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Determination of *N*-glycans in glycoproteins using chemoenzymatic labeling with Endo-M N175Q

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ABSTRACT

A novel approach for the determination of *N*-glycans in glycoproteins has been developed based on a combination of deglycosylation, chemoenzymatic labeling, and high-performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS). Glycoproteins were digested with PNGase F and then the released *N*-glycans were enriched and separated from the digesting solution using ultrafiltration units according to the molecular mass differences. The *N*-glycans were analyzed by HPLC/ESI-MS after chemoenzymatic labeling with Endo-M N175Q an Endo-M mutant. We have shown that the Endo-M N175Q demonstrates a noticeable transglycosidase-like activity for natural *N*-glycans. The productivity of Endo-M N175Q is estimated to be 2.0-fold higher than that of Endo-M. The experimental strategy was validated using glycoproteins with known glycan structures such as ovalbumin and ribonuclease B. The proposed method offers a promising advance for determining *N*-glycans in glycoproteins.

1. Introduction

Glycosylation is one of the most common post-translational modifications in proteins. It has been estimated that over 50% of human proteins are glycosylated [1]. Glycans play a vital role in protein conformation, activity, and localization, which occur in many biological processes, and many diseases related to anomalous intercellular glycans have been reported, for example, cancer and rheumatism [2–4]. Therefore, the development and establishment of determination methods of glycans are expected to result in great progress in biological and pathological studies. The most convenient approach to determine the structures of the glycoprotein glycans present in a given protein preparation regardless of their position on the protein backbone(s) requires their release by enzymes or by chemical procedures [4], and the resulting glycans can then be analyzed. Much research has already been carried out for the resolution of carbohydrate structures. Although the most reliable method seems to be by nuclear magnetic resonance (NMR) spectroscopy [5], the main drawback of the method, however, is that it requires a large amount of pure carbohydrate prior to the measurement. High-performance liquid chromatography (HPLC) has been suggested as an alternative mean for analysing carbohydrate chains [6]. Because HPLC itself does not provide an information about the structures, the elucidation of the structures is usually performed by comparison with reference carbohydrates whose structures have been established by other means.

Generally, carbohydrates do not have effective functionalities of absorption and fluorescence for sensitive detection. Furthermore, the available amounts in most analyses are minute, and hence, the direct resolution of carbohydrate chains in real samples is very difficult to achieve. The analysis by HPLC has usually been carried out after derivatization with a suitable compound possessing a sensitive function. Various types of tags including 2-(and 3)-aminobenzoic acid (AA) [7,8], 2-(and 3)-aminobenzamides (AB) [7–9], 8-amino-1,3,6-naphthalenetrisulfonic acid (ANTS) [10], and 2-aminopyridine (AP) [11] have been introduced for the sensitive detection of carbohydrates using either fluorescence or UV absorbance. Among them, the 2-aminopyridine (AP) method has been accepted as a standard method for the resolution of glycan sequences although it requires rather drastic conditions with a strong acid catalyst and a long reaction time at high temperature. The procedure can permit the release of labile moieties such as sialic acid and sulfate group [12]. In spite of such limitations, the AP method is still used for the resolution of carbohydrate chains because no other/simpler reliable method has been reported yet.

Enzymatic labeling is one of the most promising approaches for the glycosylation analysis due to its simplicity: labeling occurs during enzymatic digestion, and all that is required is avoiding extra reagents, additional steps, and side reactions. Endo-M, an *endo*-β-*N*-acetylglucosaminidase from *Mucor hiemalis*, is a family

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of 85 glycoside hydrolases. This enzyme is unique in that it can transfer en bloc the oligosaccharide of various types of *N*-glycans onto various acceptors having an *N*-acetylglucosamine (GlcNAc) residue, and thereby it enzymatically generates diverse glycoconjugates [13,14]. The transglycosylation activity seems to be useful for the determination of the glycans in the glycopeptides, because glycan derivatives are easily obtained from the glycopeptides by the enzyme reaction with Endo-M in the presence of an acceptor having asparaginyl-*N*-acetyl-D-glucosamine (Asn-GlcNAc) residue. According to this strategy, several fluorescent-Asn-GlcNAcs and permanently positively charged Boc-Asn- GlcNAcs were synthesized based on the labeling of the amino (—NH_2) and carboxyl functional groups (—COOH) in the Asn residue. The transglycosylation efficiency of these acceptors was studied in the previous investigations [15,16]. However, there is a bottleneck for its practical application because the transglycosylation reaction competes with the hydrolysis reaction. To overcome this limitation of Endo-M, Umekawa et al. [17] performed mutational studies focusing on a key catalytic asparagine 175 of Endo-M. They have shown that most of the Asn-175 mutants had significantly diminished hydrolysis activity but acted as glycosynthases capability using the synthetic sugar oxazoline for transglycosylation. Among the mutants of Endo-M, the Endo-M N175Q mutant was found to possess significantly enhanced transglycosylation activity for activated sugar oxazoline, whereas its hydrolysis activity for the product was diminished. Surprisingly, this mutant was also capable of efficiently transglycosylation of *N*-glycans with a significantly diminished hydrolysis activity, behaving as a typical "transglycosidase". The highly efficient syntheses of glycopeptides/glycoproteins by Endo-M N175Q combined with synthetic sugar oxazolines or natural *N*-glycan substrates were exemplified. However, this novel enzymatic method utilizing Endo-M N175Q have never been implemented in glycan analysis.

In this report, we described the enzyme labeling of *N*-glycans from glycanpeptide/glycanprotein using the transglycosylation activity of Endo-M N175Q for the first time and their resolution by HPLC/ESI-MS. The practical analysis of the *N*-glycans in glycoproteins (ovalbumin and ribonuclease B) was also attempted and evaluated the analytical performance by the proposed HPLC-MS method.

2. Experimental

2.1. Reagents and materials

Endo-β-*N*-acetylglucosaminidase (NEGase) from *Mucor hiemalis* (Endo-M), Endo-M-N175Q (a mutant derived from Endo-M) and biantennary complex-type sialylglycopetide (SGP) were purchased from Tokyo Chemical industry Co. (Japan). PDPZ-Boc-Asn-GlcNAc was synthesized according to a previous paper [16]. Ribonuclease B (bovine pancreas) was purchased from Sigma (St. Louis, MO, USA), and peptide-*N*-glycosidase F (PNGase F) was from New England Biolabs Inc. Other chemical reagents were obtained from commercial suppliers and used without further purification. All of the solutions were prepared with ultra-pure water obtained from a Milli-Q 50 SP Reagent Water System (Millipore Corporation, USA). PGC cartridges for desalting were purchased from Alltech Associates, Inc. (Deerfield, IL). Amicon Ultra-0.5 3 kDa devices were obtained from Millipore (Billerica, USA).

2.2. Transglycosylation reaction of SGP and PDPZ-Boc-Asn-GlcNAc with endo-β-N-acetylglucosaminidases (Endo-M N175Q and Endo-M)

The transglycosylation procedures were modified to consolidate the activities from the previous papers [16,17]. In brief, SGP (2.0 mM; 20 μL), an equal volume of 4.0 mM PDPZ-Boc-Asn-GlcNAc in 50 mM potassium phosphate buffer (pH 6.0) and 5 μL of Endo-M N175Q (5 mU) or 10 μL of Endo-M (200 μU) in water were mixed in 0.5-mL Eppendorf tube and incubated at 32 °C for 3 h (Endo-M N175Q) or 10 min (Endo-M). An aliquot (2 μL) of the reaction solution was injected into the HPLC/ESI-MS system.

2.3. Release of glycans from ribonuclease B by PNGase F and the separation of the glycans

The digestion with PNGase F was done according to the manufacturer's instructions. Denaturation of the glycoprotein (ovalbumin and ribonuclease B) (5 mg) was achieved by heating at 100 °C for 10 min with addition of the glycoprotein denaturing buffer (300 μL).

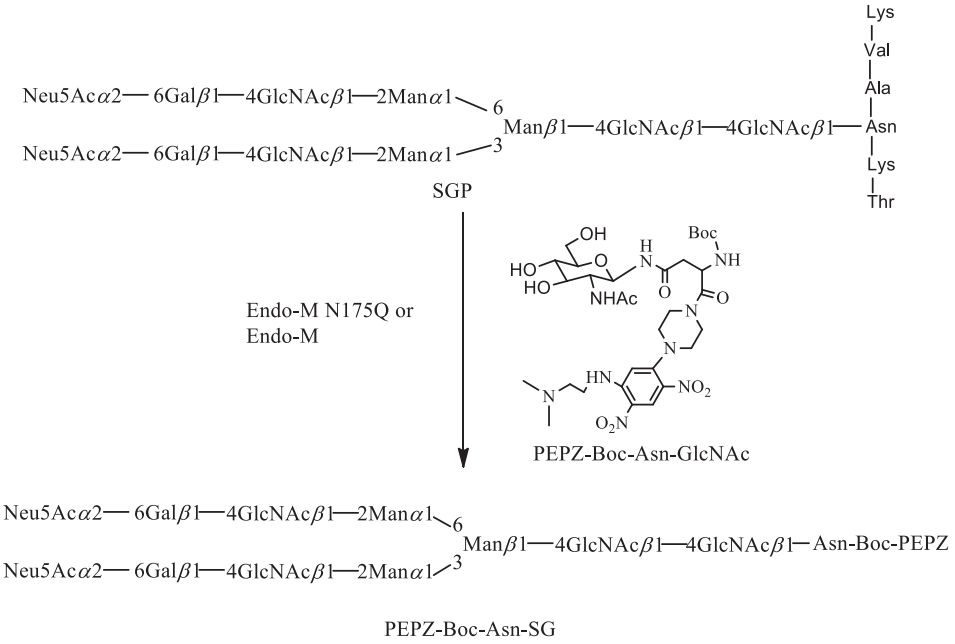

Fig. 1. The reaction scheme for transglycosylation reaction between a donor (SGP) and an acceptor (PDPZ-Boc-Asn-GlcNAc) with *endo*-β-*N*-acetylglucosaminidase (Endo-M and Endo-M N175Q).