

In-Gel β -elimination

Goal: Release of O-linked glycans from proteins resolved by SDS-PAGE. Will focus on how to prepare permethylated glycans from an excised gel band and how to analyze glycans by NSI-LTQ/Orbitrap MSn.

Procedures:

- A. Gel excision and wash
- B. β -elimination
- C. Desalting using DOWEX 50WX8-200 ion exchange resin
- D. Borate Removal
- E. C18 Cleanup
- F. Base preparation for permethylation
- G. Glycan permethylation
- H. C18 for sulfated O-glycan

Materials

- Acetonitrile (ACN)
- Nanopure water
- 5% Acetic Acid (AcOH)
- 10% AcOH
- Ethyl Acetate (EtOAc)
- 100mM Sodium Hydroxide (NaOH)
- Sodium Borohydride (NaBH₄)
- Glass Wool
- Dowex 50WX8-200 H⁺ form
- LC-MS Methanol (MeOH)
- 10% AcOH in MeOH
- Iodomethane
- 50% NaOH
- Anhydrous Dimethyl Sulfoxide (DMSO)
- Anhydrous MeOH
- Dichloromethane (DCM)
- 50% ACN

A. Gel excision and wash

1. Add 1ml H₂O to each tube. **NOTE:** This prevents accidental tears in the gel piece.
2. Excise the region of interest using a clean scalpel, cut the gel piece into 2 mm cubes, and transfer into glass tube. **TIP:** Try to remove the smallest piece possible without sacrificing sample.
3. Invert tube until gel piece is at the bottom of tube and pipette off liquid
4. Add 1ml ACN and let stand for 10min
5. Pipette off liquid
6. Add 1ml H₂O and let stand for 5min
7. Pipette off liquid
8. Repeat steps 4-7 for three total washes
9. Add 2ml EtOAc and place on nutator O/N at 4°C

B. β-elimination

10. Pipette off liquid
11. Wash with 2ml H₂O for 10min
12. Repeat Steps 10-11 for three total washes
13. Add 250ul 100mM NaOH and let stand for 5min
14. Add 250ul 2M NaBH₄ and place at 45°C for 18hr
15. Remove from incubator and immediately place on ice.
16. Slowly add 10% AcOH dropwise to neutralize. **NOTE:** The addition of acid will produce a “volcano” of bubbles. Add acid SLOWLY! **TIP:** Centrifugation will remove the bubbles and prevent spillover. Keep samples on ice to prevent excessive heat production.

C. Desalting using DOWEX

17. Break the tip off a Pasteur pipette. **TIP:** Scoring the glass will produce a cleaner break
18. Insert glass wool and push to bottom. Wash with MeOH. **TIP:** Using a “long” pipette to push the glass wool will make this much easier.
19. Swirl Dowex solution prior to pipetting into “column”. Let resin settle before adding more. Fill until ½ full.
20. Rinse column using five volumes of 5% AcOH. **TIP:** Check wash to ensure that no Dowex has gone through column.
21. Place column in new tube and add sample. Collect flow through and wash with at least eight volumes of 5% AcOH.
22. Place samples at -80°C to freeze. Lyophilize O/N

D. Borate Removal

23. Add 300ul of 10% AcOH in MeOH to sample. Vortex.
24. Dry under N₂ stream
25. Repeat steps 23-24 at least 3 times.
26. Add 500ul 5% AcOH to sample

E. C18 Cleanup

27. Equilibrate column using three volumes of ACN and five volumes of 5% AcOH
28. Load sample onto column and collect flow through.
29. Wash column with 2ml 5% AcOH.
30. Place samples at -80°C to freeze. Lyophilize O/N.

F. Base Preparation for permethylation

31. Add 200ul of 50% NaOH to glass tube
32. Add 400ul of anhydrous MeOH and vortex
33. Add 4ml of anhydrous DMSO and vortex very well
34. Spin at 2500 rpm for 1min
35. Pipette off supernatant and white precipitate
36. Repeat steps 33-35 five times
37. Add 2ml anhydrous DMSO and gently mix with glass pipette

G. Permethylation

38. Add 200ul anhydrous DMSO to sample, vortex
39. Add 250ul Base to sample, vortex
40. Add 100ul Iodomethane to sample
41. Vortex for 5min
42. Add 2ml H₂O (5% AcOH), vortex **NOTE:** This stops the reaction
43. Add 2ml DCM, vortex
44. Spin at 2500 rpm for 1min
45. Transfer aqueous layer (top layer) into a new glass tube
46. Add 2ml H₂O into the original tube, vortex
47. Spin at 2500 rpm for 1 min
48. Remove aqueous layer
49. Repeat steps 46-48 three more times (four times total)
50. Transfer organic phase into new tube and dry under N₂ stream
51. Cover with parafilm and store at -20°C

H. C18 for sulfated O-glycan

52. Equilibrate C18 as described above in E (C18 cleanup)
53. Load aqueous phase obtained from G, step 45
54. Wash column with 10 ml H₂O
55. Elute permethylated sulfated O-glycan with 50% ACN
56. Dry under N₂ stream