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# アルギニン残基修飾/除去によるMALDI-MS/MS解析における糖ペプチドのフラグメンテーションの改善

## Improving Fragmentation of Arginine-Containing Glycopeptide Through Modifying or Removing Arginine Residue in MALDI Mass Spectrometry-Based Sequence Analysis


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## 1. INTRODUCTION

Glycosylation is one of the most abundant post-translational modifications, and more than 50% of human protein is estimated to be glycosylated. Profiling glycoprotein requires the structural characterization of both glycan and peptide moieties, and the clarification of glycan modification position. Arginine (Arg)-containing peptides exhibit a low degree of structurally informative fragmentation when investigated using the commonly used collision-induced dissociation (CID) analysis. This is also true with glycopeptides. Here we present an approach for enhancing the structurally informative fragmentation of Arg-containing glycopeptides[1].

## 2. EXPERIMENTAL

### Material

High-purity MS grade 2,5-dihydroxybenzoic acid (DHB) was purchased from Shimadzu GLC (Tokyo, Japan). Human $\alpha_1$-acid glycoprotein, GluC and methylenediphosphonic acid (MDPNA) were purchased from Sigma (St. Louis, MO, USA). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI, USA). Carboxypeptidase B was purchased from Worthington Biomedical Corp. (Lakewood, CO, USA). N-Succinimidyl 4-aza-6-(2,6-dimethyl-1- piperidinyl)-5-oxohexanoate (Tandem Mass Tag, (TMT)) was purchased from Thermo Fisher Scientific (San Jose, CA, USA). PAD4 (human recombinant) was purchased from Cayman Chemical Company (Lakewood, CO, USA)

### Glycopeptide Purification

The digest solutions derived from Trypsin or GluC digestion of $\alpha_1$-acid glycoprotein were subjected to size exclusion chromatography using a 2.1mm × 220mm Superdex Peptide column (GE Healthcare, Uppsala, Sweden) and a Nexera™ high-performance liquid chromatography (HPLC) system (Shimadzu, Japan), with 2mM $NaHCO_3$ as an eluent at a flow rate of 150μl/min. Fractions were collected for 5 to 7min. Under this condition, the N-linked glycopeptides were well separated from the non-glycosylated peptides.

### Glycopeptide Derivatization

Each dried glycopeptide fraction was dissolved in 19 μl of 80% acetonitrile/water 0.4M 4-methylmorpholine. A volume of 1.5 μl of 57mM N-succinimidyl 4-aza-6-(2,6-dimethyl-1-piperidinyl)-5-oxohexanoate (in acetonitrile) was then added to the solution. The mixture was incubated at room temperature for 30min, and 50 μl of water was added to stop the reaction. After the evaporation, the amino group-derivatized glycopeptides were dissolved in 19 μl of 100mM $NaHCO_3$ solution. Carboxypeptidase B solution ( 1μl, 5.7μM) was added, and the resulting solution was incubated at 37 °C for 2 h. For citrullination, the glycopeptides were dissolved in 20 μl of 50mM Tris–HCl buffer (pH 7.4) containing 10mM $CaCl_2$ and 2 mM DTT. Five microliters of PAD4 was added to this solution and incubated at 37 ℃ for overnight.

### RP-HPLC Separation and Mass Spectrometry

The amino group-derivatized and Arg-removed glycopeptides were separated by RP chromatography on a Nexera™ system (Shimadzu, Japan). A C18 RP-HPLC column (Shim-Pack FC-ODS, 2.0mm × 150mm, Shimadzu, Japan) was used for the separation at a flow rate of 200 μl/min with a linear gradient profile of 0 to 60% solvent B over 35min. Solvent A was water containing 0.1% (v/v) acetonitrile and 0.05% (v/v) TFA, and solvent B was 80% acetonitrile containing 0.05% (v/v) TFA. The fraction was spotted onto a DHB and MDPNA[2]-precoated u-focus MALDI target plate (700 um diameter) which was purchased from Hudson Surface Technology, Inc. (Old Tappan, NJ, USA). The mass spectra were acquired using an AXIMA Performance™ mass spectrometer and MS/MS spectra were obtained using an AXIMA Resonance™ UV-MALDI mass spectrometer (Shimadzu/Kratos, UK).

### Peak Processing

The peak lists for the database search were generated from MS spectra using Mass++ (version 2.3.0). The peaks in the MS spectrum were detected using the advanced peak-picking function MWD in Mass++. The neutral-loss peaks were then removed by the peak-filtering function that was originally developed in Mass++. Mass++ can be freely downloaded from the website http://www.first-ms3d.jp/english/.

### MS/MS Ion Search

MS/MS ion searches were performed by Mascot (version 2.4.0, Matrix Science, UK) on a local server with some customization. SwissProt (released January 11, 2011) was used as the database, and the taxonomy was set to Homo sapiens (human). Trypsin, Trypsin + CarboxypeptidaseB-R (customized), or None was selected as the enzyme, with no potential cleavage. The mass tolerances of peptide and fragment ion were 0.3 Da. Fixed modifications were carboxamidomethylation of cysteine (+57 Da), citrullination of Arg (+1 Da, customized) and lysine TMT labeling (+224 Da). The variable modifications were HexNAc (+203 Da), N-terminal TMT labeling (+224 Da) and N-terminal glutamine deamination (-17 Da). MALDI-QIT-TOF was selected as the instrument type.

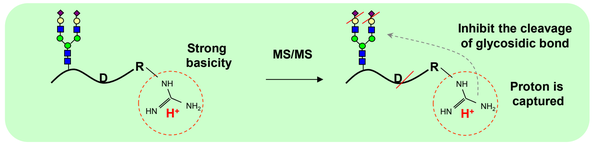
## References


[1] K.Taniguchi, H.Kuyama, S.Kajihara, K.Tanaka, *J. Mass Spectrom.*, 2013, *48*, 951–960.

[2] H.Kuyama, K.Sonomura, O,Nishimura, *Rapid Commun. Mass Spectrom.*, 2008, *22*, 1109–1116.


## 3. RESULTS and DISCUSSIONS

### Less informative fragmentation




### Solution

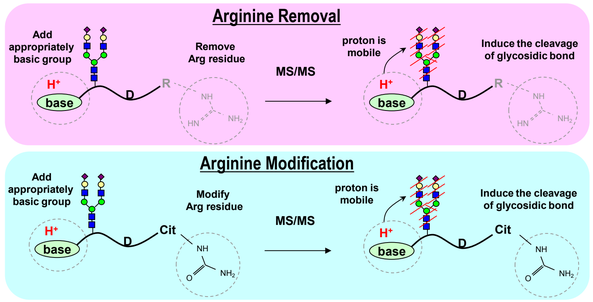



**Fig.1** Deteriorated fragmentation of Arg-containing glycopeptide and its potential solution

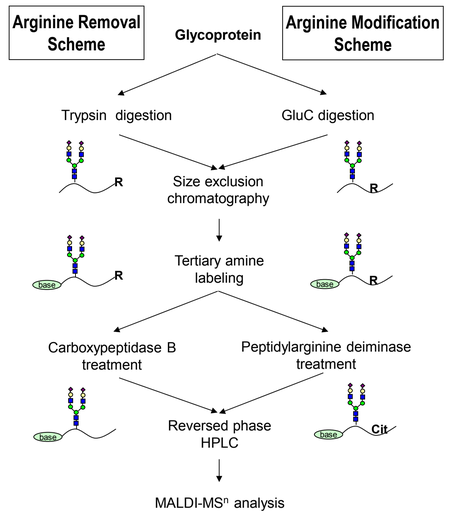



**Fig.2** Schematic depiction of enzymatic removal or modification of Arg residue.

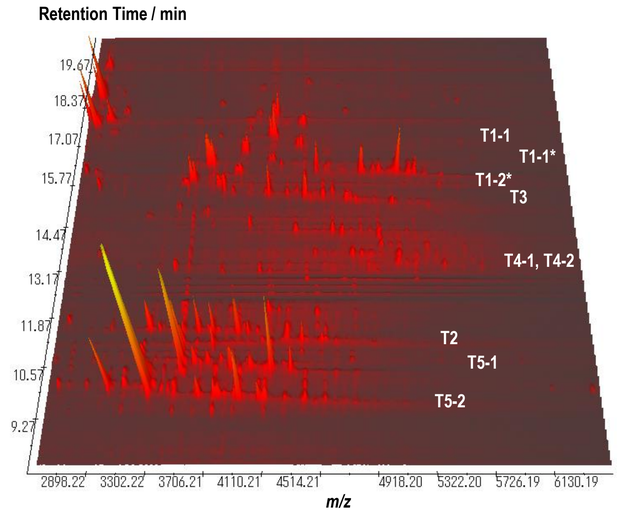



**Fig.3** LC-MALDI-MS spectrum of derivatized glycopeptides derived from trypsin digestion of human $\alpha_1$ acid glycoprotein using Mass++.

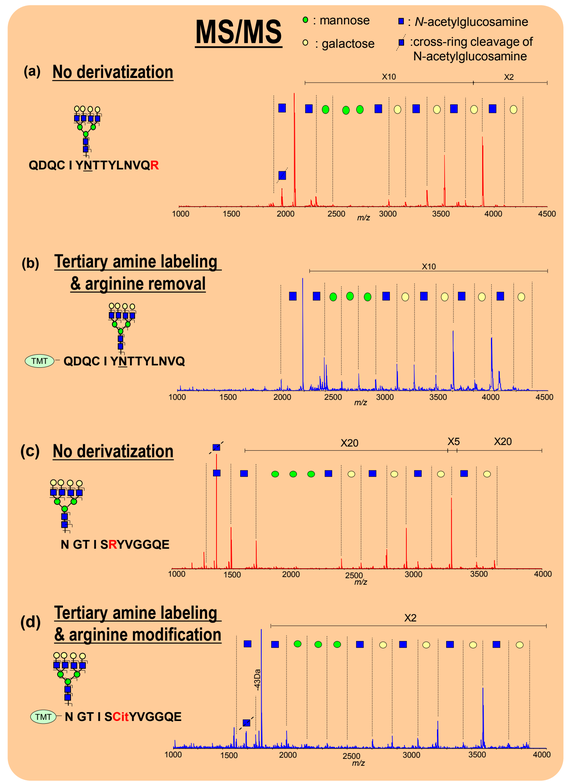



**Fig.4** Glycan sequencing of T4-1 and G5-1. The effect of Arg removal or modification and tertiary amine labeling on MS/MS fragmentation is presented. MS/MS spectrum of (a) intact T4-1, (b) labeled and Arg-removed T4-1, (c) intact G5-1 and (d) labeled and Arg-modified G5-1.

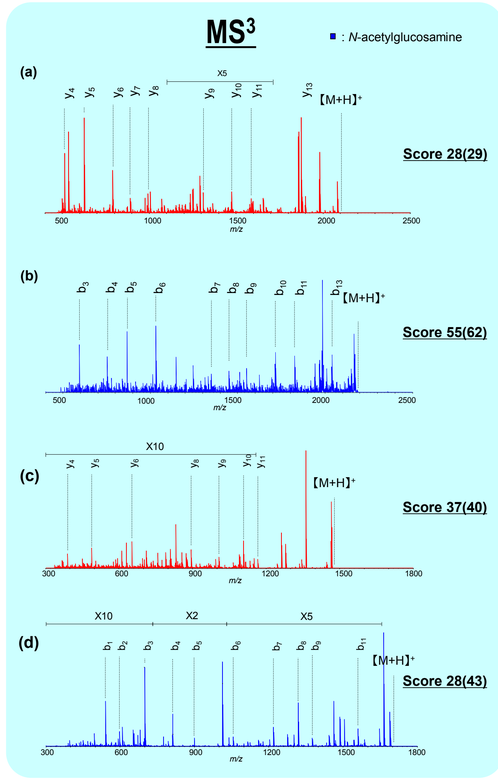



**Fig.5** Peptide sequencing of T4-1 and G5-1. The effect of Arg removal or modification and tertiary amine labeling on $MS^3$ fragmentation is presented. $MS^3$ spectrum of (a) intact T4-1, (b)labeled and Arg-removed T4-1, (c) intact G5-1 and (d) labeled and Arg-modified T4-1. The score in parentheses indicates the value obtained after neutral-loss filtering of 203Da peaks.

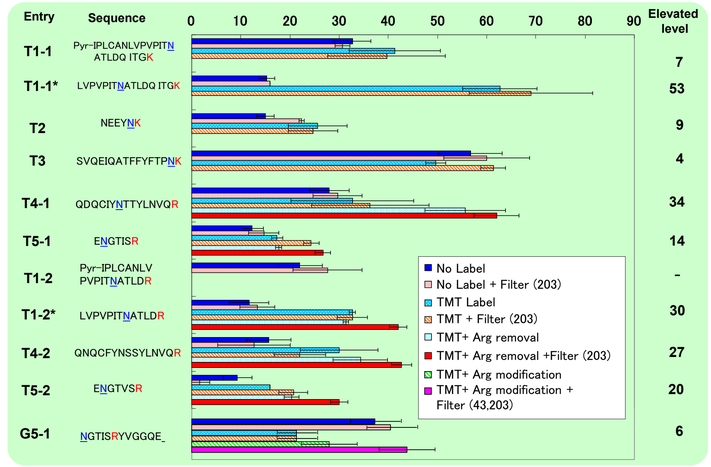



**Fig.6** Mascot score comparison of glycopeptides from trypsin or GluC digest of human $\alpha_1$-acid glycoprotein. The entries from T4-1 to G5-1 are Arg-containing glycopeptides; entries from T1-1 to T3 are glycopeptides containing lysine instead of Arg residue. The Mascot scores are the average of three experiments. Error bars represent standard deviation of the mean. Elevated levels were calculated as: "Score (TMT + Arg removal/modification + Filter (203)) - Score (No Label)" in Arg-containing glycopeptides and "Score (TMT + Filter (203)) - Score (No Label)" in lysine-containing glycopeptides.

## 4.CONCLUSIONS

Removal or modification of the Arg residue, tertiary amine labeling and neutral-loss filtering are useful for analyzing glycan structures, identifying glycoproteins and determining N-glycan binding positions.

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