOOH; 0.1% CH_3COOH. The O-glycans were released using anhydrous hydrazine, [9] labelled with 2-aminobenzamide (2-AB) and analysed by HILIC-HPLC. To find out if the amount of degradation product was reduced when performing a solvent exchange prior to hydrazinolysis, three control experiments were performed which included using the dried fetuin glycoprotein sample without a wash step and washing a fetuin glycoprotein sample with only water, or with 0.1 M PBS.

The HPLC profiles obtained from the fetuin sample dried without cleanup prior to hydrazinolysis and from the fetuin samples cleaned up with water, 0.1 M PBS and 0.1% TFA using a centrifugal filtration device (MWCO membrane,10 kDa) were compared (Figure 1). Structures of intact O-glycans as well peeling products were assigned on the basis of previously reported GU values [9,23] and were consistent with published results.[24,25] The fetuin profile contained core 1: Galβ1-3GalNAc (peak 1), mono-sialylated core 1 O-glycans: Neu5Acα2-3Galβ1-3GalNAc (peak 3) and Neu5Acα2-6(Galβ1-3)GalNAc (peak 4); di-sialylated core 1 O-glycan: Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc (peak 5); and di-sialylated core 2 O-glycan:

Neu5Acα2-3Galβ1-3(Neu5Acα2-3Galβ1-4GlcNAcβ1-6)GalNAc (peak 6). The peeled product Neu5Acα2-3Gal (peak 2, Figure 1) was also detected.

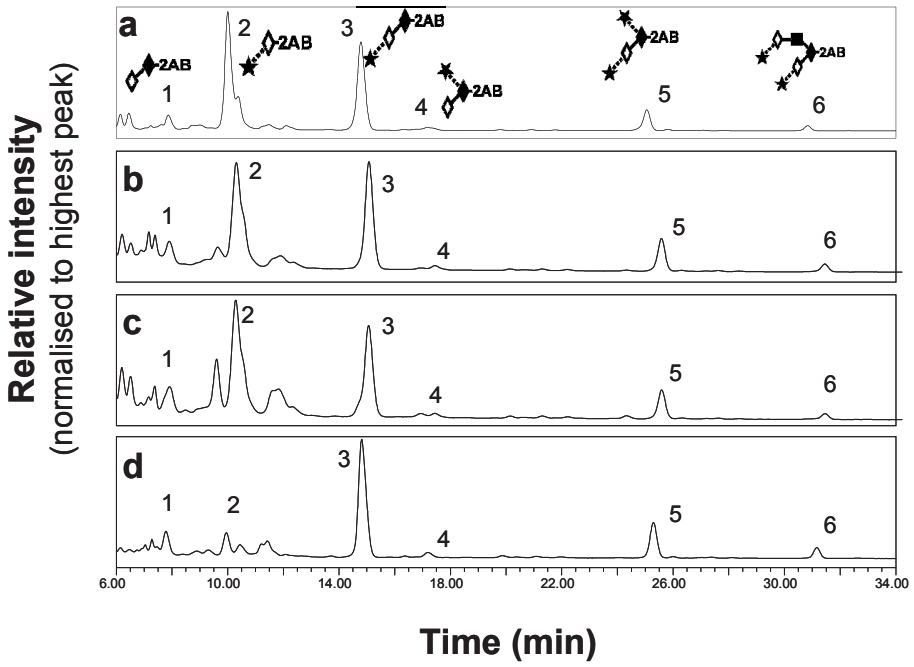

Figure 1. Comparison of HPLC O-glycan profiles of bovine fetuin following buffer exchange with a range of solutions, hydrazinolysis, and 2-AB labelling. The following buffer exchange procedures were applied: (a) no washing, (b), water wash, (c) 0.1 M PBS wash, (d) 100 mM EDTA wash. The O-glycans released by hydrazinolysis were 2-AB labelled and compared by HILIC-HPLC with fluorescence detection. Peak 2 is the peeled product. The following symbols are used to depict glycan structures [26]: ◊, galactose; ♦, *N*-acetylgalactosamine; ◈, fucose; ■, *N*-acetylglucosamine; ★, *N*-acetylneuraminic acid; ☆, *N*-glycolylneuraminic acid; dashed line, α-linkage; solid line, β-linkage.

The highest degree of peeling was observed for samples which were not cleaned up. Under these conditions, the peeled product Neu5Acα2-3Gal (peak 2, Figure 1a) had an average relative abundance of 58% (Table 2). The samples that were cleaned up in water or 0.1 M PBS showed a lower degree of peeling (36% or

45% relative intensity for peak 2, respectively; Figure 1b-c, Table 3). The most pronounced reduction of peeling products was apparent in the samples that were washed with 0.1% TFA. These samples showed a significantly reduced amount of peeled product (19% peak 2, Figure 1d, Table 3) as compared to the samples that were not cleaned up or were washed with water.

Fetuin samples not cleaned up prior to hydrazinolysis		Glycan structure					
							
		Peak area [%]					
Experiment 1	A	9.7	68.3	15.0	0.7	5.4	1.0
	B	10.3	71.7	12.1	0.7	4.3	0.9
	C	9.7	72.0	12.3	0.5	4.5	0.9
Experiment 2	A	4.2	49.9	34.2	2.2	7.7	1.8
	B	4.0	57.0	29.6	1.3	6.7	1.4
	C	7.7	30.1	46.8	2.8	10.1	2.5
Average % Area		7.6	**58.2**	25.0	1.4	6.5	1.4
STDev		2.6	14.9	13.0	0.9	2.0	0.6

Table 2. Average relative abundance of O-glycans from fetuin samples not cleaned up prior to hydrazinolysis.

Following the findings that TFA significantly reduced the occurrence of peeling we decided to investigate other inorganic and organic acids to see their effect, particularly with regard to the degree of peeling. A number of different acids were investigated. The samples were buffer exchanged by washing with: 0.1% HCl, 0.1% H_2SO_4, 0.1% HCOOH and 0.1% CH_3COOH (Table 1). The HPLC profiles obtained from the fetuin samples cleaned up by using a centrifugal filtration device (10 kDa MWCO membrane) with the different acids were compared (Figure 2). The samples that were prepared using 0.1% HCl, 0.1% H_2SO_4 and 0.1% HCOOH, showed degrees of peeling which were very similar to those of the 0.1% TFA washes (Table 3). However, three of the acid-treated samples (0.1% HCl, 0.1% H_2SO_4 and 0.1% CH_3COOH) also showed an increase in de-sialylation (peak 1, Figure 2a, b and d,

Table 3). The incidence of the non-sialylated core 1 structure (peak 1, Figure 2a-d) relative to the sialylated structures (peaks 3-6, Figure 2a-d) was much higher for these acid washed samples than for samples cleaned up with water or 0.1% TFA. From these results, it is clear that although organic and inorganic acid washes, with the exception of 0.1 % H_2SO_4, decrease the degree of peeling, they also increase the degree of de-sialylation. De-sialylation is assumed to occur during the vacuum centrifugation step which would explain why only the highly volatile acids (HCOOH and TFA) do not cause desialylation.

Structure		Fetuin samples buffer exchanged by washing with:											
		no cleanup	water	100mM PBS	0.1% TFA	0.1% HCl	0.1% H_2SO_4	0.1% HCOOH	0.1% CH_3COOH	100mM EDTA	50mM EDTA	20mM EDTA	5mM EDTA
2AB	**Average % area**	**7.6**	**8.2**	**8.2**	**9.2**	**13.0**	**35.4**	**8.6**	**7.5**	**7.1**	**8.0**	**6.9**	**6.5**
	STDEV	*2.6*	*1.5*	*0.1*	*2.2*	*4.8*	*7.8*	*4.5*	*1.7*	*1.2*	*0.1*	*0.4*	*0.2*
	p-value	-	0.9	0.8	0.4	0.2	**0.0**	0.9	0.4	0.4	0.9	0.8	0.7
2AB	**Average % area**	**58.2**	**36.0**	**45.2**	**19.1**	**20.4**	**17.6**	**24.6**	**27.4**	**17.4**	**16.4**	**23.4**	**22.1**
	STDEV	*14.9*	*6.5*	*3.1*	*7.3*	*6.0*	*6.1*	*14.7*	*10.9*	*3.0*	*1.8*	*2.5*	*3.9*
	p-value	-	**0.0**	0.4	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	0.2	0.2	0.3
2AB	**Average % area**	**25.0**	**40.0**	**33.8**	**49.8**	**46.9**	**31.9**	**46.5**	**42.5**	**51.8**	**54.6**	**48.7**	**48.3**
	STDEV	*13.0*	*3.6*	*2.1*	*4.3*	*4.0*	*9.0*	*10.5*	*13.1*	*2.9*	*0.5*	*1.4*	*4.8*
	p-value	-	**0.0**	0.6	**0.0**	**0.0**	0.2	**0.0**	**0.0**	**0.0**	0.2	0.3	0.4
2AB	**Average % area**	**1.4**	**1.7**	**1.4**	**2.6**	**3.9**	**9.8**	**2.3**	**2.1**	**2.7**	**2.6**	**2.4**	**2.4**
	STDEV	*0.9*	*0.7*	*0.0*	*1.0*	*1.7*	*12.1*	*0.6*	*0.5*	*0.4*	*0.3*	*0.4*	*0.1*
	p-value	-	**0.0**	1.0	0.1	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	0.4	0.5	0.4
2AB	**Average % area**	**6.5**	**11.3**	**9.4**	**14.1**	**12.7**	**5.9**	**14.2**	**3.0**	**16.8**	**14.9**	**14.9**	**16.4**
	STDEV	*2.0*	*1.8*	*0.8*	*2.6*	*1.3*	*1.8*	*14.2*	*3.0*	*1.7*	*2.1*	*1.2*	*0.5*
	p-value	-	**0.0**	0.2	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	0.2	0.1
2AB	**Average % area**	**1.4**	**2.7**	**2.1**	**3.6**	**3.1**	**3.5**	**3.7**	**3.6**	**4.2**	**3.6**	**3.8**	**4.4**
	STDEV	*0.6*	*0.5*	*0.3*	*1.0*	*0.6*	*3.6*	*0.9*	*3.6*	*0.6*	*0.5*	*0.1*	*0.6*
	p-value	-	**0.0**	0.2	**0.0**	**0.0**	0.3	**0.0**	**0.0**	**0.0**	**0.0**	0.1	**0.0**

Table 3. Comparison of the average relative abundance, standard deviation and significance level (p-value) of O-glycans from fetuin samples that had been buffer exchanged prior to hydrazinolysis. The significance level was calculated comparing the control condition (Table 2) with various treatments. P-values are given in bold for samples where changes were significant (p-value ≤ 0.05).

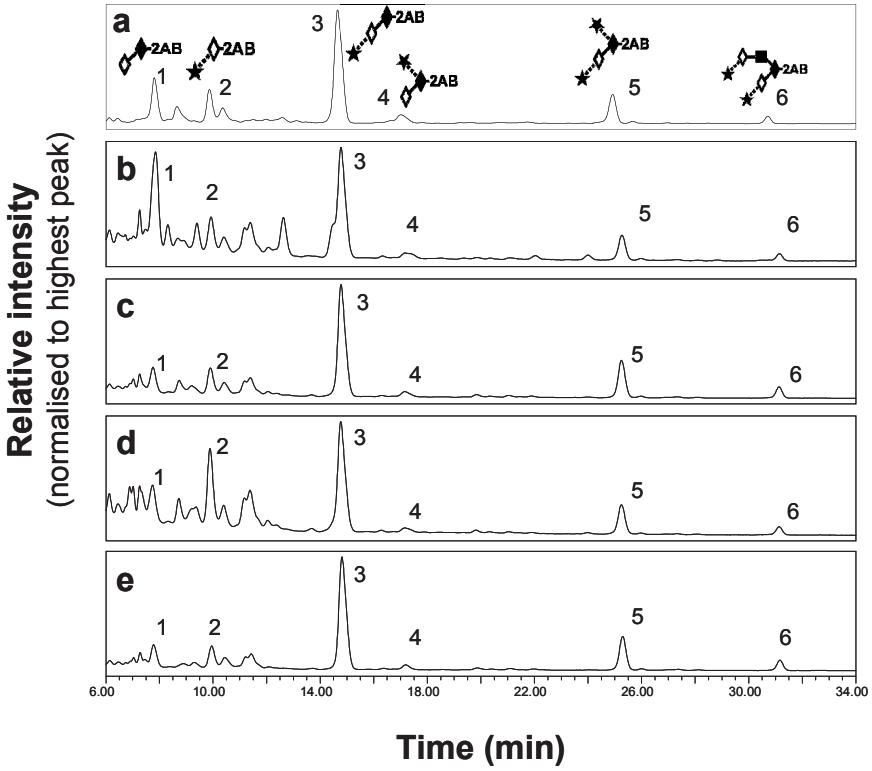

Figure 2. Fetuin O-glycan profiling following buffer exchange with a range of acids. The following buffer exchange procedures were applied: (a) 0.1% HCl, (b) 0.1% H_2SO_4, (c) 0.1% HCOOH, (d) 0.1% CH_3COOH, (e) 0.1% TFA. Subsequently, glycans were released by hydrazinolysis, 2-AB labelled and compared by HILIC-HPLC with fluorescence detection. Peak 2 is the peeled product.

We hypothesize that the suppression of peeling achieved by the acid washes could be due to the removal of salts that could possibly interfere with the release of glycans and stimulate the peeling reaction. We assume that the chelation of cations may promote salt removal. Therefore, we decided to study the effect of washes with solutions of the chelating agent ethylenediaminetetraacetic acid (EDTA) on the degree of peeling.

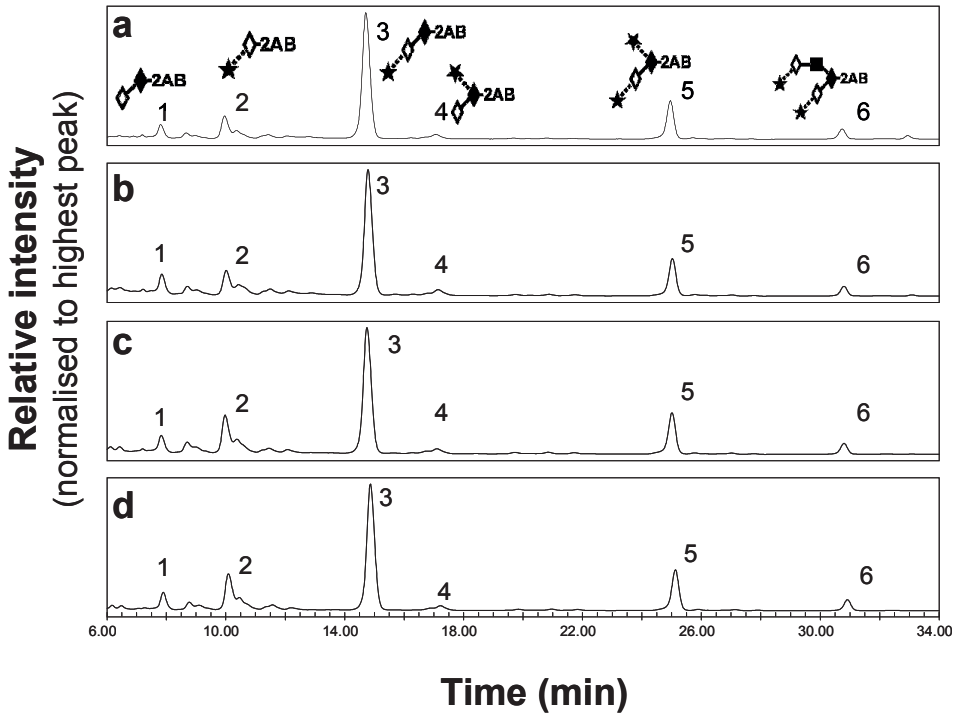

Figure 3. Fetuin O-glycan profiling after hydrazinolysis and buffer exchange with EDTA solutions. Sample washing was performed using (a) 100 mM EDTA, (b) 50 mM EDTA, (c) 20 mM EDTA, (d) 5 mM EDTA. Subsequently, glycans were released by hydrazinolysis, 2-AB labelled and compared by HILIC-HPLC with fluorescence detection. Peak 2 is the peeled product

Fetuin glycoprotein was cleaned up by centrifugal filtration with a range of solutions of EDTA with different concentrations (100, 50, 20 and 5 mM). The HPLC profiles (Figure 3) show that there were no significant differences in the relative abundance of peeling products between the samples washed with these different concentrations of EDTA solutions and that the relative intensity of the degradation product is similar to the 0.1% TFA cleanup (peak 2, Table 3). We therefore postulate

that the EDTA (and possibly the TFA) could be reducing peeling by removing cations, thereby preventing their interference during hydrazinolysis.

The repeatability of these cleanup procedures was tested over a 12 month period for 0.1% TFA and over a 3 month period for 100 mM EDTA. The O-glycans were released in triplicate from fetuin and labelled with 2-AB prior to HILIC-HPLC analysis. We used different batches of the fetuin glycoprotein, TFA and hydrazine to check the repeatability of the cleanup method (Figure 4). The relative amounts of released O-glycans are consistent across all the experiments and the ratio of the sialylated core 1 (peak 3, Figure 4) to its peeled product (peak 2, Figure 4) is much higher when 0.1% TFA or 100 mM EDTA were used instead of water to prepare the samples for hydrazinolysis.

In order to evaluate the effect of cations on the release of O-glycans and to demonstrate the role of EDTA washes in cation removal and suppression of peeling, a further experiment was performed. Fetuin was dissolved in 100 µL of a 100 mM $CaCl_2$ solution. Half of this fetuin solution was buffer exchanged by washing with 100 mM EDTA prior to hydrazinolysis and the other half was dried down without further manipulation. The two samples were then subjected to hydrazinolysis and O-glycan analysis. Experiments were performed in triplicate providing consistent results. The HPLC profiles showed that peeling is higher (peak 2, Figure 5a) and the yield of O-glycans lower (Figure 5a) for the sample that had not been buffer exchanged after addition of $CaCl_2$ compared to the sample that had been buffer exchanged with 100 mM EDTA (Figure 5b). This experiment showed that the presence of calcium chloride interferes with the O-glycan release and that the removal of calcium prior to hydrazinolysis reduces peeling.

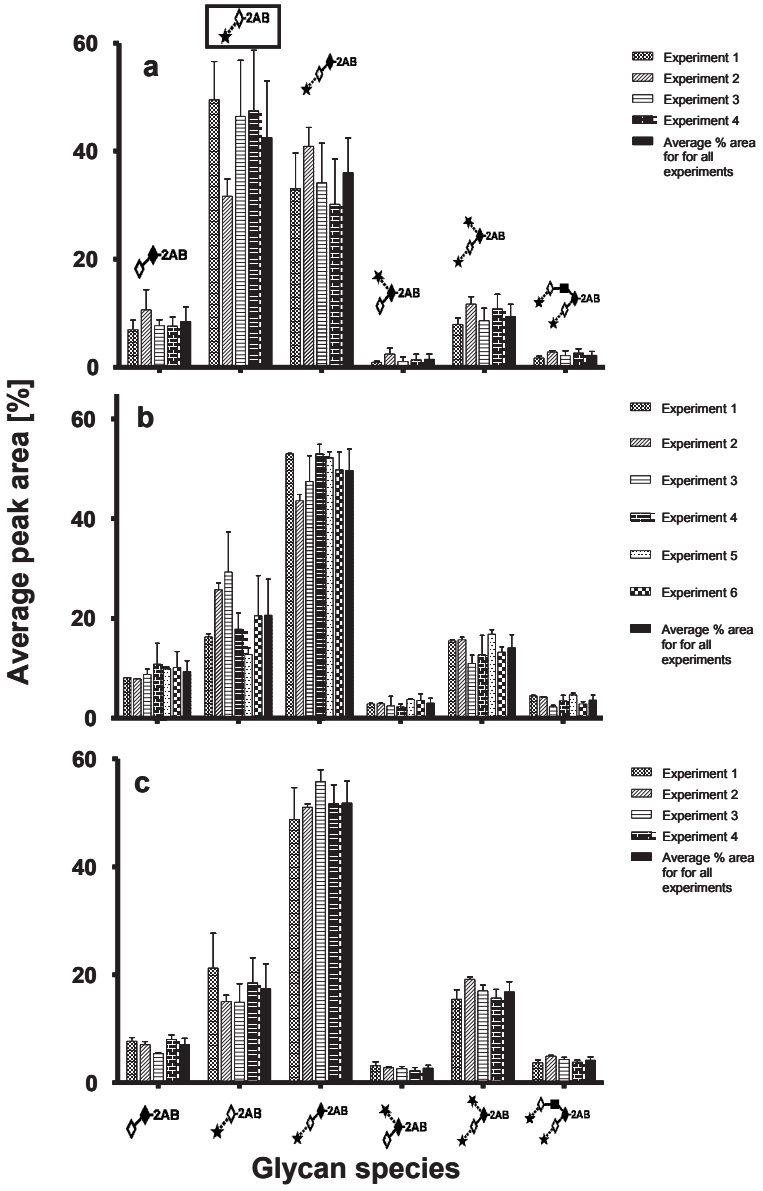

Figure 4. Comparison of fetuin O-glycan patterns obtained after applying various washing conditions. Samples were washed using (a) water, (b) 0.1% TFA, (c) 100 mM EDTA, followed by hydrazinolysis, 2-AB-labelling and HILIC-HPLC analysis with fluorescence detection. The average abundance of O-glycans was determined from experiments performed over a 3 month period for water, a 12 month period for 0.1% TFA and 3 month period for 100 mM EDTA.

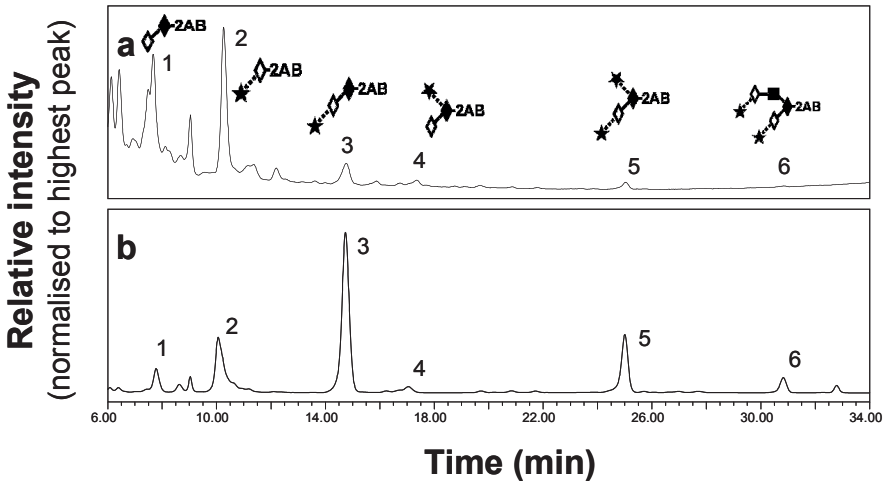

Figure 5. O-glycan peeling caused by the addition of calcium chloride may be prohibited by EDTA washes. Fetuin samples were dissolved in 100 mM CaCl2 and subjected to hydrazinolysis either directly (a) or after buffer exchange with 100 mM EDTA (b) followed by 2-AB-labelling and HILIC-HPLC analysis with fluorescence detection. Peak 2 is the peeled product.

Both the 0.1% TFA and 100 mM EDTA methods were also tested on bovine submaxillary gland mucin (BSM) as the O-glycans from mucins are also prone to peeling. Mucin samples were cleaned by centrifugal filtration with 0.1% TFA or 100 mM EDTA. The BSM O-glycans were released using anhydrous hydrazine,[9] labelled with 2-aminobenzamide (2-AB) and analysed by HILIC-HPLC.

The HPLC profiles obtained from the BSM sample without cleanup prior to hydrazinolysis and from the BSM samples cleaned up with water, 0.1% TFA or 100 mM EDTA using a centrifugal filtration device (MWCO membrane,10 kDa) were compared (Fig. 6). The profiles of the O-glycan pools from BSM show 11 peaks (Figure 6).

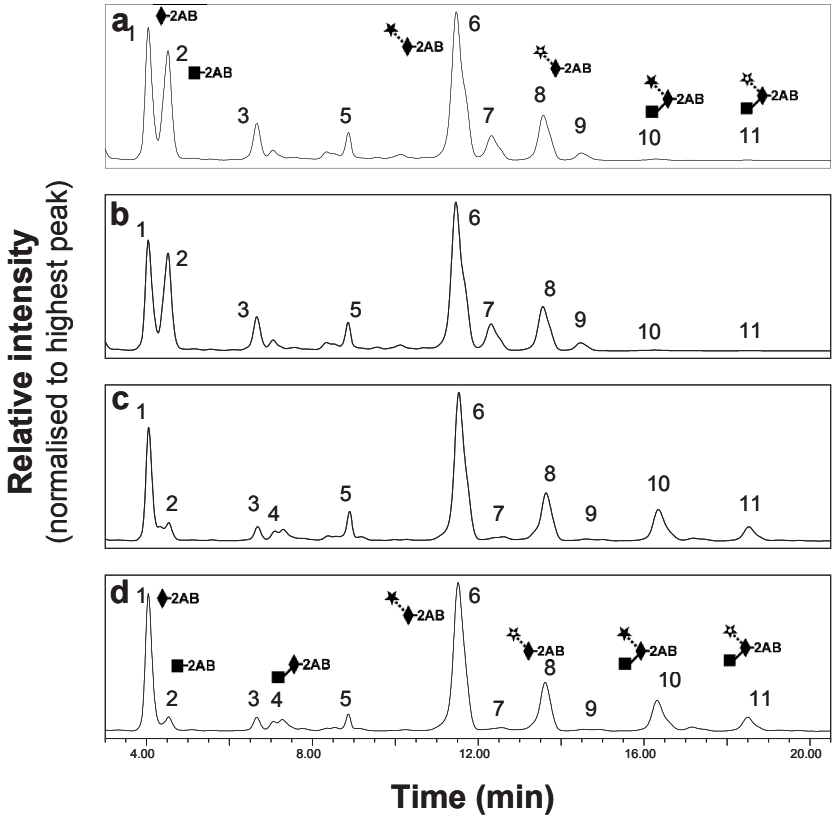

Figure 6. Comparison of HPLC O-glycan profiles of bovine submaxillary mucin following buffer exchange with a range of solutions, hydrazinolysis, and 2-AB labelling. The following buffer exchange procedures were applied: (a) no washing, (b), water wash, (c) 0.1% TFA wash, (d) 100 mM EDTA wash. The O-glycans released by hydrazinolysis from BSM were 2-AB labelled and compared by HILIC-HPLC with fluorescence detection. Peak 2 is the peeled product.

In order to characterise unknown mucin O-glycan structures, information from published LC/MS/MS analyses [17,27] was combined with the results of exoglycosidase treatments as determined by HILIC-HPLC (Figure 7). Initial digestion with sialidase showed that peaks 5 – 11 contained sialic acids (Neu5Ac or Neu5Gc) by the change in their elution position (Figures 7a-b). Digestion with bovine kidney α-fucosidase

which preferably cleaves α1-6 linked fucose showed that peak 3 contained fucose (Figures 7a and 7d). Digestion with a beta galactosidase specific for β1-4 galactose did not show any changes (Figures 7a and 7c). Six different glycan structures 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 some peeled product GlcNAc (peak 2). BSM was found to contain also other sialylated structures (peaks 5, 7 and 9) and a fucosylated structure (peak 3) which were not fully structurally elucidated.

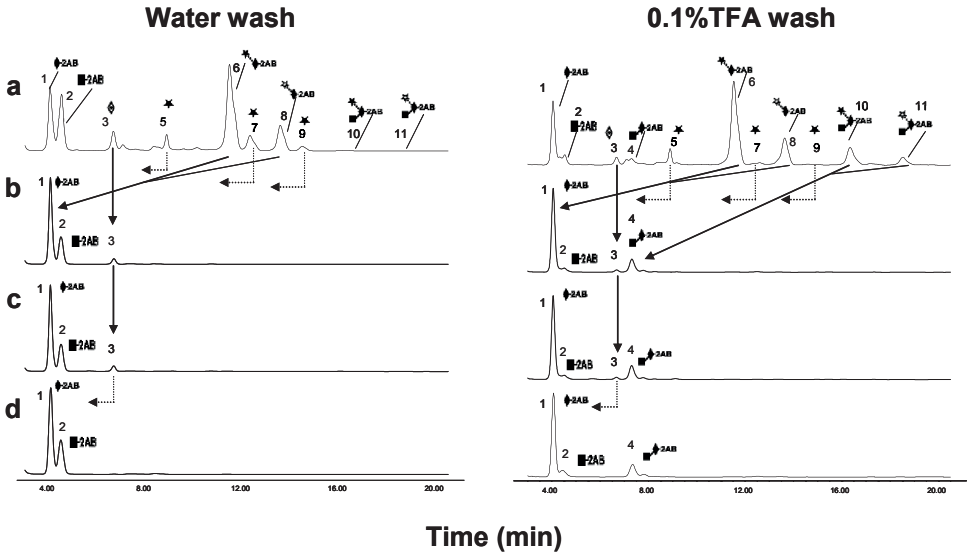

Figure 7. Glycan sequencing of BSM O-glycans before and after digestion with exoglycosidases. Samples were subjected to water wash (left) or 0.1% TFA wash (right) followed by hydrazinolysis and 2-AB labelling. Aliquots of the total 2AB-labelled O-glycan pool were incubated with different exoglycosidases, as shown in each panel, (a) before digestion, (b) sialidase, (c) sialidase + β-galactosidase, (d) sialidase + α-fucosidase. Following digestion, the products were analysed by HILIC-HPLC. Arrows indicate digestion pathways.

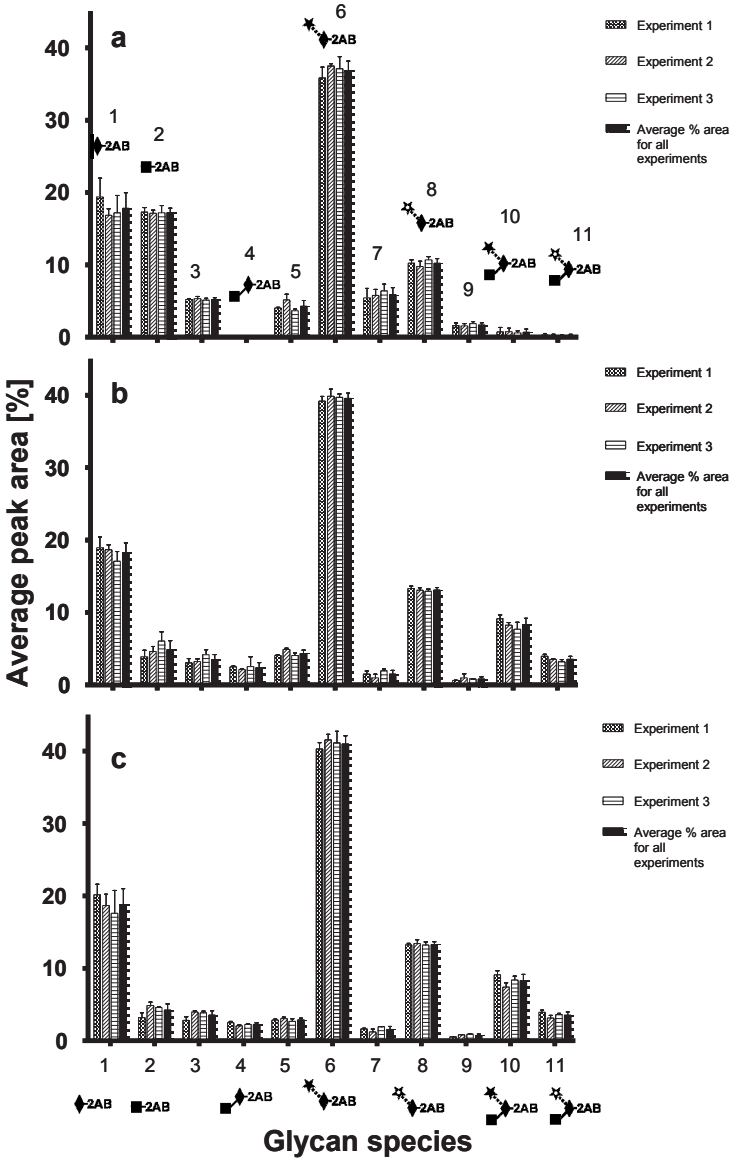

Figure 8. Comparison of BSM O-glycan patterns obtained after applying various washing conditions. Samples were washed using (a) water, (b) 0.1% TFA, (c) 100 mM EDTA, followed by hydrazinolysis, 2-AB-labelling and HILIC-HPLC analysis with fluorescence detection. The average abundance of O-glycans was determined from experiments performed over a 2 month period.

BSM samples not cleaned up prior to hydrazinolysis		Glycan structure										
		♦-2AB	■-2AB	fucosylated structure	■♦-2AB	sialylated structure	✶♦-2AB	sialylated structure	✶✶♦-2AB	sialylated structure	✶♦■-2AB	✶✶♦■-2AB
		Peak area [%]										
Experiment 1	A	18.2	17.9	5.5	-	3.8	35.6	5.9	10.4	1.9	0.5	0.2
	B	20.0	17.6	5.3	-	4.0	35.3	5.7	9.9	1.6	0.4	0.1
	C	17.7	16.5	5.2	-	4.1	37.2	6.6	10.5	1.9	0.2	0.1
Experiment 2	A	18.7	17.8	5.1	-	3.7	36.1	5.8	10.5	1.7	0.4	0.2
	B	18.7	17.3	4.9	-	4.3	36.6	5.9	10.2	1.7	0.3	0.1
	C	17.1	17.4	5.2	-	4.1	36.9	6.6	10.5	1.9	0.3	0.1
Average % Area		18.4	17.4	5.2	-	4.0	36.3	6.1	10.3	1.8	0.4	0.1
STDev		0.9	0.5	0.2	-	0.2	0.7	0.4	0.2	0.1	0.1	0.0

Table 4. Average relative abundance of O-glycans from BSM samples not cleaned up prior to hydrazinolysis.

A large difference in the relative abundance of peeling products was apparent between the different sample clean ups. The highest occurrence of peeling was observed for samples which were not cleaned up (17% peak 2, Figure 6a, Table 5) and for samples washed with water (17%, peak 2, Figure 6b, Table 5). For the samples washed with 0.1% TFA (5%, peak 2, Figure 6c, Table 5) or 100 mM EDTA (4% peak 2, Figure 6d, Table 5) there was much less peeled product. The samples which were not cleaned up or cleaned with water also showed a significantly reduced amount of GlcNAcβ1-3(Neu5Acα2-6)GalNAc (peak 10, Figure 6a) and GlcNAcβ1-3(Neu5Gcα2-6)GalNAc (peak 11, Figure 5a) when compared to the samples that were cleaned up with 0.1% TFA or 100 mM EDTA.(Figures 6c-d).

Alongside the comparisons of the relative abundance of O-glycans present in both fetuin glycoprotein and BSM glycoprotein (Tables 3 and 5), the absolute abundance of O-glycans was also compared by making use of the quantitative response of the HPLC fluorescence detector (Supplementary Tables 1 and 2). These results showed that there was no major difference in the overall yield of O-glycans from fetuin or BSM from either the acid-washed or EDTA-washed samples when compared to the control sample (no cleanup). This indicated that there were no

major glycoprotein losses occurring during the sample washing step using the centrifugal filtration device (10 kDa MWCO membrane).

Structure		BSM samples buffer exchanged by washing with:			
		no cleanup	water	0.1% TFA	100mM EDTA
♦-2AB	**Average % area**	**18.4**	**17.8**	**18.2**	**18.8**
	STDEV	*0.9*	*2.2*	*1.4*	*2.2*
	p-value	-	0.5	0.8	0.5
■-2AB	**Average % area**	**17.4**	**17.2**	**4.8**	**4.2**
	STDEV	*0.5*	*0.6*	*1.3*	*0.9*
	p-value	-	0.7	**0.0**	**0.0**
fucosylated structure	**Average % area**	**5.2**	**5.2**	**0.2**	**3.5**
	STDEV	*0.2*	*5.2*	*0.2*	*0.6*
	p-value	-	0.8	**0.0**	0.1
■♦-2AB	**Average % area**	**-**	**-**	**2.4**	**2.3**
	STDEV	-	-	*0.7*	*0.2*
	p-value	-	-	**0.0**	**0.0**
sialylated structure	**Average % area**	**4.0**	**4.3**	**4.3**	**2.9**
	STDEV	*0.2*	*0.8*	*0.5*	*0.3*
	p-value	-	0.6	0.4	**0.0**
✶♦-2AB	**Average % area**	**36.3**	**36.8**	**39.5**	**41.0**
	STDEV	*0.7*	*1.4*	*0.7*	*1.2*
	p-value	-	0.4	**0.0**	**0.0**
sialylated structure	**Average % area**	**6.1**	**5.9**	**1.5**	**1.6**
	STDEV	*0.4*	*1.0*	*0.5*	*0.4*
	p-value	-	**0.0**	**0.0**	**0.0**
✶♦-2AB	**Average % area**	**10.3**	**10.2**	**13.1**	**13.3**
	STDEV	*0.2*	*0.6*	*0.3*	*0.4*
	p-value	-	0.9	**0.0**	**0.0**
sialylated structure	**Average % area**	**1.8**	**1.7**	**0.8**	**0.7**
	STDEV	*0.1*	*0.3*	*0.3*	*0.2*
	p-value	-	**0.0**	**0.0**	**0.0**
✶♦-2AB	**Average % area**	**0.4**	**0.7**	**8.3**	**8.3**
	STDEV	*0.1*	*0.4*	*0.9*	*0.9*
	p-value	-	**0.0**	**0.0**	**0.0**
✶■♦-2AB	**Average % area**	**0.1**	**0.2**	**3.5**	**3.5**
	STDEV	*0.0*	*0.2*	*0.4*	*0.4*
	p-value	-	**0.0**	**0.0**	**0.0**

Table 5. Comparison of the average relative abundance, standard deviation and significance level (p-value) of O-glycans from BSM samples that had been buffer exchanged prior to hydrazinolysis. The significance level was calculated comparing the control condition (Table 4) with various treatments. P-values are given in bold for samples where changes were significant (p-value ≤ 0.05).

These studies show that buffer exchange into 0.1% TFA or 100 mM EDTA prior to hydrazinolysis significantly reduces the amount of undesirable peeling. Peeled product went down from 58% for the fetuin sample without cleanup to <20% for the fetuin samples washed with 0.1% TFA or EDTA and from 17% for the BSM sample without cleanup to <5% for the BSM samples washed with 0.1% TFA or EDTA. As TFA is volatile it will be removed during the vacuum centrifugation step that is applied prior to hydrazinolysis, while EDTA will stay within the sample. The two sample cleanup methods used, 0.1% TFA and 100 mM EDTA washes, appear to work equally well. Both of them result in less peeling, more robust O-glycan profiles and, therefore, greatly improve O-glycan analysis employing hydrazinolysis.

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Supplementary information

Structure		Fetuin samples buffer exchanged by washing with:							
		no cleanup	water	100mM EDTA	0.1% TFA	0.1% HCl	0.1% H_2SO_4	0.1% HCOOH	0.1% CH_3COOH
	Absolute average area (/1000000)	2.4	1.5	3.4	2.8	3.5	3.9	2.5	2.4
	Absolute average area (/1000000)	19.5	9.1	8.8	7.4	5.1	2.6	7.4	7.7
	Absolute average area (/1000000)	14.8	7.9	24.5	15.2	12.5	3.5	17.1	16.4
	Absolute average area (/1000000)	0.9	0.3	1.3	0.8	1.2	0.4	0.8	0.7
	Absolute average area (/1000000)	3.4	2.1	8.2	4.0	3.3	0.9	5.0	4.8
	Absolute average area (/1000000)	0.8	0.5	2.1	0.9	0.8	0.2	1.3	1.3

Supplementary Table 1. Comparison of the average absolute area under the curve of O-glycans from fetuin samples that had been buffer exchanged prior to hydrazinolysis. The corresponding relative abundances are presented in Table 3.

Structure		BSM samples buffer exchanged by			
		no cleanup	water	0.1% TFA	100mM EDTA
	Absolute average area (/1000000)	402.4	262.2	233.9	221.8
	Absolute average area (/1000000)	375.0	259.4	60.5	50.5
fucosylated structure	Absolute average area (/1000000)	113.7	79.3	43.6	42.7
	Absolute average area (/1000000)	0.0	0.0	30.1	27.0
sialylated structure	Absolute average area (/1000000)	88.6	64.6	55.9	34.9
	Absolute average area (/1000000)	802.8	558.5	508.8	495.4
sialylated structure	Absolute average area (/1000000)	139.7	92.2	18.7	17.9
	Absolute average area (/1000000)	226.3	155.3	168.5	160.8
sialylated structure	Absolute average area (/1000000)	40.8	26.5	10.3	8.9
	Absolute average area (/1000000)	7.5	8.8	108.3	99.6
	Absolute average area (/1000000)	2.6	8.8	45.9	42.5

Supplementary Table 2. Comparison of the average area under the curve of O-glycans from BSM samples that had been buffer exchanged prior to hydrazinolysis. The corresponding relative abundances are presented in Table 5.