Studies on Salivary Glycosylation

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Introduction

A large proportion of the human proteome consists of glycoproteins. Many studies have shown that the glycosylation patterns of these reflect the physiological status of the body and that these patterns change with pathological state. This phenomenon is currently being exploited with new clinical diagnostics based on changes in glycosylation patterns of specific serum glycoproteins[1]. Many of these blood glycoproteins are also found in saliva which, in addition, contains several heavily glycosylated proteins secreted by the salivary glands[2]. Saliva could therefore possibly be used as an alternative to blood for diagnostics of both oral and systemic diseases.

Changes in saliva glycosylation pattern have been shown to correspond with sex, approximate age and blood group, as well as with various disease states (including cancer) of individuals [3-4]. However, protein glycosylation in saliva still remains underinvestigated. Indeed, despite many apparent advantages over blood (simplicity of collection and handling), several factors make saliva challenging for clinical diagnostics. Firstly, it is a very complex matrix containing proteins, hormones, DNA, natural and pathogenic microflora, food debris and other interfering substances. Secondly, it contains low concentrations of many possible disease biomarkers (the concentration of some target biomolecules in saliva can be three orders of magnitude lower that in the serum)[5].

In our laboratory we have developed saliva collection and processing methods in order to analyze saliva protein glycosylation, with the emphasis on O-linked glycosylation. Throughout these preliminary studies we have focused on overcoming some of the challenges of performing glycomics studies on human saliva. These include dealing with the complexity of saliva components and ways of purifying the salivary glycoproteins easily, keeping the glycosylation patterns intact. The sample preparation and chromatographic methods combined with mass spectrometric analysis enabled us to determine the structures of O-linked glycans present on salivary proteins. These structures were confirmed with exoglycosidase digestions, providing a comprehensive view of total glycosylation.

Methods and Results

1. Saliva collection and processing

Saliva samples were collected from healthy participants in the morning, before breakfast and without any stimulation. In order to obtain full and comprehensive profiles of the saliva glycans, a few different approaches to saliva processing were tested: 1. Preservation of the saliva supernatant using protease inhibitor cocktail and antimycotic solution with/without buffer exchange with 0.1% TFA solution prior to glycans release; 2. Protein precipitation with ammonium sulphate with/without buffer exchange with 0.1% TFA solution; 3. Protein precipitation with ethanol with/without buffer exchange with 0.1% TFA solution; 4. Protein precipitation with acetone with buffer exchange with 0.1% TFA solution. The last method was used as a method of choice for the detailed glycan analysis due to the high efficiency and good repeatability of results (data not shown).

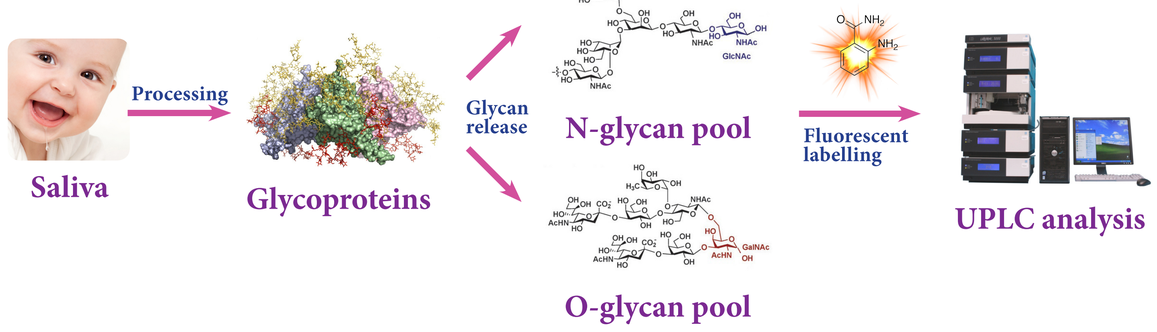


Figure 1: Workflow for saliva N- and O-glycans profiling.

2. N- and O-glycan profiling

O-glycans were released from the salivary proteins using Ludger Liberate™ ORela kit, whereas N-glycans were enzymatically released using peptide-N-glycosidase F enzyme (PNGase-F). Released glycans were separated from the proteins, then subjected to fluorescent labelling with 2-aminobenzamide (Ludger Tag™ 2-AB kit) and subsequently cleaned-up using Ludger Clean™ T1 cartridges. High resolution profiles of the saliva glycan pool were obtained by hydrophilic interaction liquid chromatography (HILIC-UPLC).

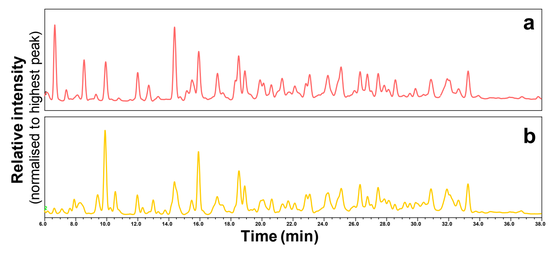


*Figure 2: Comparison of the HILIC-UPLC fluorescence profiles of the 2-AB labelled **N-glycans** released from a) male and b) female saliva.*

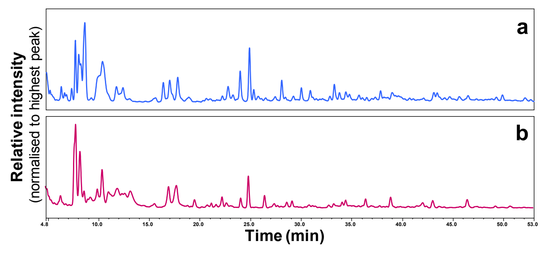


*Figure 3: Comparison of the HILIC-UPLC fluorescence profiles of the 2-AB labelled **O-glycans** released from a) male and b) female saliva.*

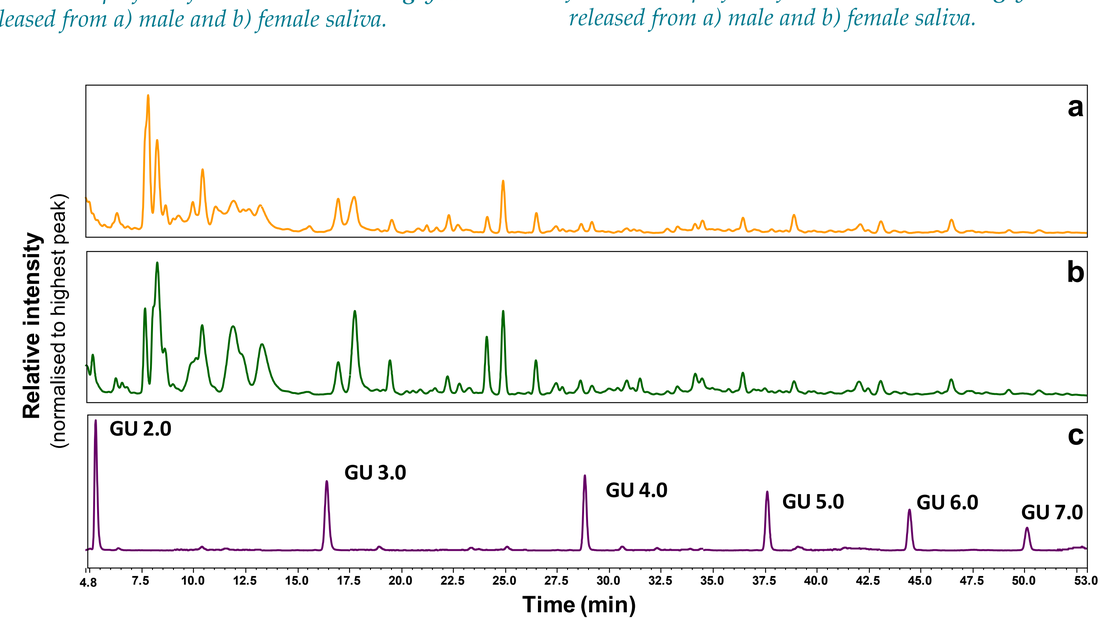


Figure 4: a) and b) Comparison of the HILIC-UPLC profiles of O-glycans released from female saliva, collected at 2 weeks of time interval; c) Glucose homopolymer (GHP) standard profile. Peaks are annotaded with corresponding numbers of glucose units.

3. O-glycans detailed structural analysis

Detailed study was performed in order to obtain O-glycan structures of salivary glycoproteins. The O-glycans were fractionated with weak anion exchange chromatography (WAX-HPLC) and HILIC-UPLC, respectively, and characterised using MALDI-TOF/TOF mass spectrometry. Obtained structures were confirmed with digestions by specific exoglycosidases. Strategy for the analysis of O-glycans is shown in Figure 5.

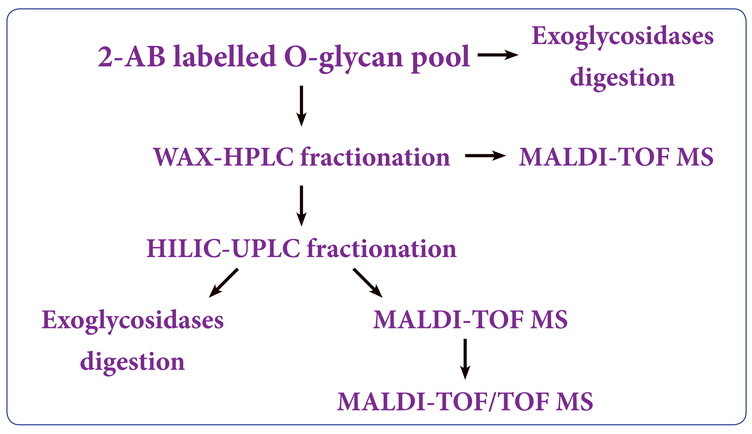
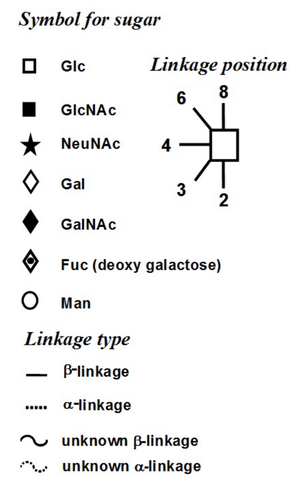


Figure 5: Strategy for the analysis of salivary O-glycans.



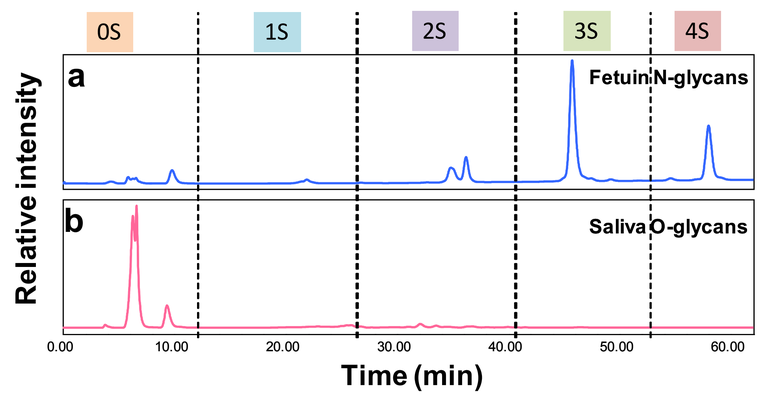


Figure 6: Weak anion exchange (WAX) profile of saliva O-glycans: a) Fetuin N-glycans used as a positive control to identify sialylated speciation; b) total O-glycan pool released from female saliva;

0S - neutral glycans; 1S - mono-sialylated glycans; 2S - di-sialylated glycans; 3S - tri-sialylated glycans; 4S - tetra-sialylated glycans.

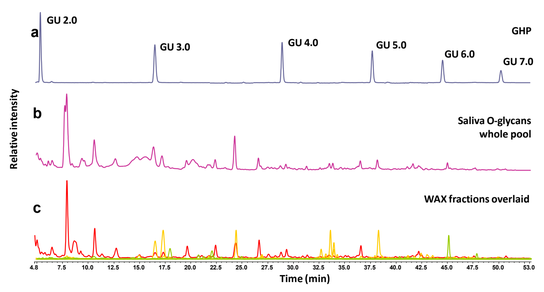


Figure 7: Summary of HILIC-UPLC profiles of saliva O-glycans: a) GHP with annotated numbers of glucose units; b) total profile of O-glycans released from female saliva; c) HILIC-UPLC overlaid profile of WAX O-glycan fractions.

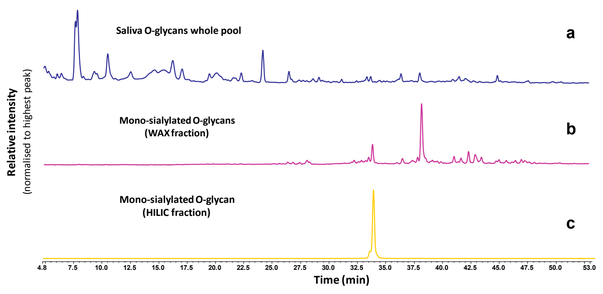


Figure 8: HILIC-UPLC analysis of O-glycans from female saliva: a) total profile of O-glycans released from saliva proteins; b) HILIC profile of WAX fraction corresponding to mono-sialylated glycans; c) HILIC separation of mono-sialylated glycan.

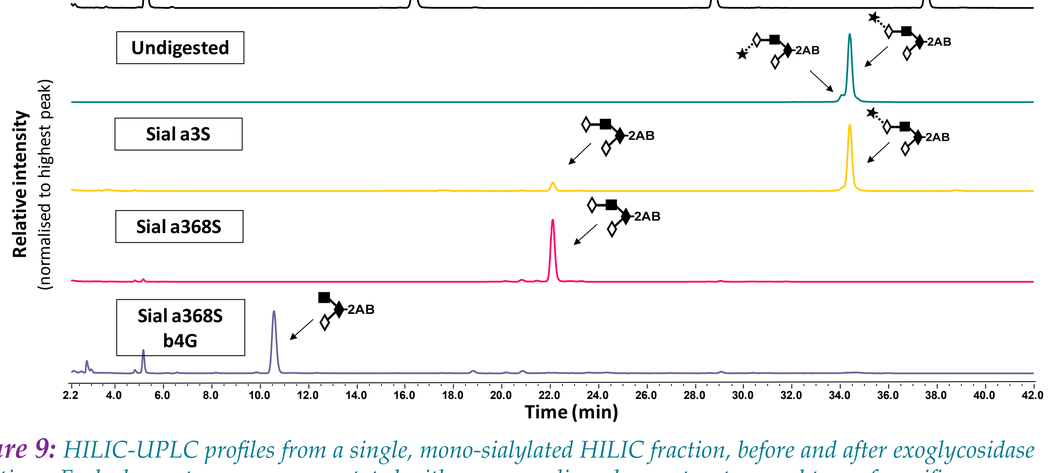


Figure 9: HILIC-UPLC profiles from a single, mono-sialylated HILIC fraction, before and after exoglycosidase digestions. Each chromatogram was annotated with corresponding glycan structure and type of specific enzyme used: Sial a3S - α(2-3)-Sialidase; Sial a368S - α(2-3,6,8)-Sialidase; b4G - β(1-4)-Galactosidase.

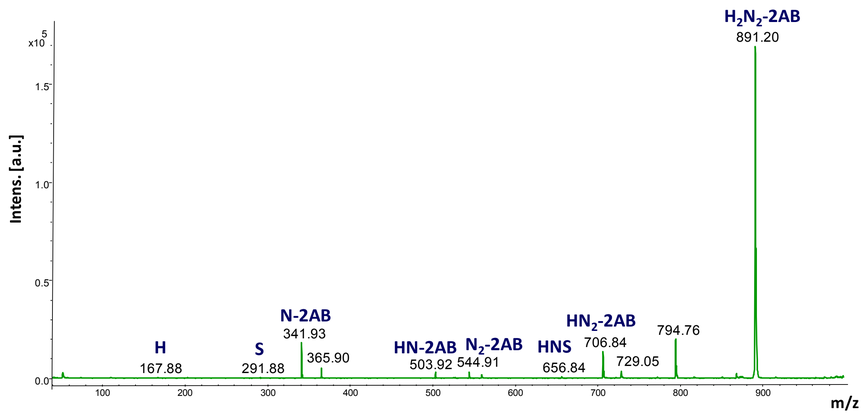


Figure 10: MS/MS spectrum of mono-sialylated O-glycans from single HILIC fraction. Recognized glycan fragments were annotated. Abbreviations: H - hexose; N - HexNAc; S - sialic acid; 2-AB - 2-aminobenzamide.

Conclusions

- **We demonstrate an affordable, easy to perform and reliable system for saliva N- and O-glycosylation profiling based on UPLC and MS analysis**
- **Our preliminary data suggests that the saliva glycosylation profiles of healthy individuals are relatively constant over several weeks and that the glycan patterns differ between individuals**
- **We now plan to expand these studies to include larger cohorts of healthy individuals and patients with chronic diseases (including inflammatory conditions and cancers).**

References

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