

Isolation of Keratan Sulfate Disaccharide-branched Chondroitin Sulfate E from *Macra chinensis*

Kyohei Higashi* and Toshihiko Toida

Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan

*For correspondence: higase@faculty.chiba-u.jp

[Abstract] Glycosaminoglycans (GAGs) including chondroitin sulfate (CS), dermatan sulfate (DS), heparin (HP), heparan sulfate (HS) and keratan sulfate (KS) are linear, sulfated repeating disaccharide sequences containing hexosamine and uronic acid (or galactose in the case of KS). Recently, a keratan sulfate (KS) disaccharide [GlcNAc6S(β1-3)Galactose(β1-)]-branched CS-E was identified from the clam species *M. chinensis*. Here, we report the isolation protocol for KS-branched CS from *M. chinensis*.

Keywords: *Macra chinensis*, Glycosaminoglycan, Chondroitin sulfate, Keratan sulfate, Galactose

[Background] GAGs are found in tissues as the glycan moieties of proteoglycan (PG) glycoconjugates. CS is a GAG type composed of linear, sulfated repeating disaccharide sequences of *N*-acetyl- β -galactosamine (GalNAc) and glucuronic acid (GlcA). Another GAG type, DS, is biosynthesized through the action of glucuronyl C5-epimerase on CS, converting its GlcA to the CS epimer, iduronic acid (IdoA). The other GAG types HS and HEP are consisted of sulfated repeating disaccharide sequence of *N*-acetyl- β -glucosamine (GlcNAc) and GlcA/IdoA. KS is composed of sulfated repeating disaccharide sequences of GlcNAc and galactose. Among them, structural variations of CS, such as sulfation patterns and fucosylation, depend on the species and tissue of origin. For example, the A-unit with the structure [-4]GlcA(β1-3)GalNAc4S(β1-) (where S designates a sulfonate residue) is a predominant disaccharide found in mammalian or chicken tracheal cartilage CS, while the C-unit with the structure [-4]GlcA(β1-3)GalNAc6S(β1-) is a major disaccharide found in shark cartilage or salmon nasal cartilage CS. In contrast, a significant amount of the D-unit disaccharide [-4]GlcA2S(β1-3)GalNAc6S(β1-) is characteristically found in CS isolated from shark cartilage, while the E-unit disaccharide [-4]GlcA(β1-3)GalNAc4S,6S(β1-) is characteristic in squid cartilage. In sea cucumber, a sulfated fucose branches at the 3-OH position of GlcA. The disaccharide composition of CS governs its biological activities, including cell proliferation, migration, differentiation, cell-cell crosstalk, adhesion and wound repair through the interaction with growth factors, receptors, and other CS-binding proteins. Evidence suggests that CS structure is tightly correlated with function. For example, consecutive and disulfated disaccharide units including B, D and E-units in CS are critical for the interaction between CS and binding proteins (Hikino *et al.*, 2003). A branched fucose at the 3-OH position of GlcA is also required for the anticoagulant activity of fucosylated CS (Mourão *et al.*, 1996).

In general, isolation of GAGs is carried out as follows. 1) acetone defatting, 2) proteolysis, 3) collection of the GAGs, 4) fractionation of GAGs by anion-exchange chromatography and 5) desalting (Maccari *et al.*, 2015). In our protocol, actinase E from *Streptomyces griseus* (step 2) and cetylpyridinium chloride precipitation (step 3) were used for the isolation of GAGs.

Materials and Reagents

1. 200 and 1,000 µl pipette tips (Thermo Fisher Scientific)
2. Centrifuge tube
3. Spectra/Por®7 Dialysis Membrane Pre-treated RC Tubing MWCO: 3,500 (Spectrum, catalog number: 132111)
4. HiPrep™ DEAE FF 16/10 (GE Healthcare, catalog number: 28936541)
5. Dry powder of hot water extract from *M. chinensis* viscera obtained from Futtsu City Fishery Association in Chiba, Japan
6. Acetone (Wako Pure Chemical Industries, catalog number: 011-00357)
7. Acetic acid (NACALAI TESQUE, catalog number: 00212-43)
8. Perchloric acid (60%, w/v) (NACALAI TESQUE, catalog number: 26502-85)
9. Ethanol (99.5% w/v) (NACALAI TESQUE, catalog number: 14713-53)
10. Tris (hydroxymethyl)aminomethane (Tris) (NACALAI TESQUE, catalog number: 35406-91)
11. Actinase E (Funakoshi, catalog number: KA-001)
12. Sodium hydroxide (NaOH) (NACALAI TESQUE, catalog number: 31511-05)
13. Sodium tetrahydroborate (Wako Pure Chemical Industries, catalog number: 192-01472)
14. Cetylpyridinium chloride monohydrate (99.0-102.0% w/v) (Wako Pure Chemical Industries, catalog number: 086-06683)
15. Hydrochloric acid (HCl) (35.0% w/v) (NACALAI TESQUE, catalog number: 18321-05)
16. Sodium chloride (NaCl) (NACALAI TESQUE, catalog number: 31320-05)
17. Sodium dihydrogenphosphate, anhydrous (NaH₂PO₄) (NACALAI TESQUE, catalog number: 31720-65)
18. Di-sodium hydrogenphosphate (Na₂HPO₄) (NACALAI TESQUE, catalog number: 31801-05)
19. Tris acetate buffer (pH 8.0) (see Recipes)
20. NaOH buffer (see Recipes)
21. Cetylpyridinium chloride solution (see Recipes)
22. 50 mM sodium phosphate (pH 6) (see Recipes)
23. 2.0 M NaCl in 50 mM sodium phosphate (pH 6) (see Recipes)

Equipment

1. Refrigerated centrifuge (Sakuma, model: M200-IVD)
2. Fume hood
3. Erlenmeyer flask (AGC, catalog number: 4980FK500)
4. Stainless steel spoon (180 mm)
5. Pipettes (Gilson, models: P20, P200 and P1000)
6. Corning® reusable low form beaker, polypropylene, size 3 L (Corning, catalog number: 1000P-3L)

7. Stir bar
8. BioShaker (TAITEC, model: BR-23FP MR)
9. Freeze drier (TOKYO RIKAKIKAI, Eyela, model: FDU-830)
10. Gradient pump (Bio-Rad Laboratories, model: ECONO GRADIENT PUMP)
11. Analytical balance (Shimadzu, model: ATX224)

Procedure

A. Isolation of crude GAGs

1. To remove lipids from the sample, suspend the dried powder (5 g) in a centrifuge tube with 20 ml of acetone and then centrifuge sample at $2,300 \times g$ for 15 min at room temperature. Remove supernatant. Repeat this defatting step three times. After centrifugation, dry samples overnight in fume hood at room temperature. In general, 30 g of dried powder was defatted.
2. To digest the core proteins, dissolve the defatted sample in 120 ml of Tris acetate buffer (pH 8.0) (see Recipes) containing actinase E in an Erlenmeyer flask and maintain for 18 h at 45 °C with shaking at 100 rpm.
3. Add 600 ml of NaOH buffer (see Recipes) to the sample and leave to stand at 4 °C for 18 h.
4. Neutralize the sample with 100 ml of 2.0 N acetic acid at room temperature and precipitate the degraded proteins by adding perchloric acid (to a final concentration of 5%). Separate the degraded proteins by centrifugation at $2,300 \times g$ for 15 min at 4 °C.
5. Transfer the supernatant into a dialysis membrane (MWCO: 3,500) and dialyze it against distilled water at room temperature for 16 h.
6. Transfer the dialyzed sample solution into a new Erlenmeyer flask. Add CPC solution (see Recipes) (final concentration 0.1%) and suspend well. Cetylpyridinium chloride (CPC), a cationic surfactant, interacts with the sulfates and carboxylates of GAGs. After storage at 4 °C for 16 h, GAG-CPC complex in the Erlenmeyer flask can be observed.
7. Collect the GAG-CPC complex by centrifugation at $10,000 \times g$ for 15 min at 4 °C and discard the supernatant.
8. Transfer the precipitate (GAG-CPC complex) into a new conical tube using a stainless steel spoon (180 mm). To remove CPC from GAG-CPC complex, resuspend the precipitate in 4 ml of 2.5 M NaCl using a 1,000 μ l pipette. Centrifuge the solution at $10,000 \times g$ for 15 min at 4 °C and transfer the GAG-containing supernatant into a new conical tube.
9. Suspend with 11 volumes of 85% ethanol and store at 4 °C for 16 h. After incubation, collect crude GAGs by centrifugation at $10,000 \times g$ for 15 min at 4 °C and remove the supernatant. Dissolve the precipitate with 10 ml of distilled water.
10. Transfer the supernatant fluid (dissolved precipitate) into a dialysis membrane (MWCO: 3,500) and dialyze it against 2 L of distilled water in a polypropylene beaker (3 L) with a stir bar at room temperature for 16 h to remove excess NaCl from crude GAGs. Distilled water is changed 5 times during dialysis.

11. Transfer the dialyzed sample into a new conical tube and freeze.
12. Lyophilize overnight using freeze drier to obtain the crude GAG powder.

B. Fractionation of KS-branched CS-E

1. Dissolve the crude GAGs (30 mg) into 2 ml of distilled water.
2. The dissolved crude GAGs are fractionated using low-pressure liquid chromatography. Fractionation condition: ECONO GRADIENT PUMP (Bio-Rad Laboratories) is applied at a flow rate of 2 ml/min on a HiPrep DEAE FF at room temperature. The eluent buffers are as follows: (A) 50 mM sodium phosphate (pH 6) (see Recipes), (B) 2.0 M NaCl in 50 mM sodium phosphate (pH 6) (see Recipes). The gradient program is 0-30 min (5% B), 30-150min (5-100% B), and 150-180 min (100% B).
3. Collect fractionated samples at 30 min intervals. Dry the fractionated samples using a freeze drier and keep samples at 4 °C.

Data analysis

The dried powder (crude GAGs or fractionated CS) after freeze-drying was weighed using analytical balance.

Notes

1. Dry powder of hot water extracted viscera from *M. chinensis* was used.
2. Crude GAGs contain heparan sulfate and chondroitin sulfate.
3. 353.8 mg of crude GAG/g of dry powder was recovered from the viscera of *M. chinensis* (Higashi *et al.*, 2016).
4. CS usually elutes at 90 to 150 min when fractionation is performed by anion-exchange chromatography, whereas elution time of hyaluronic acid (HA) and HS is faster than that of CS. HA eluted at 0 to 30 min, and HS mainly eluted at 60 to 90 min.
5. In case of *M. chinensis*, fractionated CS was obtained in fraction 3 (18.5 mg) and in Fraction 4 (3.73 mg) from 30 mg of crude GAGs (Higashi *et al.*, 2016).

Recipes

1. Tris acetate buffer (pH 8.0)
50 mM Tris acetate (pH 8.0)
2.5 mg/ml of actinase E
2. NaOH buffer
0.5 N NaOH
0.3 M sodium tetrahydroborate

3. Cetylpyridinium chloride solution
5% hexadecylpyridinium (Cetylpyridinium) chloride monohydrate
30 mM NaCl
4. 50 mM sodium phosphate (pH 6)
50 mM sodium dihydrogenphosphate, anhydrous (NaH₂PO₄)
50 mM di-sodium hydrogenphosphate (Na₂HPO₄)
5. 2.0 M NaCl in 50 mM sodium phosphate (pH 6)
50 mM sodium dihydrogenphosphate, anhydrous (NaH₂PO₄)
50 mM di-sodium hydrogenphosphate (Na₂HPO₄)
2.0 M NaCl

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