

1. Protocol for N-glycoprotein enrichment by hydrazide resin

1. Suspend the Protein pellet (about 1 mg) in 250 μ L coupling buffer (100mM Sodium Acetate, 150mM Sodium Chloride, pH 5.5) .
2. Add 100 μ L 50 mM sodium perodate for glycoprotein oxidation at room temperature (RT), protect from light and agitate for 1 h.
3. hydrazide chemistry equilibration
 - 3.1 250 μ L hydrazide+750 μ L ddH₂O, 14,000 g for 5min, discard the supernatant; repeat for three times.
 - 3.2 Add 750 μ L coupling buffer, 14,000 g for 5min, discard the supernatant; repeat for three times.
 - 3.3 Add 250 μ L coupling buffer, agitate thoroughly.
- 4 Mix the protein solution with the 500 μ L equilibrated hydrazide solution, agitate at RT for 24 h, 14, 000 g for 5 min, discard the supernatant.
- 5 Desalting
 - 5.1 Add 1 ml ddH₂O, mixed and centrifuged at 14 000 g, 4 °C for 3×5 min, discard the supernatant.
 - 5.2 Add 1 ml 40 mM NH₄HCO₃, mixed and centrifuged at 14 000 g, 4 °C for 3×5 min, discard the supernatant.
- 6 Suspend the precipitation with 250 μ L, 40 mM NH₄HCO₃.
- 7 Add 50 μ L, 100 mM DTT for protein reduction, RT for 60 min.
- 8 Add 250 μ L, 100 mM IAA for protein alkylation, RT for 60 min, keep in darkness.
- 9 Trypsin digestion. trypsin: protein=1:50 (w:w) and digest at 37 °C overnight, less than 14 h.
- 10 Add 1 μ L formic acid to stop the digestion, centrifuge at 14, 000 g, 4 °C for 5 min, discard the supernatant.
- 11 Wash the precipitation with 1 ml ddH₂O, centrifuge at 14, 000 g, 4 °C for 5 min, keep the precipitation and repeat for three times. Followed by 30% ACN, ddH₂O and 40 mM NH₄HCO₃, respectively.
- 12 Add 200~300 μ L, 40 mM NH₄HC¹⁸O₃ (NH₄HCO₃ dissolved in H₂O¹⁸) to the filter-unit, agitate.
- 13 Add 5 μ l PNGase F to release the N-linked glycans, incubate at 37 °C overnight (12~14h).

- 14 Add 1 μL formic acid to stop the digestion, centrifuge at 14,000 g, 4 °C for 15 min, keep the supernatant.
- 15 Wash the precipitation with 80% ACN (diluted by H_2O^{18}), centrifuge at 14,000 g, 4 °C for 15 min, keep the supernatant. Repeat twice.
- 16 Pool the supernatant and concentrate for MS analysis.

2. Protocol for N-glycopeptide enrichment by lectin resin.

1. Suspend the Protein pellet (about 1 mg) in 200 μL UA buffer (8 M urea dissolved in 100mM Tris, pH 8.5), 14,000 g, 4 °C for 15 min, pipette the supernatant to the 0.5 ml filter unit.
2. Add 200 μL UA buffer and centrifuge at 14,000 g for 15 min.
3. Discard the flow-through from the collection tube.
4. Add 50 μL , 100mM DTT solution and mix for 1 min, then incubate for 60 min;
5. Centrifuge the filter unit at 14,000 g for 15 min.
6. Add 250 μL , 100mM IAA solution and mix for 1 min, then incubate for 60 min in darkness.
7. Centrifuge the filter unit at 14,000 g for 15 min.
8. Add 200 μL UA to the filter unit and centrifuge at 14,000 g for 15 min. Repeat this step twice.
9. Add 200 μL , 40 mM NH_4HCO_3 to the filter unit and centrifuge at 14,000 g for 15 min. Repeat this step twice.
10. Add trypsin (enzyme :protein= 1:50) to the filter unit and put the filter unit to a new collection tube, mix for 1 min, incubate at 37 °C overnight.
11. Add 1 μL formic acid to stop the reaction, centrifuge at 14,000 g for 15 min.
12. Add 50 μL 1 \times binding buffer to the filter unit and centrifuge at 14,000 g for 2 \times 15 min.

Transfer the flow-through solution to a new filter unit, add 36 μL lectin CWR [15 μL , 6mg/ml Concanavalin A, prepared with 2 \times Binding buffer); 15 μL , 6mg/ml Wheat germ agglutinin, prepared with 2 \times Binding buffer); 6 μL RCA120 agglutinin solution], agitate for 1 h at RT.
13. Add 100 μL 1 \times binding buffer to the filter unit, centrifuge at 14,000 g for 15 min. Repeat this step for 4 times.
14. Add 200 μL , 40mM NH_4HCO_3 to the filter unit, centrifuge at 14,000 g for 15 min. Repeat this step for 3 times.
15. Add 50 μL , 40 mM $\text{NH}_4\text{HC}^{18}\text{O}_3$ (dissolved NH_4HCO_3 in H_2O^{18}), centrifuge at 14,000 g, for 5

min.

16. Add 100-200 μL , 40 mM $\text{NH}_4\text{HddC}^{18}\text{O}_3$ to the filter-unit and agitate.
17. Add 5 μL PNGase F to release the N-linked glycans, incubate at 37 °C overnight.
18. Add 1 μL formic acid to stop the digestion, transfer the filter unit to a new collection tube, centrifuge at 14, 000 g 15 min.
19. Add 50-100 μL , 40 mM $\text{NH}_4\text{HC}^{18}\text{O}_3$, centrifuge at 14, 000 g, for 10 min, repeat twice.
20. Collect the solution in the collection tube, concentrate for MS analysis.

3. Protocol for purification of N-glycans.

21. Release glycans a by PNGase F.
22. Conditioning C8 cartridge with 4 mL nanopure water, followed by 4 mL 0.1% trifluoroacetic acid (TFA) in 80% acetonitrile in water (v/v)
23. load the proteins/glycans solution to cartridge, wash with 12 mL of ddH₂O to remove the salts.
24. Using 10% acetonitrile in water (v/v), 20% acetonitrile in water (v/v), and 0.05% TFA in 40% acetonitrile in water (v/v) to elute the glycans.
25. Collect the elution and dried.

4. Protocol for purification of major royal jelly proteins 1, 2 and 3

1. Dilute fresh RJ 1:20 with 20 mM Tris-HCl buffer (pH 7.6).
2. Vortex for 30 min.
3. Centrifuge at 12,000 rpm for 30 min, collect the supernatant.
4. Ion exchange
 - 4.1 Purify proteins by ion exchange chromatography the column of DEAE-cellulose by linear gradient elution with 1M NaCl in 20mM Tris-HCl buffer (pH7.6).
 - 4.2 Collect chromatographic peak.
 - 4.3 Desalt Sample by dialysis with 20mM Tris-HCl (pH 8.0).
5. Molecular sieve chromatography
 - 5.1 Separate MRJP1 using SephadexG-100, and for MRJP2 using Superdex 200.
 - 5.2 The elusion buffer used for each column was PBS (20mM Na_2HPO_4 , 2mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 150mM NaCl, pH 7.5).