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Molecular Cloning, Expression, and Purification of the Human Sialyltransferase ST8Sia6

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Abstract

Glycans linked to cell surface proteins are one of the most significant and complex biological molecules that play a role in human health and disease. Sialic acid (Sia) is found at the outermost position of glycan chains, facilitating a key role in cell-to-cell signaling and ligand-receptor interactions. PolySia is a long homopolymer consisting of 8 to 400+ residues of α2,8-linked Sias, which differs from widely expressed α2,3- and α2,6-linked monoSia. There is considerable knowledge surrounding the critical roles of polySia in cell migration, and more recently, the relationship between sialylation and immune attenuation. However, oligoSia, which consists of 2 to 8 Sia residues, is often overlooked. Due to the lack of tools, such as commercial antibodies, there is very limited data on this glycan. This project aims to shed light on the mystery that is oligoSia by investigating the biosynthesis of oligosialylated proteins. The sialyltransferase enzymes ST8Sia3 and ST8Sia6 are likely responsible for synthesizing oligoSia. ST8Sia3 is mostly limited to the nervous system, whereas ST8Sia6 mRNA is more broadly expressed and increases in many cancers, including breast cancer and melanoma, suggesting a potential role in cancer progression. However, the endogenous protein targets of ST8Sia6 remain unknown. Here, ST8Sia6 truncations were cloned and expressed using both a bacterial and eukaryotic system. The ST8Sia6 constructs were tested for activity on various Core 1 substrates to evaluate substrate specificity. The protein expression from the bacterial system was strong, however, little to no activity on the test proteins was observed. Expression in the eukaryotic system yielded insight into the potential post-translational modifications of the enzyme and the folding efficiency of the truncated variants. Together, elucidating the substrate specificity of ST8Sia6 will advance our understanding of the role of oligoSia in both health and disease.

Keywords: glycans; sialyltransferase; sialic acid

Introduction

Sialic acids (Sia) are one of the most significant and structurally diverse molecules in vertebrate biology [1]. *N*-acetylneuraminic acid (Neu5Ac) is the most common member of the Sia family found in human and many other mammalian cells [1]. Sia occupies the terminal position on cell surface glycoproteins, allowing for the modulation of various biological and pathological processes, including cell-to-cell signalling, protein-carbohydrate interactions, and cell migration [1]. There are three lengths of Sia, the most abundant is monoSia, where one residue is attached to an underlying galactose or *N*-acetylgalactosamine via an α2,3 or α2,6-linkage [2]. PolySia differs from widely expressed monoSia as it is a homopolymer of α2,8-linked Sias, ranging from 8-400+ residues [2]. OligoSia, or short chain Sia, is 2-8 residues long and also α2,8-linked [2]. The biological functions of monoSia and polySia have been studied extensively and significant endogenous protein targets have been identified. For example, method development surrounding the capture of polysialylated proteins has recently revealed QSOX2, a secreted protein from the breast cancer cell line MCF-7, as a novel polysialylated protein [3]. However, owing to the lack of commercially available antibodies or identified lectins for capturing oligoSia, glycoproteins containing oligoSia groups have received considerably less attention.

The biosynthesis of oligo- and polySia is primarily determined by the sialyltransferase that catalyzes the transfer of a Sia moiety from CMP-Neu5Ac (the sugar donor) to various protein acceptors [4]. Human sialyltransferases (STs) are disulfide-containing, type II transmembrane glycoproteins [5]. The ST8Sia gene family encodes α2,8-sialyltransferases, which transfer a Sia from CMP-Neu5Ac to an underlying Sia, forming an α2,8-linkage [6]. Of the ST8Sia family, ST8Sia3 and ST8Sia6 are suspected to be responsible for synthesizing oligoSia [7]. According to The Human Protein Atlas, ST8Sia3 is limited to the nervous system. In contrast, ST8Sia6 has been shown to make oligoSia on serine- and threonine-linked glycans (O-linked) on fetuin and bovine

submaxillary mucin [7–10]. Furthermore, ST8Sia6 expression is widespread amongst cell types and the mRNA is upregulated in many diseases. For instance, The Human Protein Atlas deems ST8Sia6 a prognostic marker in Kidney renal clear cell carcinoma as it has been detected in some cancer cell lines and tissues. Despite these significant findings, little is known about the expression, activity, and substrate specificity of ST8Sia6.

This project aims to clone, express, and purify soluble ST8Sia6 and identify any preferential underlying Sia linkage on the substrate acceptor. Elucidating the structure of the substrate acceptor will shed light on the mechanism of ST8Sia6 and pave the way for further analysis of the underlying proteins. This will not only advance our understanding of Sia in biology, but also yield important information into the mechanism of ST8Sia6 in health and disease.

Methods

Cloning

First, a bacterial codon-optimized plasmid containing the human ST8Sia6 gene (UniProt ID: P61647) in the VEK-06 backbone (from the Wakarchuk Lab) was digested using *Nde1* and *Sal1*-HF restriction enzymes to produce the ST8Sia6 fragment and backbone. VEK-06 contained a maltose binding protein (MBP) tag in frame with the gene of interest, and a *lac* repressor was engineered into the plasmid for regulatory control in *Escherichia coli.* The ST8Sia6 fragment was truncated at serine 99 (S99) using PCR cloning, ligated back into the vector, and transformed into competent 46C cells, an engineered *E.coli* SHuffle T7 Express strain (New England Biolabs) with a PDI-QSOXb fusion under an IPTG-inducible promoter (a gift from the Wakarchuk lab). This new construct was named HUST172. Next, a mammalian codon-optimized plasmid adapted from pGen2.1, was engineered to contained a superfolder green fluorescent protein (GFP) and 8x His-tag in frame with ST8Sia6 for expression and purification from cell media. The same PCR method was used to create two different truncations using *EcoR1*-HF and *Sal1*-HF, one at S99 to complement what had been done in the bacterial construct and one at threonine 65. These new constructs were named HUST179 and HUST178, respectively. Whole Plasmid Sequencing was done for all constructs using Plasmidsaurus using Oxford Nanopore Technology.

Sialyltransferase Expression and Purification

HUST172 verified transformants were cultured overnight in Terrific Broth (TB) media at 37 °C. The overnight cultures were diluted 1/100 in TB media supplemented with 100 µg/mL ampicillin. Cultures were grown at 37°C with shaking at 200 rpm until the OD600 reached 0.6. To induce the recombinant protein expression, a final concentration of 0.5mM isopropyl-thio-β-galactoside (IPTG) was added and the cultures were incubated at 20 °C for 26 hours with shaking. The cells were lysed in 50mM HEPES (pH 7.4), 200mM NaCl, 10mM CaCl2, Complete Mini Ethylenediaminetetraacetic acid-free protease inhibitor tablet (Fischer), and benzonase. The supernatant was cleared by 30 minute centrifugation at 15,000 x*g* at 4°C then passed through a 0.45µm filter. The protein was purified using a 5 mL MBPTrap HP (Cytiva) on the ÄKTA go protein purification system. The protein was eluted in 50mM HEPES (pH 7.4), 200mM NaCl, 10mM maltose, and 10% glycerol. The purity of the fractions was assessed by SDS-PAGE. The fractions were pooled, and final protein concentration was measured by Bicinchoninic Acid (BCA) assay.

A medium-scale transfection of HUST178 and 179 was performed using HEK293F cells. HEK293F cells were cultured in complete medium, containing Gibco Freestyle Expression Medium supplemented with 10% EX-CELL 293 serum free medium. Cells were grown at 37°C with 8% CO_2, shaking at 90 rpm. The day prior to transfection, the media was changed and the cells were diluted to 1x10^6 cells/mL. On the day of transfection, cells were spun down and resuspended at 2x10^6 cells/mL. In a 250 mL Erlenmeyer flask, 20 mL of cells were transfected with 4.5ug/mL DNA and 13.5ug/mL of linear polyethylenimine (PEI 25K). The cells were incubated for 24 hours at 37 °C, 8% CO_2, and shaking at 90 rpm. After 24 hours, cells were diluted 2-fold with complete media and 2.2mM valproic acid. Cell viability was measured by staining cells with Trypan blue and counting the live and dead cells using a hemocytometer. GFP fluorescence was measured (excitation 488 nm and emission 507 nm) using a microplate reader to serve as a proxy for transfection efficiency by subtracting day 1 fluorescence of cell-free media. The recombinant proteins were harvested and purified on day 4 or 5 post-transfection. The media was cleared by 10 minute centrifugation at 360 x*g* followed by a 20 minute centrifugation at 2000 x*g*. The media was diluted with 10X buffer (50mM HEPES (pH 7.4), 200mM NaCl, 10mM imidazole) to a final concentration of 1X and filtered through a 0.45µm filter. HUST178 and HUST146 were purified using a 5 mL HisTrap HP column (Cytiva) on the ÄKTA go, while HUST179 was purified via a manual purification using a 1ml Ni-NTA resin column. The purity of the fractions was assessed by SDS-PAGE.

Synthesis of GB1 Test Proteins

Sialylated test proteins were synthesized using a lab strain of *E.coli* which contains one plasmid encoding a GB1 protein, and another plasmid encoding a human GalNAc transferase. The GB1 protein with a GalNAc was purified using a 5ml HisTrap on the ÄKTA go. Various sialylated constructs were created via subsequent reactions. The workflow of synthesis for the test proteins is as follows 1) Core 1: GB1 + GalNAc + Gal 2) S3 Core 1: GB1 + GalNAc + Gal + α2-3 linked Sia 3) S6 Core 1: GB1 + GalNAc + Gal + α2-6 linked Sia and 4) S3,6 Core 1: GB1 +

GalNAc + Gal + α2-3 linked and α2-6 linked Sia (synthesized using S3C1).

Sialyltransferase Assays and High Performance Liquid Chromatography (HPLC)

Sialyltransferase activity was tested at various conditions for optimization. Purified enzyme was combined with 50mM of buffer, 10mM of a co-factor, 2mg/ml of substrate, and 1mM of CMP-Neu5Ac. Tested buffers include HEPES at pH 7 and 2-(N-morpholino)ethanesulfonic acid (MES) at pH 6.3. Tested co-factors include, $MnCl_2$, $MgCl_2$, and $CaCl_2$. Activity assays were incubated overnight at 30 C°. The reactions were monitored via SDS-PAGE and HPLC analysis was performed using Waters BioResolve RP mAb Polyphenyl column, where the mobile phase was acetonitrile with 0.1% trifluoroacetic acid.

Immunoblotting

Standard immunoblot protocol was used. To detect the 8x His-Tag, a purified anti-His tag antibody (Biolegend) was used at 1/10000 dilution. The secondary antibody used was anti-mouse IgG HRP conjugated (R&D), at a 1/1000 dilution.

Liquid-Chromatography Mass Spectrometry (LC-MS)

The mass spectrometer used was an Agilent 6220 orthogonal acceleration (oaTOF). The ionization mode was positive (+) ESI. The protein deconvoluted results of the mass spectrums are shown.

Results

Truncations of the ST8Sia6 gene were made at serine 99 (ΔS99) and threonine 65 (ΔT65) using PCR cloning, effectively removing the single-pass transmembrane and unstructured region (Fig.1). The ΔS99 truncation was made in both bacterial and mammalian codon-optimized vectors, while the ΔT65 truncation was only made in the mammalian codon-optimized vector. While the protein expression of ΔS99 in 46C cells (HUST172) was strong (Fig. 2A), the activity assay monitored by SDS-PAGE did not show an increase in molecular weight of the substrate in the positive donor sugar reactions (Fig. 2B). This suggests that under these conditions, ST8Sia6 is not active, as we would expect to see the substrate run at a higher molecular weight had the enzyme added on Sias.

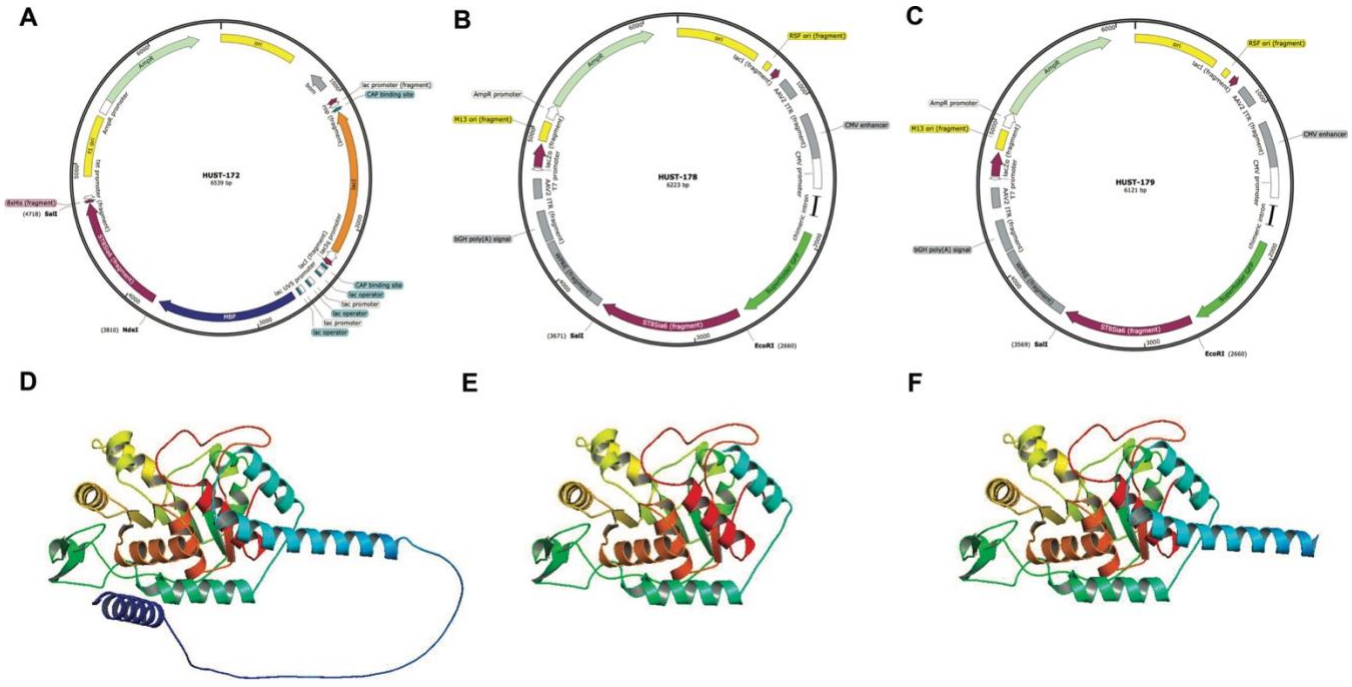

Figure 1. Plasmid maps and predicted 3D structures of ST8Sia6. Plasmids are from SnapGene viewer and 3D structures are PyMol renditions based on the predicted protein structure from the AlphaFold protein data bank (PDB) file. A) Plasmid map of HUST172, showing the ΔS99 ST8Sia6 fragment in the VEK-06 backbone. B & C) Plasmid maps of HUST178 and HUST179, showing the ΔT65 and ΔS99 fragments in the re-engineered pGen2.1 backbone, respectively. D, E &F) Predicted 3D structures of full length, ΔS99, and ΔT65 truncated ST8Sia6 structures, respectively.

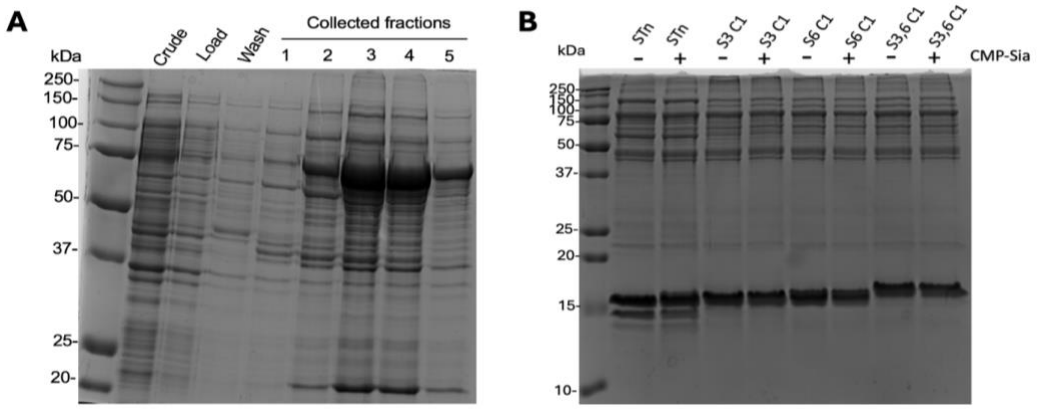

Figure 2. HUST172 expression, purification and activity assay. A) Coomassie Blue SDS-PAGE showing the 8x HisTag protein purification. The expected molecular weight of HUST172 is ~75.6 kDa. Fractions 3 and 4 were pooled, the concentration was measured via BCA assay (1.5mg/mL) and tested for activity. B) Coomassie Blue SDS-PAGE of the activity assay, showing the reaction with each substrate in the presence and absence of donor sugar CMP-Sia. If the enzyme were active, the substrate is expected to be at a higher molecular weight in the positive donor sugar reaction compared to the negative donor sugar reaction.

Of the ST8Sia6 constructs transfected into HEK293F cells, HUST178 had the highest GFP fluorescence (2.4x10^8 RFU) and total cell viability (450%) on the day of purification (Fig. 3A-C). Followed by HUST146 which had a GFP fluorescence of 5.3x10^7 RFU and total cell viability of 131% on the day of purification (Fig. 4A-C). The HUST179 construct had the lowest GFP fluorescence (4.7x10^7 RFU) and a total cell viability of 190% (Fig. 5A-C). The protein purifications were analyzed via SDS-PAGE and both HUST178 and HUST146 showed a band present at approximately the expected molecular weights of ~64.5 kDa and 69.8 kDa, respectively (Fig. 3D; Fig. 4D). However, the eluted fractions of HUST179 did not show the presence of the purified enzyme, which has an expected molecular weight of ~ 61.7 kDa (Fig. 5D), consequently, this construct was not tested for activity. The anti-His tag blot revealed the presence of the His-Tag in the flow through for the HUST179 construct but not the others, potentially suggesting that the ΔS99 truncation did not fold properly (Fig 6).

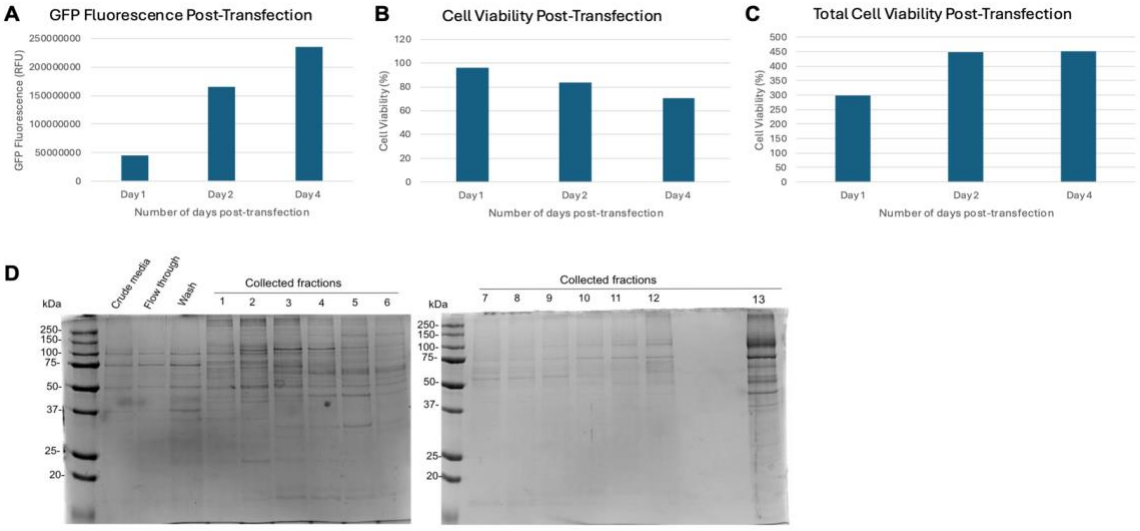

Figure 3. HUST178 transfection, expression, and purification from HEK293F cells. A) GFP fluorescence was measured from cell-depleted media using a microplate reader B) Cell viability post-transfection was measured by staining with Trypan blue and counting the cells using a hemocytometer. Percentage of cell viability was calculated (viable cells / viable cells + dead cells) C) Total cell viability was calculated (viable cells/ total number of transfected cells) D) His-Tag gradient purification using a 5mL HisTrap column on the AKTA Go. Expected molecular weight of HUST178 is ~64.5 kDa. Fraction 13 was collected and tested for activity.

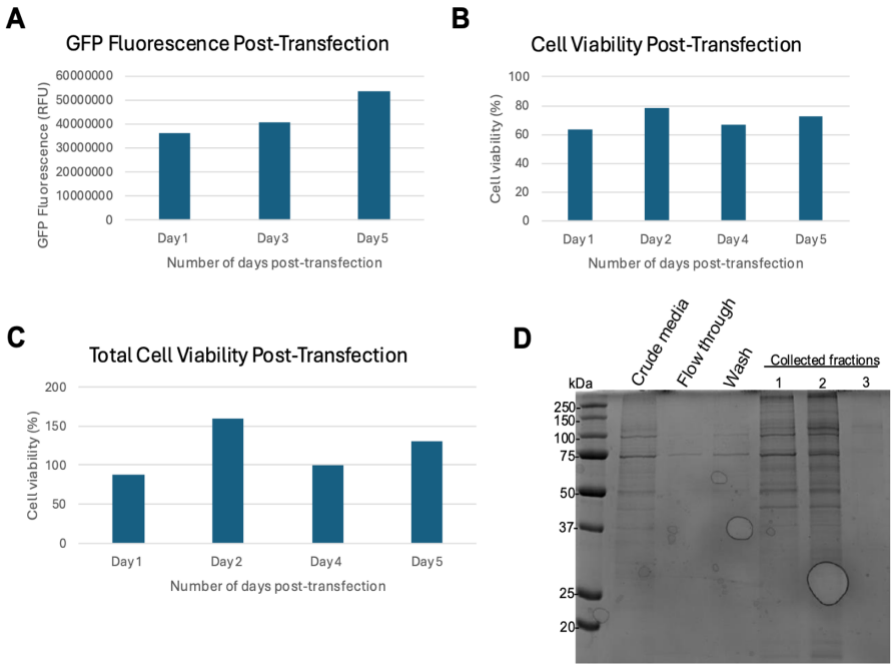

Figure 4. HUST146 transfection, expression, and purification from HEK293F cells. A) GFP fluorescence was measured from cell-depleted media using a microplate reader B) Cell viability post-transfection was measured by staining with Trypan blue and counting the cells using a hemocytometer. Percentage of cell viability was calculated (viable cells / viable cells + dead cells) C) Total cell viability was calculated (viable cells/ total number of transfected cells) D) His-Tag gradient purification using a 5mL HisTrap column on the AKTA Go. Expected molecular weight of HUST146 is ~69.8 kDa. Fraction 2 was tested for activity.

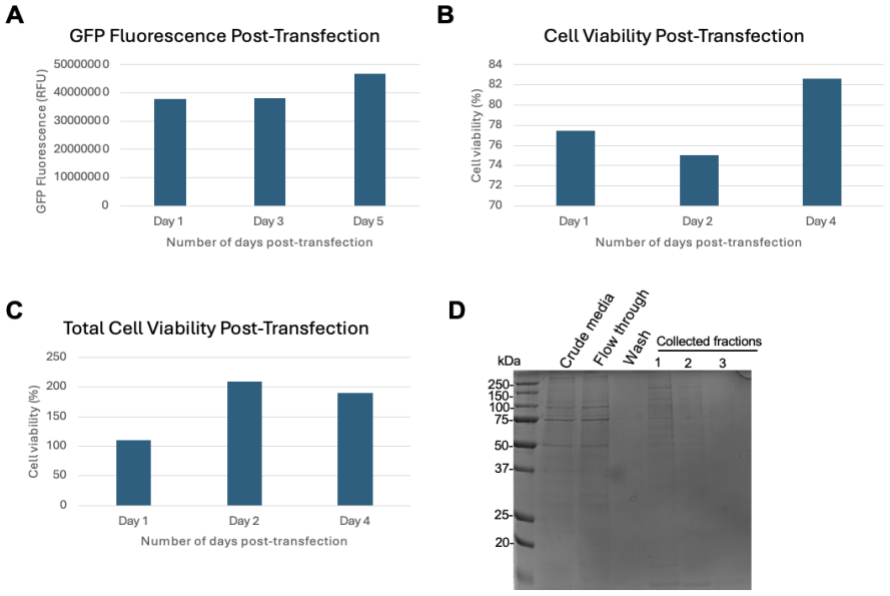

Figure 5. HUST179 transfection, expression, and purification from HEK293F cells. A) GFP fluorescence was measured from cell-depleted media using a microplate reader. B) Cell viability post-transfection was measured by staining with Trypan blue and counting the cells using a hemocytometer. Percentage of cell viability was calculated (viable cells / viable cells + dead cells) C) Total cell viability was calculated (viable cells/ total number of transfected cells) D) His-Tag manual purification using 1mL Ni-NTA resin. Expected molecular weight of HUST179 is ~61.7 kDa

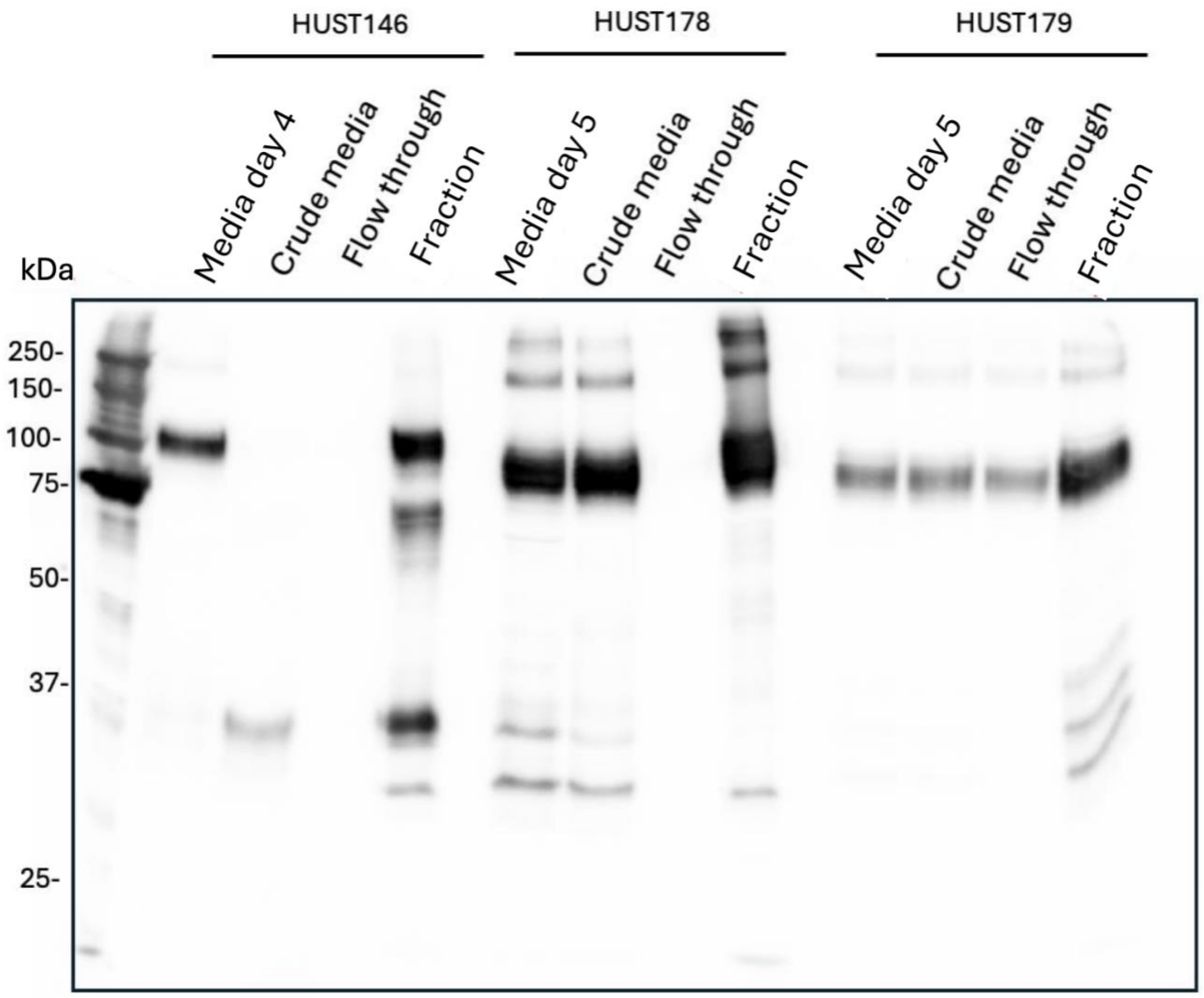

Figure 6. Western blot of ST8Sia6 purifications from HEK293F cells. Anti-His tag blot of ST8Sia6 constructs secreted from HEK293F cells into cell media. Cells were harvested on day 4 or 5 post-transfection and the media was filtered through a 0.45µm filter then purified using a Ni-NTA column.

In addition to the conditions used to test the activity of HUST172, we tested the activity of HUST178 and HUST146 at a of pH 6.3 as well. HPLC was used to detect any shifts in substrate polarity, which would be observed with an active enzyme. With a reverse-phase column, the retention time of the substrate is expected to decrease as it becomes more polar (i.e. if Sias had been added). The activity assay for HUST178 at a pH of 7 does not show a shift in substrate retention time between negative and positive donor sugar reactions, suggesting that the enzyme is not active under these conditions (Fig. 7A). The HPLC chromatogram does however show a shift in the positive donor sugar reactions for HUST146 and HUST178 at a pH of 6.3 - the shift exhibited is an increased retention time of the S3,6 Core 1 substrate compared to the negative control (Fig. 7B). There is no observed shift for the S3 Core 1 substrate and a minimal shift observed for the S6 Core 1 substrate at pH 6.3 (Fig. 7B). The S3,6 Core 1 substrate from the HUST146 reactions was sent for liquid-chromatography mass spectrometry (LC-MS) analysis for more information. The LC-MS revealed minimal differences in the spectrum from the – CMP-Sia reaction (Fig. 8A) compared to the + CMP-Sia reaction (Fig. 8B). Furthermore, the masses observed on the deconvoluted spectrum do not align with the expected mass of the S3,6 Core 1 substrate.

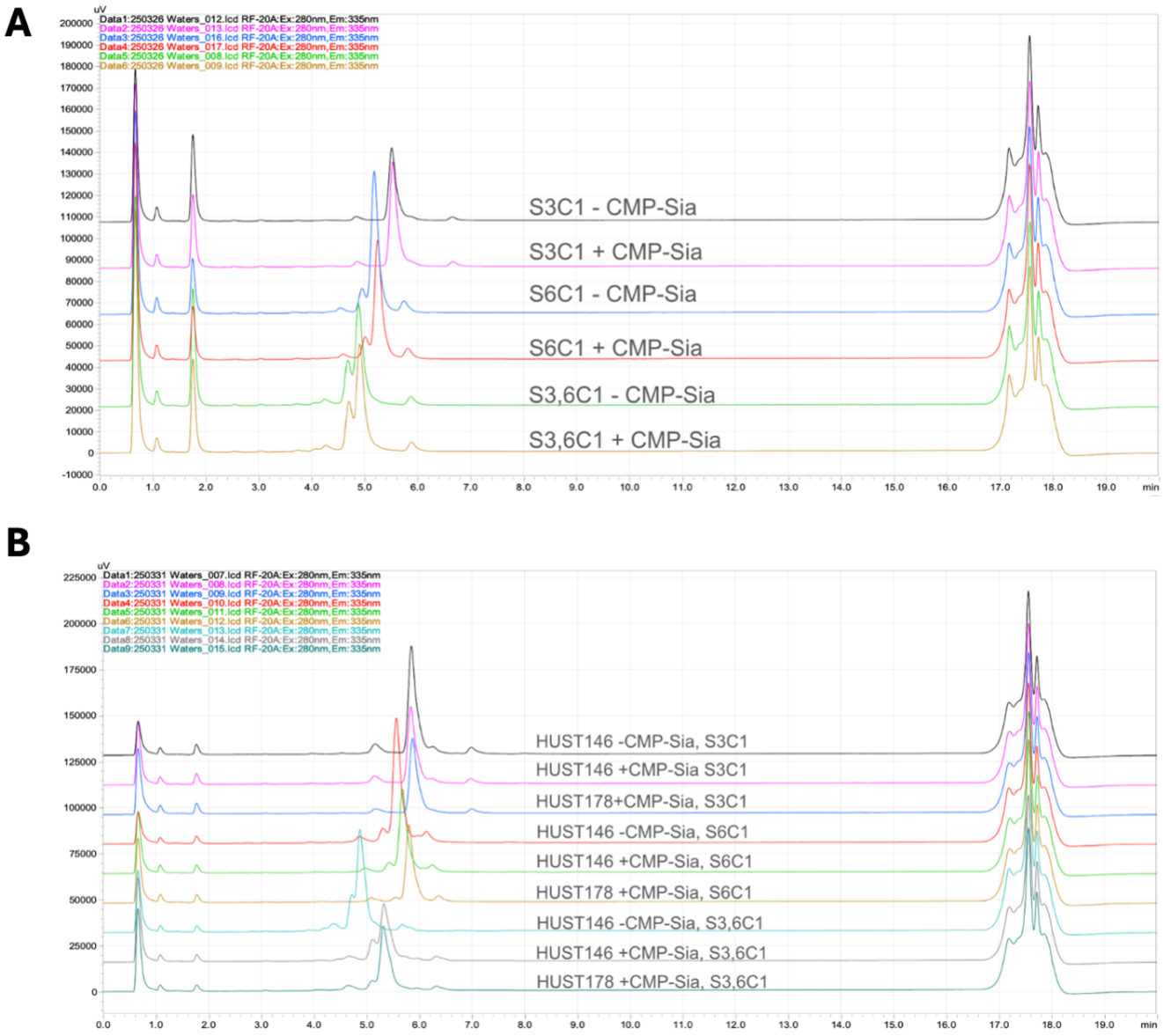

Figure 7. High performance liquid chromatography (HPLC) chromatogram of HUST146 and HUST178 activity assays showing relative absorbance as a function of retention time. The purified ST8Sia6 enzymes were tested for activity on various Core 1 substrates with and without donor sugar CMP-Sia. A) Chromatogram showing the activity assay of HUST178 on S3 Core 1, S6 Core 1, and S3,6 Core 1 substrates at pH 7 in HEPES buffer. B) Chromatogram showing the activity assay of HUST146 and HUST178 activity assay on S3 Core 1, S6 Core 1, and S3,6 Core 1 substrates performed at pH 6.3 in MES buffer.

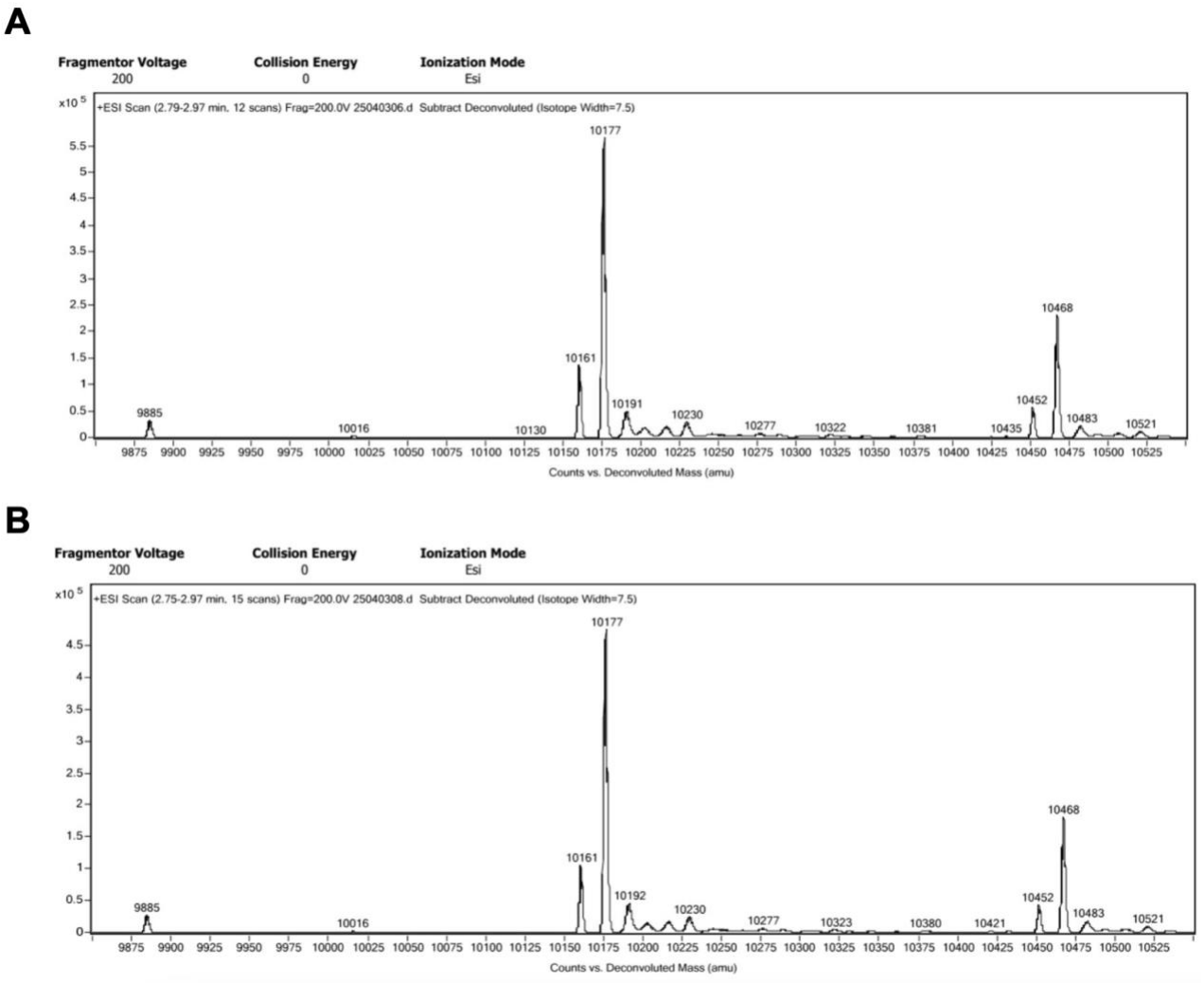

Figure 8. Liquid chromatography-mass spectrometry (LC-MS) deconvoluted protein mass spectrums. LC-MS was performed on the S3,6 Core 1 substrate activity assay with HUST146 + CMP-Sia and – CMP-Sia using the Agilent 6220 (oaTOF) mass spectrometer. The expected mass of the S3,6 Core 1 substrate is 10620.227 Dalton A) Deconvoluted mass spectrum of S3,6 Core 1 substrate from HUST146 – CMP-Sia reaction. B) Deconvoluted mass spectrum of S3,6 Core 1 substrate from HUST146 + CMP-Sia reaction.

Discussion

ST8Sia6 was successfully cloned, expressed, and purified using both a bacterial and eukaryotic expression system. The bacterial system yielded strong recombinant protein expression, however, despite testing various reaction temperatures, lengths, and co-factors, including $MnCl_2$, $MgCl_2$, and $CaCl_2$, little to no enzyme activity was observed. Notably, the activity assays were performed at a pH of 7.4, which is physiological pH, however, considering ST8Sia6 is located in the Golgi apparatus, a pH of 6.3 may have been more promising. Furthermore, 46C cells have been shown to be successful for expressing active recombinant proteins [11]; therefore, it is possible that activity was not observed due to other factors, such as post-translational modifications of the enzyme.

The eukaryotic expression system revealed a band at a higher molecular weight than expected for both HUST178 and HUST146. The increase in molecular weight may suggest the enzymes are glycosylated, which is consistent with the predicted glycosylation of ST8Sia6 by the GlycoDomainViewer Database. If ST8Sia6 is glycosylated and requires this post-translational modification for activity (such as binding the substrate) this may provide insight on why activity was not detected using the bacterial expression system. Moreover, the His-tag blot revealed inefficient binding of the HUST179 construct to the Ni-NTA resin, as the His-Tag was detected in the flow through. Improper folding of the HUST179 construct is an alternate plausible explanation for the lack of enzyme activity.

Lastly, both HUST146 and HUST178 positive donor sugar reactions show a shift on the HPLC compared to the negative control at pH 6.3. Specifically, the S6 Core 1 and S3,6 Core 1 substrates show an increase in retention time when the donor sugar is present. This is especially interesting as it is not observed with the S3 Core 1 substrate, potentially indicating the relevance of the α2-6-linked Sia. To investigate this further, liquid chromatography-mass spectrometry (LC-MS) was performed on the S3,6 Core 1 substrate reaction with HUST146 + CMP-Sia and -CMP-Sia at a pH of 6.3. While the mass spectrometry does not reveal a difference in spectrum between the two reactions, it does provide insight into the integrity of the substrates. Lastly, HPLC analysis revealed the substrate concentrations were 10X lower than expected in the activity assay. This is likely due to measuring the concentration of the substrates using a spectrophotometric measurement (NanoDrop, Thermo Scientific) and the extinction coefficients. Instead, the concentration would more accurately be measured by BCA assay.

While promising, there remains barriers to expressing and purifying active ST8Sia6. In addition to the reasons mentioned above, it is also possible that the GB1 Core 1 substrates were not suitable, and therefore, resulted in a lack of enzyme activity detection. Taken together, optimization is still required for both the enzyme expression and test substrate synthesis.

Abbreviations

HPLC: high performance liquid chromatography
HUST: human sialyltransferase
MonoSia: monosialic acid
OligoSia: oligosialic acid
PolySia: polysialic acid
Sia: sialic acid
ST8Sia6: ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6

Conflict of Interest

They authors declare they have no conflict of interests.

Ethics Approval and/or Participant Consent

This study did not require ethics approval and/or participant consent as our project does not involve collecting input and/or information from people, the use of human biological materials, data about people collected by someone else or involve animals.

Authors Contributions

AP: Made contributions to the design of the study, collected and analysed data, drafted the manuscript, and gave final approval of the version to be published.

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