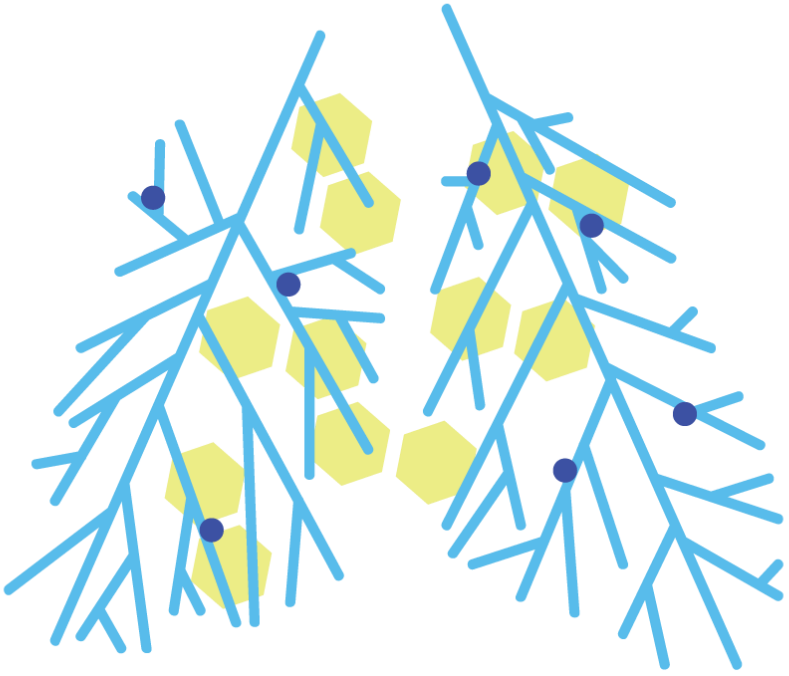

PROLUNG

DEGRADATION

ACTIVITY TESTING

LAB BOOK 3

iGEM Stockholm

Sialidase activity measurement on BSM

Background

After our experiences with PGM we decided to perform the same experiment on a substrate with a higher content of sialic acid.
Commercially available sialidase can digest terminal sialic-acid residues that are α2,3-, α2,6-, or α2,8-linked to Gal, GlcNAc, GalNAc, AcNeu, GlcNeu, oligosaccharides, glycolipids, or glycoproteins. This experience is designed to test the amount of sialic acid released from Bovine submaxiliary mucin after being digested with sialidase overnight.

Preparation of BSM stock

Aim

To prepare the necessary stock of Bovine Submaxillary Mucin (BSM) to carry out the following experiments.

Procedure

In order to prepare the necessary Bovine Submaxillary gland Mucin (BMS) stock 2,5 mg of BMS were dissolved in reaction buffer (Sodium acetate (0.05 M), 1mM calcium chloride, pH 5,5) to a concentration of 10 mg/ml. The solution was mixed with pipette and no additional steps were required.

Digestion of BSM by sialidase

Aim

To set the digestion of BSM with commercially available sialidase from *Arthrobacter ureafaciens* purchased from Sigma Aldrich (10269611001)

Procedure

25 µl of 10mg/ml BSM solution were added in a 1.5 ml eppendorf tube along with 25 µl of reaction buffer (Sodium acetate (0.05 M), 1mM calcium chloride, pH 5,5).

12.5 µl of sialidase stock solution (1U/100 µl) plus 138 µl of reaction buffer were added to the mix and left overnight at 37ºC in an incubator.

Additional slurry enzyme digestion
An additional digestion using sialidase in a micro bead slurry was performed. To do so 50 µl of 5mg/ml BSM solution were mixed into the micro bead slurry containing the enzyme and left overnight at 37ºC in an incubator.

Sialic acid concentration measurement

Aim
To measure the concentration of sialic acid released after digestion using High Performance Anion Exchange Chromatography.

Procedures
Creation of a positive control
A positive control was created to be used as a frame of reference for a total digestion, in order to do so a strong acid (0.6N H_2SO_4) was employed.
25 µl of 10 mg/ml BSM solution were added in a 1.5 ml eppendorf tube along with 25 µl of reaction buffer and 33 µl of 0.6N H_2SO_4, the mixture was then incubated in a heat block at 80ºC. After two hours the acid was neutralized with 66 µl of 0.2N NaOH.

Creation of the calibration curves
In order to translate the signals from the HPAEC into concentration values we used calibration curves to create a linear regression model. Two different calibration curves, one for the enzyme digestion and one for the positive control, where employed since values for sialic acid and acid digested sialic acid can vary.

To achieve the **positive control curve** 1.4 mg of commercially available sialic acid purchased from Sigma Aldrich was dissolved into 1.4 ml of MilliQ water (final concentration 1 mg/ml). We added 200 µl of this sialic acid solution into and 1.5ml eppendorf tube along with 200 µl of 0.2N H_2SO_4 and incubated the mix for two

hours in a heat block at 80ºC. After two hours the acid was neutralized with 400 µl of 0.1N NaOH. The resulting solution named Solution A was then filtered using a HPLC filter and mixed with filtered reaction buffer to create the following seriated dilution:

Name	**Concentration (mg/ml)**	**Dilution**
Solution I	0.1	Add 100 µl of **Solution A** into 150 reaction buffer (MiliQ)
Solution II	0.075	Add 75 µl of **Solution A** into 175 µl of water (MiliQ)
Solution III	0.05	Add 50 µl of **Solution A** into 200 µl of water (MiliQ)
Solution IV	0.025	Add 25 µl of **Solution A** into 225 µl of water (MiliQ)
Solution V	0.01	Add 25 µl of **Solution I** into 225 µl of water (MiliQ)
Solution VI	0.005	Add 25 µl of **Solution III** into 225 µl of water (MiliQ)

To achieve the **enzyme digestion calibration curve** 1.5 mg of sialic acid were dissolved in 1.5 ml of reaction buffer, then diluted to a concentration of 0.25 mg/ml using reaction buffer. The resulting solution named Solution B was was then filtered using a HPLC filter and mixed with filtered reaction buffer to create the following seriated dilution.

Name	**Concentration (mg/ml)**	**Dilution**
Solution 1	0.1	Add 100 µl of **Solution B** into 150 µl of reaction buffer
Solution 2	0.075	Add 75 µl of **Solution B** into 175 µl of reaction buffer
Solution 3	0.05	Add 50 µl of **Solution B** into 200 µl of reaction buffer
Solution 4	0.025	Add 25 µl of **Solution B** into 225 µl of reaction buffer
Solution 5	0.01	Add 25 µl of **Solution 1** into 225 µl of reaction buffer
Solution 6	0.005	Add 25 µl of **Solution 3** into 225 µl of reaction buffer

Measurements of sialic acid concentrations

The positive control, the enzyme digestion and the calibration curves solutions where filtered using HPLC filters into HPLC vials and analyzed with an HPAEC machine to observe the peaks registered with a retention time of approximately 5 minutes. The sialidase present in the bead slurry required a preliminary filtering using an Amicon Ultra 0.5 Centrifugal Filter 3K Device previously rinsed with ethanol to remove glycerol from the column.

Results

Enzyme digestion

Enzyme digestion was carried according to protocol. The results showed a calibration curve with an **R^2= 0.968** and a sialic concentration of **0,0811 µg/µl.** When extrapolated to the percentage of sialic acid from BSM (considering that the sample had a total amount of 250 µg of BSM in a volume of 200 µl) the percentage was **6,49 %.**

Positive control

On the initial experience the positive control had some slight modifications in the protocol in order to have a higher concentration of H_2SO_4 (0.6 N) and NaOH (0.3 N) and a higher amount of BSM (500 µg in 199 µl). The results showed a calibration curve with an **R^2= 0.9823** and a sialic acid concentration of **0,1039 µg/µl**. When extrapolated to the percentage of sialic acid from BSM (considering that the sample had a total amount of 500 µg of BSM in a volume of 199 µl) the percentage was **4,159 % (w/w)**.

As can be observed the activity presented by the positive control is smaller than the enzyme digestion. This contradicts the initial hypothesis. To obtain a better perspective the positive control was repeated using the initial conditions (0,2 N H_2SO_4, 0,1M NaOH and 250 µg of BSM).

Slurry digestion

The digestion was also performed with a different sialidase presented as slurry. The HPAEC analysis showed a concentration of sialic acid of **0.185 µg/µl.**

This concentration translates in a percentage of sialic acid (w/w) of **14.85%** which is much higher than the one obtained from either positive control.

Repeated positive control

Aim

To recreate the positive control using less concentrated reagents

Procedure

25 µl of 10 mg/ml BSM solution were added in a 1.5 ml eppendorf tube along with 25 µl of reaction buffer and 50 µl of 0.2N H_2SO_4, the mixture was then incubated in a heat block at 80ºC. After two hours the acid was neutralized with 100 µl of 0.1N NaOH.

The resulting mixed was then filtered using HPLC filters and analyzed through HPAEC.

Results

After repeating the positive control with the initial conditions (0,2 N H_2SO_4 and 0,1M NaOH) and 250 µg of BSM in 200 µl the results showed a sialic concentration of **0,104 µg/µl**. This concentration is similar to the seen in our other positive control but since the amount of BSM employed was less (250 µg of BSM in a volume of 200 µl) the percentage of sialic acid to BSM is **8,303% (w/w)**.

Discussion

The HPAEC analysis showed a significant concentration of sialic acid after enzyme digestion.
The observed activity was higher than the positive control which suggested some sort of error in our experience so we decided to repeat the positive control using the conditions described in the original protocol. The repeated control showed an increased activity, higher than the enzyme digestion, which could be the consequence of the following circumstances:

- The use of a more concentrated acid causes the mucin to coil decreasing the digestion.
- The use of a higher concentration of BSM made the amount of H_2SO_4 insufficient.
- Other causes such as mishandling or contamination.

The slurry digestion showed an abnormally high concentration of sialic acid, surpassing both positive controls. This could be due to the filter used prior to the HPAEC analysis. Instead of the filters typically used for HPAEC the sample was

filtered using an Amicon centrifugal filter (3kD). This filter has preservatives that might interact with the HPAEC analysis (such as glycerol which appears at retention time 2 approximately). The filter was washed twice, once with ethanol (70%) and once with MiliQ water previously to the experience but this might not have been enough to prevent an alteration on the results.