Proteomics

Goal: Identification of human saliva proteins from a gel piece by using LC-MS/MS technique. Will focus on how to prepare peptide samples from a gel and how to analyze peptide samples with using LC-MS/MS instrument.

Procedures

A. 1D separation of saliva sample
B. Coomassie Brilliant Blue (CBB) staining
C. In-gel digestion with trypsin
D. Peptide purification by C_{18} resin
E. Peptide analysis by LC-MS/MS
F. Data analysis

A. 1D-gel separation of saliva protein

Reagents:

- Acetone
- 2x sample buffer (2-mercaptoethanol added)
- SDS-PAGE gel

1. Take 400uL saliva and add 800uL of ice-cold acetone, mix and keep at -30C to precipitate proteins
2. Spin down at max speed for 15 min, remove sup, and dry
3. Add 200uL of 2x sample buffer, mix, and boil for 5 min
4. Load 40uL (equivalent to 80uL saliva) onto a SDS-PAGE gel (4-20% gradient)
5. Run the gel
6. Subject to staining

B. CBB staining

Reagents:
CBB G-250 (Bio-Safe Coomassie G-250 Stain, Bio-Rad)

1. Wash the gel in water for 5 min x3
2. Incubate in CBB stain solution for at least 1 hour
3. Change to water and incubate with gentle shaking until destained

*You can use normal CBB R-250 stain. You could also use a microwave for quick staining. But, note that high-temperature might destroy some glycan modifications such as sialic acid, which may not be compatible with glycan analysis.

C. In-gel digestion with trypsin

Reagents:

- 40mM Ammonium bicarbonate (Ambic), C_{18}-passthrough
- Acetonitrile
- 0.5M Dithiothreitol (DTT)
- Iodacetamide (freshly prepared)
- Sequence Grade Trypsin (Promega)
- Trypsin buffer (100mM Ambic, pH8/10mM $CaCl_2$)
- 20%, 50% and 80% acetonitrile/5% formic acid

(Excision of the target protein band)

1. Excise the target protein band(s) using a clean surgical blade
2. Cut the gel piece into 1mm cubes on a spatula
3. Transfer them into a clean glass tube

(Wash and destain)

4. Add 40mM Ambic ~1mL
5. Stand for 20 min
6. Change to acetonitrile ~1mL
7. Stand for 20 min
8. Repeat steps 4~7 until the gel turns white
9. Remove acetonitrile
10. Add ~500ul of 10 mM DTT and incubate at 55C for 1 hour
11. Remove DTT and add freshly prepared 25 mM iodacetoamide
12. Incubate at RT for 45 min in the dark (wrap the tubes in foil)
13. Remove sup
14. Repeat steps 4~7 twice
15. Remove acetonitrile completely
16. Dry the gels up in a desiccator for ~10 min

(Trypsin digestion)

17. Prepare 20ug/mL Sequence Grade Trypsin in Trypsin buffer, cool on ice
18. Add 100uL of Trypsin (~2ug) and incubate for 45 min on ice to let it soaked into the gels
19. Add another 100uL buffer and incubate at 37C overnight

(Extraction of peptides from the gel)

20. Transfer the sups in a clean plastic tube
21. Add ~0.5mL of 20% acetonitrile/5% formic acid, incubate for ~30 min
22. Combine the sup to the same tube
23. Add ~0.5mL of 50% acetonitrile/5% formic acid, incubate for ~30 min
24. Combine the sup to the same tube
25. Add ~0.5mL of 80% acetonitrile/5% formic acid, incubate for ~30 min
26. Combine the sup to the same tube
27. Dry peptides down by Speed Vac

D. Peptide purification by C_{18}-resin

Reagents:

- C_{18} MicroSpin column
- Buffer A, 0.1% formic acid
- Buffer B, 80% acetonitrile/0.1% formic acid

(Prepare peptide)

1. Reconstitute the peptide in 300uL of Buffer A

(Wash and equilibrate C_{18}-resin)

2. Add 300uL of Buffer B to spin column and spin at 1,000 x*g* for 30 sec
3. Discard the flow-through
4. Repeat wash one more time
5. Add 300uL of Buffer A to spin column and spin at 1,000 x*g* for 30 sec
6. Discard the flow-through
7. Repeat equilibration with Buffer A two times (total three times)

(Binding and elution)

8. Apply the peptide sample to spin column and spin at 800 x*g* for 1 min
9. Put the flow-through back to the same column and spin again
10. Discard the flow-through
11. Wash the column with 300uL of Buffer A, spin at 800 x*g* for 1 min
12. Discard the flow-through
13. Repeat wash step two more times
14. Add 250uL of Buffer B and spin at 1,500 x*g* for 1 min
15. Transfer the flow-through (eluted peptide) to a clean Eppendorf tube
16. Repeat elution with 250uL of Buffer B
17. Combine the elution and evaporate to dryness by Speed Vac

E. Peptide analysis by LC-MS/MS

Reagents:

- Buffer A, 0.1% formic acid
- Buffer B, 80% acetonitrile/0.1% formic acid

Equipment:

- LTQ-Orbitrap (ThermoScientific)
- Sample loader (nitrogen bomb)
- Capillary (PicoTip Emitter, PF360-75-15-N-5)
- C_{18} resin (MICHROM, MAGIC C18 300A)

1. Warm up LTQ instrument (FT mode) for 1 hour
2. Reconstitute the peptide in 20uL of Buffer A, mix and spin down
3. Load onto C_{18}-packed capillary by using Sample loader with a nitrogen pressure at 1,000 psi for 5 min
4. Set the capillary onto the stage
5. Start spraying (Buffer A 100% with flow rate at 110 uL/min)
6. Run the program (Top6 program)

F. Data analysis

Equipment:

- ProteomeDiscoverer (ThermoScientific)

1. Transfer the data to the PC
2. Set the mass parameters
3. Specify the proteome database that you want to search against
4. Run a search program
5. Apply a filter to the peptide data with False Discovery Rate (FDR)