



A Recent Advance Making O-Glycan Preparation Flawless

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INTRODUCTION

It has been widely recognized that glycosylations of proteins are responsible for their functions in many aspects. Therefore, the glycosylation of glycoconjugates are of interest for understanding their structure-function relationships. Analysis of N-linked glycans has been facilitated by means of N-glycosidases such as PNGase F, while that of O-linked glycans is left behind due to lack of practical releasing method. Each technique to prepare free O-glycans from glycoproteins appears to possess pros and cons in terms of safety, yield, processing time, higher rate of undesired side reaction (peeling), and so on.

Here we introduce a newly developed research tool for O-glycan analysis of glycoproteins; **EZGlyco® O-Glycan Prep Kit**. The kit operation will be accomplished within 5 to 6 hours prior to data acquisition, allowing one-day O-glycan analysis. The kit utilizes totally a new combination of chemical reagents and O-glycan enrichment bead, enabling a rapid and reliable recovery of O-linked sugars from glycoproteins. The method utilized in the kit generates a minimum amount of peeling products and efficiently liberates O-linked sugars. Sugars are recovered as a reducing form so that the kit granted 2-aminobezamide labeling of the recovered carbohydrates for efficient detection with fluorescent detector equipped with LC system such as HPLC, UHPLC, and LC-MS.

We have incorporated features such as simple operation, safety of reagents, rapid process of releasing O-linked glycans, a negligible amount of peeling, effective enrichment of released oligosaccharides, and easy fluorescent labeling of purified O-linked glycans. While conventional processes take 2-3 days to complete a series of treatments, the whole procedure of the kit could be carried out only in about 5 hours.

Avoiding complicated manipulations and disadvantages of currently available methods, the well-designed kit would finally convince researchers for the choice of O-glycan preparation kit.

In this note, we present a detailed investigation of the new kit including its robustness and flawless integration in the O-glycan analysis. We believe that the **EZGlyco® O-Glycan Prep Kit** is far more practical choice than any other conventional methods in all aspect.

OVERVIEW OF THE KIT

Easy handling

- Streamlined process

Minimal decomposition

- Suppression of "peeling" during O-glycan release

High Recovery

- Comparable results to hydrazinolysis and reductive β -elimination

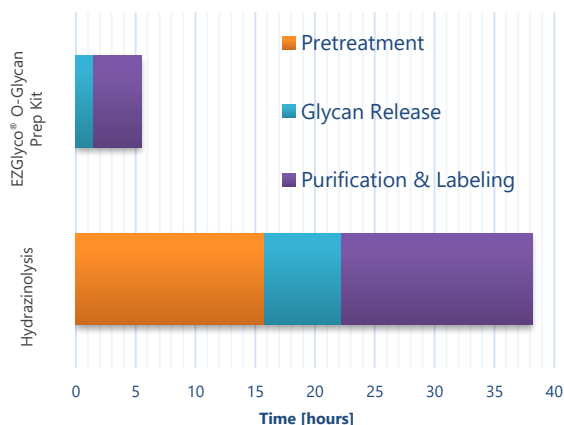
Improved safety

- No hazardous reagent handling

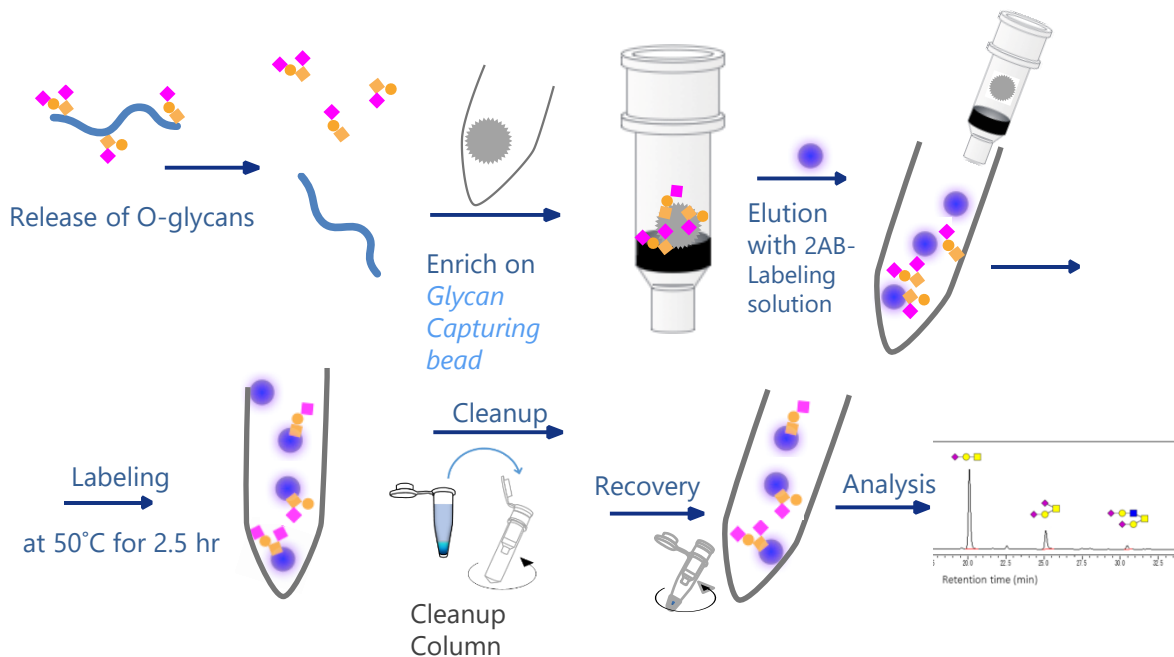
Other features include:

- The EZGlyco® O-Glycan Prep Kit utilizes novel chemical reagents for stable liberation of O-linked oligosaccharides in the form of hemiacetal reducing sugars.
- Released, small-sized O-glycans are efficiently enriched with a propriety developed Glycan Capturing Bead.

Comparison of required time



Workflow of EZGlyco® O-Glycan Prep Kit for HPLC and LC-MS-ready sample preparation in around 5 hours



Required Equipment, Labware, and Reagents

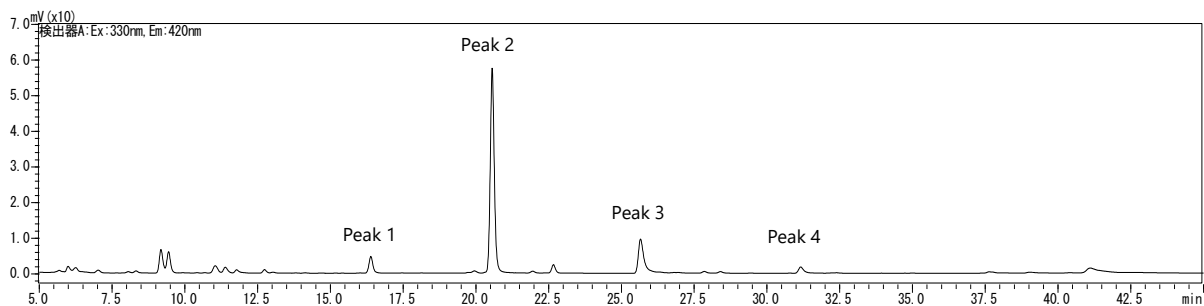
- Acetic acid (AcOH), reagent grade
- Acetonitrile (ACN), reagent grade
- Methyl alcohol (MeOH), reagent grade
- 1.5-mL microcentrifuge tubes
- Pipette and tips for 1000, 200, 20, 10, and 2 μL
- Heating block for use at 37°C and 50°C
- Vortex mixer
- Microcentrifuge (used at 500 and 3,000 $\times g$)

EXPERIMENTAL CONDITIONS

- All samples were subjected to O-glycan sample preparation using EZGlyco O-Glycan prep Kit or other commercially available kits.
- Recovered glycans were analyzed with a HILIC mode UHPLC as follows;
 - LC system: Waters ACQUITY UPLC system equipped with UV and Fluorescence detectors
 - Column: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 μm , 2.1 mm X 150 mm, Waters
 - Column Temp: 40°C
 - Flow rate: 0.2 mL/min
 - Fluorescence detection: Ex 330 nm/Em 420 nm
 - Injection volume: 1 μL out of 50 μL recovery
 - Gradient:
 - Mobile Phase A: aq. 40% acetonitrile containing 0.1% formic acid
 - Mobile Phase B: aq. 90% acetonitrile containing 0.1% formic acid

Time (min)	%A	%B
0.0	0	100
50.0	100	0

RESULTS - PRIMARY DATA -



Intra-assay variability

Peak ratio of Day1

Peak#	entry1	entry2	entry3	CV
1	6.3%	6.3%	6.3%	0.2%
2	73.1%	73.1%	73.0%	0.1%
3	17.6%	17.6%	17.6%	0.3%
4	3.1%	3.0%	3.1%	0.8%

Total peak area of Day1

	entry1	entry2	entry3	CV
Total peak area	605921	609083	606215	0.3%

Inter-assay variability

Average of peak ratio (N=3)

Peak#	Day1	Day2	Day3	CV
1	6.3%	6.2%	6.3%	0.4%
2	73.1%	73.1%	72.8%	0.3%
3	17.6%	17.5%	17.7%	0.6%
4	3.1%	3.1%	3.2%	2.4%

Average of total peak area (N=3)

	Day1	Day2	Day3	CV
Total peak area	607073	613145	710713	9.0%

Table 1. Reproducibility data of major O-glycans in bovine fetuin prepared by EZGlyco® O-Glycan Prep Kit.

Peak #1: peeling product (Hex)₁(NeuAc)₁, Peak #2: (Hex)₁(HexNAc)₁(NeuAc)₁, Peak #3: (Hex)₁(HexNAc)₁(NeuAc)₂, Peak #4: (Hex)₂(HexNAc)₂(NeuAc)₂

- 20 µg of bovine fetuin was subjected to O-glycan preparation using EZGlyco O-Glycan Prep Kit (N=3 x 3 days).
- Providing data with low CV and high reproducibility
 - glycan profile: less than 5% of CV
 - deviation in total recovery: less than 10%

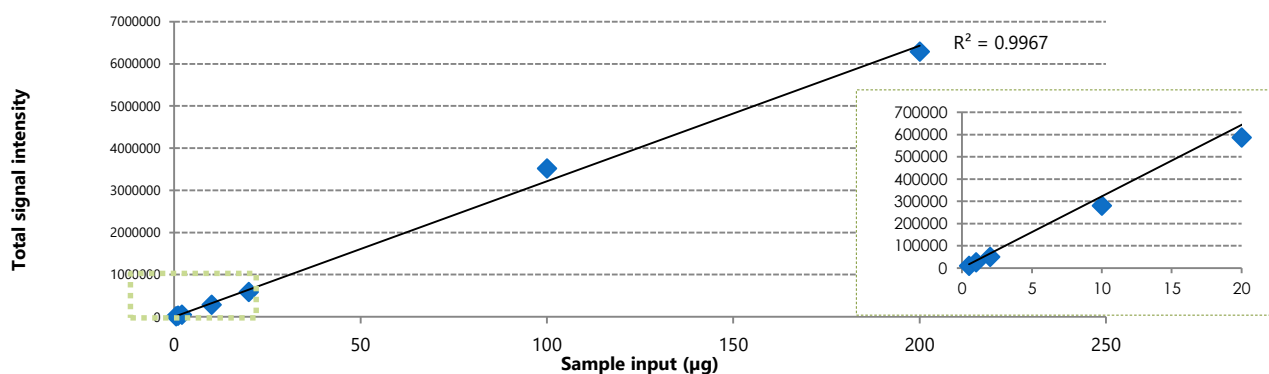


Figure 1. Linear recovery of 2-AB-labeled O-glycan in bovine fetuin.

- A varying amount of bovine fetuin (0.5 - 200 µg) was subjected to O-glycan preparation using EZGlyco O-Glycan Prep Kit (N=1).
- 1 µL of the recovered solution containing O-glycans were analyzed using a HILIC mode UHPLC.
- Recovery (intensity: sum of the area of peaks 1-4 in table 1) plotted for each input.
- EZGlyco O-Glycan Prep Kit gives a good linear recovery over a wide-range of sample input.

RESULTS - APPLICATION DATA -

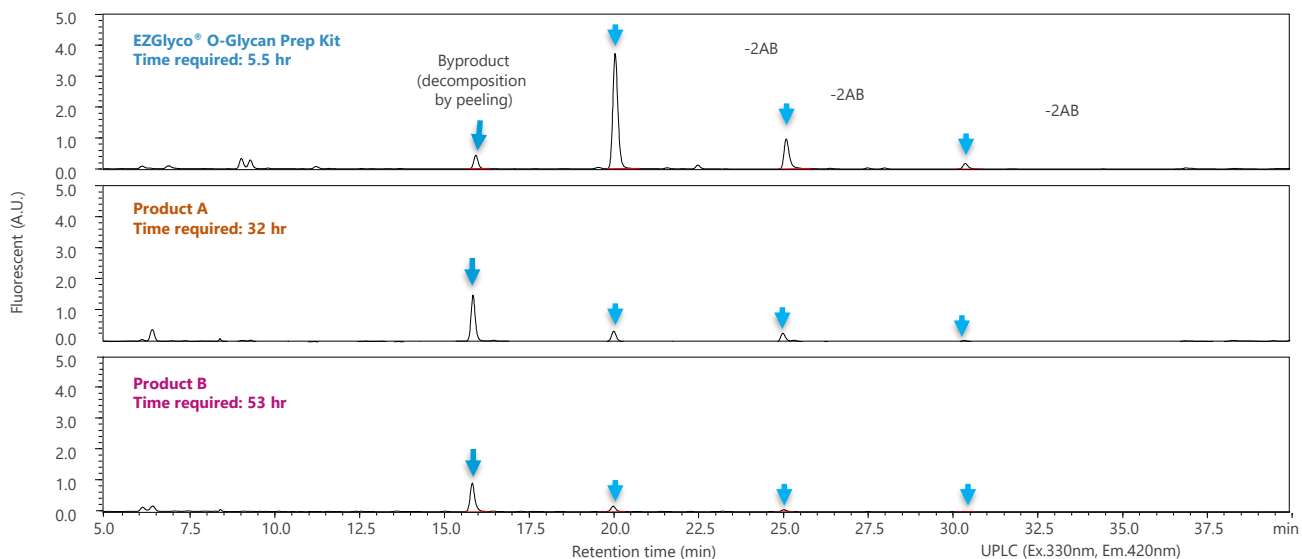


Figure 2. 2-AB-labeled bovine fetuin O-glycan analysis prepared with different sample preparation kits.

- 200 μ g of bovine fetuin was subjected to O-glycan preparation using
 - a) EZGlyco® O-Glycan Prep Kit
 - b) Product A (Alkaline β -elimination.)
 - c) Product B (Alkaline β -elimination.)
- Recovered O-glycans were analyzed using a HILIC mode UHPLC.
- As shown in the chromatograms, O-glycan total recovery and the suppression of “peeling” appear to be the best with EZGlyco® O-Glycan Prep Kit.

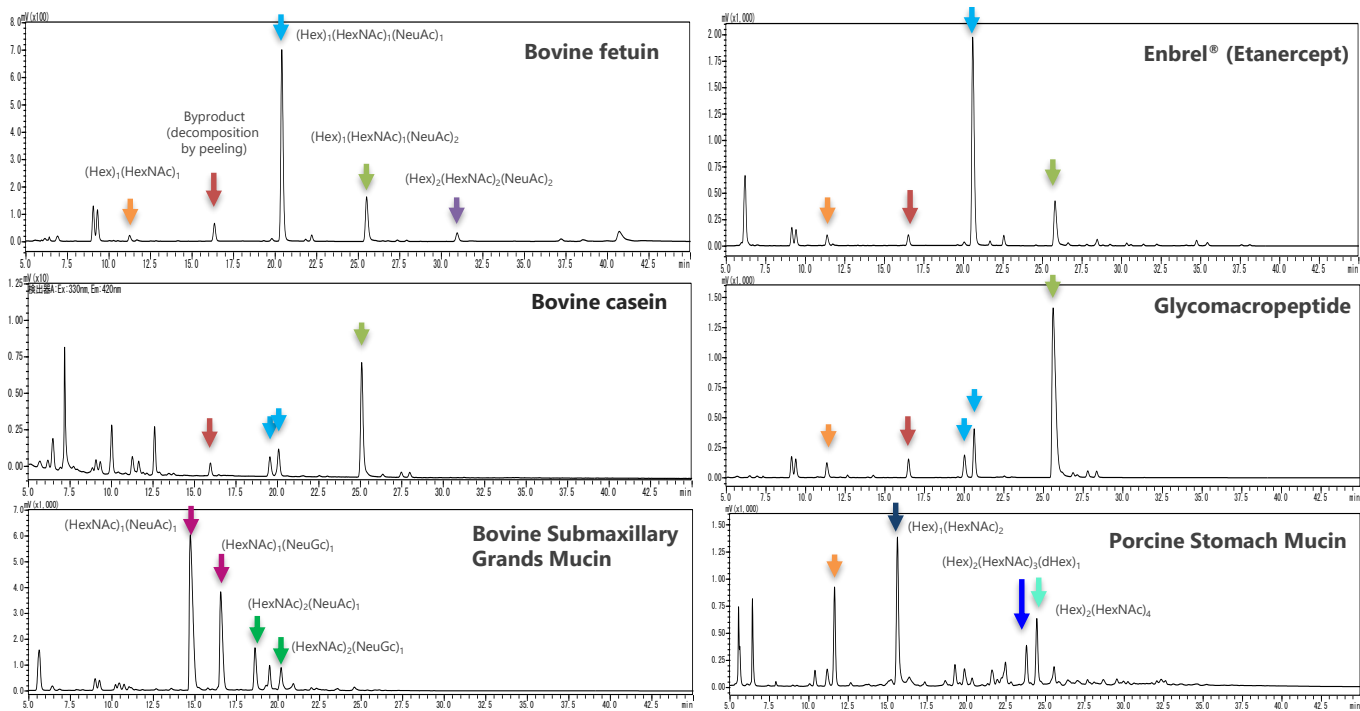


Figure 3. LC chromatograms of 2-AB-labeled O-glycans derived from various glycoproteins prepared using EZGlyco® O-Glycan Prep Kit.

- Each sample was dissolved in 10 μ L of pure water and subjected to O-glycan preparation using EZGlyco® O-Glycan Prep Kit.
- 1 μ L of the recovered solution containing O-glycans were analyzed using a HILIC mode UHPLC.
- As shown in the chromatograms, O-glycans were detected in all samples with a low peeling ratio (less than 10%).

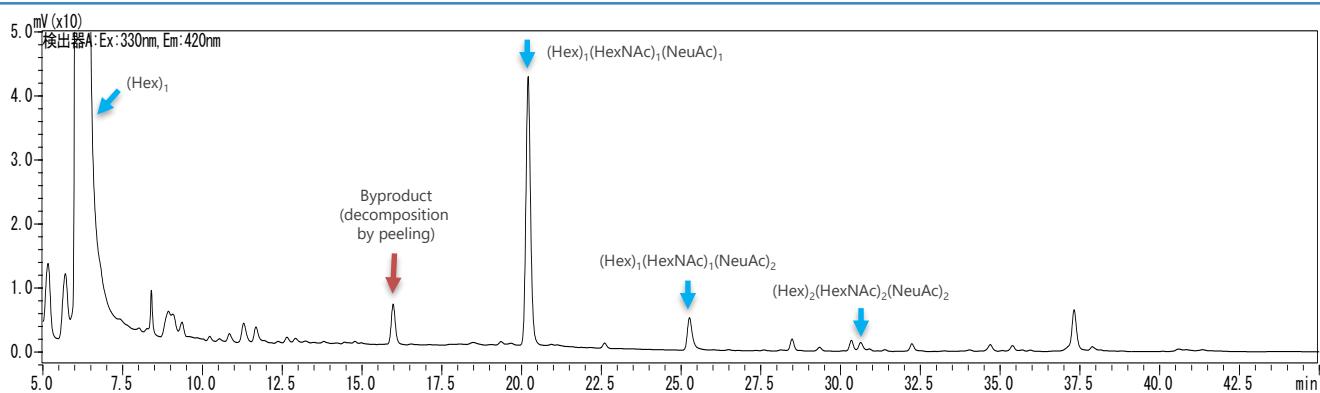


Figure 4. LC chromatograms of 2-AB-labeled O-glycans derived from human serum prepared using EZGlyco® O-Glycan Prep Kit.

- Following 20 μL of human serum was dried using centrifugal evaporator, the dried human serum was dissolved in 10 μL of pure water and subjected to O-glycan preparation using EZGlyco® O-Glycan Prep Kit.
- 1 μL of the recovered solution containing O-glycans were analyzed using a HILIC mode UHPLC.
- Even from a small amount of human serum, O-glycans were recovered with simple operation using EZGlyco® O-Glycan Prep Kit.

CONCLUSIONS

- A streamlined concept of **EZGlyco® O-Glycan Prep Kit** has been proven with a reproducible releasing and enrichment of O-glycan.
- By using the novel Kit, highly efficient O-glycan liberation has been achieved with a negligible amount of peeling from various samples including human serum.
- We believe that the novel kit has a great potential to be applied in a wide range of O-glycan studies that requires a precise preparation of oligosaccharides from target glycoproteins. Thus, we expect that the kit would be widely employed by researchers in the field of glycobiology, O-glycan-related biomarker discovery, quality control of biopharmaceuticals, and development of new diagnostics in the near future.