Sialidase assay (Quantification of sialic acid digestion)

Aim

To measure the amount of sialic acid release from a substrate after digestion with sialidase.

Procedure

Positive control (H_2SO_4 digestion)

Calibration curve for H_2SO_4 digestion

1. Dissolve sialic acid in water to prepare 200 µl of 1 mg/ml sialic acid solution.
2. Add 200 µl of H_2SO_4 (0.2 N).
3. Incubate at 80ºC for 2 hour
4. Add 400 µl of NaOH (0.1 M). The resulting solution will be referred to as Solution A
5. *NOTE: At this point total volume should be 800 µl of solution with a sialic acid concentration of 0.25 mg/ml*
6. Filter the Solution A using HPAEC filters into an HPAEC vial.
7. Pipette the filtered Solution A into HPAEC vials as follows to create the calibration curve.

Name	Concentration (mg/ml)	Dilution
Solution I	0.1	Add 100 µl of Solution A into 150 µl of water (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)

NOTE: Pipette up and down three times before taking the desired volume to acclimatize the pipette tip. When mixing by pipette action pipette up and down 20 times to assure even distribution. 30 times for the smaller volumes (Solution V & VI)

8. Measure each concentration with HPAEC and create a regression curve (**Calibration curve for positive control**).

H_2SO_4 digestion (positive control)

1. Weigh 2.5 mg of BSM and dissolve them in 500 µl of water (MiliQ). **BSM concentration**: 5 mg/ml
2. Pipette 50 µl of the previous BSM solution into an eppendorf tube
3. Add 50 µl of H_2SO_4 (0.2 N) to the tube.
4. Incubate at 80ºC for 2 hour.
5. Add 100 µl of NaOH (0.1 M) into the tube to neutralize pH.
6. Filter into an HPLC vial and label sample.
7. Measure with HPAEC.

ENZYME DIGESTION

Calibration curve for enzyme digestion

1. Dissolve sialic acid in reaction buffer to a concentration of 0.25 mg/ml (**Solution B**).
2. Filter the resulting Solution B into an HPAEC vial
3. Pipette the filtered Solution B into HPAEC vials as follow to create the calibration curve

NOTE: For this calibration curve we do not digest the sialic acid with H_2SO_4. As a result we have a calibration curve for the H_2SO_4 digestion and another one for the enzyme digestion.

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

4. Measure each concentration with HPAEC and create a regression curve (**Calibration curve for enzyme digestion**)

Enzyme digestion

1. Add 50 µl of dissolved BSM (5 µg/µl). **Substrate**: 250 µg
2. Add 12,5 µl of sialidase stock solution (1U/100 µl).
3. Add 138 µl of reaction buffer.
4. Add a negative control containing 50 µl of dissolved BSM (5 µg/µl) and 150 µl of reaction buffer.
5. Label samples and incubate over night at 37ºC.
6. Filter in HPLC vials and label samples
7. Measure with HPAEC.

NOTE: Final volume 200 µl, final PGM concentration 1.25 µg/µl. For details see ***CALCULATIONS****.*

DATA INTERPRETATION

As a result of the HPAEC analysis we obtain peaks of intensity in the band corresponding to the sialic acid. Magnitude is estimated by calculating the area under the curve (AUC) of the peaks.
With the values obtained from the HPAEC analysis we perform regression curve to obtain the two different calibration curves (**Positive control** and **enzyme digestion**).
HPLC values obtained from **H_2SO_4** and **enzyme digestion** samples are then extrapolated into their corresponding calibration curve to have a measurement of the activity.

CALCULATIONS

Enzyme/substrate ratio

According to manufacturer's specifications (See **ANNEX**) the optimal enzyme/substrate ratio for our enzyme is in the range of 0.04 U/25-80 µg. We chose as a ratio 0.04/50 µg .Packaging of the stock solution of the enzyme is 1 U/ 100 µl.

- We take 12,5 µl of sialidase stock solution. **Total enzyme: 0.125 U**
- We prepare a PGM solution with concentration 5 µg/µl (5 mg/ml) and add 50 µl of the solution in an eppendorf tube. **Total substrate: 250 µg**
- **Enzyme substrate ratio: 0.125 U/250 µg = 0.04 U/80 µg**
- Dilute to 200 µl with 125 µl of solution buffer.

- **Ratio remains within optimal range.**

Positive control internal control

- **Total volume of internal control**: 140 µl.
- **Desired sialic acid concentration**: 0.01 µg/µl.
- **Required sialic acid**: 1.5 µg.
- Required **Solution A** to achieve 1.5 µg of sialic acid: 6 µl

Enzyme digestion internal control

- **Total volume of internal control**: 110 µl
- **Desired sialic acid concentration:** 0.01 µg/µl
- Required sialic acid: 1.1 µg
- Required Solution B to achieve 1.5 µg of sialic acid: 4.4 µl

REFERENCES

- Temperature obtained from Sigma Aldrich website:
  - http://www.sigmaaldrich.com/technical-documents/protocols/biology/roche/neuraminidase-sialidase.html
- Substrate ratio and pH obtained from Sigma Aldrich website:
  - http://www.sigmaaldrich.com/technical-documents/protocols/biology/roche/neuraminidase-sialidase.html