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Improved Sialic Acid Quantitation Assay Suitable for Robotics and Process Analytical Technology Applications

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Abstract

We previously developed a sensitive, high-throughput, enzyme-based sialic acid assay suitable for application in a 96-well plate format. This method relies on the detection of hydrogen peroxide, generated through a coupled enzyme system in which sialic acid molecules are converted to mannosamine and pyruvate via the neuraminyl aldolase followed by a subsequent conversion of pyruvate into hydrogen peroxide and acetylphosphate with pyruvate oxidase. Although this method has been proven to be useful for routine monitoring of the sialic acid content of therapeutic proteins, the execution is moderately complex and time consuming, involving multiple steps and reagents. For example, three sets of reagents along with distinct buffers or organic solvent (DMSO) are added at different stages.

We have now simplified the method so that only one reagent mixture is required to carry out the two-step enzyme process with all enzymes acting simultaneously. These modifications significantly reduce the reagent preparation time, eliminate the need for the DMSO solvent, and streamline the method by consolidating multiple pipetting operations into a single step. Furthermore, this new simplified method has greater precision, higher signal-to-background ratio compared to the previous method, and is much easier to adapt to robotics. For several well-known glycoproteins, relative assay errors were reduced by half (2 – 3% for replicate analysis, compared with about 5 – 7% with the previous method) and signal-to-background ratios were tripled (>30:1 at 1000 pmol levels, compared with 10:1 with the previous assay). Detection with fluorescence can be made at 50 – 1000 pmol of sialic acid and absorbance detection can be made at 1000 – 5000 pmol.

Introduction

Although a number of methods for quantitation of sialic acid have been developed, one of the most frequently employed methods is analysis by high-performance liquid chromatography (HPLC) of pre-column derivatized fluorescent-labeled samples. HPLC analysis is advantageous because it offers an extremely high level of sensitivity (femtomole range) and allows the identification of individual sialic acid species. However, HPLC analysis is cumbersome and time consuming, particularly when analysis of multiple samples is required. Moreover, the method is not readily adaptable to a high-throughput modality.

We previously developed a sensitive enzyme-based method for high-throughput sialic acid quantitation on therapeutic proteins. Our method utilizes neuraminyl aldolase coupled with pyruvate oxidase to convert released sialic acid to hydrogen peroxide. The resulting peroxide reacts stoichiometrically with a dye that is intensely fluorescent (or absorbance). This method allows sample digestion, conversion, detection, and quantitation to be performed in a single well of a 96-well microtiter plate for fast and simple processing.

This method has proven to be extremely valuable for monitoring sialic acid on therapeutic proteins during process development campaigns, but the number of operator manipulations are extensive, requiring multiple pipetting operations and the preparation of multiple assay components.

We have reformatted and simplified the previous assay, combining all reaction components into a single reagent mixture and eliminating the need for an organic solvent and different buffers. The reformatted assay greatly reduces the hands-on time and requires only two manipulations by the operator, reconstituting the lyophilized assay mixture in cold water and adding the resulting mixture to the sialic acid standards and protein samples.

The progress of the assay can be monitored directly by following the formation of the fluorescence or absorbance of the reporter dye. In most cases, the reaction is complete after 20 minutes at 37°C or 40 minutes at room temperature.

Materials and Methods

Release Enzyme: Glyko® Sialidase A™-51 (recombinant gene from *Arthrobacter ureafaciens*, expressed in *E. coli*; ProZyme product code GK80045). Assay mixture: pyruvate oxidase (*Aerococcus* sp.); N-acetylneuraminic acid aldolase *(E. coli)*; horseradish peroxidase; and dye. Sialic Acid Standard: Sialic acid (E. coli, 98% pure) was thoroughly dried over P_2O_5 for 1 week under vacuum. A stock solution, in water, was prepared at a concentration at 100 m*M*. Instruments: Absorbance determinations were made on a Multiskan microtiter plate reader (Labsystems). Fluorescence determinations were made on a Victor² 1420 Multilabel Counter (PerkinElmer). Thermomixer R microtiter plate shaker with temperature control (Eppendorf) was used for plate incubation before measurements. Microtiter Plates: Clear, flat bottom plates (Corning) were used for absorbance measurements. Black, flat bottom plates, non-treated, polystyrene (Corning-Costar) were used for fluorescence measurements.

The Method: Sialic acid was released from the target proteins by enzyme digestion using 20 mU – 40 mU of Sialidase A-51, from 2 – 40 µg of protein substrates. Digestions were performed in a 96-well microtiter plate in 50 m*M* phosphate buffer (pH 6.0) in a total volume of 50 µl for 0.5–1 hour at 37°C. Solutions of the Sialic Acid Standard were added to empty wells at various levels and water was added to bring them to a total volume of 50 µl. The assay mixture, once reconstituted, was added immediately to the wells with the sialic acid standards and the protein samples. After adding the assay mixture, the plate was mixed for ~30 seconds on a shaker. Then, the plate was incubated, with and without shaking, at room temperature and at 37°C for varying periods of time, with a total volume of 120 µl in each well. Fluorescence or absorbance of the samples was monitored over the course of the reaction.

Figure 1 Enzyme-Coupled Assay for Sialic Acid Quantitation on Glycoproteins Using Sialidase Release

Step 1: Release of Sialic Acid

Glycoprotein + Sialidase ⟶ Sialic Acid + Glycoprotein

Digestion times for optimal release of sialic acid can be determined independent of the detection step. This allows for variations in the accessibility of the sialidase to the sialic acids residues on different types or classes of proteins.

Step 2: Detection of The Released Sialic Acid Using Coupled-Enzymes

N-acetylneuraminic acid aldolase catalyzes the reversible reaction:

Sialic Acid ⟷ Mannosamine + Pyruvic Acid

Variants of sialic acid, such as N-glycolyl- and some O-acetylneuraminic acids, are also converted to pyruvic acid and the corresponding mannosamine. Then pyruvate oxidase catalyzes the reaction:

Pyruvic Acid ⟶ Acetylphosphate + H_2O_2

Under the proper conditions, the forward aldolase reaction predominates; and when coupled with H_2O_2 generation, the reaction will go to completion.

Hydrogen peroxide forms a 1:1 molecular complex with the selected dye when catalyzed by horseradish peroxidase; the complex is intensely fluorescent (or absorbant) and can be readily quantitated.

Dye + H_2O_2 ⟶ Reporter Dye

Table 1 Comparison of the Improved Assay Method and the Previous Assay Method

	Signal to Background Ratio (Fluorescence Detection at the 1000 pmol level)	Relative Error Between Replicates	Assay Time (for duplicate sialic acid standards, triplicate protein blanks and samples of 3 different proteins)
Improved Assay	~30:1	1 – 3%	• Add protein samples and blanks – 5 min • Protein digest – 30 min • Add sialic acid standards – 2 min • Add assay mixture and incubate – 30 min • Read plate – 1 min **Total time: 68 minutes**
Previous Assay	~10:1	5 – 7%	• Hydrate conversion reagents – 2 hours • Add protein samples and blanks – 5 min • Protein digest – 30 min • Add sialic acid standards – 2 min • Add conversion reagents and incubate – 30 min • Add dye and HRP – 15 min • Incubate plate – 10 min • Read plate – 1 min **Total time: 213 minutes**

Figure 2 Sialic acid standard curve at the 200 – 1000 pmol scale using fluorescence detection.
(y = 7785.2 x + 13.85, R2 = 0.9991)

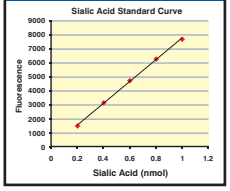

Figure 3 Sialic acid standard curve at the 1 – 5 nmol scale using absorbance detection.
(y = 0.0991 x – 0.007, R2 = 0.9990)

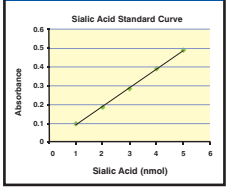

Figure 4 Change in Slope of Sialic Acid Standard Curves Over Time: Comparing Various Plate Incubation Methods

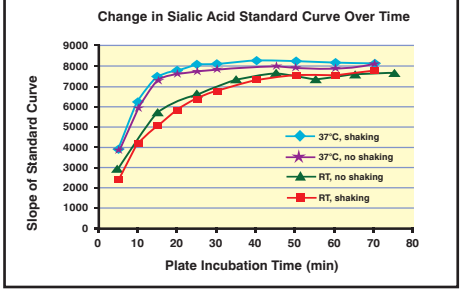

Table 2 Sialic Acid Content of Typical Glycoproteins: Comparison between Previous Assay, Improved Assay, and Standard Reported Values Using Fluorescence Detection

Glycoprotein	MW (kDa)	Amount of Protein Analyzed	Sialic Acid (mol/mol protein)*		
			Reported	Improved Assay	Previous Assay
Fetuin (bovine)	48,000	0.037 nmol/1.8 µg	13 - 17[a,c]	14.8 ± 0.40	15.6 ±0.25
Transferrin (human)	78,000	0.119 nmol/9.3 µg	3.8[d]	4.6 ± 0.05	4.9 ± 0.18
IgG (monkey)[1]	150,000	0.260 nmol/39 µg	1.1[e]	0.9 ± 0.02	0.8 ± 0.02

* Shown are sialic acid values from triplicate determinations ± S.D.
[1] Sialic acid is almost exclusively NGNA.
[a] (Townsend *et al.*, 1997); [c](Spiro, 1960); [d](Rohrer *et al.*, 1997); [e](Regoeczi *et al.*, 1979); and [a](Raju *et al.*, 2000).

Table 3 Comparison of Assay Performance Between Previous and Improved Assay: Comparing Relative Error Rate between Replicates

Glycoprotein	Relative Error (%)	
	Improved Assay	Previous Assay*
Bovine Fetuin	2.1	6.3
Human Transferrin	0.8	3.6
Monkey IgG	1.5	2.0

* Based on historical data

Results

The overall scheme for the formation of free sialic acid and its subsequent conversion to hydrogen peroxide is shown in Figure 1. Sialidase A-51 (a 51 kDa isoform of Sialidase A) was selected as a means to release the sialic acid from the protein substrates because its smaller size allows access to more sterically hindered sialic acid residues and its broad substrate specificity allows cleavage of all molecular species of sialic acids, including most O-acetylated and N-glycolyl forms **(Step 1)**. After the complete release of sialic acid, the sample was treated with sialic acid aldolase, pyruvate oxidase, and horseradish peroxidase in the presence of the reacting dye **(Step 2)** for color development for fluorescence or absorbance detection. A comparison of the improved and previous assay methods is summarized in Table 1.

Linearity of response using sialic acid standard – A linear response for the sialic acid standard was observed with both fluorescence and absorbance measurements using the improved assay method. In most cases, the R-squared values were about 0.999+ (Figure 2 and Figure 3). The time course for the assay was monitored with fluorescence detection by following the change in slope of the linear regression curve of the standard at room temperature or 37°C. The maximum signal was obtained after 40 minutes of incubation at room temperature and 20 minutes at 37°C (Figure 4). The signal at 1000 pmol, relative to the background fluorescence, remained relatively constant at about 30:1 for up to 70 minutes.

Determination of sialic acid levels on glycoproteins – Several well-characterized proteins were analyzed for sialic acid content using the improved assay method. The improved assay method results correlated with published values and previous assay results for all proteins, including an immunoglobulin (Table 2).

The relative error rate between replicates for the improved assay method was less than half of the rate for the previous method. The error rate for the improved assay method was 1 – 3% compared to 5 – 7% for the previous assay method (Table 3). Similarly, intra-assay variation was reduced by 3 – 5%.

Conclusions

The new format of the Sialic Acid Quantitation Assay greatly simplifies the execution of the assay and is more adaptable to a robotics system for high throughput screening. The improved format requires only a single reaction component and two pipetting operations. As a result, this simplified method has been shown to have increased assay precision and a significantly higher signal to background ratio (about 30:1 versus about 10:1) compared to the previous assay. The simplified format is also easier to adapt to a robotics system to screen multiple samples of recombinant protein during a process development effort or potentially for glycomic applications to screen for variations in sialic acid content of families of proteins in different disease states or in response to environmental stimulus.

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