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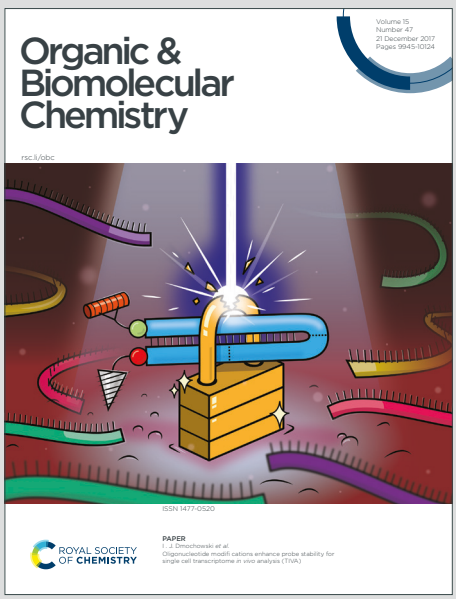

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Determinants of undesired α2-6-sialoside formation by PmST1 M144D

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Abstract

Sialyltransferases catalyze the regioselective glycosidic bond formation between sialic acid and a glycan acceptor. *Pasteurella multocida* α2-3-sialyltransferase 1 (PmST1) is a widely used enzyme that has gained widespread use in chemoenzymatic synthesis. In particularly, the PmST1 M144D mutant is routinely employed as an α2-3-sialyltransferase, although low levels of α2-6-sialyltransferase activity have been reported. Here, we discover that for certain acceptors, the formation of the undesired α2-6-sialoside can reach up to 20% of the product. To elucidate the factors that influence this regioselectivity, we systemically examined the effects of (i) sulfation of the acceptor, (ii) chemical nature of the aglycone, (iii) pH, and (iv) extent of reaction completion. Results indicate that sulfation at the 6-position of the GlcNAc or an β-ethyl-NHCBz aglycone are factors that can increase the amount of α2-6 sialoside product. Surprisingly, pH only had a small impact, and the amount of α2-6 sialoside product did not differ over the course of a reaction. These findings provide insights into the enzymatic specificity of PmST1 M144D and inform its optimized use in chemo-enzymatic synthesis of defined sialosides.

Introduction

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Sialyltransferases are a class of glycosyltransferase that catalyze the transfer of a sialic acid residue to the terminal position of oligosaccharides on glycoproteins, glycolipids, or free oligosaccharides (1-3). Sialyltransferases use an activated donor as their source of sialic acid in the form of cytidine-5′-monophospho-sialic acid (CMP-sialic acid). According to the Carbohydrate-Active enZYmes (CAZy) database, all eukaryotic sialyltransferases are classified in a single glycosyltransferase family, GT29 (4, 5). In contrast, bacterial sialyltransferases are categorized into four CAZy glycosyltransferase families: GT38, GT42, GT52, and GT80 (http://www.cazy.org).

Campylobacter Jejuni (Cst-I) from GT42 family and *Pasteurella multocida* (PmST1) are GT80 family members routinely used in chemoenzymatic synthesis (6). PmST1 has seen wide-spread use in chemoenzymatic synthesis due to desirable properties that include high expression levels in *E.*coli (100 mg/L culture), high catalytic activity, promiscuity toward a wide range of acceptors and modified CMP-sialic acid donors (6-12), and activity towards acceptors on the cell surface (13). PmST1 was initially characterized as an α2-3-sialyltransferase, exhibiting activity across a broad pH range (6.0-10.0) with an optimal pH range between 7.5 and 9.0. In addition to its main function, it also displays secondary enzymatic activities, including weak α2-6-sialyltransferase activity at pH < 7, α2-3-sialidase activity at pH values of 5.0–5.5 that is effectively the back reaction, and α2-3-trans-sialidase activity at pH values of 5.5–6.5 (6, 7, 10, 14, 15). The α2-6-sialyltransferase activity of WT PmST1 was reported to be minimal at pH values above 7.5 (8). However, the donor hydrolysis and α2-3-sialidase activities of PmST1 can significantly reduce the efficiency of sialyltransferase-catalyzed reactions, resulting in low product yields. To address this limitation, the PmST1 M144D mutant was engineered to reduce the α2-3 sialidase and donor hydrolysis activities (14). Other advantages of PmST1 M144D are that it is used as a versatile enzyme for synthesizing more complicated trisaccharides with acceptors that are not necessarily on the non-reducing end of the carbohydrate (16).

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In WT PmST1, deep donor binding induces closure of the active site and positions Trp270 to shape a well-defined acceptor pocket, enforcing specific acceptor orientations that favor either α2-3 or α2-6 linkage formation (14, 17). In the M144D mutant, the donor binding is shallower in the active site, preventing Trp270 from moving into place and leaving the acceptor region less organized. We speculate that such differences in the active site architecture could cause the mutant to have different regioselectivity depending on the nature of acceptor.

In this study, PmST1 M144D-catalyzed sialylation reactions were performed using a variety of acceptors and CMP-Neu5Ac as the donor. We discovered that PmST1 M144D can generate the undesired α2-6 sialoside product to significant extents when certain acceptors are used, and even under basic conditions. This observation led us to investigate the variables that influence the ratio of the desired α2-3 sialoside products versus the undesired α2-6 sialoside product. Specifically, a sulfation on the underlying GlcNAc residue and a certain aglycone structure such as, β-ethyl-NHCbz, can significantly enhance the undesired α2-6 sialoside product. Reactions were analyzed across different extents of completion and pH values, with basic pH only showing a modest decrease in the undesired α2-6 product. Notably, CST-06, a version of Cst-I that is a fusion protein with maltose binding protein (18, 19), and human ST3Gal4 did not show any α2-6-linked product under any conditions tested. Overall, our findings caution that chemoenzymatic reactions with PmST1 M144D requires purification to remove significant amounts of the α2-6-linked product for certain acceptors, with CST-06 being a suitable alternative in these cases.

Results and Discussion

PmST1 M144D activity on *O*-sulfated and non-*O*-sulfated disaccharide

We previously used PmST1 M144D for the synthesis of a series of sulfated sialosides on a LacNAc-β-ethyl-NH_2 core, which were purified by HPLC (20). To expedite the purification

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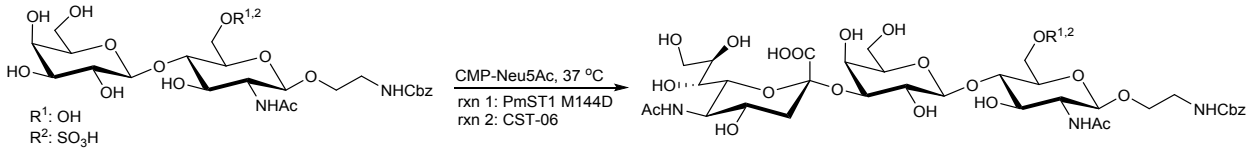
process, we began using size-exclusion chromatography (SEC; P-2 and LH-20 resins) to purify the Neu5Ac-α2-3-Gal-β(1→4)-6-*O*-sulfo-GlcNAc-β-ethyl-NHCbz product (**Scheme 1**). The β-ethyl-NHCbz aglycone was used to have an orthogonal protecting group for potential use in combination with azido sugars. These reactions went to completion and were readily cleaned up from other products (e.g. excess CMP-Sialic acid and CTP) by SEC. However, upon close analysis of the product by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, two sets of peaks were apparent for



Scheme 1. Enzymatic synthesis of sulfated and non-sulfated Neu5Ac-α2-3-LacNAc-β-ethyl-NHCbz. Reagents and conditions: CMP-Neu5Ac (2 eq), 100 mM Tris-HCl (pH 8.5), 20 mM $MgSO_4$, α2-3-sialyltransferases (PmST1 M144D and CST-06), 37 °C.

the H3 chemical shifts (**Figure 1A**), with only one mass observed by high resolution mass spectrometry (**Figure S1, SI**). In this experiment and the subsequent ones, the ^{1}H NMR spectra of all PmST1 M144D-catalyzed reactions were obtained after purification by SEC and were zoomed in on the $H3_{eq}$ region to clearly resolve and distinguish the distinct peaks corresponding to the different chemical shifts (ppm) of α2-3- and α2-6-linked sialosides. Importantly, comparison with acquired spectra of CMP-Neu5Ac and free Neu5Ac (**Figure S10)** confirmed that the additional peaks did not arise from these species; their chemical shifts were consistent only with formation of the α2-6-linked sialoside. The extent of α2-6-linked sialylated product was 19.3 ± 1.3% over four independent experiments. These results were observed despite using a pH of 8.5 in the enzymatic assay, which was reported to limit the amount of the α2-6 sialoside product using WT PmST1 (8). Parallel reactions were performed using two other sialyltransferases: CST-06 α2-3-sialyltransferase and *Photobacterium damselae* α2-6-sialyltransferase (Pd2,6ST). The

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products of these reactions were purified in the same manner as above, and only one set of H3 chemical shifts were observed with the anticipated linkage (**Figure 1A**). We were curious if the significant amount of α2-6 product was due to the 6-*O*-sulfation in the acceptor, therefore, we repeated experiments on non-sulfated LacNAc-β-ethyl-NHCbz and in this case the % of α2-6 product was 13.0 ± 1.0% over four independent experiments (**Figure 1B**). These findings indicate that the

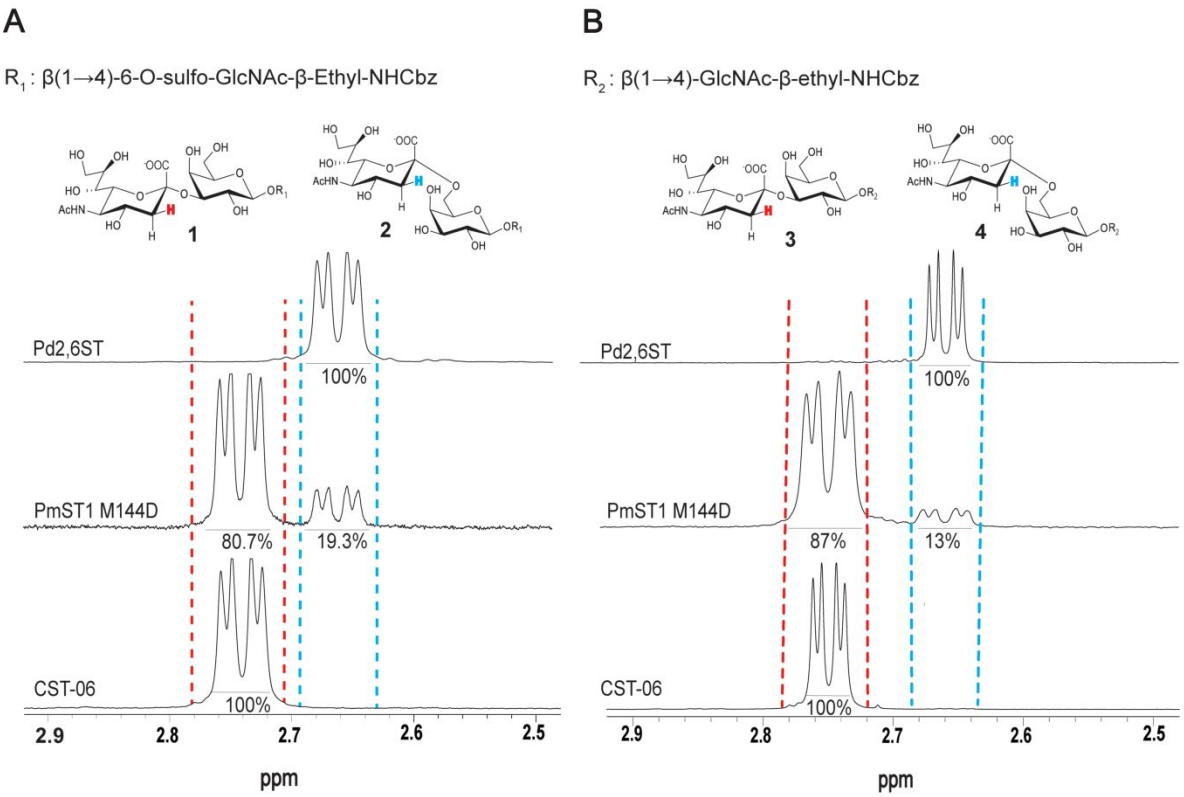

Figure 1. ^{1}H NMR analysis of product formation using different sialyltransferases. (A) The bottom spectrum shows the control compound Neu5Ac-α2-3-Gal-β(1→4)-6-*O*-sulfo-GlcNAc-β-ethyl-NHCbz, enzymatically synthesized using CST-06. The middle spectrum displays the product mixture obtained from a sialylation reaction catalyzed by PmST1 M144D, which contains both α2-3- and α2-6-linked sialylated products. The top spectrum shows the control compound Neu5Ac-α2-6-Gal-β(1→4)-6-*O*-sulfo-GlcNAc-β-ethyl-NHCbz, synthesized enzymatically using Pd2,6ST. **(B)** The bottom spectrum shows the control compound Neu5Ac-α2-3-LacNAc-β-ethyl-NHCbz, enzymatically synthesized using CST-06. The middle spectrum displays the product mixture obtained from a sialylation reaction catalyzed by PmST1 M144D, which contains both α2-

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3- and α2-6-linked sialylated products. The top spectrum shows the control compound Neu5Ac-α2-6-LacNAc-β-ethyl-NHCbz, synthesized enzymatically using Pd2,6ST.

presence of a sulfate group on the acceptor does influence the regioselectivity of PmST1 M144D, leading to formation of more of the undesirable α2-6 product. Therefore, we went on to examine additional parameters governing the regioselectivity of PmST1 M144D.

Influence of the aglycone structure on PmST1 M144D regioselectivity

To investigate the influence of aglycone structure on the regioselectivity of PmST1 M144D, two additional acceptors were investigated with different groups at the anomeric center: LacNAc in its reducing form and LacNAc-β-ethyl-N_3. As before, the trisaccharide products were analyzed by ^{1}H NMR for the H_{3eq} chemical shifts in the Neu5Ac of the trisaccharide products as a readout of α2-3 and α2-6 products. LacNAc-β-ethyl-N_3 averaged 8.2 ± 0.2% of the α2-6 product over three independent experiments while LacNAc averaged 4.7% ± 0.6% over three independent experiments (**Figure 2A,B**). This observation suggests that the β-ethyl-NHCbz aglycone, shown above (**Figure 1B**), negatively influenced PmST1 M144D regioselectivity as it had the highest amount of α2-6 product. The increased formation of the α2-6-linked product in the presence of the bulky NHCbz group may be attributed to steric effects introduced by the larger, more hydrophobic carbobenzyloxy, which could alter substrate orientation within the enzyme's active site. In summary, structural variations at the aglycone (reducing end) can significantly modulate the regioselectivity of PmST1 M144D, with more bulkier groups favouring more of the undesired α2-6 product.

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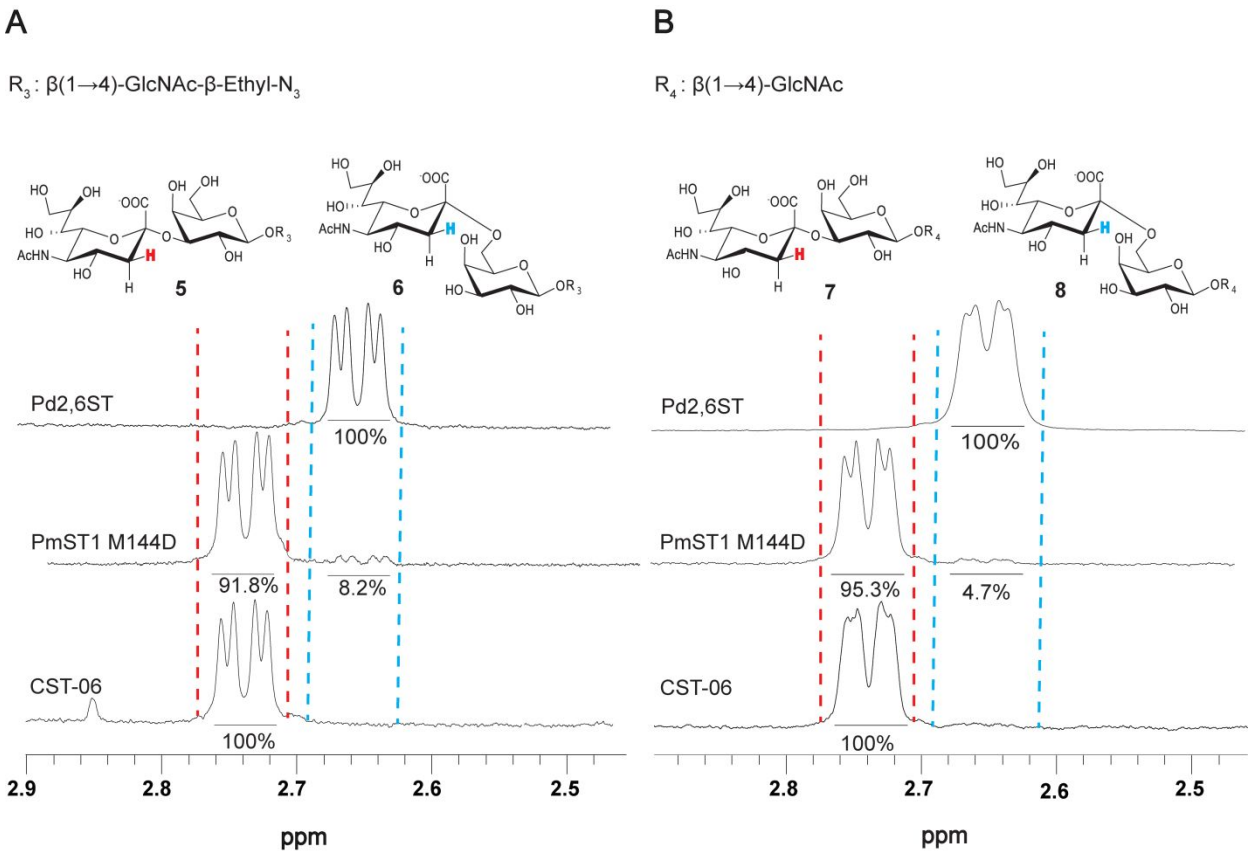

Figure 2. ^{1}H NMR analysis of sialylated products with acceptors bearing different aglycones. **(A)** The bottom spectrum shows the control compound Neu5Ac-α2-3-LacNAc-β-ethyl-N_3 enzymatically synthesized using CST-06. The middle spectrum displays the product mixture obtained from a sialylation reaction catalyzed by PmST1 M144D, which contains both α2-3- and α2-6-linked sialylated products. The top spectrum shows the control compound Neu5Ac-α2-6-LacNAc-β-ethyl-N_3, synthesized enzymatically using Pd2,6ST. **(B)** The bottom spectrum shows the control compound Neu5Ac-α2-3-LacNAc, enzymatically synthesized using CST-06. The middle spectrum displays the product mixture obtained from a sialylation reaction catalyzed by PmST1 M144D, which contains both α2-3- and α2-6-linked sialylated products. The top spectrum shows the control compound Neu5Acα2-6LacNAc, synthesized enzymatically using Pd2,6ST.

Influence of pH on PmST1 M144D regioselectivity

Previously, it was reported that the α2-6-sialyltransferase activity of WT PmST1 is significantly reduced at pH values above 7 (8). Despite using a pH of 8.5 in all experiments described above, which should have minimized the α2-6 product, we still felt that it was warranted to investigate how pH influences PmST1 M144D regioselectivity. Therefore, a series of reactions

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were conducted across a pH range of 5.0 to 9.0 using LacNAc-β-ethyl-N_3 as the acceptor. Product formation was monitoring over a 15-minute reaction time, and all reactions reached completion at every pH. Product formation was analyzed by ^{1}H NMR, as described above, to quantify the percentage of α2-6-sialylated product (**Figure 3**). Formation of Neu5Ac-α2-6-LacNAc-β-ethyl-N_3 decreased progressively with increasing pH but remained detectable at pH 9. Specifically, the

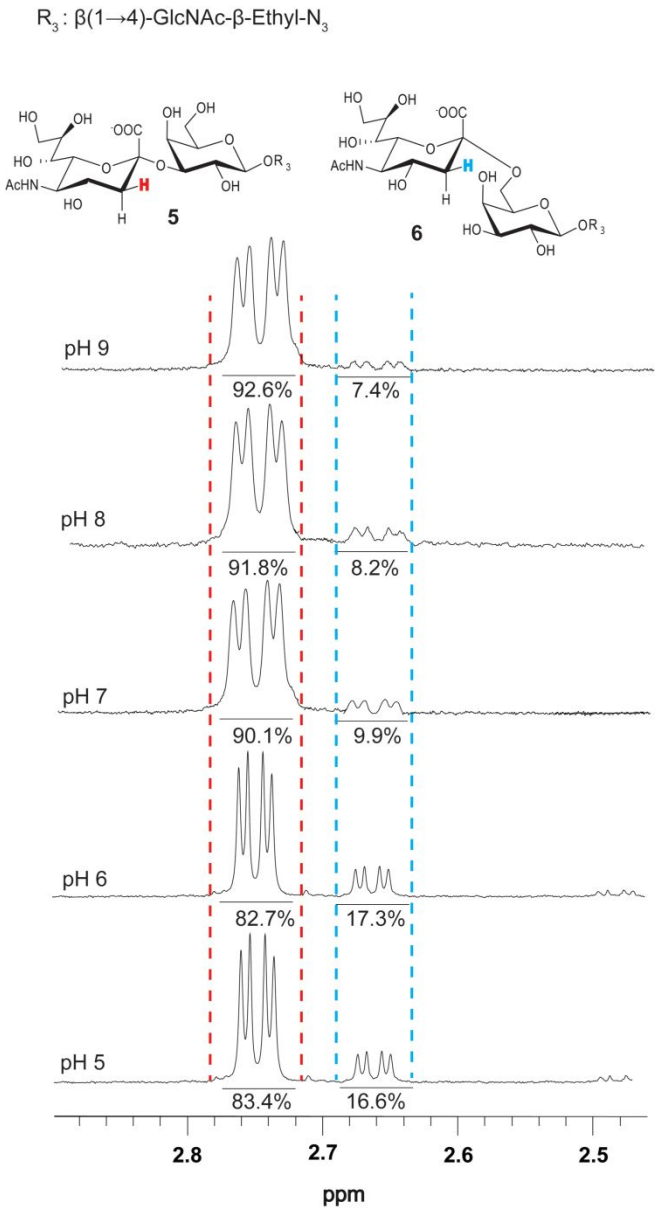

Figure 3. ^{1}H NMR analysis of PmST1 M144D-catalyzed sialylation of LacNAc-β-ethyl-N_3 across a pH range of 5.0 to 9.0. Formation of desired and undesired sialylated products, including Neu5Acα2-6LacNAc-β-ethyl-N_3, with pH-dependent changes in α2-6-sialylation peak intensities.

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average values of α2-6 product were: 16.6 ± 0.5% at pH 5.0, 17.3 ± 0.9% at pH 6.0, 9.9 ± 0.6% at pH 7.0, 8.2 ± 0.2% at pH 8.0, and 7.4 ± 0.1% at pH 9.0 over three independent reactions at each pH. These findings indicate that while α2-6-sialyltransferase activity is pH-sensitive and diminished under basic conditions, PmST1 M144D retains significant residual catalytic capacity for α2-6-linkage formation across a broad pH range. These results contrast with results reported with WT PmST1 where there was almost complete loss of α2-6-activity above pH 7 (8).

Regioselectivity of PmST1 M144D as function of reaction completion.

WT PmST1 possesses both sialidase and trans-sialidase activities, which are markedly reduced in the M144D mutant. Given that the benefits of suppressing these side activities in PmST1 M144D outweighs the drawback of slower sialylation kinetics (21), we sought to confirm that any residual activities did not influence the outcomes of reactions allowed to proceed to completion. Therefore, a series of sialylation reactions were conducted over varying time intervals. The reactions employed LacNAc-β-ethyl-NHCbz and Gal-β(1→4)-6-*O*-sulfo-GlcNAc-β-ethyl-NHCbz as the acceptor. Reactions were stopped at different time points, purified by SEC, and product purity was assessed by ^{1}H NMR for the ratio of α2-3 and α2-6 products (**Figures 4A, B**). After five minutes, approximately 25% of the total reaction had occurred, while the reaction was approximately 50% and 75% complete at ten and twenty minutes, respectively, and by 30 minutes, the reaction had gone to completion. The results show the percentage of α2-6 product was relatively constant throughout the course of the reaction. These results rule out any confounding effects form sialidase activity and demonstrate that the α2-3 and α2-6 products are formed with similar reaction rates.

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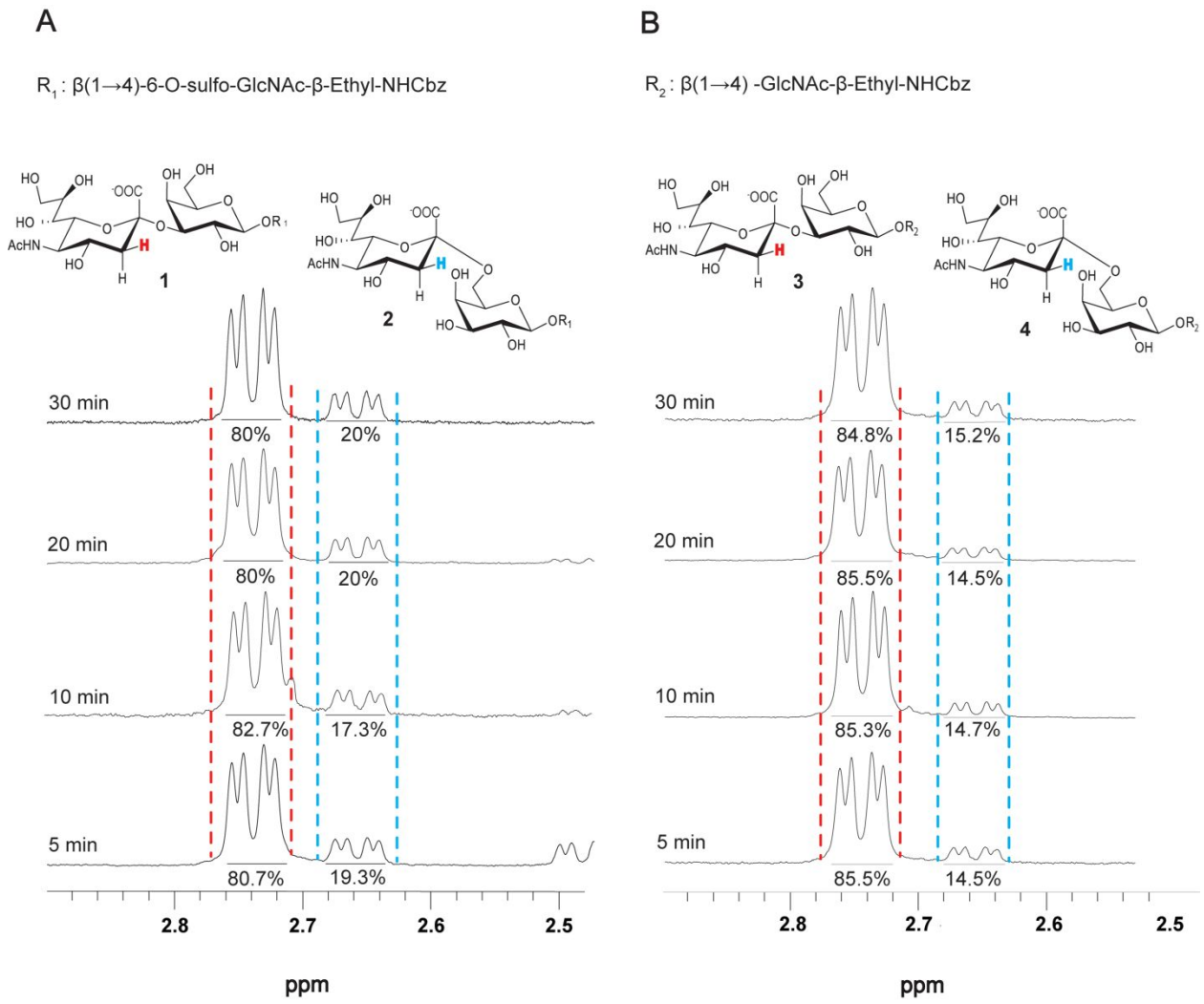

Figure 4. Time-course study of PmST1 M144D-catalyzed sialylation. (A) ^{1}H NMR analysis of H_{3eq} of Neu5Ac spectra show the integration of α2-3- and α2-6-sialylation reaction catalyzed by PmST1 M144D using Gal-β(1→4)-6-*O*-sulfo-GlcNAc-β-ethyl- as the acceptor substrate in the time-course spans from 5 to 30 minutes. **(B)** ^{1}H NMR analysis of H_{3eq} of Neu5Ac spectra show the integration of α2-3- and α2-6-sialylation reaction catalyzed by PmST1 M144D using LacNAc-β-ethyl-NHCbz as the acceptor substrate in the time-course spans from 5 to 30 minutes.

Conclusions

In conclusion, we have demonstrated that the structure of the acceptors significantly affects the ratio of α2-3- to α2-6-sialylated products formed by PmST1 M144D. Specifically, Gal-β(1→4)-6-*O*-sulfo-GlcNAc-β-ethyl-NHCbz led to a higher proportion of α2-6-sialylated products

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compared to the non-sulfated LacNAc-β-ethyl-NHCbz. Among the tested aglycone variants, LacNAc with β-ethyl-NHCbz resulted in a higher α2-6-sialylation ratio than both β-ethyl-N_3 and LacNAc without an aglycone. Although higher pH levels reduce the overall percentage of α2-6-sialylated products, they do not mitigate the α2-6-sialyltransferase activity of PmST1 M144D.

CRediT authorship contribution statement

FM: Writing – Original draft, Conceptualization, Data curation, Investigation, Validation, Visualization

MJ: Resources, Writing – review & editing.

WW: Resources, Writing – review & editing.

PW: Resources, Writing – review & editing.

MM: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Conflict of interest

There are no conflicts to declare.

Data availability

The ^{1}H NMR data of the compounds reported in this article are available in the article and Supplementary Information (SI).

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Data Availability

Data for this manuscript are available in the main text and supplementary information. Raw data underlying the manuscript include compound characterization and mass spectrometry raw chromatograms are securely archived at the University of Alberta and are available upon request.