Suppression of Peeling during the Release of O-glycans by Hydrazinolysis

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Introduction

The analysis of glycoprotein O-glycans is important in biological, clinical and biopharmaceutical research. Of the many techniques developed for release of O-glycans from glycoproteins, hydrazinolysis is one of the best for producing O-glycans with free reducing termini in high yield. However, in common with hydrazinolysis release conditions, a side reaction is observed and causes the loss of monosaccharides from the reducing terminus of the glycans (known as peeling).

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).

Methods

- Bovine fetuin samples were dissolved in water or a range of solvents; 0.1M phosphate-buffered saline (PBS); 0.1% TFA; 0.1% HCl; 0.1% H_2SO_4; 0.1% HCOOH; 0.1% CH_3COOH and 100, 50, 20 and 5mM EDTA. Bovine submaxillary gland mucin (BSM) samples were dissolved in water or a range of solvents; 0.1% TFA and 100 mM EDTA.
- Each solution of glycoprotein was transferred to a separate centrifugal filter device (10kDa MWCO membrane) and centrifuged at 4000 rpm for 10 to 12 minutes. The washing was then repeated a further five times with 5 ml of the appropriate washing solution for each sample. The remaining solution was transferred to pyrolyzed glass vials and dried down for 16h by vacuum centrifugation.
- Fetuin and BSM O-glycans were released by optimised manual hydrazinolysis and fluorescently labelled with 2-aminobenzamide (2-AB).
- A high resolution profile of the glycan pool was obtained by HILIC-HPLC using the LudgerSep-N2 column. Waters GPC software with cubic spline fit was used to allocate GU values to peaks. 2-AB-labeled glucose homopolymer was used as a system suitability standard as well as an external calibration standard for GU allocation. In order to characterise unknown mucin O-glycan structures, information from published liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses was combined with the results of exoglycosidase sequencing.

Results

- The highest degree of peeling was observed for samples that were not cleaned up. Peeling had an average relative abundance of 58% (peak 2, Figure 1a, Table 1).
- The fetuin samples that were cleaned up in water or 0.1M PBS showed a lower degree of peeling, 36% and 45% respectively (peak 2, Figure 1b and c, Table 1).
- The most pronounced reduction of peeling was apparent in the samples that were washed with 0.1%TFA. These samples showed a significantly reduced amount of peeling, 19% (peak 2, Figure

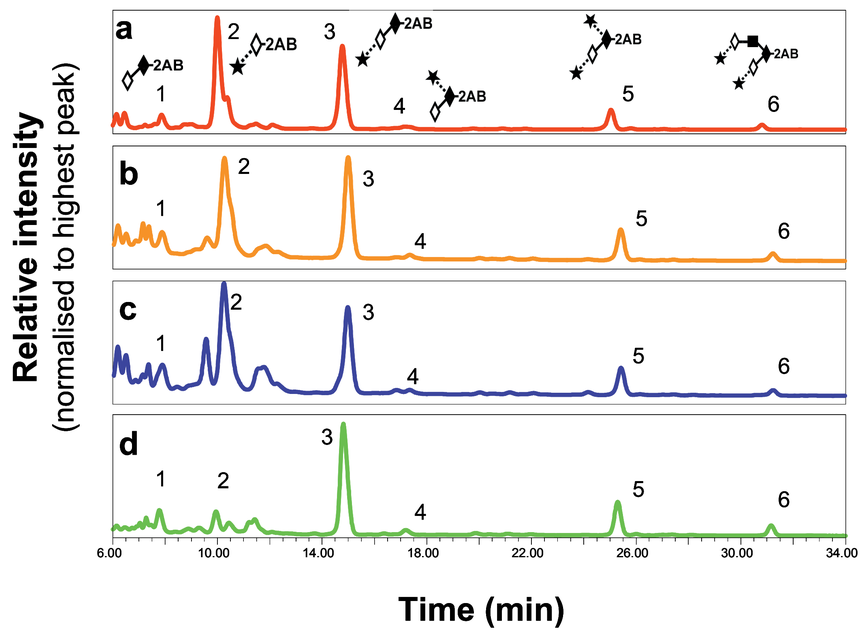
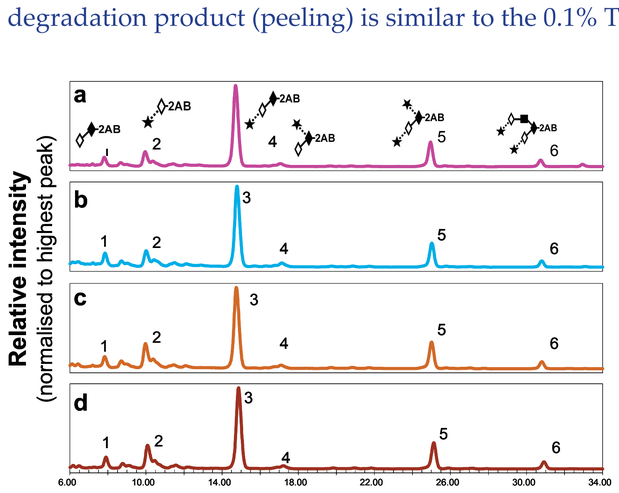


Figure 1: *Comparison of HPLC O-glycan profiles of bovine fetuin following buffer exchange prior to hydrazinolysis with a range of solutions: (a) no washing, (b), water wash, (c) 0.1 M PBS wash, (d) 100 mM EDTA wash. The O-glycans released by hydrazinolysis were 2-AB labelled and compared by HILIC-HPLC with fluorescence detection.* ***Peak 2 is the peeled product.***

- Samples that were prepared using 0.1% HCl, 0.1% H_2SO_4 and 0.1% HCOOH showed similar amounts of peeling to the 0.1% TFA wash. However, these three different acid-treated samples also showed an increase in de-sialylation (Table 1).
- There were no significant differences in the relative abundance of peeling between the samples washed with 100, 50, 20 and 5 mM EDTA solutions and that the relative intensity of the degradation product (peeling) is similar to the 0.1% TFA cleanup (Figure 2, Table 1).



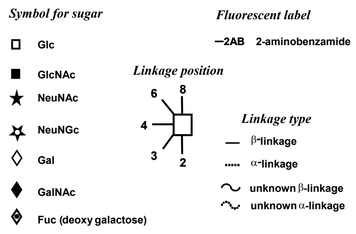


Figure 3: *Key for the glycan structure diagrams*

Figure 2: *Fetuin O-glycan profiles 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.* ***Peak 2 is the peeled product****.*

- 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, fetuin was dissolved in 100 µL of a 100 mM CaCl2 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 amount of peeling is higher and the yield of O-glycans lower for the sample that had not been buffer exchanged after addition of CaCl2 compared to the sample that had been buffer exchanged with 100 mM EDTA. (peak 2, Figure 4).

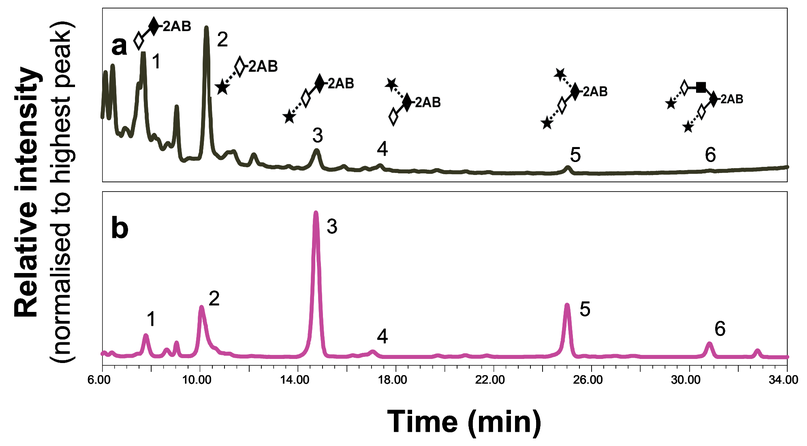


Figure 4: *Increased formation of peeling by the addition of calcium chloride may be prohibited by EDTA washes. Fetuin was dissolved in 100 mM CaCl2 and subjected to hydrazinolysis either directly (a) or after buffer exchange with 100 mM EDTA (b). Peak 2 is the peeled product*

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 1: *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).*

- Both the 0.1% TFA and 100 mM EDTA methods were also tested on bovine submaxillary gland mucin (BSM). Mucin samples were cleaned by centrifugal filtration with 0.1% TFA or 100 mM EDTA.

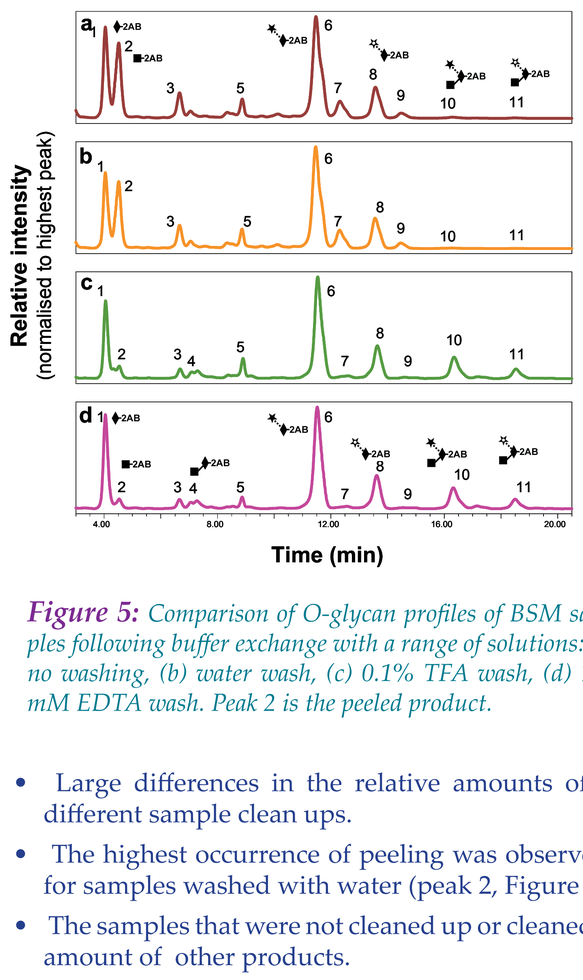


Figure 5: *Comparison of O-glycan profiles of BSM samples following buffer exchange with a range of solutions: (a) no washing, (b) water wash, (c) 0.1% TFA wash, (d) 100 mM EDTA wash. Peak 2 is the peeled product.*

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 2: *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. P-values are given in bold for samples where changes were significant (p-value ≤ 0.05).*

- Large differences in the relative amounts 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 and for samples washed with water (peak 2, Figure 5b, Table 2).
- The samples that were not cleaned up or cleaned with water also showed a significantly reduced amount of other products.

Conclusions

These studies show that:

1. **Buffer exchange into 0.1% TFA or 100 mM EDTA prior to hydrazinolysis significantly reduces the amount of undesirable peeling.**
   - » Peeling decreased from 58% for the fetuin sample without cleanup to <20% for the fetuin samples washed with 0.1% TFA or EDTA.
   - » Peeling decreased from 17% for the BSM sample without cleanup to <5% for the BSM samples washed with 0.1% TFA or EDTA.
2. **The presence of calcium chloride interferes with O-glycan release. The subsequent removal of calcium cations prior to hydrazinolysis reduces peeling.**
3. **The repeatability of these cleanup procedures was tested over a twelve month period for 0.1% TFA and over a three month period for 100 mM EDTA for the fetuin samples and over a two month period for 0.1% TFA and 100 mM EDTA for the BSM samples.**

References

1. T. Patel, J. Bruce, A. Merry, C. Bigge, M. Wormald, A. Jaques, R. Parekh (1993) Use of hydrazine to release in intact and unreduced form both N- and O-linked oligosaccharides from glycoproteins, Biochemistry **32**:679–693
2. A. H. Merry, D. C. A. Neville, L. Royle, B. Matthews, D. J. Harvey, R. A. Dwek, P. M. Rudd (2002) Recovery of Intact 2-Aminobenzamide-Labeled O-Glycans Released from Glycoproteins by Hydrazinolysis, Anal. Biochem. **304**: 91–99
3. R.P. Kozak, L. Royle, R.A. Gardner, D.L. Fernandes, M.Wuhrer (2012) Suppression of peeling during the release of O-glycans by hydrazinolysis. Anal.Biochem **423**:119-128

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