



## **GAG Release and Purification**

### **MATERIALS**

- Poly-Prep Columns (BioRad, 731-1550)
- DEAE Sephacel (Sigma, 16505)
- Protease Enzyme Solution (stock 20mg/mL in H<sub>2</sub>O) (Sigma, P5147)
- PD10 Desalting Columns (GE Healthcare, 17-0851-01)

### **Lysis/Extraction Buffer:**

1X PBS Buffer Solution (without Ca<sup>+</sup> and Mg)

- 4g of NaCl
- 0.1g of KCl
- 0.72g of Na<sub>2</sub>HPO<sub>4</sub>
- 0.12g of KH<sub>2</sub>PO<sub>4</sub>

Dissolve in 400 mL of water, adjust pH to 7.4, and bring volume to 500 mL.

### **DEAE Pre-Wash Buffer:**

50 mM NaOAc/150 mM NaCl with 0.1% Triton X-100 pH 6.0

- 6.8 g NaOAc·3H<sub>2</sub>O
- 4.4 g NaCl
- 0.5 g Triton X-100

Dissolve in 400 mL Milli-Q water, adjust pH to 6.0 and bring volume to 500 mL

### **DEAE Wash Buffer:**

50 mM NaOAc/150 mM NaCl with pH 6.0

- 6.8 g NaOAc·3H<sub>2</sub>O
- 4.4 g NaCl

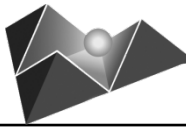
Dissolve in 400 mL Milli-Q water, adjust pH to 6.0 and bring volume to 500 mL

### **DEAE Elution Buffer:**

50 mM NaOAc/1M NaCl pH 6.0

- 6.8 g NaOAc·3H<sub>2</sub>O
- 58.4 g NaCl

Dissolve in 400 mL Milli-Q water, adjust pH to 6.0 and bring volume to 500 mL



**PROCEDURE:**

1. For cells re-suspend the cell pellet in Lysis/Extraction Buffer and sonicate. For tissue add Lysis/Extraction Buffer and homogenize.
2. Next add enough Protease Enzyme Solution to a final concentration of 100  $\mu\text{g/mL}$ , incubate at 37  $^{\circ}\text{C}$  under rotation overnight. After overnight digestion check solution to make sure that the material has dissolved. If large particles still remain add more protease and incubate overnight a second time. If particles are still present use 0.2  $\mu\text{m}$  syringe filter.
3. Pack a polyprep column with 200  $\mu\text{L}$  of DEAE Sephacel and equilibrate with 5 mL of DEAE Pre-wash buffer.
4. Load the sample onto the column. Rinse the sample tube with Milli-Q water and add to the column.
5. Wash Column with 5mL DEAE Wash Buffer.
6. Elute the GAGs by adding 1mL of DEAE Elution Buffer and collect.
7. Next prepare PD10 Desalting column by washing 6X with 10% Ethanol.
8. Load sample and wash with 2.5 mL of 10% Ethanol.
9. Avoid collecting the first 2.5 mL as it is the void volume of the column.
10. Collect the next 3.5 mL of 10% Ethanol and lyophilize.
11. Sample can be immediately digested with enzyme or stored at -20  $^{\circ}\text{C}$  until digestion can be performed

**ENZYMATIC DIGEST:**

**Heparin Lyase Buffer:**

100 mM Sodium Acetate/0.1 mM Calcium Acetate pH 7.0

- 3.4 g NaOAc $\cdot$ 3H $_2$ O
- 4.4 mg Ca(OAc) $_2$  $\cdot$ H $_2$ O

Dissolve in 200 mL of Milli-Q water, adjust pH to 7.0 and bring volume to 250 mL

**Chondroitin ABC Lyase Buffer:**



50 mM Tris-Cl/50 mM Sodium Acetate pH 8.0

- 1.5 g Tris Base
- 3.4 g NaOAc·3H<sub>2</sub>O

Dissolve in 200 mL of Milli-Q water, adjust pH to 8.0 and bring volume to 250 mL

### Heparin Lyase Digestion

1. Resuspend dried sample in 100 µL Heparin Lyase Buffer and add cocktail enzyme (I, II, and III) to a final amount of 10 mU each.
2. Incubate overnight at 37<sup>0</sup>C with rotation.
3. Terminate the reaction by heating at 100 °C for 5 minutes.
4. To avoid contamination of the column sample can be passed through a 10K MWCO spin filter and dried. If no large particles exist sample can be dried straight away.
5. Make the sample ready for injection by re-suspending in 100 µL of Milli-Q water.

### Chondroitinase ABC Digestion

1. Resuspend dried sample in 100 µL Chondroitin ABC Lyase Buffer and add cocktail enzyme to a final amount of 10 mU.
2. Incubate overnight at 37<sup>0</sup>C with rotation.
3. Terminate the reaction by heating at 100 °C for 5 minutes.
4. To avoid contamination of the column sample can be passed through a 10K MWCO spin filter and dried. If no large particles exist sample can be dried straight away.
5. Make the sample ready for injection by re-suspending in 100 µL of Milli-Q water.

## **HPLC OF GAG DISACCHARIDES WITH POST COLUMN LABELLING:**

### Heparin Sulfate Disaccharides

- Colum:
  - Dionex Analytical ProPac1 column 4.6 mm x 250 mm Particle size 4µm
- Solvents:
  - A: Acidic Water pH 3.5
  - B: 1M NaCl pH 3.5
  - Initial conditions of 100% A at 1.0 mL/min
- Post Column Solvent:
  - A: 1% 2-Cyanoacetamide (10 g/L of 2-cyoacetamide)
  - B: 250 mM NaOH (13 mL of 50% NaOH in 1L Milli-Q water)
  - Initial conditions of 50% A/50%B at 1.0 mL/min
- Heater Controller:
  - Reactor: 130 °C
- Fluorescence Detector:
  - Excitation: 346 nm



- Emission: 410 nm
- Gain: 1
- Sensitivity: High
- Gradient Settings:

Time	%A	%B
0	100	0
1	100	0
10	95	5
20	65	35
40	55	45
60	0	100
70	0	100
72	100	0
90	100	0

## Chondroitin Sulfate Disaccharides

- Colum:
  - Dionex Acclaim 120 C18 column 4.6 mm x 250 mm Particle size 4µm
- Solvents:
  - A: Water
  - B: 10mM TBAHS (Tetrabutyl Ammonium Hydrogen Sulfate)
  - C: 50% Acetonitrile
  - D: 0.2 M NaCl
  - Initial conditions of 78% A, 12% B, 10% C at 1.0 mL/min
- Post Column Solvent:
  - A: 1% 2-Cyanoacetamide (10 g/L of 2-cyoacetamide)
  - B: 250 mM NaOH (13 mL of 50% NaOH in 1L Milli-Q water)
  - Initial conditions of 50% A/50%B at 1.0 mL/min
- Heater Controller:
  - Column: 45 °C
  - Reactor: 130 °C
- Fluorescence Detector:
  - Excitation: 346 nm
  - Emission: 410 nm
  - Gain: 1
  - Sensitivity: High



- Gradient Settings:

Time	%A	%B	%C	%D
0	78	12	10	0
1	78	12	10	0
8	78	12	10	0
10	68	12	20	0
40	48	12	40	0
60	40.5	12	40	7.5
70	33	12	40	15
75	18	12	40	30
80	0	12	40	48
80.50	78	12	10	0
100	78	12	10	0

### Standard Preparation

1. Prepare a mg/mL solution of each heparin sulfate or chondroitin sulfate disaccharide and take 20  $\mu$ g for hydrolysis.
2. Analyze 1  $\mu$ g of each hydrolyzed standard by HPAEC-PAD and determine the amount, in nanomoles, of GlcA and GlcNAc for heparin sulfate or the amount of GlcA and GalNAc for chondroitin sulfate.
3. Using the amounts determined in Step 2 calculate the molarity of the mg/mL stock solution. Once the molarity is known determine the dilution of the mg/mL stock standard solution needed to make 1 mL of a 5.0  $\mu$ M solution containing either all of the HS or all of the CS standards.
4. The 5.0  $\mu$ M solution is equivalent to 50 pmoles per 10  $\mu$ L. Inject 50 pmoles per standard run.