Extraction and Purification of Sialic Acid from Submandibular Mucin of Goat

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Abstract: In this study, Sialic acid (Neuraminic acid) was extracted, purified and partially characterized from submandibular mucin of goat. The extraction involved homogenization, treatment with enzymes (phospholipase and lipase) and acid hydrolysis. Ion exchange chromatography was used to purify the sialic acids. The partial characterization involved Fourier transform infrared spectroscopy (FTIR). Treatment of the submandibular mucin with phospholipase led to an increase in release of sialic acid with optimum release of sialic acids at 90 minutes while treatment of the homogenate with lipase led to a slight increase in sialic acids released. Acid hydrolysis of the homogenized submandibular gland led to a 15 fold increase in sialic acids released. The purification fold after carrying out ion exchange chromatography on the sialic acid extract of the goat submandibular mucin was 12.11. The FTIR spectra of the purified sialic acid and that of the standard sialic acid had wavenumbers that are indicative of alkyl halide, alkenes, allene, alkane and carboxylic acid which are the functional groups that are found in sialic acids. The results have indicated that sialic acids are extractible from the submandibular mucins with the aid of phospholipase and lipase.

Keywords: Sialic acid, goat submandibular gland, phospholipase, lipase, FTIR
1. Introduction

Sialic acid is a derivative of a nine-carbon monosaccharide. It comprises of a large family of \( N \)- and \( O \)-substituted neuraminic acids. The amino group of sialic acid is linked to either an \( N \)-acetyl or \( N \)-glycolyl group, which yields \( N \)-acetylenuraminic acid (Neu5Ac) or \( N \)-glycolylenuraminic acid (Neu5Gc) respectively. The hydroxyl groups of both sialic acids are often modified. It is a naturally widespread carbohydrate with numerous biological functions, including blood protein half-life regulation, variety of toxin neutralization, starting reagent of biochemical derivatives for the synthesis of pharmaceuticals, cellular adhesion and glycoprotein lytic protection (Varki et al., 2009; Schauer, 2004a). Sialic acid is covalently bound to the side chains of mucin by a 2-6' glycosidic bond; however, the amount and types of sialic acids present in mucin vary by species of animal and system of isolation. For example, 22% of the dry weight of bovine submaxillary mucin is sialic acid, but the vast majority of the sialic acid is \( N \)-acetylenuraminic acid, with minor amounts of \( N \)-glycolylenuraminic acid (Mizan et al., 2000). Their first level of diversity results from the different alpha linkages that may be formed between the Carbon-2 (C-2) of sialic acid and underlying sugars by specific sialyltransferases, using cyclic monophosphate bound to sialic acid (CMP-Sias) as high-energy donors. The most common linkages are to the C-3 or C-6 positions of galactose residues or to the C-6 position of \( N \)-acetylgalactosamine residues (Varki and Schauer, 2009). Sialic acid analogs are antiviral therapeutics as well as crucial tools in bacterial pathogenesis research, immunobiology and development of cancer diagnostic imaging. The scarce supply of sialic acid hinders production of these materials (Lundgren and Boddy, 2007).

2. Methodology

2.1. Collection of Submandibular Tissue

The submandibular gland was acquired from the slaughter immediately after the animal was slaughtered. The submandibular tissue was submerged in ice cold dextrose saline and brought to the laboratory for use. All subsequent experiments were carried out at room temperature. The unwanted fat and connective tissue were cut off with a knife then, the gland was weighed and the value was recorded.

2.2. Homogenization of Submandibular Gland

The gland (13.4g) was chopped into small pieces then 100 ml of 50mM phosphate buffered saline, pH 6.8 was added to it and it was homogenized. Thereafter, the homogenate was centrifuged at 1500 x g for 5 minutes then the supernatant was assayed for sialic acids.
2.3. Assay for Sialic Acid

The free sialic acids were quantified using the method described by Aminoff (1961). The sample (500µl) was pipetted into a test tube. Distilled water (500µl) was pipetted into a different test tube as blank. Periodate reagent (250µl) was added to the test tubes. The tubes were then incubated at 37°C for 30 minutes in a water bath. The excess periodate was reduced with 200µL of sodium arsenite reagent. As soon as the yellow colour of the liberated iodine disappeared, 2000µL of thiobarbituric acid reagent was added and the test tubes were covered with aluminum foil. The test tubes were incubated in a boiling water bath for 7.5 minutes in a boiling water bath. The colored solutions were then cooled and shaken with 5000µL of acid butanol reagent. The test tubes were then centrifuged at 3000 x g for 5 minutes to facilitate rapid separation of two phases. The colour intensity in the butanol layer was read at 549 nm after zeroing the spectrophotometer with the blank.

2.4. Treatment of Homogenized Submandibular Gland with Enzymes

Twenty milliliters of the homogenate was incubated with 0.15mLs of phospholipase (0.1U) and 3mg of lipase respectively for 3 hours and at 30 minutes intervals. The resulting enzyme-treated homogenate contained free and maybe bound sialic acids. After this, the enzyme-treated homogenate was assayed again for sialic acid concentration.

2.5. Acid Hydrolysis

Acid hydrolysis was done using 0.1N HCl in a 1:1 ratio with the enzyme-treated homogenate. A fraction (10mLs) of the sample was taken and an equal volume of 0.1N HCl was added. The pH was adjusted to 3.5 from 5.8 with concentrated formic acid. The resulting solution was heated at 80°C in a water bath for 1hr. The resulting hydrolysate was assayed for the released sialic acids. The hydrolysate was centrifuged at 300 x g for 40 minutes. The supernatant was retained and the pellet was discarded. The supernatant was put into a dialysis bag and dialyzed against phosphate buffer (pH 7.2) for 48 hours respectively. Total sialic acid was assayed using the method described by Aminoff (1961).

2.6. Purification of Sialic Acids

The extracted sialic acids were purified using ion exchange chromatography. The dialysate, 20mLs was applied unto an anion exchange resin (Dowex-50). The column was pre-equilibrated with 50mM phosphate buffer, (pH 7.2). The sialic acids were eluted with 10mLs of step-wise gradient from 0.0-0.5M sodium chloride (NaCl). The fractions obtained were collected in 2mL capacity Eppendoff tubes. Fractions obtained were assayed for sialic acid concentration to determine the fractions.
containing the sialic acids. These fractions were pooled together, the sialic acid concentration requantified and used for partial characterization.

2.7. Fourier Transform Infrared Spectroscopy (FTIR)

An FTIR-8400S spectrometer equipped with a standard detector connected to an IR solution software was used for FTIR data collection. FTIR spectra were collected from 10 scans, at a resolution of 2 cm\(^{-1}\) and at mid infrared region (4700-340 cm\(^{-1}\)) against a polystyrene background.

3. Results and Discussion

The purification data is shown in table 1.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Volume (ml)</th>
<th>Sialic acid conc. (mg/ml)</th>
<th>Total sialic acid (mg)</th>
<th>Purification fold</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>100</td>
<td>0.046</td>
<td>4.6</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Phospholipase</td>
<td>25</td>
<td>0.125</td>
<td>3.13</td>
<td>2.72</td>
<td>68.04</td>
</tr>
<tr>
<td>Lipase</td>
<td>25</td>
<td>0.127</td>
<td>3.18</td>
<td>2.76</td>
<td>69.13</td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>20</td>
<td>0.546</td>
<td>10.92</td>
<td>11.87</td>
<td>42.13</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>30</td>
<td>0.557</td>
<td>16.71</td>
<td>12.11</td>
<td>27.53</td>
</tr>
</tbody>
</table>

The representing the effect of incubating the homogenized submandibular mucin of goat with phospholipase is shown in figure 1. After the incubation of the homogenized goat submandibular mucin with phospholipase, the linear increase in the quantity of sialic acid hydrolyzed in the first 90 minutes may indicate that the hydrolytic action of the enzyme within that time is first order. Typical of enzyme catalyzed reactions, initial periods of interaction of enzymes and substrates is usually characterized by linear increase in the release of products to a certain level, after which the linearity diminishes. The loss of linearity after 90 minutes could be due to the fact that most of the phospholipids bound to sialic acid molecules have been completely hydrolyzed or that there is a saturation of the active site of the phospholipase by the phospholipids available.
Figure 1: Effect of incubation of goat submandibular mucin with phospholipase at various time intervals.

In Figure 2, after incubation with phospholipase and lipase consecutively, the slight increase in sialic acid concentration during the incubation is an indication that either lipase has a lesser effect on sialic acid hydrolysis from submandibular mucin after treatment with phospholipase or the action that lipase would have carried out has already been carried out by phospholipase. The enzymes had optimum activity after incubation with submandibular mucin for 120-150 minutes. The sialic acid was hydrolysed using 0.1N HCl (Aminoff, 1961).

Figure 2: Effect of incubation of goat submandibular mucin with phospholipase and lipase at various time intervals.
During ion exchange chromatography (Figure 3), sialic acids were eluted using 0.1M, 0.2M and 0.3M concentrations of NaCl because the sialic acid molecules were not tightly bound to the anionic exchanger.

![Graph showing ion exchange elution profile for sialic acid from submandibular mucin of goat.](image)

**Figure 3:** Ion exchange elution profile for sialic acid from submandibular mucin of goat.

The FTIR analyses of the sialic acid extract (Figure 4) and the standard sialic acid solution in 0.2M NaCl revealed the presence of an alkyl halide, secondary amine group, primary amide, hydroxyl group, alkene, alkane and allenes (Johny et al., 2011).

For FTIR, the analysis time was less than five minutes and required the use of a minute quantity of the sample. In the FTIR spectrum obtained for standard freeze dried sialic acid (Figure 5), 13 bands were seen. However, when the standard sialic acid was dissolved in 0.2M NaCl, the FTIR spectrum obtained was similar to that obtained from submandibular mucin of goat (Figure 6). This is due to the fact that the sialic acid extract from the mucin was eluted with NaCl solutions during ion exchange chromatography. However, in the FTIR spectrum obtained for a solution of standard sialic acid in 0.2M NaCl, a sixth absorption band was observed.
Figure 4: FTIR spectrum for sialic acid extract from goat submandibular mucin

Figure 5: FTIR spectrum for standard sialic acid
4. Conclusion

Phospholipase cleaved off more bound sialic acid molecules than lipase during incubation with submandibular mucin. The percentage of sialic acid per gram of goat submandibular gland was 2.56%. This study was not time consuming and it led to the extraction and partial purification of sialic acids. The FTIR spectrum for a solution of standard sialic acid in 0.2M NaCl was almost identical with that obtained from the extracted sialic acid from goat submandibular mucin. The percentage of sialic acid extracted is higher than calculated because after the submandibular gland gotten from the goat is immersed in dextrose saline, the absorbed water will result in a higher weight of the submandibular gland.

5. Recommendation

High performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is the best method of chromatography for characterizing charged amino sugars. It should be carried out on sialic acid extracts so one can tell the ratio of NeuAc to NeuGC it contains.

References


