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Quantitative analysis of *O*-GlcNAcylation in combination with isobaric tag labeling and chemoenzymatic enrichment

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ABSTRACT

Protein *O*-GlcNAcylation regulates various biological processes, and is associated with several diseases. Therefore, the development of quantitative proteomics is important for understanding the mechanisms of *O*-GlcNAc-related diseases. We previously reported selective enrichment of *O*-GlcNAcylated peptides, which provided high-selectivity and effective release by a novel thiol-alkyne and thiol-disulfide exchange. Here, we describe a new approach using initial isobaric tag labeling for relative quantification followed by enrichment and β-elimination/Michael addition with dithiothreitol for identification of both proteins and modification sites. The approach was validated using model proteins and peptides. This novel strategy could be used for quantitative *O*-GlcNAcome of biological samples.

O-Linked β-*N*-acetylglucosamine (*O*-GlcNAc) modification is a post-translational modification that occurs on serine (Ser) and threonine (Thr) residues of cytosolic, nuclear, and mitochondrial proteins. Like phosphorylation, this modification is reversible under the control of *O*-GlcNAc transferase and *O*-GlcNAcase, which catalyze the addition and removal of the GlcNAc moiety, respectively. Alterations of the *O*-GlcNAc modification are associated with several diseases, such as diabetes,[1] Alzheimer's disease,[2] and cancer.[3] Therefore, qualitative and quantitative analyses of *O*-GlcNAcylated proteins are important for the development of biomarkers and for understanding the pathological mechanisms underlying various *O*-GlcNAc-related diseases.

Combining an enrichment method for *O*-GlcNAcylated proteins/peptides with mass spectrometry (MS) offers a powerful approach for the efficient identification of *O*-GlcNAcylated proteins. Various enrichment methods have been developed using an anti-*O*-GlcNAc antibody,[4] wheat germ agglutinin lectin,[5] chemical derivatization,[6–8] and chemoenzymatic labeling (using mutant galactosyltransferase 1, Gal-T1 (Y289L)).[9,10] For MS-based relative quantitation, incorporation of stable isotopic tags, such as isobaric tags for relative and absolute quantitation (iTRAQ),[9] tandem mass tag,[8] and dimethyl labeling,[10] are available. Therefore, several enrichment methods coupled with isotopic labeling have been reported for relative quantitation of *O*-GlcNAcylation.[6–10]

As a method of incorporation of stable isotopic labeling, β-elimination/Michael addition (BEMA), a chemical derivatization method using nucleophilic reagents, such as isotope-labeled dithiothreitol (DTT)[6] and biotin-cystamine,[7] has been readily coupled with thiol- and avidin-affinity enrichment. BEMA involves alkaline-induced β-elimination of the *O*-GlcNAc moiety from Ser/Thr to give an α,β-unsaturated carbonyl and subsequent Michael addition with nucleophilic reagents. Consequently, the resulting derivatives through BEMA are stable during collision-induced dissociation (CID)-MS/MS analysis. Conversely, the glycosidic linkage between Ser/Thr and the GlcNAc moiety is highly susceptible to collisional activation and cleaved easily in the CID-MS/MS analysis, which hampers identification of glycosylated peptides and the associated glycosylation sites. Therefore, BEMA may be useful for detecting *O*-GlcNAcylated proteins and modification sites, as well as the incorporation of isotopic labeling. Since the mechanism of β-elimination is not dependent on the chemical structure of the modified Ser/Thr, BEMA should work on modified peptides through *O*-GlcNAc enrichment. Recently, Ramirez-Correa et al. performed β-elimination/Michael addition with DTT (BEMAD) and thiol-affinity enrichment combined with tandem mass tag labeling before enrichment.[8] However, since BEMA also occurs on other modified residues, such as phosphorylated Ser/Thr,[11] alkylated cysteine,[11] and *O*-linked glycans on Ser/Thr,[12] there is a possibility of false positive identifications besides *O*-GlcNAcylation. Therefore, a combination of BEMA and other enrichment methods, such as chemoenzymatic labeling/copper(I)-catalyzed alkyne-azide

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cycloaddition (CuAAC) reaction, may improve enrichment specificity.

Wang et al. reported enrichment of *O*-GlcNAcylated peptides using a combination of chemoenzymatic labeling/CuAAC reaction and BEMA with d_0/d_6-DTT.[9] Moreover, they performed relative quantitation of total protein levels by iTRAQ labeling of the flow-through (FT) of biotin-avidin affinity enrichment. This approach could determine the relative quantitation of both *O*-GlcNAcylated proteins from the ratio of d_0/d_6-DTT-labeling and total proteins from that of iTRAQ-labeling. Furthermore, it is useful to calculate relative occupancy ratios (RORs) of *O*-GlcNAcylation. However, in their approach, since the DTT-labeling procedure was the last step after enrichment of *O*-GlcNAcylated peptides, the relative quantitation of *O*-GlcNAcylated proteins was most likely affected by the efficiency and recovery of all procedures prior to DTT-labeling. Moreover, additional iTRAQ-labeling of the FT was required for the relative quantitation of total proteins.

Here, we report a new approach using initial iTRAQ-labeling and subsequent *O*-GlcNAcylated peptide enrichment and BEMAD. The advantages of our methods are as follows: (1) the relative quantitation of *O*-GlcNAcylated proteins is unaffected by the efficiency and recovery of the enrichment procedures, because iTRAQ-labeled samples are combined into one tube before enrichment and BEMAD; (2) the false positive identification is minimized by using chemoenzymatic labeling/CuAAC reaction; (3) the specific detection of *O*-GlcNAcylated peptides is available using neutral loss (NL) of the *O*-GlcNAc moiety from thiol-alkyne-labeled peptides; and (4) additional isotopic labeling of the FT is not required for the quantitation of total proteins.

Fig. 1A and B show the workflow and chemically modified structure of the *O*-GlcNAc moiety, respectively. Protein samples were reduced, alkylated, and digested with trypsin. In our enrichment, the cysteine must be completely blocked during the alkylation step because peptides containing cysteine are enriched by thiol-disulfide exchange. After labeling with each iTRAQ reagent (114, 115, 116, or 117), the iTRAQ-labeled samples were combined into one tube and subjected to enrichment of *O*-GlcNAcylated peptides using a highly specific method based on chemoenzymatic labeling, the CuAAC reaction, and thiol-disulfide exchange.[13] The eluate containing thiol-alkyne-labeled peptides was split into two samples. One was directly analyzed by liquid chromatography (LC)-MS/MS for the detection of *O*-GlcNAcylated peptides using the unique NL of 652 and 449 Da derived from thiol-alkyne-labeled *O*-GlcNAc moieties (product I in Fig. 1A). The other was subjected to BEMAD for the identification of proteins and modification sites, and the relative quantitation of *O*-GlcNAcylation (product II in Fig. 1A). Moreover, the FT of thiol-disulfide exchange was also analyzed for the relative quantitation of the total protein expression (product III in Fig. 1A).

The advantage of chemoenzymatic labeling using azide-labeled uridine 5′-diphospho -*N*-acetylgalactosamine (UDP-GalNAz) and Gal-T1 (Y289L) is that it is highly specific for the GlcNAc moiety. However, because *N*-hydroxysuccinimide esters, such as iTRAQ reagents, can also react with hydroxyl groups in addition to the primary amino group, there is a possibility that iTRAQ reagents directly react with the *O*-GlcNAc moiety and disturb the reaction of Gal-T1 (Y289L), the transfer of GalNAz onto the GlcNAc moiety. Moreover, it is unclear whether BEMAD proceeds on the thiol-alkyne-labeled *O*-GlcNAc moiety. Thus, the objectives of this study were to examine the following points: (1) iTRAQ labeling does not disturb *O*-GlcNAc enrichment; (2) DTT-labeled peptides are obtained from thiol-alkyne-labeled peptides by BEMAD; and (3) *O*-GlcNAcylated peptides can be explored by searching for the NL of thiol-alkyne peptides.

In this study, bovine α-crystallin (CRYA) was used as the *O*-GlcNAcylated protein standard. CRYA has two *O*-GlcNAc sites at Ser162 of the A chain (CRYAA) and Thr170 of the B chain (CRYAB). For the *O*-GlcNAcylated peptide standard, the Click-iT™ *O*-GlcNAc Peptide LC/MS Standard (Invitrogen, TAPTSTIAPG, $[M+H]^+$ = 1118.5), which has an *O*-GlcNAc modification at the Ser residue, was used. The sequence is present in the cyclic AMP-responsive element-binding protein.

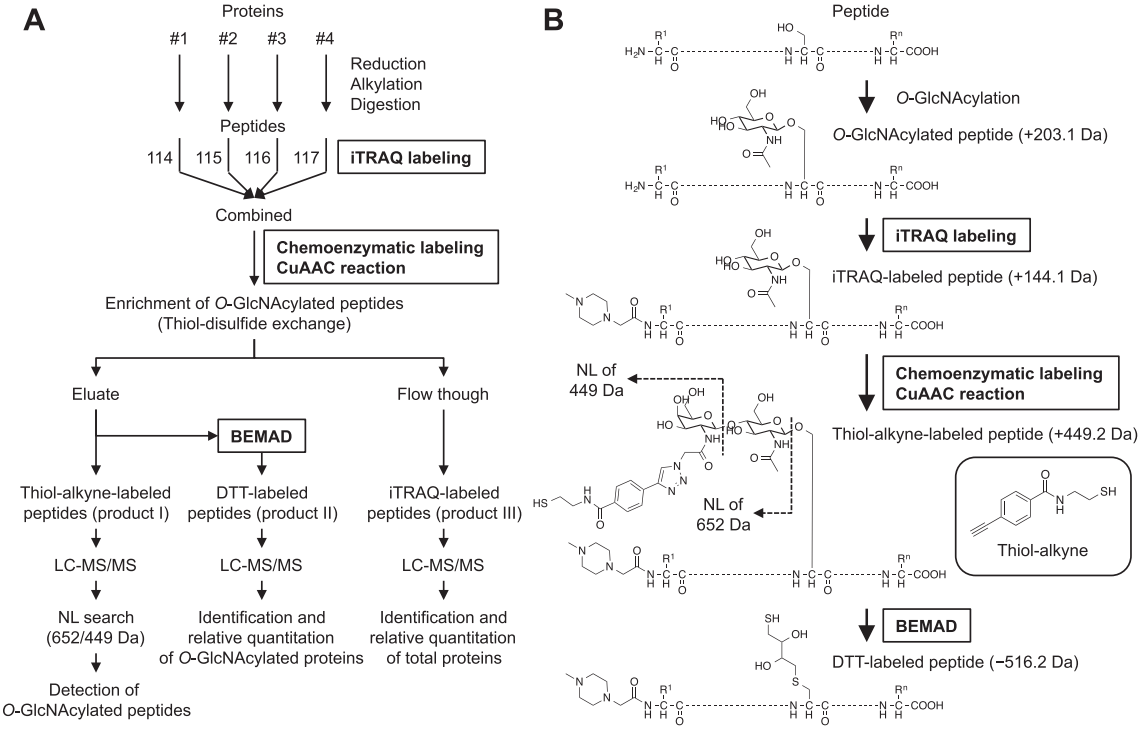

Fig. 1. Quantitative analysis of *O*-GlcNAcylation in combination with iTRAQ labeling, chemoenzymatic labeling/CuAAC reaction, and BEMAD. (A) Flow chart of the overall strategy. (B) Chemical structure and mass change of *O*-GlcNAcylated, iTRAQ-labeled, thiol-alkyne-labeled, and DTT-labeled peptides.