NatGlycan

Technical Report No.1

Synthesis of N-Glycopeptides with ENGase-catalyzed Transglycosylation using homogenous Man6GlcNAc

By

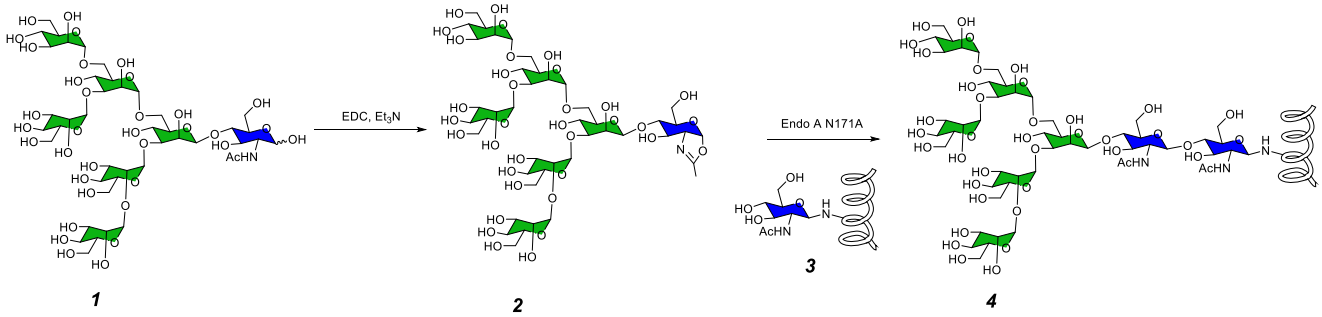
Lei Li, Ph.D., Assistant Professor, Department of Chemistry, Georgia State University, 50 Decatur St., Atlanta, GA 30303

Introduction

N-Glycosylation is a major posttranslational modification of proteins, and the carbohydrate components of glycoproteins participate in many biological processes such as cell adhesion, host-pathogen interactions, and immune responses. However, an understanding of the biological functions of glycoprotein glycans is often hampered by the heterogeneity of the N-linked oligosaccharide(s). However, the last decade has witnessed chemical and enzymatic advances in synthesis of glycopeptides with homogenous N-glycans, among which ENGase-catalyzed transglycosylation has been shown to be most promising. This method has been applied in the synthesis of a vast range of homogenous glycopeptides and glycoproteins.[1]

The endo-β-*N*-acetylglucosamindases (ENGase, EC 3.2.1.96) are a class of enzymes that hydrolyze the β-1,4-linked glycosidic bond in the *N,N'*-diacetylchitobiose core of N-linked glycans on glycoproteins to release the oligosaccharide and leave an intact GlcNAc moiety on the Asn residue in the protein. Besides hydrolyzing activity, some ENGase were found to have transglycosylation activity, i.e., the ability to transfer the released oligosaccharide moiety back onto a peptide or protein bearing a GlcNAc residue. In 2008, Wang and co-workers reported the first example of ENGase-based glycosynthases (a mutant devoid of the hydrolysis activity) by screening a series of Endo-M mutants using a Man9GlcNAc N-glycan oxazoline (reaction intermediate) as the activated donor substrate.[2] This approach, involving mutation of a key amino acid residue that promotes the oxazolinium ion intermediate formation during the catalysis, greatly increased the efficientcy of ENGase-catalyzed transglycosylation. Here we show the standard procedure for the synthesis of high-mannose glycopeptides using mg quantities of the homogenous oligosaccharide, Man6GlcNAc that is now available from NatGlycan, LLC.

Workflow



Scheme 1. Workflow of Endo A mutant N171A catalyzed synthesis of glycopeptides.

1. Oxazoline glycans preparation

In brief, 10 mg of compound **1** was dissolved in 100 μL D_2O (for NMR characterization), then triethylamine (Et_3N, 45 equiv.) and 2-Chloro-1,3-dimethylimidazolinium chloride (DMC) (15 equiv.) were added. The reaction was incubated on ice for 30 minutes, and the reaction was monitored using NMR. After the reaction was finished, the reaction mixture was directly loaded onto a G15 column (1.0 x 100 cm) that was pre-equivalented with 5 mM NaOH, and the desired fraction was collected and lyophilized to yield white powder **2** (12 mg).

2. Synthesis of glycopeptide using GlcNAc-peptide (3) as an acceptor

Compounds **2** (5 mg) and **3** (1 equiv.) were dissolved in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.5) and then incubated with Endo-A^{N171A} (150 μg/ml) at 30 °C for 10 mins. The mixture containing target compound was monitored by MALDI-TOF-MS and by HPLC using a Xbridge Peptide BEH C18 column (3.5 μm, 4.6 mm × 250 mm) (Figure 1 and 2). Then, the synthesized glycopeptide was purified using the Xbridge Peptide BEH C18 column (3.5 μm, 10 mm × 250 mm) column with conditions as follows: solvent A (H_2O with 0.1 % TFA), solvent B (ACN with 0.1 % TFA), from 0 min to 30 min, gradient solvent from 5 % to 30 %.

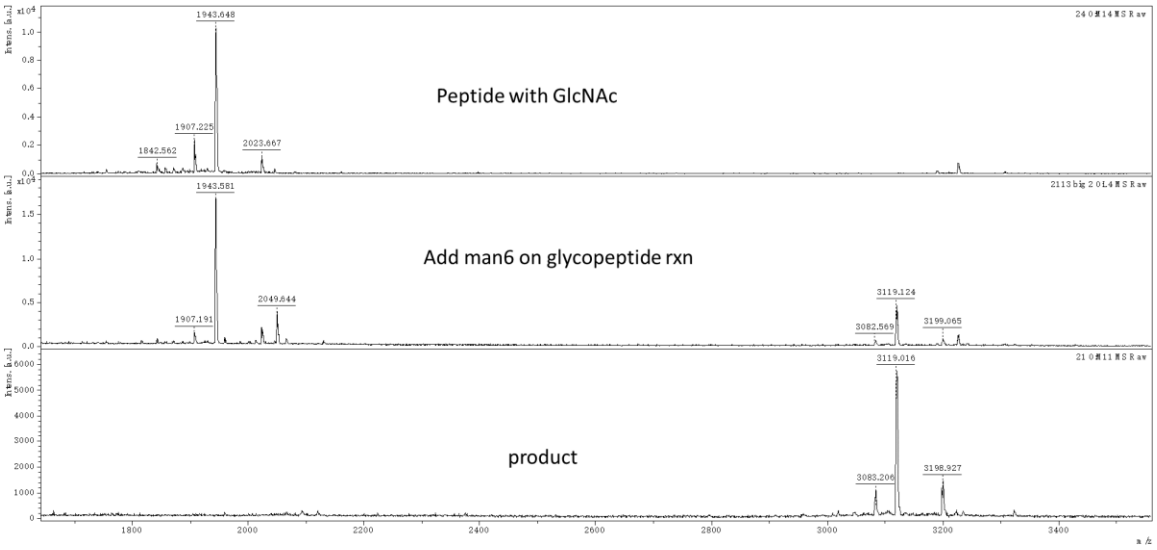


Figure 1. Reaction monitored by MALDI-TOF

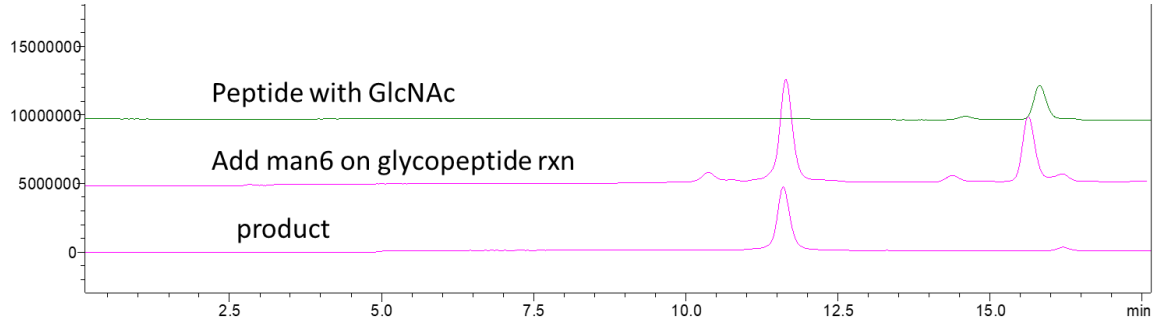


Figure 2. Reaction monitored by HPLC

Conclusion

For the preparation of oxazoline glycans, the pH of the reaction system and the purification process need to be monitored carefully. *"The reaction must be performed under basic conditions. After adding all materials, check whether the reaction has a pH >11. If the pH is <11, the conversion rate might decrease and more Et_3N will be required"* .[3] Additionally, the oxazoline glycan may be unstable in aqueous solution, it high recommended to use oxazoline glycan powder directly.

For the preparation of glycopeptides with glycosynthase, the enzyme purity and concentration should be well characterized. From our experience, the more enzyme was used, the higher yield could be achieved. The ratio of the reaction should also be optimized, normally, high ratio of glycan-oxazoline: GlcNAc-peptide (5: 1 to 10: 1 or higher) could ensure high yields (>90%). In this case, 1:1 was applied as glycan-oxazoline is limited, a moderate ratio of 50% was achieved. Excess amounts of glycan-oxazoline could also increase the pH of the reaction system, which should be carefully adjust before the addition of enzyme. The reaction time should also be optimized as glycosynthases still possess weak hydrolysis activity. Nevertheless, less than 30 mins reaction time is highly recommended. Glycan-oxazoline can be recycled and reused as described in Step 1.

References

1. Li, C.; Wang, L. X., Chemoenzymatic Methods for the Synthesis of Glycoproteins. *Chem Rev* **2018,** *118* (17), 8359-8413.

2. Umekawa, M.; Huang, W.; Li, B.; Fujita, K.; Ashida, H.; Wang, L. X.; Yamamoto, K., Mutants of Mucor hiemalis endo-beta-N-acetylglucosaminidase show enhanced transglycosylation and glycosynthase-like activities. *The Journal of biological chemistry* **2008,** *283* (8), 4469-79.

3. Tang, F.; Wang, L.-X.; Huang, W., Chemoenzymatic synthesis of glycoengineered IgG antibodies and glycosite-specific antibody–drug conjugates. *Nature Protocols* **2017,** *12* (8), 1702-1721.