Full protein sequence mapping using mass spectrometry

Donald Hunt
University of Virginia
Erin Jeffery
University of Virginia

Method Article

Keywords: protein sequencing, mass spectrometry, phosphorylation sites

Posted Date: February 25th, 2009

DOI: https://doi.org/10.1038/nprot.2009.50

Abstract

Introduction

The procedures described here are those used in the Hunt laboratory to map the full protein sequence of migration-related proteins identified in the Proteomics Initiative of the Cell Migration Consortium, with the ultimate goal of identifying phosphorylation sites on these proteins.

Reagents

DTT – dithiothreitol, Iodoacetamide, Endoproteinase Lys-C, Trypsin, Chymotrypsin, Glu-C, C-18 packing material \(YMC Corporation), 2 M methanolic hydrochloride, ascorbic acid

Equipment

LTQ-FTMS and ETD mass spectrometry technologies and associated equipment, "microcapillary" HPLC columns constructed from fused silica glass capillaries \(Polymicro Technologies, Phoenix, AZ), a laser puller, a pressurized stainless steel bomb and Helium gas, HPLC machine, a Finnigan LTQ-FTMS \(ThermoElectron Corp., San Jose , CA) instrument, Immobilized Metal Affinity Column \(IMAC) chromatography

Procedure

Sample Preparation: reduction/alkylation with DTT/Iodoacetamide 1. Treat protein sample with dithiothreitol \(DTT) to break disulfide linkages between cysteine residues. An 8mM aliquot in ammonium bicarbonate buffer to 3nmol of cysteine residues is sufficient. 2. Heat sample at 51 degrees Celsius for 1.5 hrs. 3. Let cool to room temperature. To prevent the disulfide bonds from re-forming, add iodoacetamide \(16mM in ammonium bicarbonate buffer for 3nmol of cysteine residues is sufficient) and place in the dark at room temperature for 30-45 minutes. 4.To quench excess iodoacetamide, add a second aliquot of DTT \(same amount as in step1) and let heat at 51 degrees Celsius for 1 hr. Let cool to room temperature. For further info, see "http://www.ionsource.com/Card/cmc/method.htm":http://www.ionsource.com/Card/cmc/method.htm **Protease digestion** 1. Use Endoproteinase Lys-C \(Lys-C) or trypsin to digest proteins into a population of peptides that can be identified by the mass spectrometer. Add enzyme at 1:20 enzyme: protein ratio and let incubate at room temperature for 6-9hrs. For detailed information and digestion protocols, see "http://www.roche-applied-science.com/pack-insert/1420429a.pdf":http://www.roche-applied-science.com/pack-insert/1420429a.pdf \(Lys-C) or "http://www.promega.com/tbs/9piv511/9piv511.pdf":http://www.promega.com/tbs/9piv511/9piv511.pdf\(trypsin). 2. For maximal amino acid sequence coverage, digest with several proteases. The selection of proteases depends greatly upon the amino acid sequence of the target protein. Choices of additional enzymes include Glu-C and chymotrypsin. Again, add at 1:20 enzyme: protein ratio and let incubate at room temperature for 6-9 hrs. For detailed information and digestion protocols, see \(Glu-C)

"http://www.roche-applied-science.com/pack-insert/1420399a.pdf":http://www.roche-applied-science.com/pack-insert/1420399a.pdf and \(chymotrypsin) "http://www.roche-applied-science.com/pack-insert/1418467a.pdf":http://www.roche-applied-science.com/pack-insert/1418467a.pdf . **LC-MS Analysis** 1. Construct microcapillary RP-HPLC separation interfaced separation columns from fused silica glass capillaries \(Polymicro Technologies, Phoenix, AZ). Cut off a segment of 360x50um fused silica about 15cm in length. 2. Burn off the polyimide coating of about 2cm of the capillary \(about 6cm from the top) using a small flame. 3. Use a laser puller to pull a bottleneck into the clear glass section such that the diameter of the bottleneck is approximately 4um. 4. Make up a slurry of 5um C18 packing material \(YMC Corporation) in 80:20 acetonitrile:isopropanol. Load this packing material behind the bottleneck using a pressurized stainless steel cylinder. Pack approximately 6-7cm of packing material behind the bottleneck. 5. Use the laser puller to pull an electrospray tip approximately 2-3mm after the bottleneck such that the tip is approximately 5um in diameter. 6. Condition the column by loading 5pmol of angiotensin onto the column and running three short RP-HPLC gradients. 7. Load an aliquot of the proteolytic digest onto the microcapillary column \(for aliquots of 1-3uL) using the pressurized stainless steel cylinder. 8. For larger sample volumes \(>3uL), load onto a “pre-column”. These are constructed from 360x75um fused silica capillaries. 9. Cut a piece of 360x75um fused silica that is approximately 7-8cm in length. 10. Burn the polyimide coating off the very end of the capillary \(about 3-5mm) using a small flame. 11. Pack licrosorb material into the clear end of the capillary and then burn with a micronox flame until licrosorb is melted but still porous. 12. Make a slurry of 5-20um C18 packing material \(YMC Corporation) in 80:20 acetonitrile:isopropanol. 13. Pack approximately 3-5cm of the packing material behind the frit using a pressurized stainless steel cylinder. 14. Condition the column as in step 6. 15. After loading the proteolytic digest onto the pre-column, wash with 0.1% acetic acid to remove any salts. 16. Connect the pre-column to the analytical column using a Teflon sleeve. 17. Connect the column apparatus to the HPLC solvent line. 18. Adjust HPLC pump flow such that the flow out of the ESI tip is close to 57nL/min. 19. For electrospray mass spectrometry, apply a high voltage \(1.8-2kV) from the mass spectrometer to the stainless steel union on the HPLC waste line. 20. Depending on complexity of the sample, run a RP-HPLC gradient of 0-80% organic solvent over a period of 40min-1.5hrs. The low flow rate and shallow gradient help minimize the number of peptides being analyzed by the mass spectrometer at any given time, effectively decreasing the complexity of the sample and maximizing the coverage of peptides selected for MS/MS analysis. **MS/MS analysis of peptide ions** 1. We use ion trap mass spectrometry for our analyses, including Recently, a LTQ-FTMS, LTQ equipped with ETD and Orbitrap instruments \(ThermoFisher Scientific, San Jose, CA). 2. MS/MS spectra are acquired in “data-dependent” mode. This enables the acquisition of mass spectra in a non-redundant manner so that as many peptides as possible are surveyed from a complex mixture. 3. Set up an experimental method during which one full range MS scan \(300-2000 m/z) is acquired followed by the acquisition of ten data-dependent MS/MS scans. **Peptide sequence identification using SEQUEST and OMSSA** 1. For full protein sequence mapping, search the MS/MS spectra against a database of the target protein sequence. For this step, it is essential that the full amino acid sequence of the target protein is accurate. The plasmid itself, used for transfection of target DNA into host cell, should be sequenced to insure confidence in the amino acid sequence that is ultimately translated. 2. Include a variable modification of +80amu \(the mass of a phospho modification) on S,T and Y residues and a static modification of +57amu on cysteine \(mass of carbamidomethylation from

DTT/Iodoacetamide treatment) in the search parameters. 3.For data sets that include ETD spectra, we prefer the OMSSA search algorithm. It is available for free download from "http://pubchem.ncbi.nlm.nih.gov/omssa/":http://pubchem.ncbi.nlm.nih.gov/omssa/ . 4. We manually validate all phosphopeptide hits to confirm the position of the phosphorylation site. All database hits below the top-scoring thresholds are manually confirmed to eliminate false positive hits. **IMAC enrichment of phosphopeptides** 1. To pack an IMAC column, use 360x100 or 360x150um fused silica \(depending on capacity needs) and frit with licrosorb as described above. 2. Make a slurry of POROS MC 20 packing material in water and pack approximately 5-8cm \(depending on capacity needs) behind the frit using a pressurized stainless steel cylinder. 3. To prepare the sample for IMAC, the peptides must be esterified. This will eliminate the binding carboxylic acid side chains to the IMAC column. 4. To esterify, prepare a solution of 2M methanolic hydrochloride by adding 160uL acetyl chloride, dropwise, while stirring under a ventilation hood, to 1mL of anhydrous methanol. 5. Let solution incubate, stirring, for 5 minutes at room temperature. 6. Dry down the peptide sample to complete dryness and add approximately 40uL of the 2M methanolic HCl to the sample tube. 7. Let sit at room temperature for 1-1.5 hrs. Dry sample again. 8. Repeat treatment with fresh methanolic HCl. Let sit at room temperature for 1hr. 9. Dry sample for last time and reconstitute in approximately 30uL of 1:1:1 acetonitrile: methanol: 0.01% acetic acid. 10. Prepare IMAC column by using a pressurized stainless steel cylinder to rinse with ddH20 for 10 min at 20uL/min. 11. Then rinse column with 50mM EDTA for 5 min at 20uL/min. 12. Rinse again with ddH20 for 5 min at 20uL/min. 13. Activate column by rinsing with 100mM FeCl3 for 10 min at 20uL/min. 14. Load entire sample onto IMAC column slowly \(approx 1uL/min). 15. Rinse column with 1:1:1 acetonitrile: methanol: 0.01% acetic acid \(15min at 1uL/min). 16. Rinse column with 0.01% acetic acid for 15 min at 1uL/min. 17. Attach the RP-HPLC pre-column to the top of the IMAC column using a Teflon sleeve. 18. Elute the phosphopeptides off of IMAC column using 15uL of 100-200mM ascorbic acid \(made fresh) at 1uL/min. 19. Detach pre-column and wash pre-column with 100% HPLC Solvent A \(aqeous solvent) for approximately 15 minutes. 20. Attach pre-column to analytical column using a Teflon sleeve and run LC-MS/MS analysis as described above.

Timing

Sample Preparation - approximately 8-9hrs LC-MS Analysis – approximately 1-2hrs Peptide sequence identification using SEQUEST – approximately 30min for database search and 12hrs for manual validation, depending on complexity IMAC enrichment of phosphopeptides – approximately 8 hrs, including esterification

References

Yates JR 3rd, Eng JK, McCormack AL, Schieltz D. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. Anal Chem. 1995;15,67\(8):1426-36. PMID: 7741214 Ficarro S, Chertihin O, Westbrook VA, White F, Jayes F, Kalab P, Marto JA, Shabanowitz J, Herr JC, Hunt DF, Visconti PE. Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation. J

Biol Chem. 2003;278\(13):11579-89. PMID: 12509440 Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM. Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. Nat. Biotechnol. 2002;20\(3):301-5. PMID: 11875433 **Web Resources** General Mass Spectometry resources "http://i-mass.com/guide/protocol.html":http://i-mass.com/guide/protocol.html - General proteomics site containing information about protocols and resources "http://us.expasy.org/":http://us.expasy.org/ - Proteomics tools and resources "http://prospector.ucsf.edu/":http://prospector.ucsf.edu/ - Mass spec data analysis "http://depts.washington.edu/yeastrc/ms_home.htm":http://depts.washington.edu/yeastrc/ms_home.htm - General tandem mass spec information Mass Spectometry Protocols and Procedures "http://www.ionsource.com/Card/ester/esters.htm":http://www.ionsource.com/Card/ester/esters.htm - Making methyl esters "http://www.ionsource.com/Card/cmc/why.htm":http://www.ionsource.com/Card/cmc/why.htm - Reduction of proteins "http://www.ionsource.com/Card/cmc/intro.htm":http://www.ionsource.com/Card/cmc/intro.htm - Reduction/alkylation of proteins "http://prowl.rockefeller.edu/recipes/sproteas.htm":http://prowl.rockefeller.edu/recipes/sproteas.htm - Proteolysis