Human neuraminidases have reduced activity towards modified sialic acids on glycoproteins

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Abstract

Multiple levels of diversity in sialic acid presentation can influence the substrate activity of sialosides for glycoside hydrolases. Few reports have investigated the specificity of human neuraminidase (hNEU) activity towards modified sialic acid residues that can occur on glycoproteins. Previously, we evaluated hNEU activity towards 9-*O*-acetylated sialic acid in glycolipid substrates and found that hNEU generally discriminated against 9-*O*-acetylated sialic acid over Neu5Ac. Here, we have investigated the substrate specificity of hNEU enzymes for a glycoprotein substrate (bovine submaxillary mucin) containing 9-*O*-acetylated and Neu5Gc residues. Using this model substrate, we observe a general trend for hNEU tolerance of Neu5Ac>Neu5Gc>>>Neu5,9Ac$_2$, consistent with our previous results with glycolipid substrates. These results expand our understanding of hNEU enzyme specificity and suggest that naturally occurring modifications of sialic acids can play a role in regulating hNEU activity.

Keywords: neuraminidase, sialidase, acetylated sialic acid, neuraminic acid

1. Introduction

The presentation of naturally occurring sialic acids feature diversity in the underlying glycoconjugate (non-terminal residues or aglycone), the glycosidic linkage, as well as the structure of the monosaccharide itself.[1] Catabolism of sialic acids by the human neuraminidase (hNEU) enzymes regulates cellular processes including cell-cell interactions, host pathogen interactions, tumor malignancy, and cell migration.[2-4] To date, four hNEU isoenzymes have been identified (NEU1-NEU4).[2] Previous work has found that sialic acid presentation influences recognition by hNEU and that isoenzymes have distinct substrate specificities. For instance, NEU1 prefers glycoprotein substrates while NEU3 prefers gangliosides.[5] The NEU2 isoenzyme has a strong preference for α(2→3) linked sialic acids over α(2→6) linked sialic acids, while NEU3 has only a moderate preference for α(2→3) linked sialosides.[6-7] Modified sialic acids are unstable or else difficult to prepare or purify which has impeded their study. As a result, hNEU substrate tolerance towards unusual sialosides remains largely undefined.

The most common sialic acid in humans is *N*-acetylneuraminic acid (Neu5Ac, **Figure 1, 1**), and most other sialic acids are biosynthetic descendants of Neu5Ac.[8] Common sialic acid modifications in mammalian systems include hydroxylation of the C5 amide to generate *N*-glycolylneuraminic acid (Neu5Gc, **2**) and acetylation at O9 to give the 9-*O*-acetylated sialic acid (Neu5,9Ac$_2$, **3**).[9] The ester linkage of Neu5,9Ac$_2$ is labile, particularly under basic conditions, which has historically resulted in this residue being overlooked.[10] We previously reported the first systematic study detailing hNEU activity towards Neu5,9Ac$_2$ and Neu5Gc on glycolipid analogs.[7] Although our results showed a general trend for hNEU substrate preference of Neu5Ac>Neu5Gc>>>Neu5,9Ac$_2$, several

outstanding questions remain for this system. We observed an increase in discrimination against Neu5,9Ac_2 for α(2→6)- versus α(2→3)-linked octyl sialyllactosides. Further, we observed that NEU4 discriminated against Neu5,9Ac_2 octyl sialyllactoside mimics of the ganglioside GM3 (**4, 5**), but had a preference for Neu5,9Ac_2 over Neu5Ac on simple fluorogenic substrates with a 4-methylumbelliferyl aglycone (**6**).[7] This result highlighted the need for studying hNEU substrate specificity on natural substrates. We reasoned that the impact of sialic acid 9-*O*-acetylation on hNEU may be dependent on the context of the sialic acid, in other words, the glycosidic linkage and reducing-end residue (or aglycone) could influence substrate activity for hNEU. As a result, we were inspired to explore the influence of modified sialic acid residues within glycoprotein substrates on hNEU activity.

Modified sialosides in glycoproteins have disctinct biological roles. Sialoglycoproteins containing 9-*O*-acetylated sialosides modulate B-cell immune response through disruption of sialic acid recognition by CD22.[11-12] Sialic acids on colonic mucins are heavily *O*-acetylated, providing protection from neuraminidases of gut bacteria.[13-14] Removal of 9-*O*-acetyl groups unmasks sialyl Lewis^x, a glycan involved in metastasis.[15-17] Sialomucin with Neu5,9Ac_2 has been identified as a differentiation marker on $CD4^+$ T-cells.[18] In cells without cell surface Neu5,9Ac_2, 9-*O*-acetylated sialoglycoproteins have been identified in the Golgi, suggesting an unexplored role for intracellular Neu5,9Ac_2.[19-20] Sialic acid *O*-acetylation of glycoproteins plays a role in pathological processes. In acute lymphoblastic leukemia (ALL) lower levels of Neu5,9Ac_2 glycoproteins were correlated with better prognosis,[21] and Neu5,9Ac_2 on glycoproteins has been implicated in the escape of cancerous lymphoblasts from the bone marrow to circulating blood.[22] Although humans lack the ability to produce Neu5Gc,[23] the residue can be incorporated into glycans from

dietary sources.[24-25] Glycoproteins and gangliosides containing Neu5Gc stimulate inflammatory response[26] and may provide a link between red meat and diseases such as atherosclerosis[27] and some cancers.[28]

Herein, we investigated hNEU activity towards Neu5,9Ac_2 and Neu5Gc sialosides on a glycoprotein substrate, the bovine submaxillary mucin. This study expands our understanding of hNEU processing of glycoproteins. Furthermore, these are the first data regarding NEU1 hydrolysis of Neu5,9Ac_2 and Neu5Gc sialosides, as previous efforts with glycolipid substrates were unable to provide insight towards NEU1 specificity.

2. Results and Discussion

A common strategy used to probe the effects of *O*-acetylated sialic acids on glycoproteins is treatment of a sialoglycoprotein with base to hydrolyze the *O*-acetyl esters, followed by comparison of the treated protein to an untreated control.[13, 29-31] Bovine submaxillary mucin (BSM) is an inexpensive commercially available glycoprotein containing α(2→6) linked sialosides enriched in *O*-acetylated sialic acids, particularly Neu5,9Ac$_2$, making it a popular model to study 9-*O*-acetylated glycoproteins.[32-33] This strategy has been used to study sialic acid released by sialidase and sialate *O*-acetylesterase activity in bacteria.[14, 34] The capacity for neuraminidase to hydrolyze acetylated sialic acids versus its non-acetylated variant is determined by comparing the amount of sialic acid released from control BSM to BSM pre-treated with NaOH to hydrolyze *O*-acetyl esters.[14] After enzyme treatment, the samples were again submitted to basic conditions to remove any *O*-acetyl groups to standardize the analysis to detection of only Neu5Ac and Neu5Gc across all samples (**Figure 2A**). Released free sialic acid was labeled with a 1,2-phenylenediamine and detected after separation using HPLC.[14] Labeling of free sialic acids with a 1,2-phenylenediamine such as *o*-phenylenediamine (OPD),[35] 1,2-diamino-4,5-dimethoxybenzene (DMB),[36] or 4,5-dimethylbenzene-1,2-diamine (DMBA),[37] followed by separation using HPLC and fluorescence or absorbance detection, is a widely used method to resolve different sialic acids.[14, 34-38]

When we implemented this assay to study hNEU, we found that the assay had insufficient sensitivity for our purposes. We calculated a limit of detection (LOD) of 1.3 μM (**Figure S1, Materials and Methods**) for Neu5Ac labeled with *o*-phenylenediamine

(OPD) with absorbance detection at 350 nm. Previous studies using this approach have reported a small but significant signal with their enzyme-free base-treated negative control.[14] The background noise was inconsequential in studying bacterial neuraminidases where the signal was approximately 10-fold higher than the background; however, our preparation of hNEU enzymes were at least 8- and 15-fold lower efficiency than bacterial neuraminidases from *C. perfringens* (NanI) and *A. ureafaciens* (siaAU), respectively (**Figure 3**, **Figure S2**). With the reduced efficiency of hNEU towards BSM, the noise resulting from hydrolysis of the *O*-acetyl groups with base was prohibitive for the study of hNEU substrate tolerance.

To account for the lower efficiency of the hNEU towards BSM we modified the assay to avoid pre-treatment of BSM with base, meaning we could not compare the amount of sialic acid released from BSM with or without *O*-acetyl modifications.[34] Instead, we calculated the ratio of modified sialic acids released by hNEU relative to Neu5Ac (**Figure 2B**). This approach eliminated noise from the negative control and allowed us to study hNEU activity; however, it complicated the analysis from detection of Neu5Gc and Neu5Ac to at least five different sialic acid species. We identified Neu5Gc, Neu5Ac, and Neu5,9Ac_2 using standards, and assigned Neu5,7Ac_2 and Neu5Gc9Ac based on the order of elution from the column.[14, 39]

To determine substrate preferences for hNEU without pre-treatment of the samples with base, we needed to determine the sialic acid composition of BSM. Two methods typically employed for the total release of *O*-acetylated sialic acids from glycoconjugates are acid hydrolysis and hydrolysis with siaAU; however, both methods have limitations. Acid hydrolysis is widely used for total sialic acid release from glycoconjugates.[40]

Conditions for acid hydrolysis of sialosides will also hydrolyze *O*-acetyl esters and may lead to migration of acetate groups from O7 and O8 to O9.[41-42] Treatment with siaAU is an alternative method, but is not as efficient as acid treatment[41, 43] and discriminates against Neu5Gc and *O*-acetylated sialic acids.[14, 44-45] In our experiments, acid hydrolysis gave a ratio of NeuGc to Neu5Ac of 0.7 ± 0.1 and treatment with siaAU gave a ratio of 0.59 ± 0.05. For the ratio of Neu5,9Ac_2 to Neu5Ac, acid hydrolysis gave 1.1 ± 0.2, and treatment by siaAU gave a ratio of 1.5 ± 0.1 (**Figure 4.**, **Figure S2**). The lower ratio of Neu5,9Ac_2 to Neu5Ac from acid hydrolysis is likely due to the loss of *O*-acetyl groups. Based on these results, we concluded that an observed ratio below 1.1 - 1.5, with respect to acid or enzyme control conditions, indicated a preference for Neu5Ac over Neu5,9Ac_2.

Due to the generally low activity of the hNEU enzymes, in some cases we quantified the minimum preference of hNEU for Neu5Ac by calculating a ratio of Neu5,9Ac_2 or Neu5Gc to Neu5Ac where the numerator was defined by the limit of detection (LOD) for the assay (**Figure 4**). In these cases the absolute preference for Neu5Ac over the modified sialic acids remains undefined, so we instead report a minimum preference. We observed that all hNEU isoenzymes had a moderate preference for Neu5Ac over Neu5Gc (**Figure 4A**), consistent with our previous study of hNEU activity on Neu5Gc-containing glycolipids **5** and **6**.[7] This preference was most pronounced in NEU3, and was shared by both bacterial enzymes tested. NanI discriminated against Neu5Gc to a greater extent than Neu5,9Ac_2 (**Figure 4**), consistent with previous reports.[44]

The discrimination of Neu5,9Ac_2 by hNEU was common to all isoenzymes. For NEU2, NEU3, and NEU4 we could quantify a greater than 10-fold preference for Neu5Ac over Neu5,9Ac_2 (**Figure 4B**). Comparison to the acid or the siaAU positive control

indicated an 11- or 16-fold preference, respectively, of NEU2 for Neu5Ac over Neu5,9Ac$_2$. Previous work from our group and others has found that NEU2 has very strict substrate tolerance which can be attributed to its constrained C9 active site pocket.[7, 45] For NEU3 we detected a 9- to 12-fold preference; and for NEU4 an 11- to 15-fold preference for Neu5Ac over Neu5,9Ac$_2$. Experiments with NEU1 found at least a 1.5- to 2-fold preference for Neu5Ac over Neu5,9Ac$_2$; however, we note that these experiments used a lower enzyme activity where the Neu5Ac signal was close to the LOD (see **Materials and Methods**) and the Neu5,9Ac$_2$ value was defined by the LOD. Previous results with an artificial substrate based on 4MU-NANA (**4**) found a 2-fold preference for Neu5Ac over Neu5,9Ac$_2$, and a selective NEU1 inhibitor developed by our group contains a C9 acetamido group, suggesting that NEU1 may be more tolerant of an acetate at C9.[46] It is important to note that the ratiosof Neu5,9Ac$_2$ to Neu5Ac released by hNEU were all defined by the limit of detection and thus indicate a lower limit, rather than absolute, substrate preference. As a result the discrimination against Neu5,9Ac$_2$ may be even greater than calculated here. Further study with more sensitive methods will be necessary to determine if the tolerance of NEU1 for Neu5,9Ac$_2$ is significantly different from the other hNEU isoenzymes.

We have expanded our study of hNEU activity towards Neu5,9Ac$_2$ to include glycoprotein substrates. This work provides the first data for NEU1 substrate tolerance towards Neu5,9Ac$_2$ on glycoprotein substrates of hNEU. Considering that the sialic acid reducing-end substituents and aglycone influence hNEU substrate tolerance, these data on sialoglycoprotein substrates add an important measure of NEU2, NEU3, and NEU4 activity. Consistent with our previous study of Neu5,9Ac$_2$ – containing glycolipids, we again observed the general trend of Neu5Ac > Neu5Gc > Neu5,9Ac$_2$. Further study on

diverse sialoside substrates, particularly α(2→8)-linked sialosides, will be important to generate a complete understanding of hNEU substrate specificity towards 9-*O*-acetylated sialic acids.

3. Materials & Methods

3.1 General Methods

HPLC was performed with a Waters Delta 600 pump, a Waters 600 controller, and a Waters 2996 photodiode array (PDA) detector with Empower 2 software (Waters Ltd., Mississauga, ON, Canada). Bovine submaxillary mucin Type I-S, 9-24 % bound sialic acids was purchased from Sigma Aldrich. Neuraminidases from *Clostridium perfringens* (NanI) and *Arthrobacter ureafaciens* (siaAU) were purchased from Sigma Aldrich. The human neuraminidase enzymes NEU2, NEU3, and NEU4 were expressed as fusion proteins with maltose binding protein, as previously reported.[47-49] NEU1 was overexpressed in HEK293E cells and used as crude cell lysate.[7] Specific activity of the neuraminidase enzymes were normalized in comparison to a standard curve with NanI using 4-methylumbelliferyl α-$_D$-*N*-acetylneuraminic acid (4MU-NANA) as a substrate. Unless otherwise stated, results reported are technical replicates, with preliminary independent replicates confirming the trends reported.

3.2 LOB and LOD determination

To determine the limit of detection (LOD) for the quinoxaline derivatized sialic acids, 30 µL of 5 µM Neu5Ac (or H_2O for limit of blank, LOB) was mixed with 30 µL of 40 mM TFA, 30 µL derivatization mixture (25 mM *o*-phenyldiamine (OPD), 18 mM $Na_2S_2O_4$, 0.5 M ß-mercaptoethanol), and 10 µL 2 mM *p*-nitrophenol (internal standard). The mixture was heated for 3 h at 50 °C after which it was analyzed by HPLC on a C-18 reverse phase column with isocratic elution with 84:9:7 H_2O:acetonitrile:methanol and detection at 350 nm. The LOB and LOD were calculating using peak areas normalized to the internal standard with $LOB = mean_{blank} + 1.645(StDev_{blank})$ and $LOD = LOB + 1.645(StDev_{sample})$.[50]

The peak areas for the LOB were obtained by integrating the baseline with the average retention times and peak widths detected for the LOD.

3.3 Acid hydrolysis of sialic acid from bovine submaxillary mucin

Bovine submaxillary mucin (250 µg) was dissolved in 40 µL of water and 30 µL acetic acid. The mixture was heated for 3 h at 80 °C to release all sialic acid.[43] Derivitization mixture (25 mM *o*-phenyldiamine (OPD), 18 mM $Na_2S_2O_4$, 0.5 M ß-mercaptoethanol) and 10 µL of 2 mM *p*-nitrophenol were added. Protein was removed using centrifugal filters (30 kDa molecular weight cut-off) for 30 min at 14,000 rpm. The filtrate was heated for 3 h at 50 °C and the labeled sialic acid was analyzed after separation by HPLC on a C-18 reversed phase column with an isocratic solvent composition of H_2O:acetonitrile:methanol 84:9:7. Elution peaks were detected at 350 nm.

3.4 Neuraminidase release of sialic acids from bovine submaxillary mucin

Bovine submaxillary mucin (250 µg) was dissolved in 20 µL sodium acetate buffer pH 4.5 (5.5 for NEU2). 20 µL (1 mU) of enzyme was added (or water as a negative control). After 5 h incubation at 37 °C, 30 µL 40 mM TFA, 30 µL derivatization mixture (25 mM *o*-phenyldiamine (OPD), 18 mM $Na_2S_2O_4$, 0.5 M ß-mercaptoethanol), and 10 µL of 2 mM *p*-nitrophenol were added. Protein was removed using centrifugal filter units (30 kDa molecular weight cut-off) for 30 min at 14,000 rpm after which the filtrate was heated for 3 h at 50 °C. The labeled sialic acid was analyzed after separation by HPLC on a C-18 reversed phase column with an isocratic solvent composition of H_2O:acetonitrile:methanol 84:9:7. Peaks were detected at 350 nm. For NEU1, 500 µg of bovine submaxillary mucin was used. The volumes of all reagents were also doubled, and 1 mU enzyme activity was used.

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Supplementary data

Supplementary data to this article can be found online including calibration curves and peak areas from hydrolysis experiments with neuraminidase enzymes.

Figures & Tables

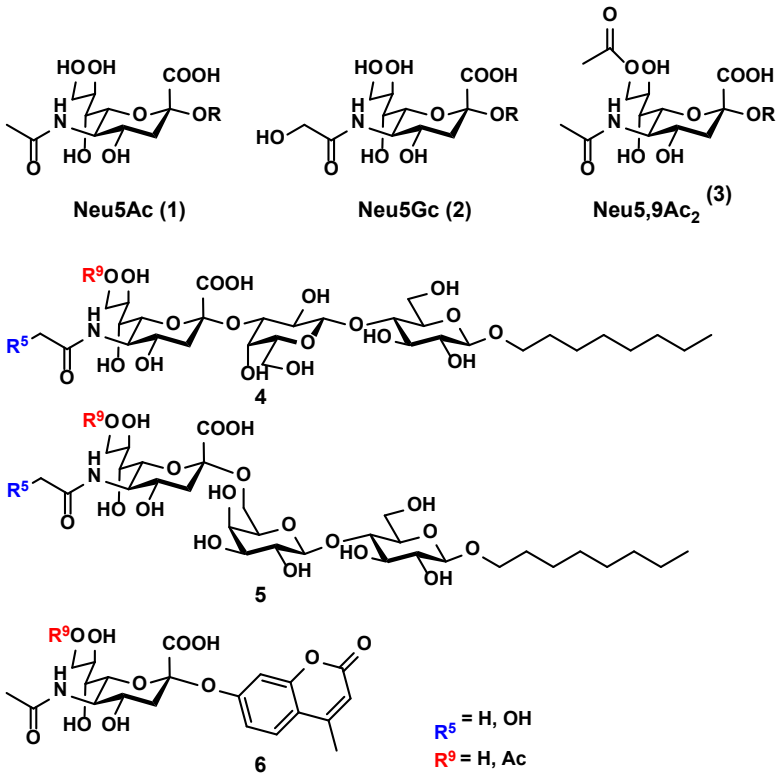

Figure 1: Human neuraminidase substrates used in previous work. Compounds **4** and **5** were tested as Neu5Ac, Neu5Gc, and Neu5,9Ac$_2$ analogs, compound **6** was tested as Neu5Ac and Neu5,9Ac$_2$ analogs.

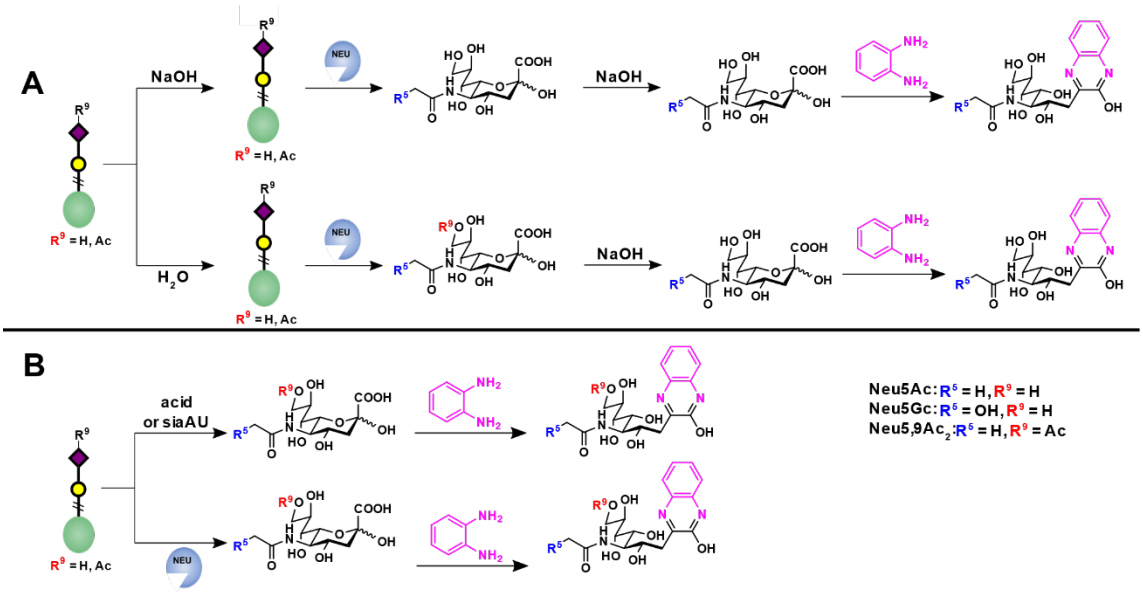

Figure 2: Assay workflow to detect modified sialic acid released from glycoproteins (A) comparing sialic acids released before and after hydrolysis of *O*-acetyl groups; and (B) comparing to total hydrolysis of sialic acids (used in this work).

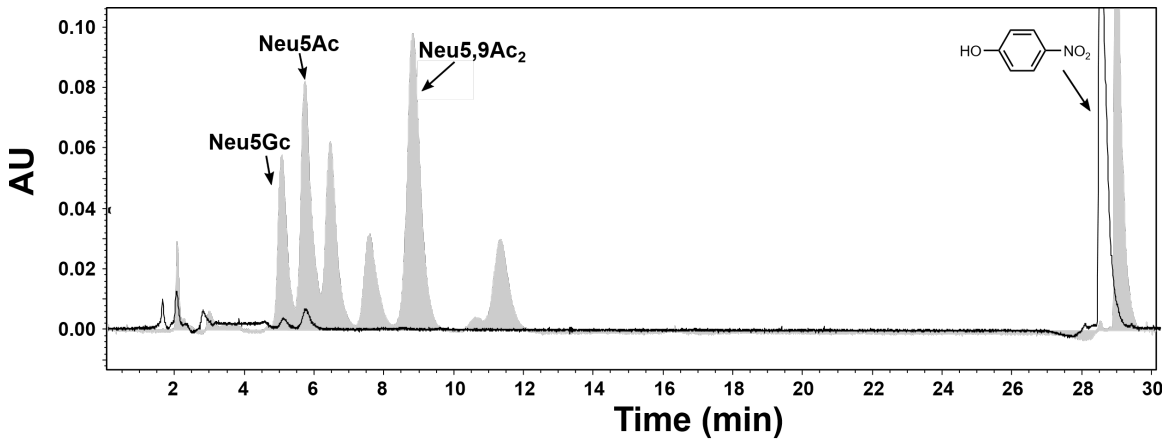

Figure 3: Representative run of sialic acid released from bovine submaxillary mucin by siaAU (grey) and by NEU2 (black). Selected assignments for Neu5Ac, Neu5Gc, Neu5,9Ac$_2$, and *p*-nitrophenol (IS) are shown by arrows. Enzyme activity was normalized to 1 mU based on hydrolysis of 4MU-NANA.

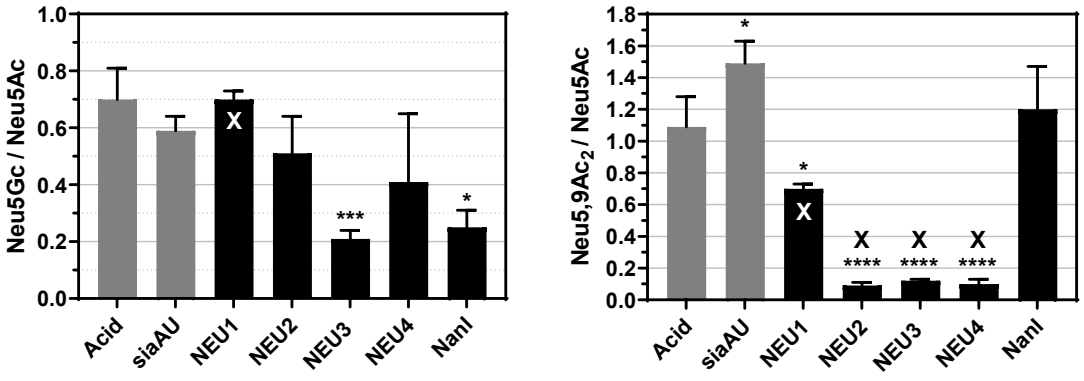

Figure 4: Release of modified sialic acids from bovine submaxillary mucin relative to Neu5Ac. Results are shown as the mean of triplicate experiments, with error bars denoting one standard deviation. Hydrolysis controls with acetic acid or siaAU are shown as grey bars. Unpaired t-tests comparing enzymatic conditions to release of sialic acid with acid were performed, and are indicated with * = $p > 0.05$, *** = $p > 0.001$, **** = $p > 0.0001$. Values marked with an "X" indicate that the nominator for the calculated ratio was defined by the limit of detection of the assay rather than by a detected peak.

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