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Preparation of the Partially Methylated Alditol Acetates Derived from CS Tetrasaccharides Containing Galactose for the Gas Chromatography/Mass Spectrometry Analysis

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[Abstract] Chondroitin sulfate (CS), a member of the glycosaminoglycan (GAG) family of carbohydrates, is composed of linear, sulfated repeating disaccharide sequences of *N*-acetyl-D-galactosamine (GalNAc) and glucuronic acid (GlcA). Recently, a keratan sulfate (KS) disaccharide [GlcNAc6S(β1-3)Galactose(β1-]-branched CS-E was identified from the clam species *M. chinensis.* This protocol details a methodology to analyze the glycosidic linkages of galactose in KS disaccharide-branched CS by GC-MS analysis. A complementary method for the identification and characterization of KS-branched CS in *M. chinensis* can be found in Higashi *et al.* (2016).
Keywords: *Mactra chinensis*, Chondroitin sulfate, Keratan sulfate, Partially methylated alditol acetates, GC-MS analysis

[Background] Gas chromatography/mass spectrometry (GC-MS) analysis of the reaction products of partially methylated alditol acetates (PMAA) derived from the polysaccharides has been shown to represent a powerful tool to investigate the glycosidic linkages. The PMAAs preparation from *M. chinensis* in this protocol was performed according to the method of Anumula and Tayler (1992) with minor modifications.

Materials and Reagents

1. Screw-cap tube (AGC techno glass, borosilicate Pyrex glass, 13 mm i.d. x 120 mm)
2. Glass measuring pipette (0.1, 0.5, 1 and 2 ml)
3. Pasteur pipette IK-PAS-5P (IWAKI, catalog number: 73-0001)
4. Keratan sulfate from Bovine Cornea (SEIKAGAKU, catalog number: 400760)
5. Dry tetrasaccharide (100 μg) is composed of ΔUA, *N*-acetyl-D-galactosamine (4S, 6S), galactose, *N*-acetyl-D-glucosamine (6S). Briefly, KS branched CS from *M. chinensis* was treated with chondroitinase ACII, and resulting tetrasaccharide was collected through the fractionation using HPLC with Docosil column. Please see details in Higashi *et al.* (2016)
6. Dimethyl sulfoxide, dehydrated (dry DMSO) (Wako Pure Chemical Industries, catalog number: 040-18032)
7. Iodomethane (CH_3I) (Wako Pure Chemical Industries, catalog number: 139-02662)
8. Chloroform
9. Nitrogen gas (> 99.995%) (Nippon Megacare)

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10. Trifluoroacetic acid (TFA) (Wako Pure Chemical Industries, catalog number: 204-02743)
11. Acetic acid (NACALAI TESQUE, catalog number: 00212-43)
12. 4-*N,N*-dimethylaminopyridine (Wako Pure Chemical Industries, catalog number: 042-19212)
13. Pyridine (NACALAI TESQUE, catalog number: 29509-25)
14. Acetic anhydride (Wako Pure Chemical Industries, catalog number: 011-00276)
15. Hexane (NACALAI TESQUE, catalog number: 17935-05)
16. Sodium hydroxide (NaOH) (NACALAI TESQUE, catalog number: 31511-05)
17. Methanol (NACALAI TESQUE, catalog number: 21915-93)
18. Dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, catalog number: 043-07216)
19. 0.5 mol/L hydrochloric acid methanolic solution (Wako Pure Chemical Industries, catalog number: 080-07725)
20. Sodium tetrahydroborate ($NaBH_4$) (Wako Pure Chemical Industries, catalog number: 192-01472)
21. NaOH-DMSO suspension (see Recipes)
22. 5% (v/v) pyridine in 50% acetonitrile/water (see Recipes)
23. $NaBH_4$ (5 mg/ml) solution (see Recipes)

Equipment

1. Test tube mixer (SEIKAGAKU, model: TM-251)
2. Centrifuge (KUBOTA, model: Model 5922)
3. Sample concentrator (Hangzhou Allsheng Instruments, model: MD200-2)
4. Time-of-flight mass spectrometer JMS-T100GCV (JEOL, model: JMS-T100GCV)
5. ZB-5ms column (0.25 μm film thickness, 0.25 μm i.d. x 30 m) (Phenomenex)
6. Agilent Technologies 7890A GC system (Agilent Technologies, model: Agilent 7890A GC)

Procedure

The preparation of partially methylated alditol acetates (PMAAs) is depicted in Figure 1. All reagents are added using glass pipettes.

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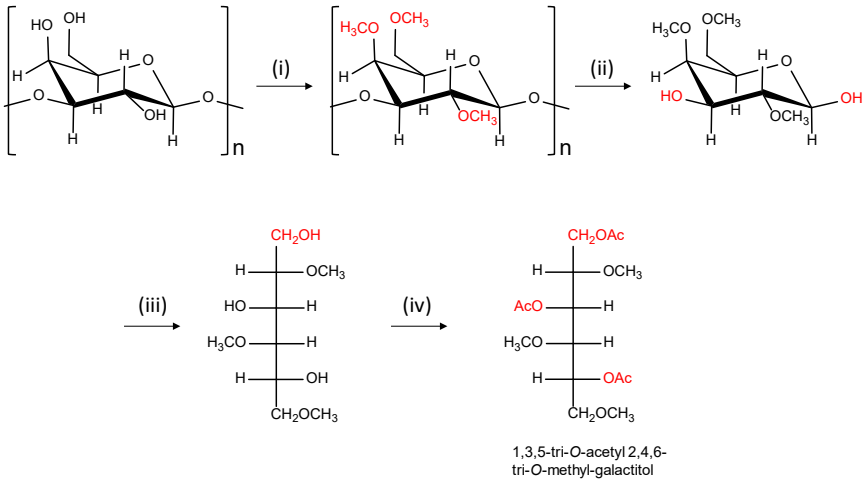

Figure 1. Scheme of the preparation of partially methylated alditol acetates (PMAAs) from polysaccharides having galactose residue. (i) DMSO, NaOH-DMSO suspension, CH_3I; (ii) TFA (2.5 M); (iii) 5% pyridine in 50% CH_3CN/H_2O, $NaBH_4$ in 30% CH_3OH containing 0.03 M NaOH; (iv) 4-*N*,*N*-dimethylaminopyridine in CH_3CN, pyridine, acetic anhydride.

A. Preparation of partially methylated sugars

1. Dry tetrasaccharide (100 µg) in a screw-cap tube.
2. Add 200 µl of dry DMSO and sonicate for 30 min.
3. Add 200 µl of NaOH-DMSO (see Recipes) and vortex.
4. Transfer the tube containing the sample onto ice and add 100 µl of iodomethane (CH_3I).
5. Sonicate for 5 min and vortex 3 times.
6. Add 50 µl of CH_3I again, and incubate for 30 min on ice.
7. Add 1 ml of distilled water and then vortex.
8. Add 1 ml of chloroform using a glass pipet (1 ml), vortex for 1 min, shake the tube well for 1 min, vortex for 1 min.
9. Centrifuge sample at 900 *x g* for 5 min at room temperature.
10. Remove water fraction (upper layer) using Pasteur pipette (aspiration).
11. To wash organic layer containing the methylated carbohydrates, add 1 ml of water and shake the tube well for 1 min, then vortex for 30 sec. Centrifuge at 900 *x g* for 5 min at room temperature and remove the water fraction using Pasteur pipette (aspiration).
12. Wash the organic layer two times using 1 ml of water.
13. Evaporate organic fraction (lower layer) for 40 min at 40 °C under a stream of nitrogen using a sample concentrator (Figure 2).
14. Dissolve sample with 150 µl of 2.5 mol/L TFA. Fill with nitrogen gas and close tightly with a screw cap. Incubate at 100 °C for 4 h.
15. Evaporate sample for 40 min at 40 °C under a stream of nitrogen using a sample concentrator (Figure 2).
16. Add 500 µl of 5% pyridine in 50% acetonitrile/water (see Recipes) to the dried, partially

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methylated sugars.

Figure 2. Evaporation of sample at 40 °C under a stream of nitrogen using sample concentrator (MD200-2). The spray nozzle was inserted into a glass tube which was placed to aluminum dry bath heating block. Sample was dried at 40 °C under nitrogen gas.

B. Preparation of partially methylated alditol acetates (PMAAs) from partially methylated sugars
   1. Add 200 µl of 5 mg/ml of $NaBH_4$ solution (see Recipes) into the sample and incubate for 4 h at 37 °C.
   2. Add 200 µl of acetic acid and evaporate sample within 1 h at 40 °C under a stream of nitrogen using a sample concentrator (Figure 2).
   3. Dissolve sample with 1 ml of 0.1% (v/v) MeOH-HCl and evaporate sample within 1 h at 40 °C under a stream of nitrogen (Figure 2). Repeat this step four times.
   4. Add 150 µl of 5 mg/ml of 4-*N,N*-dimethylaminopyridine, 50 µl of pyridine and 150 µl of acetic anhydride, respectively, and incubate at room temperature for 4 h.
   5. After incubation, add 2 ml of distilled water.
   6. Add 2 ml of chloroform, vortex, centrifuge at 900 *x g* for 5 min at room temperature and remove water (upper layer).
   7. Add 2 ml of distilled water, vortex, centrifuge at 900 *x g* for 5 min and remove water (upper layer).
   8. Evaporate sample (organic layer) for 40 min at 40 °C under a stream of nitrogen (Figure 2).
   9. Dissolve sample with 30 µl of hexane.

C. GC-MS (Agilent Technologies 7890A GC system) analysis of PMAAs
   1. Set electro impact ionization, 70 eV.
   2. Set carrier gas, helium at 1.2 ml/min.
   3. Set split less sample injection.

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4. Set column oven temperature program: 3 min at 100 °C, with an increase at 4 °C/min to 160 °C, 1 min at 160 °C followed by an increase at 0.5 °C/min to 180 °C, and a final increase at 20 °C/min to 260 °C and held for 10 min at 260 °C.
5. Submit 1 µl of PMAAs in hexane to GC-MS.

Data analysis

Electron ionization (EI) which afford the fragment-masses of small compounds is the most common form of ionization for GC-MS analysis. In general, fragmentation patterns generated by EI are compound dependent. In case of the structural analysis of glycans, electron ionization chromatogram (EIC) of certain fragment ions (*m/z* 45, 117, 161, 233) are useful for identifying peaks corresponding to PMAAs (Björndal *et al*., 1970). When EIC of *m/z* 233 was monitored, PMAA (1,3,5-tri-*O*-acetyl 2,4,6-tri-*O*-methyl-galactitol) from galactose in tetrasaccharide of *M. chinensis* was detected at 28.68 min (Higashi *et al*., 2016). However, PMAAs from ΔUA, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine were not observed.

Notes

1. For isolation of KS branched CS-E from *M. chinensis* and preparation of tetrasaccharide from KS branched CS-E, please see details in Higashi *et al.* (2016). In general, quantitation of CS was performed by post-column HPLC through the detection of unsaturated disaccharides obtained by chondroitinase (Chase). Interestingly, unknown peaks were found when CS from *M. chinensis* was treated with Chase ACII (at high concentrations) but not Chase ABC. Thus, unknown peaks were collected and analyzed by LC-MS/MS and GC-MS. In LC-MS/MS analysis, we found that unknown peaks consisted of tetrasaccharides including ΔUA, *N*-acetyl-D-galactosamine (4S, 6S), hexose, HexNAc (S). In addition, GC-MS analysis suggested that hexose in tetrasaccharide was galactose. Finally, *N*-acetyl-D-glucosamine (6S) in tetrasaccharide was suggested by 2D-NMR.
2. PMAAs from *M. chinensis* were prepared according to the method of Anumula and Taylor (1992) with minor modifications.

Recipes

1. NaOH-DMSO suspension (prior preparation)
   a. Combine 0.2 ml of 50% (w/w %) NaOH and 0.4 ml of methanol in a screw-cap tube
   b. Dilute with 6 ml of DMSO, vortex and sonicate for 3-5 min
   c. Centrifuge the fine dispersion of NaOH in DMSO at 900 *x g* for 10 min at room temperature
   d. Resuspend obtained precipitate with 6 ml of fresh DMSO and centrifuge at 900 *x g* for 10 min at room temperature. Repeat this step two times

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e. Resuspend obtained precipitate with 6 ml of dry DMSO, dehydrated and centrifuge at 900 *x g* for 10 min at room temperature. Repeat this step three times
f. Suspend precipitate with 4 ml of dry DMSO and store at 4 °C

2. 5% (v/v) pyridine in 50% acetonitrile/water (prior preparation)
   a. Prepare 1.9 ml of 50% acetonitrile with distilled water
   b. Combine 0.1 ml of pyridine and 1.9 ml of 50% acetonitrile/water
3. $NaBH_4$ (5 mg/ml) in 30% methanol containing 0.03 mol/L of NaOH (prior preparation)
   a. Prepare a solution of 0.03 mol/L NaOH in 30% MeOH
   b. Dissolve $NaBH_4$ with 30% MeOH containing 0.03 mol/L of NaOH

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