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

Radoslaw P. Kozak [a], Louise Royle [a], Richard A. Gardner [a], Daryl L. Fernandes [a], Manfred Wuhrer [b,*]

[a] Ludger Ltd., Culham Science Centre, Oxfordshire OX14 3EB, UK
[b] Biomolecular Mass Spectrometry Unit, Department of Parasitology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands

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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 labeled with a fluorophore and analyzed by fluorescence detection. Under the hydrazinolysis release conditions, a side reaction is observed and causes the loss of monosaccharides from the reducing terminus of the glycans (known as peeling). Using bovine fetuin (because it contains the sialylated *O*-glycans most commonly found on biopharmaceuticals) and bovine submaxillary gland mucin (BSM), here we demonstrate that peeling can be greatly reduced when the sample is buffer exchanged prior to hydrazinolysis with solutions of either 0.1% trifluoroacetic acid (TFA) or low-molarity (100, 50, 20, and 5 mM) ethylenediaminetetraacetic acid (EDTA). The addition of calcium chloride to fetuin resulted in an increase in peeling, whereas 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.

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

There are two main types of protein glycosylation found in eukaryotes: N-linked glycosylation and O-linked glycosylation. Although the methods for release and analysis of *N*-glycans (asparagines 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 because 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, whereas sodium borohydride reduces the terminal sugar by converting it to an alditol, thereby 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 (2AB)[1] or 2-aminobenzoic acid (2AA) for fluorescence detection or 1-phenyl-3-methyl-5-pyrazolone (PMP) for ultraviolet (UV) detection [10]. Therefore, alternative detection

* Corresponding author. Fax: +31 715266907.
E-mail address: m.wuhrer@lumc.nl (M. Wuhrer).

[1] *Abbreviations used:* 2AB, 2-aminobenzamide; 2AA, 2-aminobenzoic acid; PMP, 1-phenyl-3-methyl-5-pyrazolone; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid; MWCO, molecular weight cutoff; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; BSM, bovine submaxillary gland mucin (type I-S); HILIC, hydrophilic interaction chromatography; HPLC, high-performance liquid chromatography.

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methods such as mass spectrometry [11] and high pH anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD) [12] must be used for the analysis of alditols.

To overcome this problem with alditols, nonreductive 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 and coworkers [16]. First 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 labeled with a fluorophore or chromophore, and mass spectrometric analysis is required.

Recently, Zauner and coworkers [17], Wang and coworkers [18], and Furukawa and coworkers [19] described a combined method for *O*-glycan release and labeling using a β-elimination method with dimethylamine [17], ammonia [18], or sodium hydroxide [19] for the release and concomitant labeling with PMP. The technique is quick, provides good release and labeling, and is reported to have a reduced or very low degree of peeling. However, PMP labeling allows only UV detection, which is not as sensitive and specific as fluorescence detection or mass spectrometric analysis of the released PMP-labeled 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. Whereas *N*-glycans are released from glycoproteins using more vigorous conditions (85–100 °C, 5–16 h) [20], *O*-glycans are released and recovered under milder conditions (60 °C, 6 h) [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 and coworkers [9] reported that removal of water from the sample before hydrazinolysis is essential to minimize peeling. The presence of water may lead to the alkaline conditions that favor β-elimination and produce higher amounts of peeling. Although removal of water does reduce peeling, it still remains a significant problem that has proved to be 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 obtained from Ludger (Oxford, UK). EDTA (99.0%) was obtained from VWR (Leicestershire, UK). Acetonitrile (Romil, 190 SpS for UV/gradient quality) was obtained from Charlton Scientific (Oxon, UK). Centrifugal filter devices with a molecular weight cutoff (MWCO) membrane of 10 kDa were obtained from Fisher Scientific UK (Leicestershire, UK). Bovine fetuin was obtained from Ludger, 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 phosphate-buffered saline (PBS); 0.1% trifluoroacetic acid (TFA); 0.1% HCl; 0.1% H_2SO_4; 0.1% HCOOH; 0.1% CH_3COOH; and 100, 50, 20, and 5 mM EDTA. Each 150-μl solution of fetuin glycoprotein was then transferred to a separate centrifugal filter device (10 kDa MWCO membrane) that had been prewashed with water (5 ml). Each centrifugal filter device was centrifuged at 4000 rpm for 10 to 12 min (until 0.3–0.5 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 pyrolyzed 5-ml glass vials (for triplicate release) and dried down for 16 h by vacuum centrifugation (without heating) prior to the addition of hydrazine.

BSM samples (375 μg) were prepared as for fetuin with the 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 h as described previously by Merry and coworkers [9], Patel and coworkers [20], and Ashford and coworkers [21]. 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). The glycans were eluted off the cartridges using water (3 × 0.5 ml). Eluates were dried by vacuum centrifugation prior to fluorescent labeling.

Fluorescent labeling

Released *O*-glycans were labeled with 2AB according to Bigge and coworkers [22] using a Ludger 2AB Glycan Labeling Kit. The released glycans were incubated with labeling reagents for 3 h at 65 °C. The 2AB-labeled glycans were cleaned up using LudgerClean S Cartridges (Ludger). 2AB-labeled glycans were eluted from the LudgerClean S Cartridges with water (2 × 0.5 ml). The samples were evaporated to dryness under high vacuum using centrifugal evaporation and resuspended in water (100 μl) for further analysis.

Exoglycosidase digestions

Exoglycosidase sequencing was performed according to Royle and coworkers [23]. The released 2AB-labeled glycans were incubated with exoglycosidases at standard concentrations in a final volume of 10 μl in 50 mM sodium acetate for 16 h at 37 °C. The enzymes used were sialidase from *Arthobacter ureafaciens* (a368S), specific for α2, -3, -6, -8, and -9 sialic acids (E-S001, QABio, Palm Desert, CA, USA); β-galactosidase from *Streptococcus pneumoniae* (b4G), specific for β1–4 galactose (E-BG07, QABio); and 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

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

Acid used for washing fetuin sample prior to hydrazinolysis	Molarity (mM)	pH
0.1% HCl	3.6	1.4
0.1% H_2SO_4	10.2	1.7
0.1% HCOOH	21.7	2.7
0.1% CH_3COOH	16.7	3.3
0.1% TFA	8.8	2.0

elution of the glycans from the plate with water. The samples were analyzed by hydrophilic interaction chromatography high-performance liquid chromatography (HILIC–HPLC).

HPLC analysis

2AB-labeled samples were analyzed by HILIC–HLPC using a LudgerSep-N2 column (4.6 × 150 mm, Ludger) on a 2795 HPLC device with a 2475 fluorescence detector, controlled by Empower software (Waters, Manchester, UK). Solvent A was 50 mM ammonium formate (pH 4.4), and solvent B was acetonitrile. Samples were injected in 20% aqueous/80% acetonitrile with an injection volume of 25 µl. Gradient conditions were as follows: 20 to 38% A at a 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. 2AB-labeled glucose homopolymer (CAB-GHP-30, Ludger) was used as a system suitability standard as well as an external calibration standard for GU allocation [23].

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 whether 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, and 0.1% CH_3COOH (Table 1). The *O*-glycans were released using anhydrous hydrazine [9], labeled with 2AB, and analyzed by HILIC–HPLC. To find out whether the amount of degradation product was reduced when performing a solvent exchange prior to hydrazinolysis, three control experiments were performed: 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 (10 kDa MWCO membrane) were compared (Fig. 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 the following: core 1, Galβ1–3GalNAc (peak 1); monosialylated core 1 *O*-glycans, Neu5Acα2–3Galβ1–3GalNAc (peak 3); and Neu5Acα2–6(Galβ1–3)GalNAc (peak 4); disialylated core 1 *O*-glycan, Neu5Acα2–3Galβ1–3(Neu5Acα2–6)GalNAc (peak 5); and disialylated core 2 *O*-glycan, Neu5Acα2–3Galβ1–3(Neu5Acα2–3Galβ1–4GlcNAcβ1–6)GalNAc (peak 6). The peeled product Neu5Acα2–3Gal (peak 2, Fig. 1) was also detected.

The highest degree of peeling was observed for samples that were not cleaned up. Under these conditions, the peeled product Neu5Acα2–3Gal (peak 2, Fig. 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, Fig. 1B, C and 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, Fig. 1D and Table 3) as compared with the samples that were not cleaned up or were washed with water.

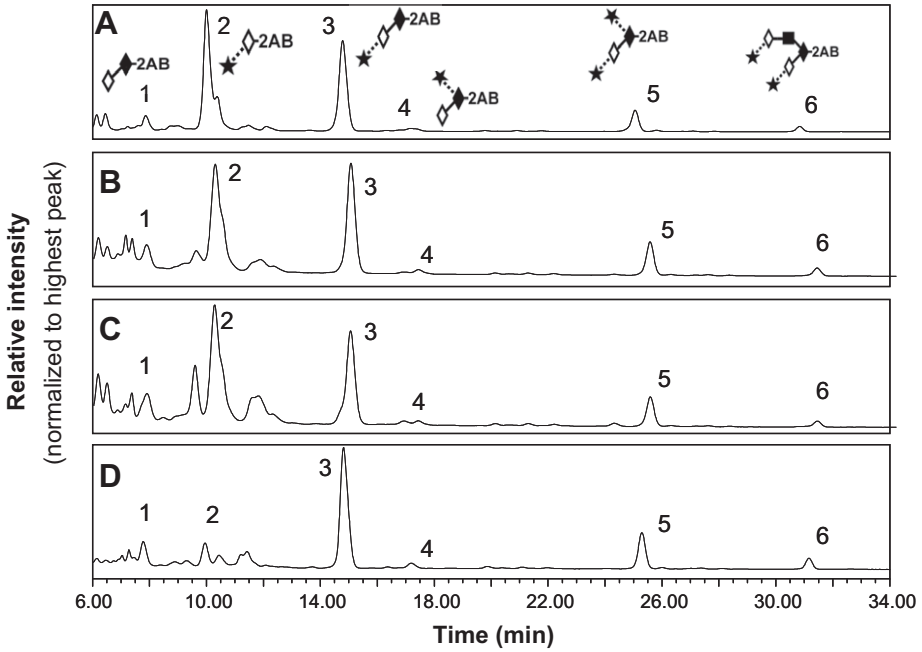

Fig.1. Comparison of HPLC *O*-glycan profiles of bovine fetuin following buffer exchange with a range of solutions, hydrazinolysis, and 2AB labeling. The following buffer exchange procedures were applied: (A) no washing; (B) water wash; (C) 0.1 M PBS wash; (D) 0.1% TFA wash. The *O*-glycans released by hydrazinolysis were 2AB labeled and compared by HILIC–HPLC with fluorescence detection. Peak 2 is the peeled product. The following symbols are used to depict glycan structures [27]: ◇, galactose; ♦, *N*-acetylgalactosamine; ◆, fucose; ■, *N*-acetylglucosamine; ★, *N*-acetylneuraminic acid; ☆, *N*-glycolylneuraminic acid; dashed line, α-linkage; solid line, β-linkage.

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

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
Standard deviation		2.6	14.9	13.0	0.9	2.0	0.6

Note. Symbols are as in Fig. 1.

Table 3
Comparison of average relative abundance, standard deviation, and significance level (p value) of *O*-glycans from fetuin samples that had been buffer exchanged prior to hydrazinolysis.

Structure		Fetuin samples buffer exchanged by washing with:											
		No cleanup	Water	100 mM PBS	0.1% TFA	0.1% HCl	0.1% H_2SO_4	0.1% HCOOH	0.1% CH_3COOH	100 mM EDTA	50 mM EDTA	20 mM EDTA	5 mM EDTA
	**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**
	Standard deviation	*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
	**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**
	Standard deviation	*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
	**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**
	Standard deviation	*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
	**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**
	Standard deviation	*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
	**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**
	Standard deviation	*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
	**Average % area**	**1.4**	**1.42.7**	**2.1**	**3.6**	**3.1**	**3.5**	**3.7**	**3.6**	**4.2**	**3.6**	**3.8**	**4.4**
	Standard deviation	*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**

Note. 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 $\leqslant 0.05$). Symbols are as in Fig. 1.

Following the finding 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 (Fig. 2). The samples that were prepared using 0.1% HCl, 0.1% H_2SO_4, and 0.1% HCOOH showed degrees of peeling that 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 desialylation (peak 1, Fig. 2A, B, D and Table 3). The incidence of the nonsialylated core 1 structure (peak 1, Fig. 2A–D) relative to the sialylated structures (peaks 3–6, Figs. 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

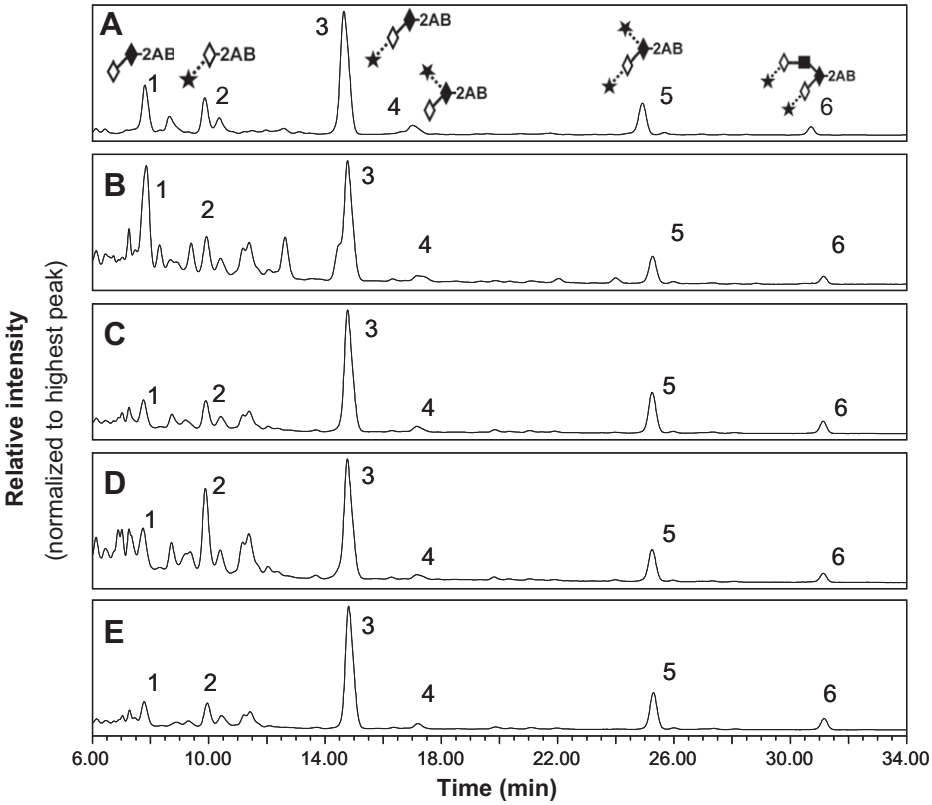

Fig.2. Fetuin *O*-glycan profiling following buffer exchange with a range of acids. The following buffer exchange procedures were applied: (A) 0.1% HCl; (B) 0.1% H_2SO_4; (C) 0.1% HCOOH; (D) 0.1% CH_3COOH; (E) 0.1% TFA. Subsequently, glycans were released by hydrazinolysis, 2AB labeled, and compared by HILIC–HPLC with fluorescence detection. Peak 2 is the peeled product. Symbols are as in Fig. 1.

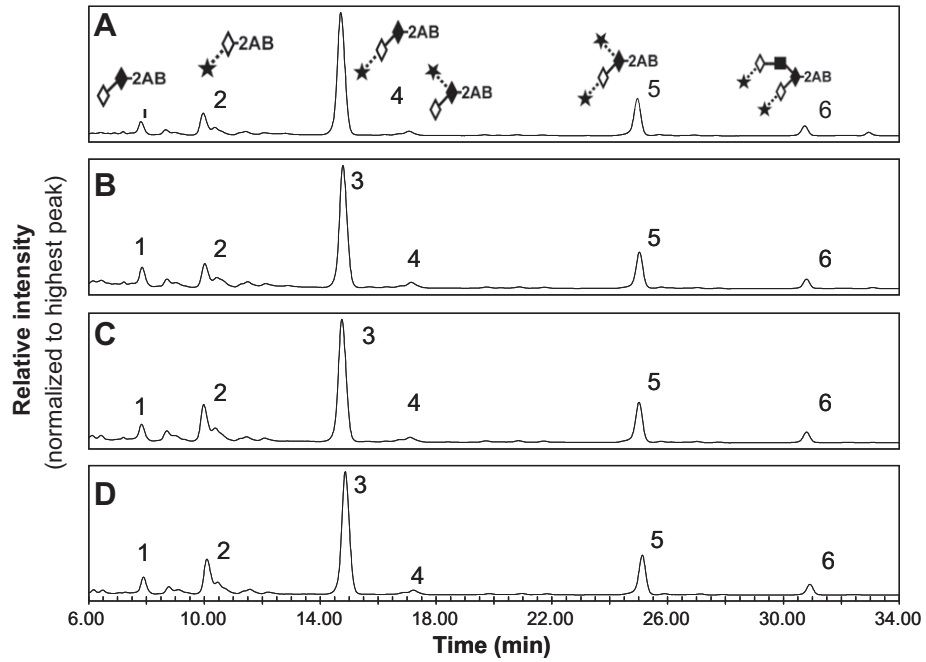

Fig.3. Fetuin *O*-glycan profiling after hydrazinolysis and buffer exchange with EDTA solutions. Sample washing was performed using 100 mM EDTA (A), 50 mM EDTA (B), 20 mM EDTA (C), and 5 mM EDTA (D). Subsequently, glycans were released by hydrazinolysis, 2AB labeled, and compared by HILIC–HPLC with fluorescence detection. Peak 2 is the peeled product. Symbols are as in Fig. 1.

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 desialylation. Desialylation 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.

We hypothesize that the suppression of peeling achieved by the acid washes could be due to the removal of salts that could

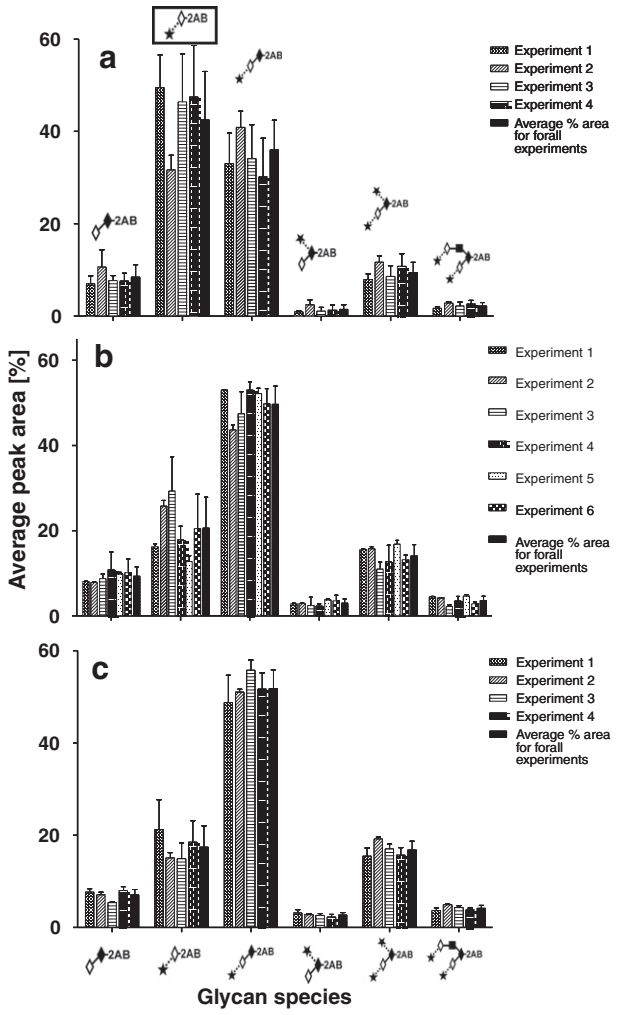

Fig.4. Comparison of fetuin *O*-glycan patterns obtained after applying various washing conditions. Samples were washed using water (A), 0.1% TFA (B), and 100 mM EDTA (C), followed by hydrazinolysis, 2AB labeling, and HILIC–HPLC analysis with fluorescence detection. The average abundance of *O*-glycans was determined from experiments performed over a 3-month period for water, a 12-month period for 0.1% TFA, and a 3-month period for 100 mM EDTA. Symbols are as in Fig. 1.

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 EDTA on the degree of peeling.

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 (Fig. 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). Therefore, we 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 labeled with 2AB prior to HILIC–HPLC analysis. We used different batches of the fetuin glycoprotein, TFA, and hydrazine to check the repeatability of the cleanup method (Fig. 4). The relative amounts of released *O*-glycans are consistent across all of the experiments, and the ratio of the sialylated core 1 (peak 3, Fig. 4) to its peeled product (peak 2, Fig. 4) is much higher when 0.1% TFA or 100 mM EDTA was used instead of water to prepare the samples for hydrazinolysis.

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, Fig. 5A) and the yield of *O*-glycans lower (Fig. 5A) for the sample that had not been buffer exchanged after the addition of $CaCl_2$ compared with the sample that had been buffer exchanged with 100 mM EDTA (Fig. 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.

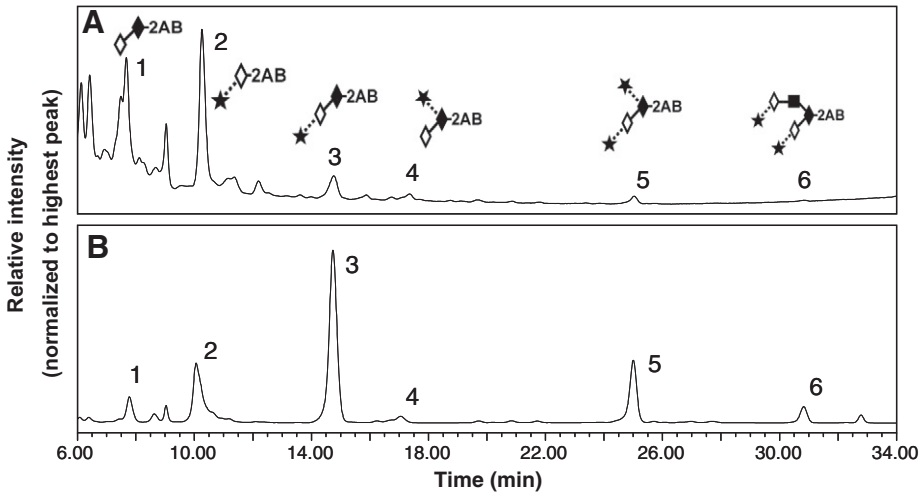

Fig.5. *O*-Glycan peeling caused by the addition of calcium chloride may be prohibited by EDTA washes. Fetuin samples were dissolved in 100 mM $CaCl_2$ and subjected to hydrazinolysis either directly (A) or after buffer exchange with 100 mM EDTA (B), followed by 2AB labeling and HILIC–HPLC analysis with fluorescence detection. Peak 2 is the peeled product. Symbols are as in Fig. 1.

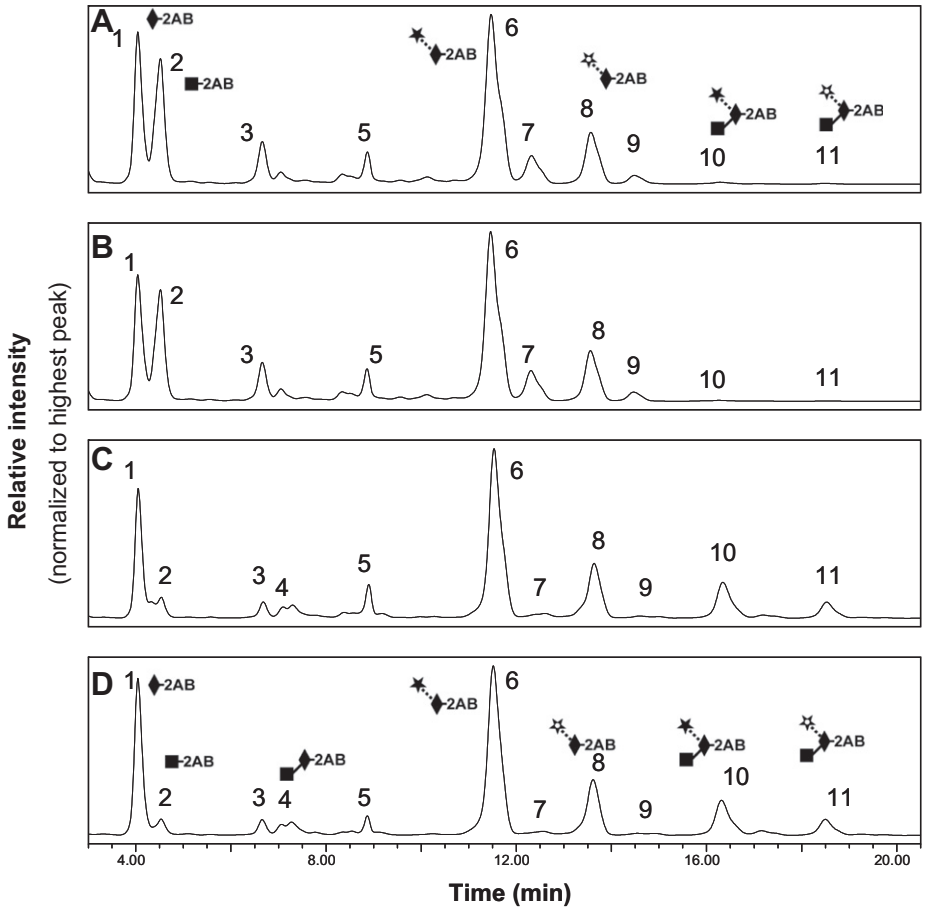

Fig.6. Comparison of HPLC *O*-glycan profiles of BSM following buffer exchange with a range of solutions, hydrazinolysis, and 2AB labeling. The following buffer exchange procedures were applied: (A) no washing; (B) water wash; (C) 0.1% TFA wash; (D) 100 mM EDTA wash. The *O*-glycans released by hydrazinolysis from BSM were 2AB labeled and compared by HILIC–HPLC with fluorescence detection. Peak 2 is the peeled product. Symbols are as in Fig. 1.

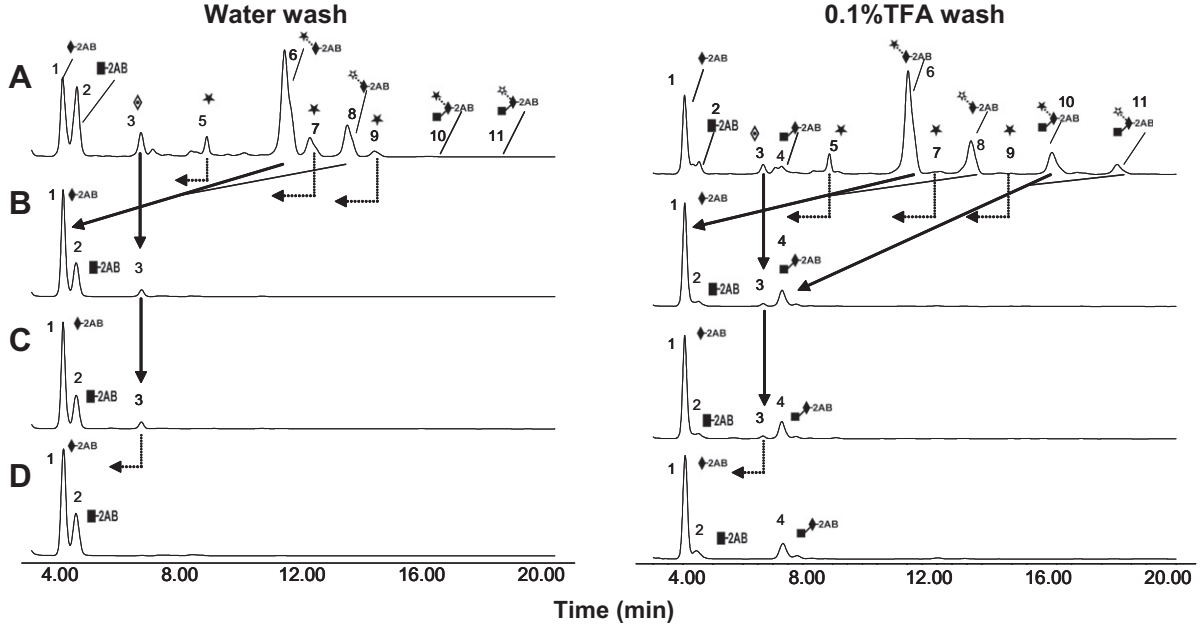

Fig.7. Glycan sequencing of BSM *O*-glycans before and after digestion with exoglycosidases. Samples were subjected to water wash (left) or 0.1% TFA wash (right), followed by hydrazinolysis and 2AB labeling. Aliquots of the total 2AB-labeled *O*-glycan pool were incubated with different exoglycosidases, as shown in each panel: (A) before digestion; (B) sialidase; (C) sialidase + β-galactosidase; (D) sialidase + α-fucosidase. Following digestion, the products were analyzed by HILIC–HPLC. Arrows indicate digestion pathways. Symbols are as in Fig. 1.

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

BSM samples not cleaned up prior to hydrazinolysis		Glycan structure										
		◆-2AB	■-2AB	Fucosylated structure		Sialylated structure		Sialylated structure		Sialylated structure		
		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
Standard deviation		0.9	0.5	0.2	–	0.2	0.7	0.4	0.2	0.1	0.1	0.0

Note. Symbols are as in Fig. 1.

Table 5
Comparison of average relative abundance, standard deviation, and significance level (*P* value) of *O*-glycans from BSM samples that had been buffer exchanged prior to hydrazinolysis.

Structure		BSM samples buffer exchanged by washing with:			
		No cleanup	Water	0.1% TFA	100 mM EDTA
◆-2AB	Average % area	**18.4**	**17.8**	**18.2**	**18.8**
	Standard deviation	*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**
	Standard deviation	*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**
	Standard deviation	*0.2*	*5.2*	*0.2*	*0.6*
	P value	–	0.8	**0.0**	0.1
	Average % area	–	–	**2.4**	**2.3**
	Standard deviation	–	–	*0.7*	*0.2*
	P value	–	–	**0.0**	**0.0**
Sialylated structure	Average % area	**4.0**	**4.3**	**4.3**	**2.9**
	Standard deviation	*0.2*	*0.8*	*0.5*	*0.3*
	P value	–	0.6	0.4	**0.0**
	Average % area	**36.3**	**36.8**	**39.5**	**41.0**
	Standard deviation	*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**
	Standard deviation	*0.4*	*1.0*	*0.5*	*0.4*
	P value	–	**0.0**	**0.0**	**0.0**
	Average % area	**10.3**	**10.2**	**13.1**	**13.3**
	Standard deviation	*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**
	Standard deviation	*0.1*	*0.3*	*0.3*	*0.2*
	P value	–	**0.0**	**0.0**	**0.0**
	Average % area	**0.4**	**0.7**	**8.3**	**8.3**
	Standard deviation	*0.1*	*0.4*	*0.9*	*0.9*
	P value	–	**0.0**	**0.0**	**0.0**
	Average % area	**0.1**	**0.2**	**3.5**	**3.5**
	Standard deviation	*0.0*	*0.2*	*0.4*	*0.4*
	P value	–	**0.0**	**0.0**	**0.0**

Note. 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 $\leqslant$ 0.05). Symbols are as in Fig. 1.

Both the 0.1% TFA and 100 mM EDTA methods were also tested on BSM because 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], labeled with 2AB, and analyzed 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 (10 kDa MWCO membrane) were compared (Fig. 6). The profiles of the *O*-glycan pools from BSM show 11 peaks (Fig. 6). To characterize unknown mucin *O*-glycan structures, information

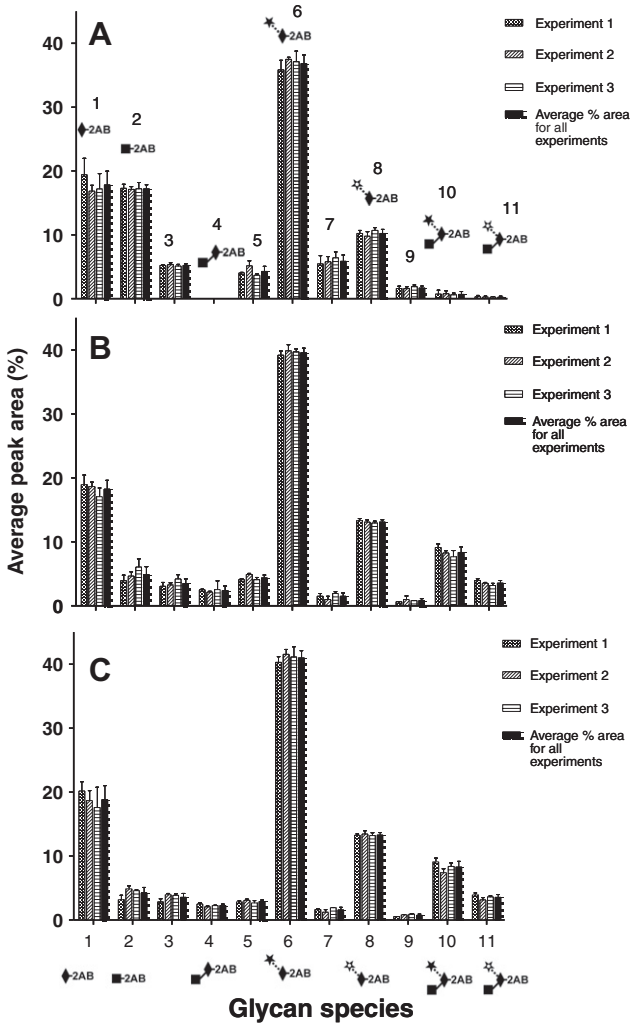

Fig.8. Comparison of BSM *O*-glycan patterns obtained after applying various washing conditions. Samples were washed using water (A), 0.1% TFA (B), and 100 mM EDTA (C), followed by hydrazinolysis, 2AB labeling, and HILIC–HPLC analysis with fluorescence detection. The average abundance of *O*-glycans was determined from experiments performed over a 2-month period. Symbols are as in Fig. 1.

from published liquid chromatography tandem mass spectrometry (LC–MS/MS) analyzes [17,26] was combined with the results of exoglycosidase treatments as determined by HILIC–HPLC (Fig. 7). Initial digestion with sialidase showed that peaks 5 to 11 contained sialic acids (Neu5Ac or Neu5Gc) by the change in their elution position (Fig. 7A, B). Digestion with bovine kidney α-fucosidase, which preferentially cleaves α1–6-linked fucose, showed that peak 3 contained fucose (Fig. 7A, D). Digestion with a β-galactosidase specific for β1–4 galactose did not show any changes (Fig. 7A, C). 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 also found to contain other sialylated structures (peaks 5, 7, and 9) and a fucosylated structure (peak 3) that were not structurally characterized in full.

A large difference in the relative abundance of peeling products was apparent between the different sample cleanups. The highest occurrence of peeled was observed for samples that were not cleaned up (17%, peak 2, Fig. 6A and Table 4) and for samples washed with water (17%, peak 2, Fig. 6B and Table 5). For the samples washed with 0.1% TFA (5%, peak 2, Fig. 6C and Table 5) or 100 mM EDTA (4%, peak 2, Fig. 6D and Table 5), there was much less peeled product. The samples that were not cleaned up or cleaned with water also showed a significantly reduced amount of GlcNAcβ1–3(Neu5Acα2–6)GalNAc (peak 10, Fig. 6A) and GlcNAcβ1–3(Neu5Gcα2–6)GalNAc (peak 11, Fig. 5A) when compared with the samples that were cleaned up with 0.1% TFA or 100 mM EDTA.(Fig. 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 (see Supplementary Tables 1 and 2 in supplementary material). These results showed that there was no major difference in the overall yield of *O*-glycans from fetuin or BSM from either the acid- or EDTA-washed samples when compared with 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).

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 less than 20% for the fetuin samples washed with 0.1% TFA or EDTA and from 17% for the BSM sample without cleanup to less than 5% for the BSM samples washed with 0.1% TFA or EDTA (Fig. 8). Because TFA is volatile; it will be removed during the vacuum centrifugation step that is applied prior to hydrazinolysis, whereas 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 and more robust *O*-glycan profiles; therefore, they greatly improve *O*-glycan analysis employing hydrazinolysis.

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