Novel Bacterial Sialidase Mutants for Site Specific Sialylation of Biologically Important Glycoproteins

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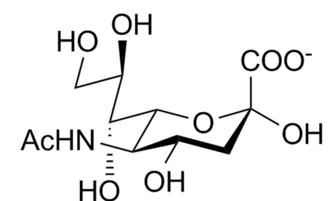
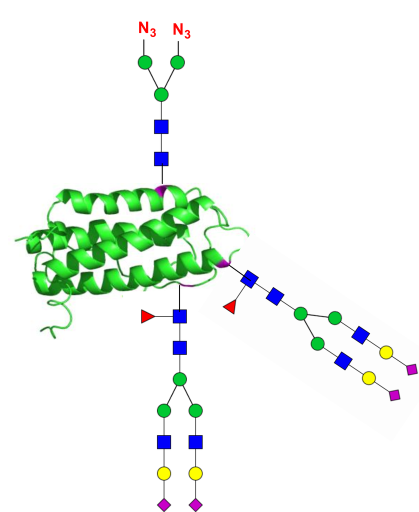
Introduction

Sialylation is a unique modification of glycans existing in different glycoproteins on cell surfaces, which significantly modulates many important physiological and pathological processes, such as anti-inflammatory and tumor metastasis. Despite tremendous efforts on the synthesis of this crucial glycan decoration using chemical and enzymatic methods, site-specific attachment of sialic acid moieties onto biologically important glycopeptides or glycoproteins is still challenging. In the last two decades, converting glycosidases into their transfer mutants has become a fascinating approach in the synthesis of glycoconjugates. In this work, a bacterial α2,6-sialidase (MvNA) from *Micromonospora viridifaciens* was selected as candidate for efficient transfer of sialic acid in a regio- and stereo-controlled manner due to its inherent trans-sialylation activity. First, wild-type MvNA was cloned and overexpressed in *E. coli*. Two types of sialidase mutants with mutation site at the nucleophile (Y370) and the general acid/base (D92) residues were then created by site-directed mutagenesis and successfully expressed. In the preliminary activity tests, the wild-type MvNA showed strong hydrolytic activity towards a natural sialylated complex-type N-glycopeptide, while terminal sialic acids from the same glycopeptide can't be removed by created MvNA mutants, including Y370A, Y370G and D92A. The potential trans-sialylation activities of those sialidase mutants will be systematically tested using various synthetic activated substrate donors, such as sialyl fluorides and nitrophenyl sialosides. We are also aiming to create sialidase mutants derived from other types of sialidases with distinct specificities, e.g. α2,3 and α2,8-linkages. The potential sialidase transfer mutants could provide a new avenue to access the structurally well-defined sialylated glycopeptides and glycoproteins for sialylation bio-function study and therapeutic applications.

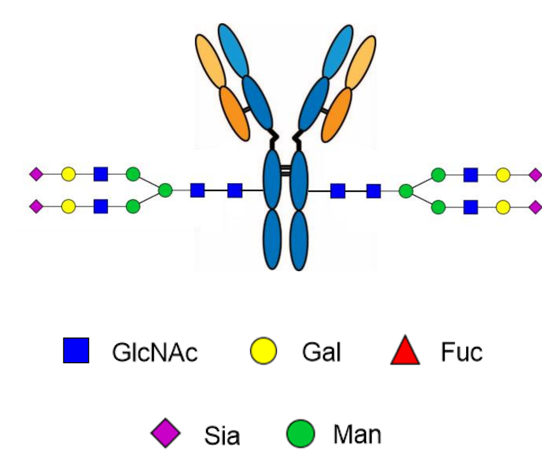
Biofunctions of Sialylation



Structure of N-acetylneuraminic acid, a major sialic acid residue produced in human glycoconjugates.



Increased terminal sialylation in therapeutic glycoproteins (e.g. EPO) is shown to have increased serum half-life[2].



Sialylation in Fc region of antibodies has shown to enhance anti-inflammatory properties[1].

Limitations of Current Synthesis Methods

Chemical methods

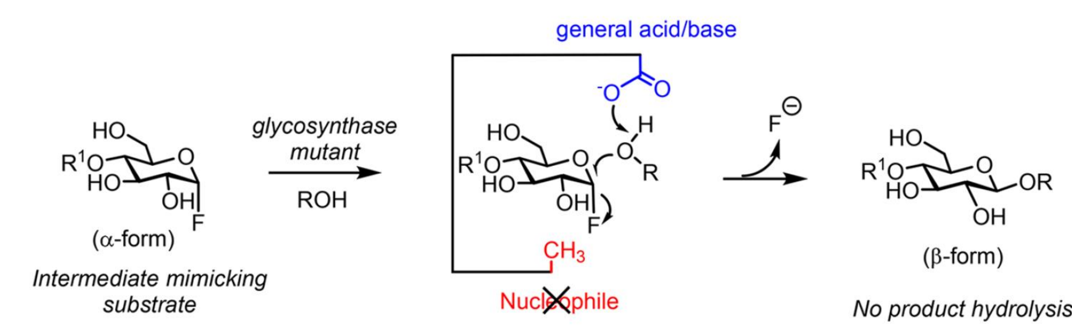
1. Need of protection and deprotection steps.
2. Hard to control stereo/regio-chemistry.

Enzymatic methods

1. Mammalian sialyltransferases are mostly membrane proteins that are not easy to express in *E. coli* system.
2. Donor substrate sources are very specific and are usually expensive (sugar nucleotides or sugar phosphate).
3. Low to moderate yields obtained usually for protein sialylation (<50%).

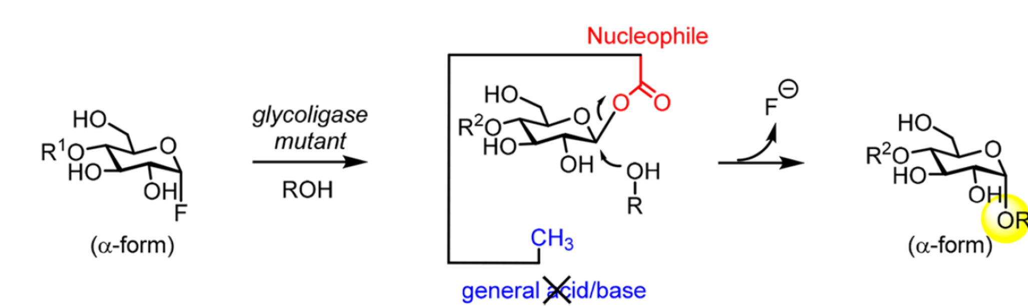
Concept of Glycosynthase and Glycoligase

Glycosynthase



Glycosynthase is a glycosidase mutant where the nucleophile residue is eliminated. An activated donor substrate mimics the enzyme-substrate complex. Shown above is reaction scheme for a retaining β-glycosynthase mutant[3].

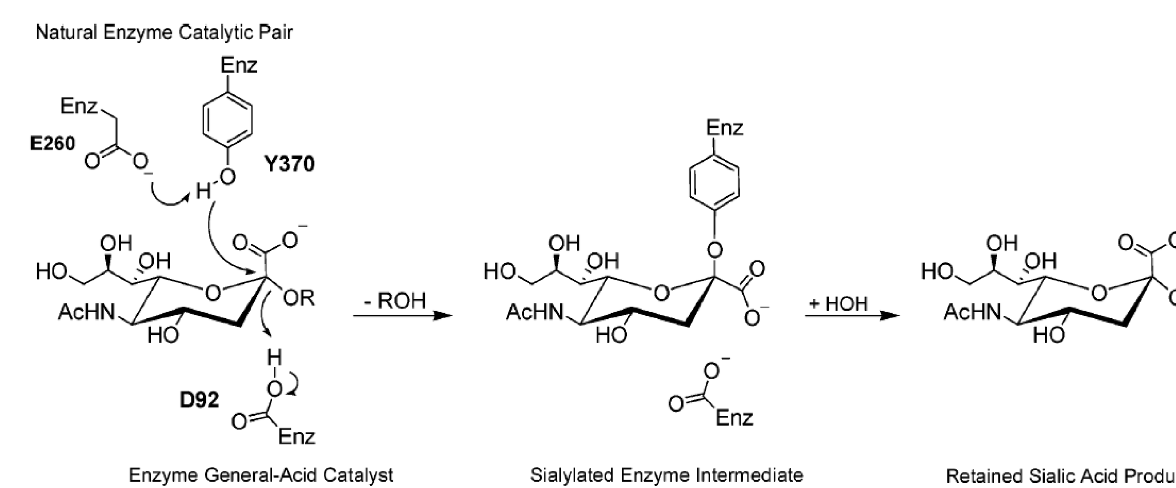
Glycoligase



Glycoligase is a glycosidase mutant where the general acid/base residue is eliminated. Donor used have same anomeric configuration as substrate used in wild type enzyme. Shown above is reaction scheme for an inverting α-glycoligase mutant[3].

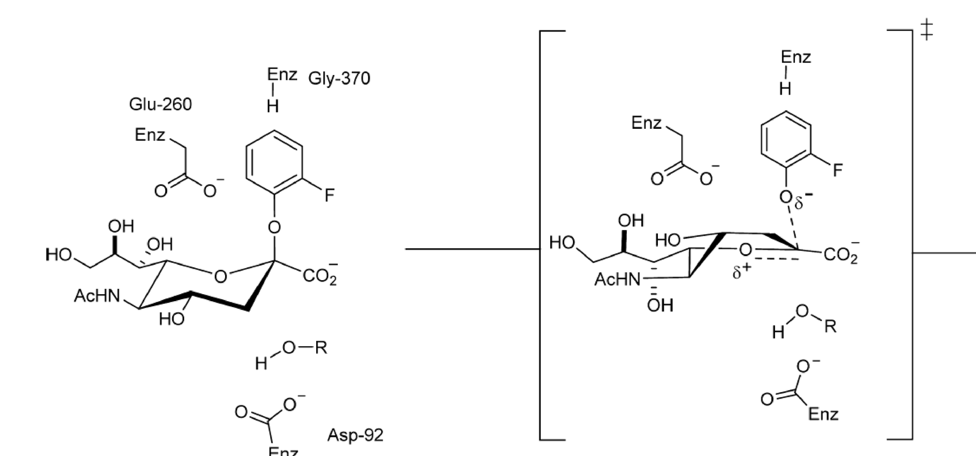
Experimental Design

MvNA WT



MvNA is a sialidase with three active sites, Y370, D92, and E260. Shown above is the mechanism of hydrolysis for MvNA WT[4].

MvNA Sialosynthase

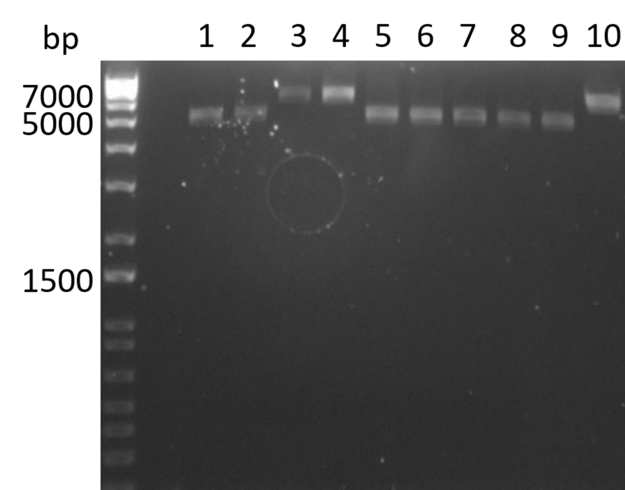


Shown above is the transition state proposed for sialosynthase Y370G mutant using 2-fluorophenyl as donor substrate[5].

MvNA Cloning & Enzyme Purification

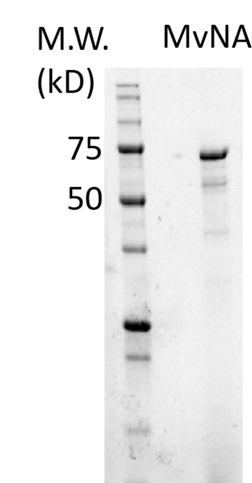
Cloning

Genomic DNA of MvNA (1944 bp) was amplified using Phusion hot start II DNA polymerase. DNA obtained was digested and cloned into digested pET28a vector (5369 bp). Obtained PCR product was amplified with DH5α competent cell. DNA extracted was loaded on DNA gel for characterization.



Above shows plasmid were digested and linearized loaded in lane 3,4, and 10 were around 7 kbp in length compared to the ladder where others appears to be at around 5 kbp in length.

Expression and purification



Successfully cloned plasmids were transformed into BL21 *E. Coli* competent cells for expression. Crude cellular protein suspension was then purified with HisTrap column. The purified protein sample was loaded onto protein gel and check for identity.

Above shows the band to be between 50 and 75 kD where MvNA WT has M.W. of 68 kD.

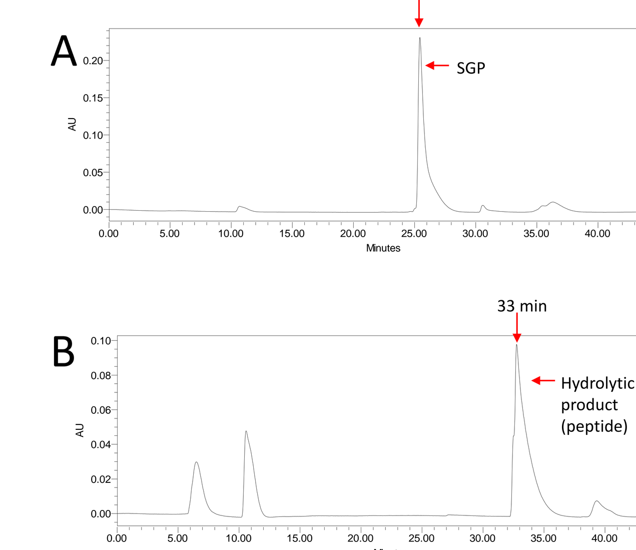
Site-directed mutagenesis

Nucleophilic active site (Y370) and general acid/base active site (D92) were mutated through site-directed mutagenesis. Mutated DNA plasmids were sent for sequencing and the correct plasmids were amplified with DH5α competent cell. The mutants were then expressed and purified.

Preliminary Test

Hydrolytic activity test

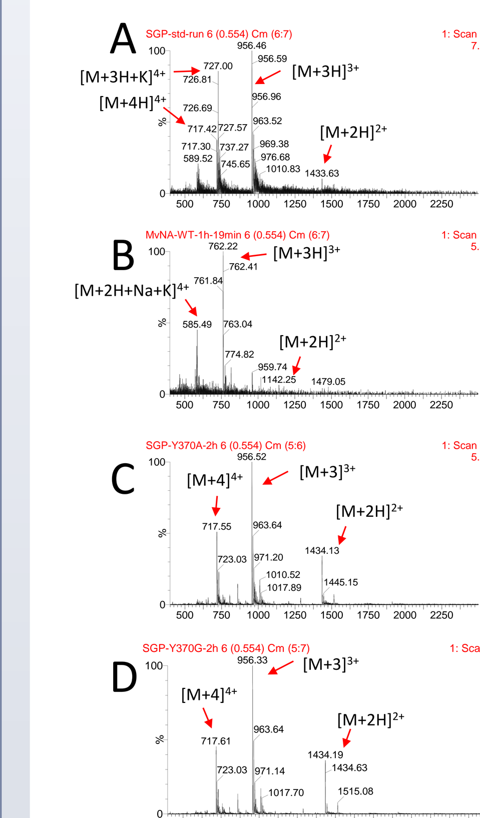
Sialyl glycopeptide (SGP) was used as substrate for hydrolytic activity test for WT MvNA through a 1:500 molecular ratio reaction. Hydrolytic activity was monitored through HPLC with 0-5% acetonitrile gradient.



Above shows A) SGP standard, B) MvNA WT hydrolysis reaction mixture.

Preliminary Test

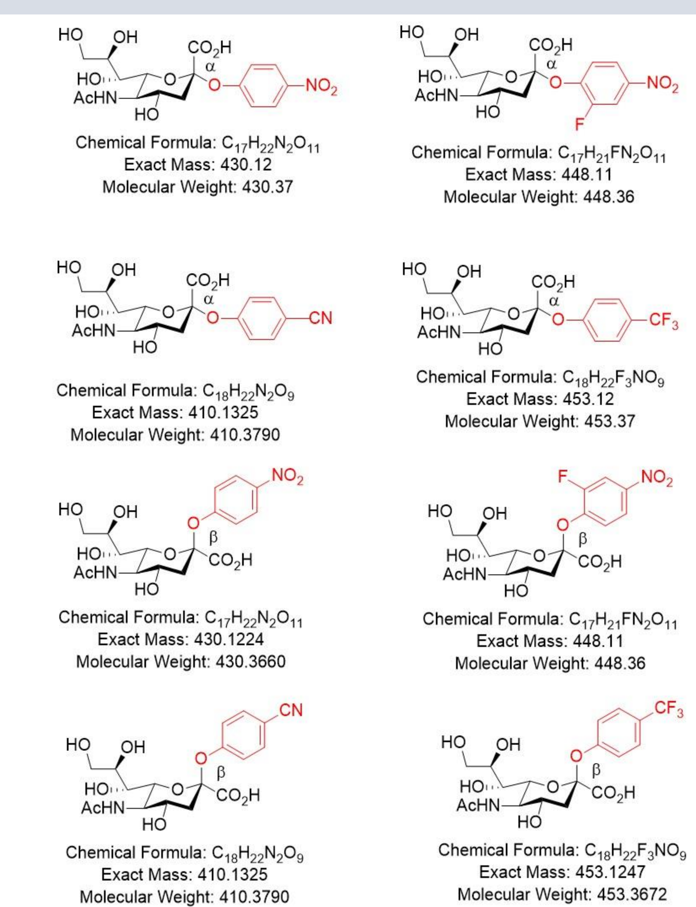
Hydrolytic activity test



SGP was used to test hydrolytic activity of WT, Y370A, and Y370G mutants and mass spectra was obtained using LC-ESI-MS.

On left shows mass spectra of A) SGP standard, B) WT reaction, C) Y370 A mutant reaction, and D) Y370G mutant reaction. No specific hydrolytic activity was found in the mutants generated.

Future Works



1. Transfer activity test on the sialosynthase using synthetic sugar fluorides and sialosides with different leaving groups. Illustrations above shows sialosides synthesized by Yuanwei Dai with α and β-configurations
2. Hydrolytic activity and transfer activity test on D92A mutant.
3. Explore α2,3 and α2,8 sialidases.

References

1) Raju TS, Lang SE. Diversity in structure and functions of antibody sialylation in the Fc. *Current Opinion in Biotechnology*. **2014,** *30*,147-152.
2) Fukuda M, Sasaki H, Lopez L, et al. Survival of recombinant erythropoietin in the circulation: the role of carbohydrates. *Blood.* **1989**, *73*, 84–89.
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4) Watson JN, Indurugalla D, Cheng LL, et al. The Hydrolase and Transferase Activity of an Inverting Mutant Sialidase Using Non-natural β-Sialoside Substrates, *Biochemistry.* **2006**, *45*, 13264-13275
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