Video Article

Enzymatic In-gel Digestion of Proteins for Identification Using Mass Spectrometry

Brett Phinney[1], Rudy Alvarado*[2], Diana Tran*[2]

[1]Genome Center, University of California

[2]Genome Center, University of California at Davis

*These authors contributed equally

Correspondence to: Brett Phinney at bsphinney@ucdavis.edu

URL: http://www.jove.com/video/2515
DOI: doi:10.3791/2515

Keywords: Proteomics, ingel digestion, protein, digestion, mass spectrometry

Date Published: 6/15/2015

Citation: Phinney, B., Alvarado, R., Tran, D. Enzymatic In-gel Digestion of Proteins for Identification Using Mass Spectrometry. *J. Vis. Exp.* (), e2515, doi:10.3791/2515 (2015).

Abstract

Preparing proteins for identification and characterization by mass spectrometry can be very challenging as proteins span a wide range of physiochemical properties. These physiochemical properties can vary dramatically from protein to protein such as, their solubility in aqueous solvents (membrane spanning regions), their three-dimensional structure (alpha helix, beta sheet) and how they are modified posttranslationally (carbohydrates, phosphorylations, etc.). Often proteins you need to identify or characterize are in mixtures of other proteins that may have different physiochemical properties. In addition to their physiochemical properties, protein samples often contain salts and detergents which are refractory to most mass spectrometry systems and must be removed before analysis.

Bottom-up proteomics is the technique of digesting proteins into peptides, typically using proteases such as trypsin and then analyzing these peptides by a mass spectrometer. Protein identification, finding post translation modifications and targeted proteomics all typically rely on this bottom-up proteomic strategy. One popular technique for generating these proteolytic peptides involves digesting proteins in a matrix of polyacrylamide gel. One-dimensional and two-dimensional SDS-PAGE gels are widely used in many proteomics and biochemistry laboratories to separate and visualize proteins. Digesting proteins or protein mixtures in a SDS-PAGE gel helps overcome a number of the challenges mentioned above. Contaminants such as salts and detergents can be washed away from the protein, which is trapped in the gel matrix, and because the samples can be solubilized in SDS sample buffer, difficult to solubilize proteins such as membrane proteins can be solubilized with greater efficiency.

In order to process these protein gel samples for bottom up proteomics several steps must be taken. The gel pieces are first washed extensively and then chemically and mechanically dehydrated to ensure that the gel pieces will be completely saturated with reagents. The protein's cysteine residues are then usually reduced with dithiothreitol and alkylated with iodoacetamide. This reduces any disulfide bonds that may be present and prevents new one from forming. Trypsin needs a basic pH to digest proteins because it will denature in acidic conditions, the optimal pH for a tryptic digestion is around pH 8 which can be achieved by generously covering the gel pieces with 50mM ammonium bicarbonate to serve as a digestion buffer before the addition of trypsin. The modified trypsin utilized in this protocol optimally requires at least four to six hours to digest. As demonstrated in this protocol, the sample is left to digest overnight.

After digestion, the digested peptides should extricate itself from the polyacrylamide gel matrix and can be extracted along with the digestion buffer. However to fully ensure that all peptides are extracted from the gel pieces, the gel pieces are soaked in an extraction solution and sonicated. The peptide extracts are now ready to be concentrated and analyzed by LC-MS/MS analysis.

Video Link

The video component of this article can be found at http://www.jove.com/video/2515/

Protocol

1. Digestion:

1. Cut the gel bands into 1mm^3 cubes to increase surface area of the gel pieces exposed to the solutions.
2. Soak the gel pieces in a 50mM ammonium bicarbonate(AmBic, M_r 79g/mol) wash for about three to five minutes, generously cover the gel pieces.
    1. AmBic wash buffer will have to be made just prior to usage by weighing out 11.859mg of AmBic solid and dissolved in 3mL of high purity optima water(Fisher Water, M_r 18g/mol).
3. Discard the AmBic buffer.

4. Repeat steps two and three.
5. Generously cover the gel pieces with 100% LCMS grade acetonitrile (ACN, M_r 41.05g/mol) for about three to five minutes.
6. Discard the ACN wash.
7. Repeat steps five and six(the gel pieces should be shrunken in size and opaque in color at this point).
8. Dry the gel pieces mechanically in a speedvac concentrator for about ten minutes(the speedvac concentrator is the source of most of the keratin contamination and care must be taken to wipe down the inside of the concentration with methanol just prior to usage).
9. Generously cover the gel pieces with 10mM dithiothreitol(DTT, M_r 154.24g/mol), take into account the volume of DTT that will be absorbed by the gel pieces as they are hydrated and incubate at 56°C for thirty minutes.
    1. DTT stock is made prior to usage by measuring out 15.4mg of DTT solid and dissolving the powder completely in 10mL of Fisher Water.
    2. The solution is then divided into aliquots of 500uL each in a 1.5mL Eppendorf test tube and frozen in the -80°C freezer until usage.
    3. DTT aliquots will need to be completely thawed by leaving it out at room temperature before adding to the gel pieces.
10. Remove the gel pieces from the incubator and let it cool down to room temperature for about five minutes.
11. Decant the DTT solution into a liquid waste container.
12. Generously cover the gel pieces with 100% LCMS grade ACN for about three to five minutes.
13. Discard the ACN wash into waste.
14. Repeat steps twelve and thirteen again(gel pieces should be shrunken in size and opaque in color) Steps 12 through 14 are optional, but recommended to maximize complete saturation of the solvent in step 16.
15. Dry the gel pieces mechanically in a speedvac concentrator for about ten minutes or until gel pieces are completely dry.
16. Generously cover the dried gel pieces with 55mM iodoacetamide(IAA, M_r 184.96g/mol). Take into account the volume of IAA that will be absorbed by the dried gel pieces so they are all sufficiently covered with solution.
    1. IAA aliquots are made prior to usage by measuring 79mg of IAA solid quickly because IAA degrades under light.
    2. The IAA is then dissolved in 10mL of high purity optima water.
    3. The stock solution is then divided into 500uL aliquots in 1.5mL Eppendorf test tubes and frozen in a -80°C freezer until usage.
17. The gel pieces covered with IAA are then incubated in the dark on a shaker for twenty minutes.
18. Discard the IAA into liquid waste.
19. Generously cover the gel pieces with 50mM AmBic for about three to five minutes.
20. Discard the AmBic wash.
21. Repeat steps nineteen and twenty.
    1. At this point the gel pieces should be completely clear and have no traces of blue color if you used a coomassie based stain. If your gel is still blue you can wash with a 1:1 mixture of ACN and 100mM AmBic at 37°C for thirty minutes with gentle mixing (Eppendorf thermo Mixer or equivalent.)
22. Generously cover the gel pieces with 100% ACN for about three to five minutes.
23. Discard the ACN wash.
24. Repeat steps twenty-two and twenty-three(gel pieces should now be opaque in color and shrunken in size).
25. Dry the gel pieces via speedvac concentrator for about ten minutes.
26. Pipette 250ng of modified sequencing grade trypsin(if necessary the amount of trypsincan be adjusted based on the estimated amount of protein in your gel piece) onto the side of the Eppendorf tube next to the dried gel pieces, but not directly touching them.
    1. A stock of 250ng/uL of trypsin is made prior to usage and stored in a -80°C freezer in aliquots of 5uL each.
    2. To make the 250ng/uL stock of trypsin, add 80uL of 100mM AmBic to 20ug vials of modified sequencing grade porcine trypsin.
    3. Trypsin should be completely thawed just before usage.
27. Generously cover the dried gel pieces and trypsin in an 50mM AmBic buffer(remember to take into account the amount of AmBic that will be absorbed by the dried gel pieces overnight)
28. Incubate the gel pieces overnight at 37°C.

2. Extraction:

1. Centrifuge the gel pieces with digestion buffer for about 30 seconds to a minute on a bench top centrifuge.
2. Aspirate all of the digestion buffer and place into a clean Eppendorf test tube and label the test tube with the sample name.
3. Add 60% LCMS grade acetonitirile(ACN, M_r 41.05g/mol) and 0.1% trifluroacetic acid(TFA, M_r114.02g/mol) solution just enough to cover the gel pieces and sonicate in a water bath for 10 minutes.
    1. The 60% ACN and 0.1% TFA solution is made prior to usage and kept in the fume hood at room temperature.
4. Combine the 60% acetonitrile from step three with the digestion buffer from step 2.
5. Reduce the volume of the liquid from step 4 using a speedvac concentrator until almost dry(drying completely can lead to sample loss depending on the initial concentration of your sample).
6. Inject into a LC-MS/MS system or analyze by a MALDI mass spectrometer.

3. Representative Results:

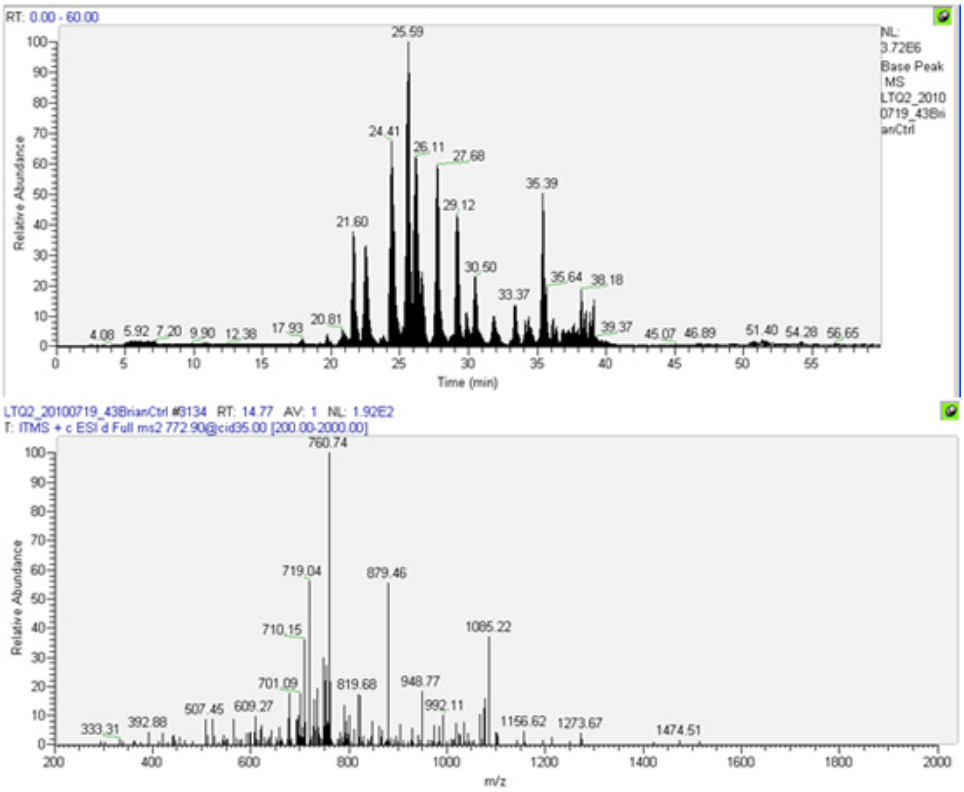

Figure 1. Base peak ion chromatogram

The number of proteins identified depends on the complexity of the sample being digested. The following picture is a representative LC-MS/MS base peak ion chromatogram of bovine serum albumin that is used as a qualitative control at UCD Proteomics Core Facility. A successful digestion should yield nicely separated peptides with no contamination from detergents or salts and little keratin contamination.

Discussion

The solution volumes used in the protocol varies from sample to sample and depends on the size of the gel piece. The only downside to adding too much DTT and IAA is wasting reagents. Also, the larger the amount of extraction buffer added to the digested gel pieces means longer vacuum centrifugation time to dry the protein extract completely. The most critical step in this procedure is the addition of the endoprotease trypsin to digest the protein into peptides, usually a 1:30 ratio of trypsin to total protein amount is used. However, UCD Proteomics Core Facility generally uses 250ng of trypsin for every digestion, because it is more than enough that is needed to digest most protein samples. If you find a large amount of keratin contamination you can omit all the vacuum centrifugation steps and just use acetronitrile to dehydrate your gel pieces (digestion steps 8,15,25 & extraction step5). If you do omit these steps, care must be taken to reduce the amount of acetonitrile from extraction step 5 to under 5% before you analyze by reverse phase LC-MS/MS. Another way to decrease keratin contamination is to work in a clean environment, such as a clean bench, and always use clean nitrile gloves (do not use natural latex as latex contains many proteins that can contaminate your samples). You will, in all likelihood, not be able to decrease keratin contamination to zero as the gel pieces themselves are often contaminated with keratin.

Disclosures

No conflicts of interest declared.

Acknowledgements

Modified from Shevchenko, Methods in Molecular biology Vol 122 1999 based on Shevchenko 1996 analytical chem... Rowley, methods 20 383-397 2000)

References

1. Shevchenko, A. *et al*. “In gel digestion for mass spectrometric characterization of proteins and proteomes”. *Nat. Protocols* 2007.1: p.2856 – 2860.