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Application News

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MALDI-TOF Mass Spectrometry

A Study on a Method for Evaluating Glycans in Biopharmaceuticals

- Suppressing Peeling Reactions in Pretreatment for *O*-glycan Analysis --

Many protein-based biopharmaceutical products, typified by antibody drugs, are synthesized in cultured cells derived from eukaryotes such as CHO (Chinese hamster ovary) cells. For this reason, there are inevitably many post-translational modifications to the biosynthesized proteins. Among these, modifications of glycans have gained attention as items for evaluating the quality of biopharmaceuticals since they are associated with the adjustment of protein functions, as well as with the unwanted development of antigenicity depending on their structure. However, there are various technical challenges in evaluating glycans. With *O*-linked glycans (*O*-glycan), it is particularly difficult to comprehensively release glycans from protein using enzymes, leading to the use of mainly the following two chemical methods: hydrazinolysis and β-elimination. However, these methods have issues that need to be improved. Hydrazinolysis requires great care since an explosive reagent is handled and therefore is not easy to implement. With the β-elimination method, a peeling reaction where glycans gradually decompose due to a continuous elimination reaction occurs. Conventionally, in analysis of *O*-glycans using β-elimination, glycans are released so as not to cause a continuous elimination reaction by using the reductive β-elimination method, which involves simultaneous releasing of glycans under alkaline conditions and reducing the root portion of the glycan with a reducing agent. However, this method completely reduces the root portion of the glycan and therefore does not allow labeling such as with fluorescent reagents after releasing glycans, thus limiting the available analysis methods. Also, in analysis by mass spectrometry of glycans obtained in this way, the high sensitivity analysis is not possible because the ionization efficiency of the glycan itself is not high. To address this, a non-reductive β-elimination/fluorescent labeling method is being examined as a method to bind a fluorescent labeling reagent such as 2-AB or PA by not reducing the root of the glycan, but this has not succeeded in significantly suppressing the continuous elimination reaction. Even so, in academic researches where *O*-glycan was the object of analysis, the existence of by-products due to this peeling reaction has not been problematic enough to hinder researches. However, glycans have to be evaluated for quality control in drugs which are to be administered to the human body, such as biopharmaceuticals, and the question of how to handle the existence of by-products during this evaluation is a major issue.

In this article, we report the results of studying a method for releasing *O*-glycans chemically in which the peeling reaction is suppressed, based on a PMP labeling method[*1].

S. Nakaya

One-pod-PMP Labeling with Modified Alkali Reagents

For the release of glycans by an alkali catalyst and PMP labeling of the released glycans, we used a method based on the One-Pod method of Wang et al[*2], which uses 28 % ammonia. We used ammonium carbamate as the alkali catalyst, anticipating an inhibitory effect on the peeling reaction. For the sample, we used fetuin (Fetuin from fetal bovine serum: Sigma-Aldrich F3004-25MG), and released *O*-glycans and accomplished labeling by the following method.

10 mg of fetuin was dissolved in 1 mL of water. The solution was then dispensed 10 µL each into 1.5 mL micro tubes with a screw cap, and dried by centrifugal evaporator at room temperature. After drying, the following two types of reagent solution were prepared as the glycan releasing/labeling reagent solution.

First, an "ammonia PMP labeling solution" (final concentration of PMP: 0.5 M, final concentration of ammonia: 28 %) was made by adding 200 µL of 28 % ammonia (Sigma-Aldrich) to 17.4 mg of PMP (1-Phenyl-3-methyl-5-pyrazolone, Sigma-Aldrich). Next, an "ammonium carbamate PMP labeling solution" (final concentration of PMP: 0.5 M, final concentration of ammonium carbamate: 2.5 M) was made by mixing equal amounts of the following two solutions: 34.8 mg of PMP with 200 µL of methanol added, and 390 mg of ammonium carbamate added to 1 mL of water.

50 µL of each of these solutions was added to dried fetuin and left to react for 16 hours at 50 °C, to simultaneously release glycans from the protein and accomplish the PMP labeling. The reacted solution was transferred to a glass micro tube, and after adding 500 µL of water and stirring well, it was dried by centrifugal evaporator at room temperature. A 1 % acetic acid solution (500 µL) and chloroform (500 µL) were added to the dried reaction sample, stirred strongly, then after separation into the water layer (upper layer) and the chloroform layer (lower layer) by centrifugation, the water layer was transferred into a new glass micro tube. After repeating the process of adding chloroform to the transferred water layer and performing liquid/liquid separation two times, the water layer containing the PMP-labeled glycans was collected in a 1.5 mL tube.

The collected solution was dried by centrifugal evaporator at room temperature then re-dissolved in 1 mL of water, and then injected into a C18 SPE cartridge (SupelClean LC-18 SPE Tube 1 mL, Supelco) that had been washed with 2 mL of acetonitrile and 2 mL of water. After washing with 3 mL of 3 % acetonitrile solution, the PMP-labeled glycans were eluted with 1 mL of a 30 % acetonitrile solution. The eluted solution was dried by centrifugal evaporator at room temperature, then re-dissolved in 50 µL of a 50 % acetonitrile solution and used for mass spectrometry and liquid chromatograph analysis.

MADLI-TOF MS Analysis of PMP-labeled *O*-glycan

A 0.1 % trifluoroacetic acid (TFA) solution (10 µL) was added to a PMP-labeled *O*-glycan sample (2 µL) and stirred well, then adsorbed into NuTip Carbon (glygen) that had been washed with 1 M sodium hydroxide (10 µL × 3), water (10 µL × 5), 50 % acetonitrile/0.1 % TFA solution (10 µL × 3), and 0.1 % TFA solution (10 µL × 5). After washing with 0.1 % TFA solution, the entire volume of the solution eluted with 50 % acetonitrile/0.1 % TFA solution (approx. 3 µL) was placed on the MALDI target plate. After the loaded sample solution dried, 0.5 µL of the matrix solution for MALDI was dried on top of this sample, then the result was recrystallized with 0.2 µL of ethanol and then analyzed. The matrix for MALDI was prepared by dissolving 5 mg of DHB (2,5-dihydroxybenzoic acid, Shimadzu GLC) in 50 % acetonitrile/0.05 % TFA solution (500 µL), and a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-7090, Shimadzu/Kratos) was used for the analysis.

The analysis result is shown in Fig. 1. The detected signal intensity of peeling reaction products was weaker when using ammonium carbamate with a final concentration of 2.5 M compared to when using 28 % ammonia.

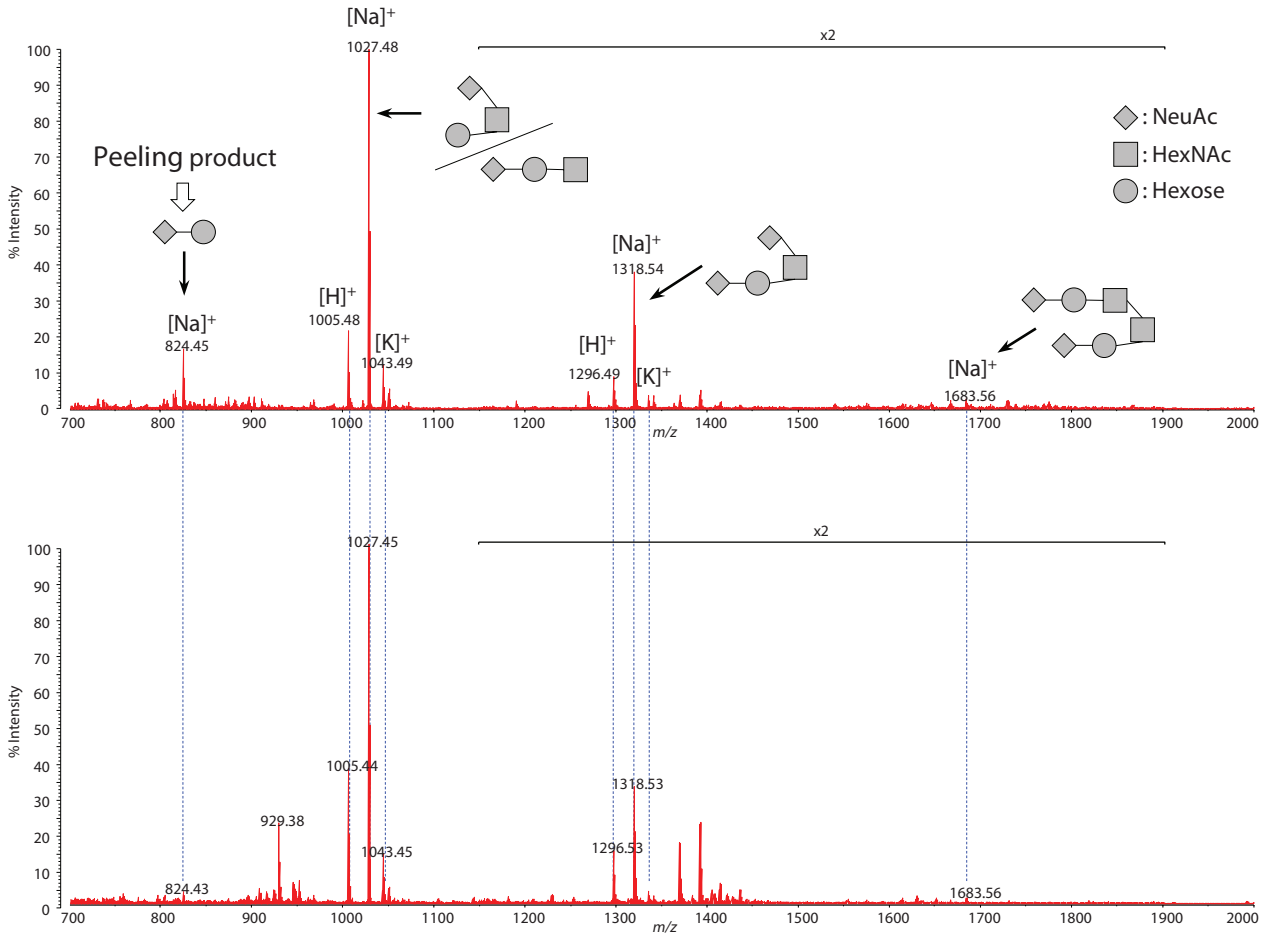

Fig. 1 MALDI-TOF Mass Spectra of the PMP-labeled *O*-glycan of Fetuin
Top: Reaction with Ammonia with a Final Concentration of 28 %,
Bottom: Reaction with Ammonium Carbamate with a Final Concentration of 2.5 M

LC Analysis of PMP-labeled *O*-glycan

The prepared PMP-labeled *O*-glycan samples were subjected to LC analysis under the conditions shown in Table 1. The samples were prepared twice each using pretreatments with different alkali catalysts.

With the sample using 28 % ammonia, 20 µL was injected and with the sample using ammonium carbamate, 40 µL was injected and then analyzed.

Table 1 LC Analysis Conditions for PMP-labeled *O*-glycan

Instrument	:	Nexera
Column	:	GlycanPac AXH-1, Analytical 1.9 µm 150 mm L 2.1 mm I.D. (Thermo Scientific)
Mobile phase A	:	100 mM Ammonium formate (pH 4.5)
Mobile phase B		Acetonitrile
Total flow rate	:	0.25 mL/min
Concentration of mobile phase B	:	0 to 15 min: 90 %, 15 to 40 min: 90 → 45 %, 40 to 45 min: 30 %, 45 to 50 min: 90 %
Column temp.	:	40 °C
Detection	:	UV absorption 245 nm (SPD-M30A)

As shown in Fig. 2, analysis results indicate that a peak derived from peeling reaction products was detected in the case of pretreatment using 28 % ammonia, whereas it was hardly detectable in the case of pretreatment using ammonium carbamate with a final concentration of 2.5 M. Table 2 summarizes the peak areas for peeling reaction products and main *O*-glycans. Peeling reaction products constituted about 12% of the total when using 28% ammonia, and about 2% when using ammonium carbamate with a final concentration of 2.5 M. This suggests that the peeling reaction was suppressed by the use of ammonium carbamate. However, it was also found that the efficiency of the *O*-glycan release/labeling reaction was lower than when using ammonia.

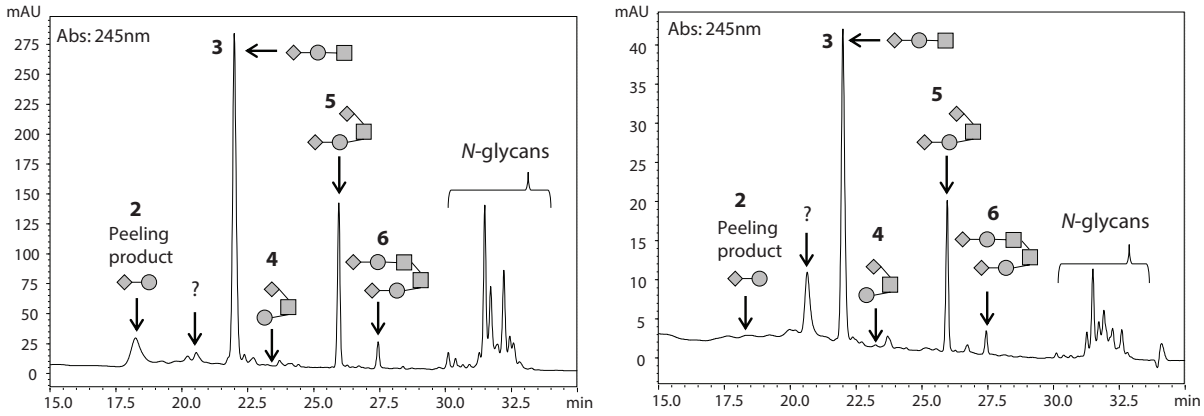

Fig. 2 Example LC Chromatograms for PMP-labeled *O*-glycans
Left: Reaction with Ammonia with a Final Concentration of 28%,
Right: Reaction with Ammonium Carbamate with a Final Concentration of 2.5 M

Table 2 LC Profiling of PMP-labeled *O*-glycans (n = 2)

28 % Ammonia

	Retention Time			Area			% Area			Height		
Peak No.	Average	SD	RSD	Average	SD	RSD	Average	SD	RSD	Average	SD	RSD
2	18.2	0.01	0.04	470403.0	174277.78	37.05	12.53	1.35	10.78	17485.5	6695.59	38.29
3	22.0	0.01	0.03	2334391.0	543258.83	23.27	63.39	2.31	3.64	239416.0	52457.42	21.91
4	23.3	0.01	0.02	2882.0	87.68	3.04	0.08	0.02	23.85	424.5	16.26	3.83
5	26.0	0.01	0.02	766212.0	241747.08	31.55	20.57	1.02	4.96	113022.0	34481.36	30.51
6	27.5	0	0.01	126936.0	32437.82	25.55	3.44	0.04	1.29	18832.0	4775.8	25.36

2.5 M Ammonium carbamate

	Retention Time			Area			% Area			Height		
Peak No.	Average	SD	RSD	Average	SD	RSD	Average	SD	RSD	Average	SD	RSD
2	18.3	0.04	0.24	13180.0	10096.07	76.6	2.21	1.48	67.07	436.5	355.67	81.48
3	22.0	0.01	0.05	401757.0	53148.97	13.23	70.18	0.29	0.41	43209.5	4999.95	11.57
4	23.2	0.00	0.01	2356.5	259.51	11.01	0.41	0.01	1.82	265.0	29.70	11.21
5	26.0	0.00	0.01	134460.0	7737.16	5.75	23.6	1.67	7.1	20017.5	979.34	4.89
6	27.4	0.01	0.03	20582.5	2150.31	10.45	3.6	0.09	2.39	3207.0	294.16	9.17

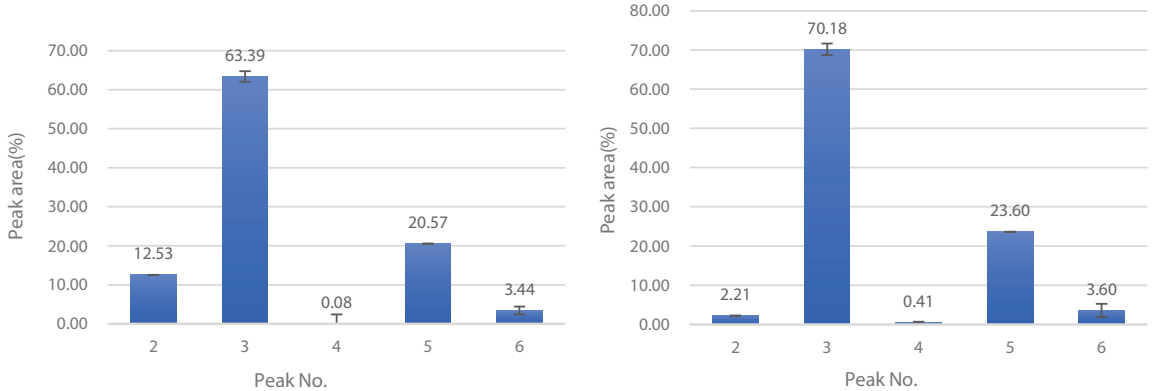

Fig. 3 LC Profiling Graphs for PMP-labeled *O*-glycans (n = 2)
Left: Reaction with Ammonia with a Final Concentration of 28%,
Right: Reaction with Ammonium Carbamate with a Final Concentration of 2.5 M

Observations

The results of this experiment confirm that the generation of peeling reaction products, which are an artefact that is problematic when attempting to perform accurate *O*-glycan profiling, can be suppressed to a few percent of the total *O*-glycans by carrying out One-pod PMP labeling using ammonium carbamate. Although there are restrictions such as the amount of samples required, this method can be used for *O*-glycan profiling.

However, the reagent and reaction conditions that were used in this study gave a glycan release efficiency and labeling efficiency that were not high when compared with other methods. The technique of simultaneously accomplishing glycan release and labeling is effective in terms of suppressing the peeling reaction. For PMP labeling, however, detection with LC is based on UV absorbance, and the detection sensitivity is low in comparison with fluorescence detection such as for PA labeling and 2-AB labeling, and there is susceptibility to background effects due to contaminant reagents. In order to eliminate these effects, it is necessary to investigate reagents with good reaction efficiency that can simultaneously perform releasing and labeling, and to conduct further studies on the reaction conditions.

References

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*2 C Wang. et al., One-pot nonreductive *O*-glycan release and labeling with 1-phenyl-3-methyl-5-pyrazolone followed by ESI-MS analysis. *Proteomics*. 2011, 11, 4229–4242

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