

In Gel Digestion for Mass Spectrometry (MS) Analysis

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INTRODUCTION

There are two major strategies for converting proteins extracted from biological material to peptides suitable for MS-based proteome analysis. The first involves solubilization of proteins with detergents, separation of proteins by SDS-PAGE (**S**odium **D**odecil **S**ulfate **P**olyacrylamide **G**el **E**lectrophoresis) and digestion of the gel-trapped proteins ("in-gel" digestion). The second is the detergent-free method comprising protein extraction with strong chaotropic reagents such as urea and thiourea, protein precipitation, and digestion of proteins under denaturing conditions ('in-solution' digestion). The one dimensional SDS-PAGE can be used not only to reduce sample complexity, but also to remove sample contaminants as detergents, salts, DNA and other non-protein compounds. However, the sample recovery in in-gel digestions is likely much less than in in-solution digestions, so this technique is more indicated for very complex samples (>300 proteins) where sample amount is not limited.

MATERIALS

- DIGESTION BUFFER: 50 mM ammonium bi-carbonate (NH_4HCO_3 , ABC) in water (pH 8.0). To make 50 mL, dissolve 200 mg ABC in 50 mL water. Store at room temperature.
- DESTAINING BUFFER: 25 mM NH_4HCO_3 / 50% ethanol (EtOH). Combine equal volumes of digestion buffer and 100% EtOH. Store at room temperature.
- REDUCTION BUFFER: 10 mM dithiothreitol (DTT) in 50 mM ABC. To make 10 mL dilute 100 μL of a 1 M DTT solution in 9.9 mL digestion buffer. Stored in small aliquots (1 ml) at -20°C .
- ALKYLATION BUFFER: 55 mM iodoacetamide in 50 mM ABC. To make 10 mL, dissolve 102 mg iodoacetamide in 10 mL digestion buffer. Store in small aliquots (1 ml) at -20°C . *Keep in the dark!*
- TRYPSIN SOLUTION: 12.5 ng/ μL trypsin in 50 mM ABC. When diluting one new vial containing 20 μg trypsin (modified sequencing grade, Promega), measure the original volume and add enough digestion buffer to reach 1600 μL final volume. Prepare IMMEDIATELY before use and keep it always on ice to minimize autocatalysis. Stored at -20°C . (Add 1.5 ml ABC-buffer to 1 vial of trypsin)
- EXTRACTION SOLUTION: 3% trifluoroacetic acid (TFA) / 30% acetonitrile (ACN). To make 10 mL, dilute 300 μL TFA and 3 mL ACN in 6.7 mL water. Store at room temperature.
- 2% TFA
- 100% ACN

METHODS

1. Place the gel on a clean glass plate or plastic foil and rinse it with water.
2. Excise the bands of interest with a clean (new, if possible) scalpel. Cut as close to the edge of the band as possible in order to reduce the included volume of gel.
3. Chop each of the excised bands into pieces of approximately 1x1 mm and transfer these cubes to a fresh microcentrifuge tube.
4. Wash the gel pieces **twice** with destaining buffer for **20 min** each at 25°C . Discard the liquid each time. Use around 1 ml of buffer, the procedure should be repeated until the staining is completely removed.

5. Dehydrate the gel pieces in 100% AcN for **10 min** at 25°C. A volume corresponding to approximately 3-4 times the total volume of gel pieces should be used and the procedure should be repeated until the gel pieces are hard and white. Discard the liquid afterwards.
6. Dry the sample in a speed-vac for **5 min** or until the gel pieces are bouncing in the tube.
7. Rehydrate* the gel pieces in reduction buffer and incubate for **60 minutes** at 56 °C. Enough solution should be used to at least cover the gel pieces. Discard ALL the liquid afterwards.
8. Add* alkylation buffer and incubate the gel pieces IN THE DARK for **45 min** at 25°C (150 µl). Enough solution should be used to at least cover the gel pieces. Discard the liquid afterwards.
9. Wash the gel pieces with digestion buffer for **20 min** at 25°C. Discard the liquid afterwards. Enough solution should be used to at least cover the gel pieces.
10. Dehydrate the gel pieces in 100% AcN for **10 min** at 25°C. Discard the liquid afterwards. A volume corresponding to approximately 3-4 times the total volume of gel pieces should be used.
11. Wash the gel pieces with digestion buffer for **20 min** at 25°C. Discard the liquid afterwards. Enough solution should be used to at least cover the gel pieces.
12. Dehydrate the gel pieces **twice** in 100% AcN for **10 min** each at 25°C. Discard the liquid each time. A volume corresponding to approximately 3-4 times the total volume of gel pieces should be used and the procedure should be repeated until the gel pieces are hard and white.
13. Dry the sample in a speed-vac for **5 min** or until the gel pieces are bouncing in the tube.
14. Rehydrate the gel pieces in the trypsin solution at 37°C. Enough solution should be used to at least cover the dehydrated gel pieces. Incubate until the gel has swelled as much as possible (~**20 min**).
15. Control amount of trypsin solution and add enough to at least cover the gel pieces. Incubate the sample **overnight** at 37°C.
16. Extract peptides from the gel matrix by incubating the gel pieces twice with extraction buffer 1:1 (v/v) for 15 min each, shaking vigorously at 25°C. After each extraction cycle spin down the gel pieces, recover the supernatant and combine it with that from step 16.
17. Dehydrate the gel pieces twice with 100% acetonitrile 1:1 (v/v) for 10 min each, shaking vigorously at 25°C. After each cycle spin down the gel pieces, recover the supernatant and combine it with those from steps 16 and 17.
18. Dry the sample in a speed-vac until 10-20% original volume to remove acetonitrile.
19. Reacidify the sample with ca. 40% (v/v) 2% TFA. Check pH of the sample.
20. Spin down at high speed to pellet left over gel pieces.
21. Proceed to peptide purification using StageTips (see protocol). Digested peptides can also be stored at -20°C until use.

BIBLIOGRAPHY

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