Isolation of Phosphopeptides by Immobilized Metal Ion Affinity Chromatography

This unit describes mass spectrometric (MS) analysis of phosphopeptides and different approaches to enrich the phosphopeptides from complex protein samples prior to MS analysis. Basic Protocol 1 involves manual or automated prefractionation of peptides by cation-exchange chromatography followed by immobilized metal ion affinity chromatography (IMAC) using commercially available spin columns. It is suitable when larger amounts of complex material are available (at least 100 μg), and can be performed in any molecular biology laboratory because it requires no specialized chromatographic or mass spectrometric equipment. The eluted samples can be sent for MS analysis at a suitable facility. The Alternate Protocol describes the use of anion-exchange chromatography and IMAC. Basic Protocol 2 describes an online IMAC method optimized for highly selective and sensitive detection of phosphopeptides from complex mixtures using methyl esterification of peptides. Basic Protocol 3 describes a new method for analysis of tyrosine phosphorylation from complex mixtures using IMAC. Prefractionation of samples by multidimensional chromatography at either the peptide or protein level considerably increases the number of identified phosphopeptides for Basic Protocols 1 and 2.

MANUAL PEPTIDE FRACTIONATION BY CATION-EXCHANGE CHROMATOGRAPHY AND IMAC

BASIC PROTOCOL 1

This protocol describes the fractionation of a complex peptide mixture by cation-exchange chromatography, followed by immobilized metal ion affinity chromatography (IMAC) of each individual fraction. No specialized large equipment (such as FPLC or HPLC) is needed, and the procedure, up to the elution of the purified phosphopeptides, can be performed in any molecular biology laboratory.

The first consideration is the preservation of the in vivo phosphorylation state in the cell extract. Total soluble proteins can be extracted under denaturing conditions to prevent dephosphorylation, but if subcellular fractionation is performed, native buffers with generous amounts of appropriate phosphatase inhibitors must be used (e.g., EDTA, sodium pyrophosphate, sodium fluoride, β-glycerophosphate, and calyculin A for serine/threonine phosphatases; sodium orthovanadate for tyrosine phosphatases). It is also worth considering the inhibition of protein kinases after breaking the cells to avoid nonspecific in vitro phosphorylation in the extract; addition of EDTA may be sufficient to block kinase activity by chelating magnesium, but more specific inhibitors like K-252a can also be used.

The use of volatile buffers in all steps from digestion to IMAC has the considerable advantage of preventing sample losses through the desalting steps (Gruhler et al., 2005). Triethylammonium bicarbonate (TEAB) is more volatile than ammonium bicarbonate and is preferred for both trypsin digestion and anion-exchange chromatography (see Alternate Protocol). The salt component in cation-exchange chromatography is ammonium formate. IMAC is very sensitive to salt, and ammonium formate is easily removed from the eluted fractions by lyophilization or by concentration in a Speedvac evaporator.

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Peptide trap cartridges are offered by a variety of vendors and allow manual sample preparation with a syringe. Optimize Technologies offers cation-exchange cartridges (Opti-Lynx) with different capacities and a holder with fittings. A high-capacity cartridge of this type is also supplied with Applied Biosystem's iTRAQ kit. This protocol using cation exchange is compatible with iTRAQ labeling for quantitative experiments. It is an adaptation of the manufacturer's protocol for manual sample cleanup after labeling with the iTRAQ reagent (an amine-labeling reagent for multiplexed relative and absolute protein quantitation). The method described here uses the ion-exchange cartridge supplied by the manufacturer with the solvents described in Gruhler et al. (2005). This provides a simple manual setup that requires little specialized equipment, but for greater reproducibility of fractionation and greater loading capacity (milligram range), the use of an HPLC or FPLC system with an SCX column is recommended (see Beausoleil et al., 2004; Gruhler et al., 2005).

A variety of IMAC materials have become commercially available, including different combinations of metals and chelating resins and options for batch or spin-column chromatography. In side-by-side tests, these were at least as good as hand-made microcolumns packed with Fe^{3+}-charged POROS-MC resin (Nühse et al., 2003). For ease of use and reproducibility, this protocol uses Phos-Select IMAC resin (Sigma).

This protocol is universally applicable to complex soluble or membrane protein samples. In the latter case, washing the membrane preparation with a high-pH buffer removes more soluble proteins and leads to more peptide identifications from integral membrane proteins.

Materials

Protein sample (100 to 500 μg)
100 mM sodium carbonate (Na_2CO_3)
50 and 500 mM triethylammonium bicarbonate (TEAB) in ddH_2O, diluted from 1 M stock solution (Sigma)
Sequencing-grade modified trypsin
2.5% (v/v) formic acid/30% (v/v) acetonitrile
Cation-exchange cartridge (e.g., Optimize Techologies Opti-Lynx or Applied Biosystems iTRAQ), with holder and syringe adapter
SCX start buffer (see recipe)
SCX step elution buffers (10, 20, 30, 40, 60, and 100 mM salt; see recipe)
SCX cleaning buffer (see recipe)
Sodium azide, optional
Phos-Select IMAC resin (Sigma)
IMAC load/wash buffer (see recipe)
IMAC elution buffer (see recipe)
10% (v/v) formic acid

11 × 34–mm polycarbonate ultracentrifuge tubes
Ultracentrifuge (e.g., Beckman Optima tabletop ultracentrifuge with a TLA120 rotor)
37°C water bath
Lyophilizer or Speedvac evaporator
1.5- and 2-ml microcentrifuge tubes
End-over-end rotator
Fritted spin columns (e.g., Mobicols, MoBiTec; Handee Mini Spin columns, Pierce)

Digest sample

For insoluble, particulate, or membrane protein samples

1a. Resuspend protein/membrane pellet in 100 mM Na_2CO_3 in a 11 × 34–mm polycarbonate ultracentrifuge tube and incubate 10 min on ice to remove peripheral proteins. Ultracentrifuge 30 min at 100,000 × *g*, 4°C.

This step is only necessary when there is a particular focus on integral membrane proteins. Peripheral membrane proteins can make up ≥50% of a microsomal pellet and are efficiently stripped off the membrane with the alkaline carbonate solution. At the same time, integral membrane proteins are partially denatured.

2a. Resuspend the pellet in 0.8 ml of 500 mM TEAB and centrifuge again 30 min at 100,000 × *g*, 4°C.

3a. Resuspend the pellet in 0.2 ml of 50 mM TEAB and measure the protein concentration.

Ammonium bicarbonate is the traditional buffer for digestion and can be used, but TEAB evaporates much more readily.

4a. Start with 100 to 500 μg protein and add sequencing-grade modified trypsin at a trypsin/protein ratio of 1:50 to 1:20 (2 to 25 μg trypsin). Digest overnight at 37°C.

For larger ion-exchange columns with a higher capacity, up to several milligrams of protein can be digested.

Sequencing-grade modified trypsin is used to avoid the low chymotrypsin-like activity that is present in unmodified trypsin.

The digested sample can be stored for several weeks at −70°C if required, but some sample loss may occur.

5a. Lyophilize or concentrate in a Speedvac evaporator to 10 to 20 μl. Proceed to step 6.

For soluble protein samples

1b. Digest proteins as described in Basic Protocol 2, steps 1c to 6c, and then desalt according to Basic Protocol 2, steps 12 to 17. Proceed to step 6.

Perform cation-exchange chromatography

6. Redissolve the dried peptide sample in 0.5 ml of 2.5% formic acid/30% acetonitrile.

The sample should ideally have zero salt and be at pH ≤2.7. Since ammonium formate does not buffer at this pH, the peptides are loaded in dilute acid rather than the start buffer.

7. Centrifuge 10 min at 15,000 × *g*, room temperature, to remove insoluble material.

8. Prepare the cation-exchange cartridge by washing at ~1 ml/min with the following:

2 ml SCX start buffer
2 ml SCX cleaning buffer
2 ml SCX start buffer
1 ml 2.5% formic acid/30% acetonitrile.

9. Slowly (~200 μl/min) load the cleared sample onto the cartridge. Collect flowthrough, if desired.

In the authors' experience, few phosphopeptides are recovered from the unbound fraction, but this may vary in different types of samples.

10. Wash with 2 ml wash buffer. Elute with 0.5 ml each of 10, 20, 30, 40, 60, and 100 mM SCX step elution buffers for highly complex samples, or with 25, 50, and 100 mM SCX step elution buffers for less complex samples.

11. Lyophilize fractions or concentrate in a Speedvac evaporator to ~20 µl.

    Lyophilzation is easier if the fractions are first concentrated in the Speedvac to half of the original volume, because the acetonitrile lowers the freezing point of the liquid. Thawed samples will "boil over" in the vacuum of the lyophilizer.

12. Clean cartridge with 2 ml cleaning buffer and then re-equilibrate with 2 ml start buffer or with 2 ml start buffer plus 3 mM sodium azide for long-term storage.

Perform IMAC

13. Resuspend Phos-Select IMAC resin to homogeneity.

14. Pipet an aliquot of the 50% slurry (40 to 80 µl per SCX fraction per IMAC experiment) into a 2-ml microcentrifuge tube. Centrifuge 2 min at 500 × *g*, room temperature. Remove as much of the manufacturer's glycerol-containing storage buffer as possible.

15. Add 1 ml IMAC load/wash buffer, mix by inverting a few times, centrifuge again, and remove wash buffer. Repeat at least three times.

16. Distribute equal aliquots of the slurry into tubes for each SCX fraction to be analyzed.

17. Redissolve each concentrated or dried peptide fraction from ion-exchange chromatography in 0.3 ml IMAC load/wash buffer. If insoluble material remains, clear samples by centrifuging 5 min at 15,000 × *g*, room temperature.

18. Add dissolved peptide fractions to the tubes with Phos-Select resin. Incubate by end-over-end rotation for 1 to 2 hr at room temperature.

19. Prepare a fritted spin column with a 2-ml collection tube for each sample. Flick down liquid droplets and resin from the lid of the tube, transfer the complete slurry to the spin columns, and centrifuge 30 sec at 8200 × *g*, room temperature. Collect flowthrough (nonphosphorylated peptides) if desired.

20. Add 200 µl IMAC load/wash buffer to the beads and resuspend by pipetting the slurry up and down. Centrifuge 30 sec at 8200 × *g*, room temperature.

    Unlike with prepacked spin columns, it is not sufficient to simply add the wash solution to the top of the tube. The beads should be resuspended by pipetting up and down.

21. Wash one additional time with IMAC load/wash buffer and then one time with ddH_2O.

22. Transfer spin columns to clean 1.5-ml microcentrifuge tubes.

23. Add 30 µl IMAC elution buffer, resuspend well, and incubate 1 to 2 min at room temperature. Centrifuge 10 sec at 8200 × *g* and collect the eluate.

24. Repeat elution with IMAC elution buffer and combine eluates.

    Take care not to leave the Phos-Select resin in the alkaline buffer for longer than ~5 min total.

25. Concentrate eluates in the Speedvac to ~10 to 20 µl and acidify with 1 to 2 µl of 10% formic acid.

    The samples are ready for mass spectrometric analysis. Rapid sample losses due to adsorption occur if the tubes are kept at room temperature or 4°C, but it is possible to store them at −70°C for a short time (up to several days).

MANUAL PEPTIDE FRACTIONATION BY ANION-EXCHANGE CHROMATOGRAPHY AND IMAC

ALTERNATE PROTOCOL

This protocol uses a microcolumn setup with POROS HQ anion-exchange resin and a volatile buffer system at alkaline pH. If the sample digest has been performed in dilute TEAB without urea or other additives (as in the case of membrane or insoluble proteins, which can be washed in trypsin buffer after denaturing as described in Basic Protocol 1), the digest can be applied directly to the column after adding acetonitrile and pelleting the insoluble material.

Additional Materials *(also see Basic Protocol 1)*

POROS HQ strong anion-exchange resin
SAX start buffer (see recipe)
SAX step buffer (see recipe)
SAX step elution buffers (50, 100, 200, and 500 mM; see recipe)
GELoader tips (Eppendorf)

1. Prepare POROS HQ resin by washing ~100 µl of settled material in 1 ml SAX start buffer.
2. Prepare a microcolumn in a GELoader tip (see Support Protocol 1). Load the slurry into the prepared column to a packed height of ~2 to 3 cm in the wider part of the tip (i.e., 3 to 10 mm below the wide neck).
3. Wash with 50 µl each of SAX start buffer, SAX step buffer, and again SAX start buffer.
4. Prepare the sample digest in SAX start buffer: if the digest is in trypsin buffer, by adding 0.75 vol each ddH_2O and acetonitrile; if it is in buffers containing urea or salts, by reversed-phase cleanup (see Basic Protocol 2, steps 12 to 14; use 500 µl of 20 mM TEAB/50% acetonitrile for elution).
5. Slowly load the sample onto the microcolumn with a syringe and adapter (see Support Protocol 1).
6. Wash three times with 50 µl SAX start buffer.
7. Elute with 50 µl SAX step elution buffers (50, 100, 200, and 500 mM salt).

   The number of steps depends on the complexity of the sample; most phosphopeptides elute between 100 and 200 mM salt.

8. Lyophilize the eluates as in Basic Protocol 1, step 11.

MANUAL CHROMATOGRAPHY WITH MICROCOLUMNS

SUPPORT PROTOCOL 1

Conventional sample preparation methods are often not appropriate for the microgram to nanogram amounts of material available for MS experiments. Gobom et al. (1999) introduced a manual microchromatographic method for reversed-phase (RP) purification and concentration of peptides for analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The RP material is packed into the narrow capillary tip of a gel-loading pipet tip with a constricted end. A column of 2 to 3 cm packing height has a volume of only a few microliters, and thus reduces sample loss from nonspecific adsorption. This format is very versatile, and virtually any chromatographic material with a bead size of ~20 µm or larger can be used. To create columns with slightly higher capacity, the wider-bore part of the pipet tip can also be packed to a volume of up to ~50 µl (e.g., for ion-exchange separation of several hundred micrograms of digest). The disadvantage of this method is that preparation of multiple samples is time-consuming and most likely not as reproducible as automated approaches.

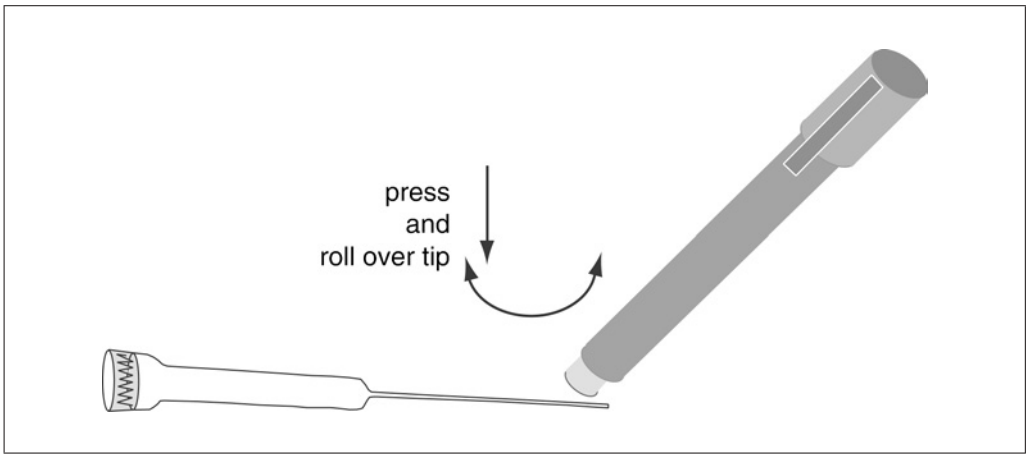

Figure 18.13.1 Constriction of a GELoader tip for use as a microcolumn.

The only pipet tips that work satisfactorily for this technique are original Eppendorf GELoader tips; the capillaries are soft enough to be constricted so they retain the chromatography material. A 1-ml plastic Luer-type syringe is also needed, and a 200-µl pipet tip trimmed on both ends with a razor blade is used as a tight adapter between the Luer syringe tip and the GELoader tip.

To constrict the GELoader tip, lay the tip flat on the benchtop and press with a semi-blunt plastic item (e.g., the back end of a pen; Fig. 18.13.1) at a point ~1 to 2 mm away from the end of the fine capillary. Use considerable pressure, but be careful not to sever the tip off. Pipet 10 µl acetonitrile into the tip and hold upright. If the tip is sufficiently compressed, the drop will not move or drop out. If it does, the tip is not narrow enough to hold the POROS beads; try again with a fresh tip. Fit the syringe and adapter onto the tip and apply full pressure. Ideally, there should be resistance at first, and then the liquid should come out. It should now be possible to load the slurry into the tip.

Pipet the slurry, sample, wash, and elution buffers as far down into the tip as possible and then flick the tip down with a quick wrist motion. Fit the syringe onto the tip and press the liquid through. Depending on the backpressure from the medium, full compression of a 1-ml syringe may be necessary. For larger column volumes, take care not to run the material dry. Microcolumns packed only into the capillary end of the tip are less sensitive to drying out briefly, provided no chromatographic resolution is required.

BASIC PROTOCOL 2

ENRICHMENT OF PHOSPHOPEPTIDES THROUGH METHYL ESTER FORMATION AND IMAC

Background binding of peptides containing a high percentage of acidic amino acids to the IMAC column can lead to significant contamination of the enriched phosphopeptide samples, and thus difficulty in applying IMAC alone to satisfactorily enrich phosphopeptides from complex cellular lysates (Brill et al., 2004). This protocol describes IMAC enrichment of phosphopeptide methyl esters for increased IMAC selectivity (Ficarro et al., 2002, 2005; Salomon et al., 2003; Brill et al., 2004; Ndassa et al., 2006). Although this procedure requires the use of an HPLC pump and practice working with capillary columns, it provides unparalleled sensitivity and efficiency due to the highly selective enrichment of phosphopeptides from complex cell-derived samples. All separations and sample transfers occur column-to-column ("online") without exposure to plastic, including microcentrifuge tubes or 96-well plates, thereby minimizing peptide loss. Sensitivity arises from the use of capillary chromatography coupled with nanospray mass spectrometry at flow rates approaching 20 nl/min. This protocol describes preparation of the phosphopeptide methyl esters, IMAC enrichment, and MS analysis. The

IMAC enrichment procedure is performed with the use of a pressure bomb and a total of four custom-made capillary columns. This IMAC protocol incorporates newly developed improvements in IMAC buffer solutions, is optimized for reduced competition on the IMAC column between singly- and multiply-phosphorylated peptides, and improves overall yield of phosphopeptides (Ndassa et al., 2006).

Protocols for elution of phosphopeptides from the precolumn over the analytical column and into the mass spectrometer will vary depending on the configuration and type of chromatography and mass spectrometry equipment available. The method described here works well for the LTQ, LTQ-FTICR, or LTQ-Orbitrap and Agilent 1100 quaternary pump.

Materials

Protein sample (1×10^8 cells or ~100 fmol purified protein)
Cell lysis buffer A (see recipe), optional
Immunoprecipitation resin: anti-phosphotyrosine agarose (e.g., clone PT66, Sigma), optional
Immunoprecipitation wash buffer (see recipe), optional
Trypsin digestion buffer with urea (see recipe)
Milli-Q water
Sequencing-grade modified trypsin (Promega)
Dialysis buffer (see recipe), optional
Trizol LS (Invitrogen), optional
DC protein assay kit (Bio-Rad) containing:
  Alkaline copper tartrate solution
  Folin reagent
  Surfactant solution
  Bovine gamma-globulin standard
Angiotensin II phosphate (EMD Biosciences) or other synthetic phosphopeptide
1 M HCl
Reversed-phase loading buffer: 0.1% acetic acid in Milli-Q water
Reversed-phase elution buffer (see recipe)
Macrotrap wash buffer 1: 20:80 (v/v) dimethylsulfoxide (DMSO)/methanol
Macrotrap wash buffer 2: 45:45:10 (v/v/v) isopropanol/water/formic acid
Methylation solution (see recipe)
Nitrogen source
Fe(III)-activated IMAC column (see Support Protocol 2)
IMAC preparation buffer 1: 50 mM EDTA, pH 8
IMAC preparation buffer 2: 100 mM $FeCl_3$ (99.99+%, Sigma)
Desalting column (see Support Protocol 2)
HPLC buffer A (IMAC): 0.01% acetic acid in Milli-Q ddH_2O
HPLC buffer B (IMAC): 0.01% acetic acid in acetonitrile (99.8%; HPLC grade)
IMAC wash buffer (see recipe)
Precolumn (see Support Protocol 2)
IMAC elution buffer: 25 mM Na_2HPO_4, pH 9.0
Analytical column (see Support Protocol 2)
HPLC buffer C (reversed-phase): 0.1 M acetic acid in Milli-Q ddH_2O
HPLC buffer D (reversed-phase): 0.1 M acetic acid in acetonitrile (99.8%; HPLC grade)

1-ml syringes and 27-G needles
50-ml conical tubes
1.5-ml microcentrifuge tubes
95°C heating block
0.45-μm, 4-mm syringe filters (e.g., Millipore Millex), optional

200- and 1000-µl pipets (Rainin)
10,000 MWCO Slide-A-Lyzer (Pierce), optional
Reversed-phase C18 peptide macrotrap (Michrom Bioresources)
1.5-ml screw-top microcentrifuge tubes (Sarstedt)
Speedvac evaporator (Thermo Savant)
Inline microfilters (Upchurch Scientific)
HPLC pump (e.g., Agilent 1100 Quaternary Pump)
Fused silica capillaries (360-µm o.d. × 50-, 75-, and 200-µm i.d.; Polymicro Technologies)
Microtee (Upchurch Scientific)
Laser Puller P2000 (Sutter Instruments), optional
Mass spectrometer (e.g., LTQ-FTICR and LTQ available from Thermo)
Valco trap holder (Michrom Bioresources)
Multiple-syringe infusion pump
500-µl Hamilton syringe
Platinum wire (Sigma)

NOTE: All sample preparation procedures involving intact proteins are carried out at 4°C unless otherwise specified.

Prepare sample

For mammalian cell phosphorylation analysis

1a. Lyse cells in 25 ml cell lysis buffer A per 1×10^9 cells.

2a. Centrifuge 20 min at 12,000 × *g*, 4°C, and collect supernatant (total cellular lysate).

3a. Store samples, if necessary, at −80°C. Proceed to step 11.

For an individual protein or a subset of total cellular phosphorylated proteins

1b. Immunoprecipitate phosphorylated proteins from the total cellular lysate using CNBr-agarose-conjugated antibodies. Add 200 µl of immunoprecipitation resin per 1×10^9 cell equivalents in 25 ml (in a 50-ml tube) and incubate 4 hr with tumbling.

CNBr linkage is preferable for coupling antibodies to agarose beads compared to noncovalent linkages such as protein A/G due to its resistance to the urea used in the elution of proteins in this protocol, and the high density of antibody on the resin resulting in reduced amounts of resin. For isolation of the tyrosine phosphoproteome, anti-phosphotyrosine agarose clone PT66 (Sigma) works well. Clone 4G10 works very poorly for immunoprecipitation of native proteins. Tyrosine phosphorylation represents only a small fraction (~0.1%) of cellular phosphorylation, necessitating an additional affinity enrichment step before IMAC to enrich the population of tyrosine phosphorylated proteins relative to serine and threonine phosphorylated proteins. For serine and threonine phosphorylation, no pre-enrichment step is necessary before IMAC.

2b. Wash beads once with 50 ml cell lysis buffer A and once with 50 ml immunoprecipitation wash buffer in a 50-ml conical tube. Centrifuge 5 min at 1700 × *g*, 4°C, and then aspirate the supernatant.

It is critical to be thorough in the removal of the wash buffer, because detergent (Triton X-100) will significantly contaminate the IMAC and reversed-phase columns, resulting in high background detergent peaks in the MS spectra and dramatic reduction of the number of phosphorylation sites identified.

3b. Add 1 ml of immunoprecipitation wash buffer to beads and transfer beads into a 1.5-ml microcentrifuge tube using a 1000-µl pipet tip with 3 mm of the tip cut off.

4b. Insert a 27-G needle attached to a 1-ml syringe along the side of the tube and withdraw residual wash buffer to thoroughly dry beads.

5b. Elute immunoprecipitated proteins by incubating with 200 µl trypsin digestion buffer with urea in a 1.5-ml microcentrifuge tube for 5 min at 95°C.

6b. Repeat elution with another 200 µl trypsin digestion buffer with urea for 5 min at room temperature, without additional heating. Insert needle along the side and to the bottom of the tube to withdraw all of the supernatant, being careful not to contaminate with resin.

7b. (Optional) Filter the eluted proteins with 0.45-µm, 4-mm syringe filters into 1.5-ml microcentrifuge tubes to remove any residual resin.

8b. Measure volume of eluant with a 200-µl pipet and dilute precisely 1:1 with Milli-Q water for a final urea concentration of 4 M.

9b. Add 10 µg sequencing-grade modified trypsin per 1×10^9 immunoprecipitated cell equivalents in a 1.5-ml microcentrifuge tube and incubate overnight at 37°C.

Do not reduce and alkylate proteins prior to trypsin digestion and IMAC enrichment because this frequently reduces the number of phosphorylation sites discovered.

10b. Store trypsin-digested samples, if necessary, at −80°C. Proceed to step 11.

For total cellular phosphorylation

1c. Dialyze total cellular lysate 1:1000 (v/v) in dialysis buffer in a 10,000 MWCO Slide-A-Lyzer three times, 3 hr each time, at 4°C.

2c. Extract proteins by Trizol LS according to manufacturer's instructions.

3c. Solubilize the Trizol pellet in trypsin digestion buffer with urea for 5 min at 95°C.

4c. Dilute 1:1 with Milli-Q water for a final urea concentration of 4 M.

5c. Measure the protein concentration using the Bio-Rad DC protein assay kit according to the manufacturer's instructions.

6c. Add sequencing-grade modified trypsin to the protein at a ratio of 1:20 (w/w) trypsin/total protein and incubate overnight at 37°C.

Do not reduce and alkylate proteins prior to trypsin digestion and IMAC enrichment because this frequently reduces the number of phosphorylation sites discovered.

7c. Store trypsin-digested samples, if necessary, at −80°C. Proceed to step 11.

Prepare peptide methyl esters

11. Add 10 pmol of angiotensin II phosphate or another synthetic phosphopeptide per 1×10^9 cell equivalents to digested peptides as a control for methylation.

Many synthetic tyrosine phosphorylated peptides are available from Anaspec.

12. Acidify peptide solution to pH 3 with 1 M HCl. Load sample on a reversed-phase C18 peptide macrotrap (placed in a Valco trap holder) that has been pre-equilibrated by washing with 500 µl of reversed-phase loading buffer (delivered by a 500-µl Hamilton syringe attached to a multiple syringe infusion pump).

13. Wash macrotrap with 1 ml of reversed-phase loading buffer.

This step removes urea and salts from samples.

14. Elute peptides with 500 µl of reversed-phase elution buffer into 1.5-ml screw-top microcentrifuge tubes.

Loss of peptides due to nonspecific absorption to tubes and plates is a major concern. In the authors' experience, Sarstedt tubes provide the lowest nonspecific peptide binding and should be used for macrotrap elution and methylation.

15. Regenerate macrotrap columns by washing with 500 µl of macrotrap wash buffer 1 followed by 500 µl of macrotrap wash buffer 2.

    Carryover of peptides on C18 columns is a persistent concern that must be monitored carefully. This regeneration procedure is designed to minimize this carryover and prevent contamination of subsequent samples.

16. Reequilibrate macrotrap with 500 µl of reversed-phase loading buffer and repeat steps 12 through 16 until all samples are processed.

17. Completely dry peptides in a Speedvac evaporator without heat for 2 hr with rotation.

    It is imperative that all water be removed at this point to optimize the yield of the methylation procedure.

18. Mix 400 µl of methylation solution with each dried peptide sample and layer nitrogen on top of the reaction prior to closing cap.

19. Mix on vortex mixer for 5 sec and allow reaction to proceed for 2 hr.

20. Dry methylated peptides for 2 hr in a Speedvac evaporator without heat and with rotation. Make sure methylation solution is fully removed after 2 hr.

21. Store dried, methylated peptides, if desired, at −80°C.

Enrich peptide methyl esters by IMAC

22. Prepare an Fe(III)-activated IMAC column by washing with IMAC preparation buffer 1, then IMAC preparation buffer 2, and finally reversed-phase loading buffer for 5 min each at a rate of 20 µl/min.

23. Connect an inline microfilter as a frit to the outlet of a desalting column and equilibrate it with reversed-phase loading buffer.

    For elution of peptides from the desalting column to the IMAC column, an inline microfilter is used as a union to attach the columns in series.

24. Reconstitute the dried, methylated peptides with reversed-phase loading buffer and immediately load on the desalting column.

    Typical reconstitution volumes are from 10 to 100 µl, but the actual volume is not critical. Peptides should only be reconstituted immediately before IMAC enrichment.

25. Wash column for 10 min at 7 µl/min with reversed-phase loading buffer.

    Column flow rates are not absolutely critical, but may be controlled by adjusting the air pressure to the pressure bomb and measuring the flow rate using a 5-µl measured capillary and a stopwatch (500 psi is a good starting point).

26. Connect the inlet of the IMAC column to the outlet of the desalting column using the inline microfilter already attached.

27. Attach the inlet of the desalting/IMAC column pair to the outlet of the HPLC pump. Elute to the activated IMAC column by washing 20 min at 1.8 µl/min with reversed-phase elution buffer or using a 17-min HPLC gradient of 0% to 70% HPLC buffer B at 1.8 µl/min.

    A step-elution method would not require use of an HPLC pump, but reveals approximately half the number of phosphopeptide species from a complex cell lysate.

28. Remove IMAC column from desalting column. Wash IMAC column with IMAC wash buffer for 5 min followed by reversed-phase loading buffer for 2.5 min, both at 20 µl/min.

29. Connect precolumn to IMAC column through the inline microfilter.

30. Elute with 12 μl IMAC elution buffer at 2 μl/min.

31. Wash precolumn with 10 μl HPLC buffer A at 5 μl/min.

Analyze by MS

32. Connect the washed precolumn to the analytical column through an inline microtee with one side coupled to a platinum wire for the application of electrospray voltage.

33. Use the "vented column" approach to control flow rate through the precolumn and analytical column. Connect an empty 50-μm capillary (called a restrictor) to a microtee junction between the incoming HPLC capillary and the precolumn. Configure the flow rate through the precolumn and analytical column by adjusting the length of the restrictor such that 0.25 ml/min HPLC pump output produces ~200 nl/min flow rate at the electrospray tip.

    This configuration allows rapid delivery of the gradient to the precolumn and is preferable to nanoHPLC, which is more expensive and results in long delays for gradient changes to be conveyed to the precolumn and analytical column.

34. Elute peptides into the analytical column with a 30-min gradient from 0% to 70% HPLC buffer D.

    Optionally, the flow rate may be reduced during the peptide elution part of the gradient by adjusting the flow rate at the HPLC pump down to 0.05 ml/min. This reduction in flow rate, called peak parking, is useful in increasing the sensitivity for detection of peptide ions and spreading out the peptides so that the time to acquire MS and MS/MS data for individual peptides is increased.

35. Acquire spectra with data-dependent scanning (one MS scan followed by five MS/MS scans of the most abundant ions). Set collision energy at 35% with a 3-Da window, and set dynamic exclusion at a repeat count of one and an exclusion time of 1.5 min.

    Dynamic exclusion prevents the redundant acquisition of MS/MS spectra.

36. Assign MS/MS spectra with the Sequest or Mascot search algorithms. Search for a variable addition of 79.9663 Da on Ser, Thr, and Tyr (indicating phosphorylation), and a static modification of 17.0342 Da on Asp, Glu, and the peptide C terminus (indicating D3-methylation).

CONSTRUCTION OF COLUMNS FOR IMAC AND REVERSED-PHASE SEPARATION OF METHYL ESTER–DERIVITIZED PEPTIDES

SUPPORT PROTOCOL 2

The enrichment of phosphopeptide methyl esters by IMAC requires the use of a total of four columns. A large-capacity desalting column is used after peptide methylation to remove residual acid from the methylation reaction for better IMAC retention. An IMAC column loaded with Fe^{3+} enriches phosphopeptides. These phosphopeptides are then eluted to a precolumn with phosphate, and the phosphate is removed by washing. Peptides are then eluted by a reversed-phase gradient from the precolumn, through the analytical column, and into the mass spectrometer. These columns should be prepared and utilized in close collaboration with a mass spectrometry facility. Generally, this technique is compatible with any electrospray mass spectrometer capable of nanospray. In the authors' experience, the LTQ-FTICR and LTQ from Thermo are excellent instruments for phosphorylation analysis due to their impressive scan rate and sensitivity. Other instruments such as quadrupole time-of-flight (QTOF) mass spectrometers would likely provide enough sensitivity and mass accuracy, but require longer gradients for complex samples due to their slow scan rate.

Flow rates or volumes are measured with a 5-μl measured capillary and a stopwatch. Collect column flowthrough in a capillary over a 1-min period and then use a ruler to

measure the number of microliters collected in the measured capillary over this time period.

Use of the pressure bomb: Pressure bombs are used both to pack slurries of chromatographic resin as well as to load samples into 50- to 250-μm-i.d. capillaries. It is not feasible to provide the necessary pressure of >500 psi to prepare a column using a syringe. Although HPLC pumps can provide these sorts of pressures, it is not convenient to pass an emulsion of resin through the flow path of the HPLC. The pressure bomb (GNF commercial systems; Fig. 18.13.2) works by application of 500 to 1000 psi of nitrogen pressure to the 1/8-in. stainless steel tubing in the side of the bomb. A Swagelok three-position 1/8-in. ball valve is attached to the tubing to allow for the controlled release of pressure from the bomb. The sample is placed inside the bomb and a small glass capillary containing a porous frit is inserted into the sample solution through a 1/16-in. to 0.5-mm Teflon one-hole ferrule (Chromatography Research) in the top. The sample can be a mixture of reversed-phase or IMAC resin in 95% acetonitrile/5% isopropanol, column wash solution, or analytical sample. The pressure bomb is placed on a standard laboratory magnetic stirrer and all emulsions of resins are stirred in the bomb in small glass vials. Various materials may be used for the resin-retaining frit, including LiChrosorb Si60 (5 μm; EMD Biosciences) or Kasil (PQ Corporation). Most conveniently, an inline microfilter from Upchurch can simply be screwed onto the end of the capillary. Once the bomb is closed with the two top screws, application of high pressure (typically through a Swagelok three-way valve) from a nitrogen gas tank causes the sample to be passed onto

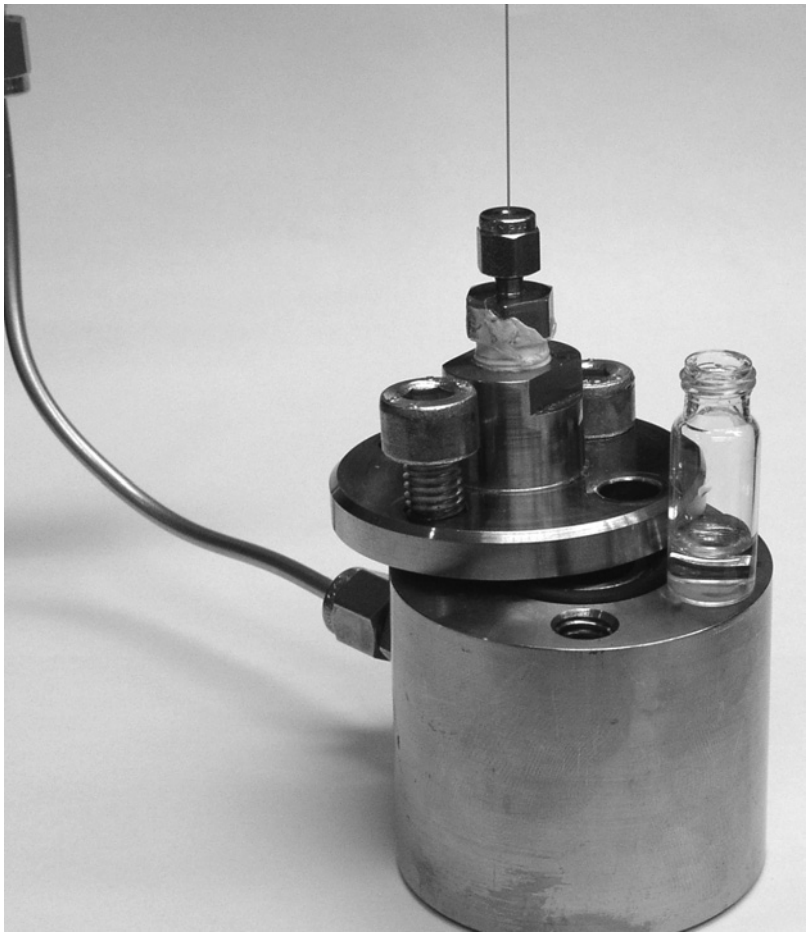

Figure 18.13.2 A pressure bomb used for packing resins and loading samples.

the column. The flow rate or volume through the column is gauged with a Drummond 5-µl calibrated pipet and can be adjusted by altering the gas pressure at the regulator.

Construction of desalting and IMAC columns: The fused silica columns may be cut to the desired length with a fused silica cutter (Alltech), being sure that all cuts are straight (check under microscope). All columns are packed on a pressure bomb as described above and fritted with inline microfilters. The desalting column is packed with 12 cm of POROS 10R2 (a C18 resin available from Applied Biosystems) in 360-µm o.d. × 200-µm i.d. fused-silica tubing, and the IMAC column is packed with 15 cm of POROS 20MC (Applied Biosystems) in a 360-µm o.d. × 200-µm i.d. fused silica capillary. After packing, both columns are washed once for 5 min under 500 psi of bomb-applied pressure with acetonitrile and then with reversed-phase loading buffer.

Construction of precolumn: The precolumn is prepared by first preparing a LiChrosorb Si60 frit in a 360-µm o.d. × 75-µm i.d. fused silica capillary. To prepare this type of frit, dip the capillary into the powdered LiChrosorb powder ten times and then flame the tip with a nitrous oxide flame three times for 1 sec each pass. A nitrous oxide flame may be created with a microflame gas torch set (VWR) by first starting a butane flame, minimizing its intensity, and then increasing the nitrous oxide until a uniform blue flame is produced. Adjust the capillary flame timing such that the LiChrosorb material is in the flame long enough to cause a brilliant white glow. Pack the precolumn with 2 cm of 5-µm Monitor C18 (100-Å, Column Engineering) on the pressure bomb and wash with acetonitrile and then reversed-phase loading buffer.

Construction of an analytical column with integrated electrospray emitter tip: There are two possible configurations of the analytical column. For optimal sensitivity, analytical columns may be prepared with integrated electrospray emitter tips (Fig. 18.13.3) on a P2000 laser puller (Sutter Instruments). Useful recommendations for the tuning of the laser puller are provided in the manufacturer's manual. Remove 3 cm of the polyimide coating from a 360-µm o.d. × 75-µm i.d. fused silica capillary by flaming with butane and removing debris with isopropanol. On the exposed section of the capillary, pull a 5-µm-i.d. tip, being careful to avoid the polyimide. Obtaining a 5-µm tip requires adjustment of the heat, velocity, delay, and pull settings on the puller. Good starting values are heat = 290, filament = 0, velocity = 15, delay = 127, and pull = 10. To maintain reproducible pulling, clean the puller mirror regularly with spectroscopy-grade isopropanol or acetone. Determine the diameter of the tip by bomb loading 3-µm silica (Bangs Labs) into the column after pulling the tip. Two 3-µm beads should have lodged into the tip. If the tip is occluded by one bead, it is too narrow; try decreasing the heat setting. After fritting the analytical column with 3-µm silica, pack 12 cm of 5-µm Monitor C18 (100-Å, Column Engineering) into the column.

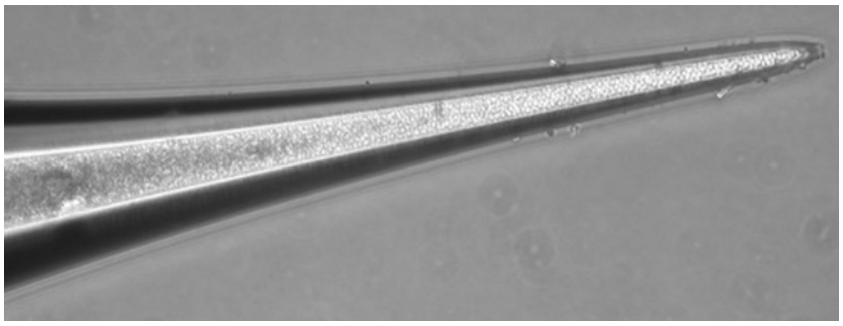

Figure 18.13.3 A 5-µm emitter tip (under a microscope at 200× magnification).

Construction of analytical column attached to a picotip: If a laser puller is not available, picotips (8-μm; New Objective) may be purchased. This configuration is less sensitive but may be adequate for some analyses. Pack a 360-μm-o.d. × 75-μm-i.d. fused silica capillary, fritted with an inline microfilter, with 12 cm of 5-μm Monitor C18 (100-Å, Column Engineering). The analytical column is then attached to the picotip through the inline microfilter.

BASIC PROTOCOL 3

ENRICHMENT OF TYROSINE-PHOSPHORYLATED PEPTIDES VIA PEPTIDE IMMUNOPRECIPITATION AND IMAC

Unlike phosphotyrosine immunoprecipitation (IP) at the protein level (see Basic Protocol 2, steps 1b to 10b), peptide IP employs phosphotyrosine-specific antibodies to isolate tyrosine-phosphorylated peptides directly from a total cell lysate of digested cellular proteins (Rush et al., 2005). The advantage of protein IP is that it permits the more extensive analysis of all serine, threonine, and tyrosine phosphorylation sites on tyrosine-phosphorylated proteins as well as all phosphorylation sites on proteins that interact in a complex with tyrosine-phosphorylated proteins. The advantage of phosphotyrosine peptide IP is that it is more sensitive for detection of tyrosine phosphorylation due to the increased efficiency of capture of the phosphorylated peptides by the PTyr100 antibody relative to the PT66 antibody. Another advantage of the peptide IP procedure is that it provides higher-quality quantitative analysis, because peptide chemical isotopic labeling such as with the popular iTRAQ reagent does not interfere significantly with the IP step. With protein IP, peptides must be labeled after the IP step.

In the peptide IP procedure, cells are lysed and digested, then the digests are desalted and immunoprecipitated by antibody-conjugated protein G beads (Rush et al., 2005). Peptides are eluted from beads by IP elution buffer (Zhang et al., 2005). A second enrichment of phosphopeptides is accomplished by IMAC as described in Basic Protocol 2, but without methylation. IMAC is necessary after peptide IP due to unacceptably high carryover levels of unphosphorylated peptides through the IP. The strategy of enhancing the selectivity of IMAC using peptide methyl esters is unnecessary in this procedure due to the selective enrichment afforded by the phosphotyrosine peptide IP. Although, in theory, methyl ester formation would diminish the low levels of nonspecific unphosphorylated peptides that appear after peptide IP and IMAC, the decrease in overall peptide yield through the methyl ester reaction results in overall net decrease in phosphopeptides identified with this method.

Materials

Anti-phosphotyrosine antibody (clone PTyr100, Cell Signalling Technology)
Antibody coupling buffer: 20 mM sodium phosphate, pH 7.0
Protein G–agarose beads (Roche)
Protein sample (3×10^7 cells or ~100 fmol purified protein)
Cell lysis buffer B (see recipe)
Dithiothreitol (DTT)
Iodoacetamide
100 mM ammonia bicarbonate, pH 8.9
DC protein assay kit (Bio-Rad) containing:
  Alkaline copper tartrate solution
  Folin reagent
  Surfactant solution
  Bovine gamma-globulin standard
Affinity purified, TPCK-treated trypsin (Promega)
Concentrated HCl
Sep-Pak C18 plus column (Waters)

100% acetonitrile
Sep-Pak loading buffer: 0.1% acetic acid in Milli-Q water
Sep-Pak elution buffer: 25% acetonitrile/0.1% acetic acid in Milli-Q water
Peptide immunoprecipitation (IP) buffer (see recipe), 4°C
Synthetic phosphopeptide external standard
pTyr-100 immobilized antibody
Peptide IP wash buffer 1 (see recipe)
Peptide IP wash buffer 2 (see recipe)
Peptide IP elution buffer: 100 mM glycine, pH 2.5
Tyrosine-phosphorylated standard peptides (e.g., angiotensin II phosphate, EMD Biosciences; or various pTyr peptides, Anaspec)

End-over-end rotator, 4°C
56°C water bath
15-ml conical tubes
1.5-ml screw-top microcentrifuge tubes (Sarstedt)
18- and 27-G needles
Speedvac evaporator (Thermo Savant)

Additional reagents and equipment for IMAC enrichment and MS analysis (see Basic Protocol 2)

NOTE: All sample preparation procedures involving intact proteins are carried out at 4°C unless otherwise specified.

Prepare antibody

1. Prepare the pTyr-100 immobilized antibody by mixing soluble pTyr-100 in antibody coupling buffer with protein G–agarose beads at a concentration of 2.25 mg antibody/ml resin. Incubate overnight at 4°C with end-over-end rotation.

   The pre-immobilized pTyr-100 currently sold from Cell Signaling Technologies is much less efficient than immobilized antibody prepared with protein G–agarose for peptide immunoprecipitation.

Prepare sample for immunoprecipitation

2. On the same day, lyse cells in 2 ml cold cell lysis buffer B per 3×10^7 cells and incubate 20 min on ice.

3. Centrifuge lysate 15 min at 14,000 × *g*, 4°C, and retain the supernatant.

4. Add DTT to a concentration of 10 mM to reduce proteins, and incubate 1 hr in a 56°C water bath.

5. Add iodoacetamide to a concentration of 55 mM and incubate 1 hr at room temperature in the dark.

6. Dilute lysate five times with 100 mM ammonia bicarbonate, pH 8.9, to a final urea concentration of 1.6 M. Measure the protein concentration using the DC protein assay kit according to the manufacturer's instructions.

7. Add affinity-purified, TPCK-treated trypsin to protein at a ratio of 1:100 (w/w) trypsin/total protein and incubate overnight at room temperature.

   A trypsin concentration of 1:40 is optimal but may be cost prohibitive. The 1:40 ratio would afford ~10% more phosphopeptides and a lower rate of missed cleavage sites. A 1:300 ratio of trypsin provides unacceptably high rates of miscleavage and greatly diminishes the number of phosphopeptides identified. Promega affinity-purified and TPCK-treated trypsin works best.

8. Acidify tryptic digests to pH 2 with concentrated HCl and centrifuge acidified digests 5 min at 2000 × *g* to remove any precipitate.

9. Pre-equilibrate a Sep-Pak C18 plus column by washing once with 5 ml of 100% acetonitrile and then twice with 3.5 ml of Sep-Pak loading buffer.

   The flow rate for steps 8 through 11 is ~1 to 10 ml/min. Within this range, a lower flow rate gives a better loading efficiency. The control of flow rate can be accomplished by adjusting the intensity of the vacuum.

10. Load precleared digested peptides onto washed Sep-Pak plus column.

11. Wash column with 10 ml Sep-Pak loading buffer to remove salts.

12. Elute peptides from column with 10 ml Sep-Pak elution buffer into a 15-ml conical tube.

13. Dispense eluents into 1.5-ml screw-top microcentrifuge tubes with their tops perforated using an 18-G needle.

14. Dry peptides in a Speedvac evaporator without heat for 6 hr with rotation. Combine eluents from all tubes when the total volume fits in one tube, before peptides are completely dried.

Perform immunoprecipitation

15. Reconstitute peptides with 500 µl cold (4°C) peptide IP buffer and add 10 pmol of a synthetic phosphopeptide external standard for quantitation of relative phosphopeptide abundance, as well as to control for variations in peptide IP efficiency.

    A variety of synthetic tyrosine phosphorylated peptides are available from Anaspec.

16. Add 20 µl (bed volume) of pTyr-100 immobilized antibody to peptide solution and incubate with gentle tumbling overnight at 4°C.

17. Wash resin two times with 400 µL peptide IP wash buffer 1 for 10 min at 4°C, and microcentrifuge 5 min at 2000 × *g*, 4°C, to bring down the resin. Remove supernatant as completely as possible by inserting a 27-G needle along the side and to the bottom of the microcentrifuge tube to remove any trace amounts of detergent.

18. Wash resin two times with 400 µl peptide IP wash buffer 2 for 5 min at 4°C.

19. Elute phosphopeptides from resin with 100 µL of peptide IP elution buffer 1 hr at room temperature. Spin down 5 min at 2000 × *g*, room temperature, and collect all eluent by inserting a 27-G needle along the side and to the bottom of the microcentrifuge tube.

20. Add 200 fmol of another tyrosine phosphorylated standard peptide such as angiotensin II phosphate to the eluent as a quantitative control for the remaining steps.

21. Perform IMAC enrichment and MS analysis as in Basic Protocol 2, steps 22 through 36, but replace the database search paramaters in step 36 with a static modification of +57.0215 Da on Cys and a variable modification of +79.9663 Da on Ser, Thr, and Tyr.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Cell lysis buffer A

20 µg/ml aprotinin
50 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
100 mM NaCl

continued

1% (v/v) Triton X-100
10% (v/v) glycerol
20 μg/ml leupeptin
1 mM Pefabloc
2 mM Na_3VO_4 (prepared fresh)
1 mM EDTA
10 mM β-glycerophosphate
Prepare fresh and keep at 4°C

Cell lysis buffer B

8 M urea
1 mM sodium orthovanadate
100 mM ammonium bicarbonate, pH 8.0
Prepare fresh and keep at 4°C

Dialysis buffer

50 mM ammonium bicarbonate, pH 8.3
4 M urea
Prepare fresh and keep at 4°C

IMAC elution buffer

0.4 M ammonium hydroxide
30% (v/v) acetonitrile

This buffer is for use with Phos-Select resin (Sigma); for other affinity resins, the manufacturers' instructions should be followed.

IMAC load/wash buffer

250 mM acetic acid
30% (v/v) acetonitrile

This buffer is for use with Phos-Select resin (Sigma); for other affinity resins, the manufacturers' instructions should be followed.

IMAC wash buffer

25% (v/v) acetonitrile
75% (v/v) ddH_2O
0.01% (v/v) acetic acid
100 mM NaCl

Immunoprecipitation wash buffer

20 mM Tris, pH 7.4 (*APPENDIX 2*)
120 mM NaCl

Methylation solution

Layer nitrogen gas over the reaction at each point in its preparation. Add 0.75 ml of 99.8% methanol-d_4 (Cambridge Isotope Labs) to a small dry glass vial with stirring (methanol-d_4 is used primarily because it is supplied very dry in ampules). Add 120 μl of 98+% acetyl chloride (Sigma) to methanol-d_4 dropwise with stirring (popping occurs if acetyl chloride is added too quickly). Incubate 5 min at room temperature and add immediately to dried peptides. The volume of this solution should be scaled up such that all related samples are methylated with the same methylation solution (use 400 μl of solution for each sample).

This solution must be very dry due to the reversible nature of the methylation reaction.

Peptide immunoprecipitation buffer

30 mM Tris·Cl, pH 7.4 (*APPENDIX 2*)
30 mM NaCl
0.3% (v/v) nonidet P-40 (NP-40)

Peptide immunoprecipitation wash buffer 1

100 mM Tris·Cl, pH 7.4 (*APPENDIX 2*)
100 mM NaCl
0.3% (v/v) nonidet P-40 (NP-40)

Peptide immunoprecipitation wash buffer 2

100 mM Tris·Cl, pH 7.4 (*APPENDIX 2*)
100 mM NaCl

Reversed-phase elution buffer

70% (v/v) acetonitrile
29.9% (v/v) Milli-Q water
0.1% (v/v) acetic acid (final concentration)

SAX start buffer

20 mM triethylammonium bicarbonate (TEAB)
30% (v/v) acetonitrile

The pH is ~8 or 9. No adjustment is necessary.

SAX step buffer

20 mM triethylammonium bicarbonate (TEAB)
1 M ammonium formate
30% (v/v) acetonitrile

SAX step elution buffers

Mix SAX start buffer (see recipe) and SAX step buffer (see recipe) to yield ammonium formate concentrations ranging from 50 to 500 mM.

These concentrations are appropriate for POROS HQ resin. For other anion-exchange resins, the concentrations may need to be adjusted.

SCX cleaning buffer

1 M ammonium formate
30% (v/v) acetonitrile
Adjust to pH 3 with formic acid

This requires a large amount of formic acid, and it is virtually impossible to bring the pH below 3.

SCX start buffer

5 mM ammonium formate
30% (v/v) acetonitrile
Adjust to pH 2.7 with formic acid

SCX step elution buffers

Mix SCX start buffer (see recipe) and SCX cleaning buffer (see recipe) to yield total ammonium formate concentrations ranging from 10 to 100 mM.

In Opti-Lynx cartridges, most phosphopeptides elute between 10 and 50 mM salt. For other column materials, the appropriate salt concentrations must be determined empirically.

Trypsin digestion buffer with urea

100 mM ammonium bicarbonate, pH 8.3
8 M urea

Urea solution MUST be prepared fresh before each use.

COMMENTARY

Background Information

Most cellular processes are regulated at some level by protein phosphorylation, be it developmental, hormonal, stress, or disease signaling. Of all posttranslational modifications, phosphorylation is the best understood, probably most widespread, and arguably the most important. To understand and investigate the role phosphorylation plays in cellular signaling, it is essential to know not only the identity of the phosphorylated proteins but also the exact site of modification. Site-directed mutagenesis of the phosphorylatable residue to a phospho-amino acid mimetic or a non-phosphorylatable residue reveals the biological significance of the modification, and ultimately the circuitry of the signaling pathway.

The determination of phosphorylation sites on individual proteins of interest was once an arduous task involving in vivo or in vitro labeling with radioactive phosphate, separation of labeled peptides by column or thin-layer chromatography, and Edman sequencing. These tools are still indispensable in some cases, but the rapid development of highly sensitive and accessible mass spectrometry techniques makes this an invaluable addition to the toolbox.

Phosphopeptides have a number of properties that make their analysis by mass spectrometry substantially more difficult than that of other peptides. Small phosphopeptides are so hydrophilic that they may not be retained on the reversed-phase matrices typically used in LC-MS (Larsen et al., 2004). In addition, phosphorylation sites of signaling proteins are often extremely difficult to detect due to their low abundance and low stoichiometry of phosphorylation in a cellular context of abundant background proteins. Several approaches have been developed to overcome these limitations by increasing the scale of the experiment through multidimensional chromatography (MudPIT; Washburn et al., 2001) or low-pH strong-cation exchange (Beausoleil et al., 2004). These unspecific methods have a lower efficiency in purification of phosphopeptides than affinity methods such as immobilized metal ion affinity chromatography (IMAC), making them better suited as a prefractionation step prior to affinity chromatography for extremely complex protein mixtures such as total cell lysates. Phosphopeptide tandem mass spectra frequently have poor quality due to a combination of poor signal-to-noise ratio (arising from the low abundance and poor ionization of phosphorylated peptides) and poor peptide backbone fragmentation (due to abundant neutral loss of phosphate from phosphoserine and phosphothreonine residues). Since a phosphoprotein is often identified from a single phosphopeptide spectrum, it is necessary to "manually" inspect the quality of the database match for every candidate MS/MS spectrum. This time-consuming limitation could potentially be overcome by increasing the quality of the data through the use of high mass accuracy spectrometers that narrow down the database search (Mann and Jensen, 2003), better fragmentation methods such as electron transfer dissociation (Syka et al., 2004), and more highly selective chromatography (Ficarro et al., 2002; Brill et al., 2004; Ndassa et al., 2006), as well as the development of new statistical algorithms (MacCoss et al., 2002). The limited dynamic range of mass spectrometers necessitates selective enrichment of phosphopeptides to obtain the highest numbers of phosphorylation sites from a complex mixture. Furthermore, peptide MS/MS spectra are routinely obtained in the positive ion mode, i.e., from fragmentation of the protonated peptide cations, where the additional negative charge of the phosphate group reduces the ionization efficiency of phosphopeptides relative to unphosphorylated peptides. While it is not possible to change the phosphate group itself when looking for definitive proof of the modification from the mass spectrum, it is possible to overcome the ionization problem by enriching the sample as much as possible for phosphopeptides.

By far the most successful method for enriching phosphopeptides is immobilized metal ion affinity chromatography (IMAC; *UNIT 10.11B*). A range of variations has been published, using different chromatographic materials, metal ions, and manual or automated setups (Neville et al., 1997; Posewitz and Tempst, 1999; Stensballe et al., 2001;

Ficarro et al., 2005; Olsen et al., 2006). Often, it is much easier to isolate phosphopeptides from complex mixtures than to identify a certain phosphopeptide(s) from individual proteins.

Affinity chromatography of proteins on chelated metal ions has been used for a long time and enjoys widespread popularity in the form of a nickel-nitrilotriacetic (NTA) or iminodiacetic acid (IDA) resin for the purification of hexahistidine-tagged proteins. Andersson and Porath (Andersson and Porath, 1986) were the first to notice that Fe^{3+}-chelated IDA has a strong affinity for highly phosphorylated proteins. While the use of IMAC for full-length proteins has been limited, at least until the recent availability of an IMAC-based commercial kit for enrichment of phosphoproteins, the isolation of phosphopeptides has been successful in a large number of cases. One of the first uses of the technique was in the study of phosphopeptides from casein in a nutritional context (Scanff et al., 1991). A large number of technical publications on new concepts for phosphopeptide isolation with IMAC or other procedures demonstrate feasibility exclusively with digests of β-casein. The reader should be wary of claims of the universality of these approaches until successfully applied to mixtures of proteins. IMAC with Fe^{3+} or other metals such as Ga^{3+}, or more recently TiO_2 (Olsen et al., 2006), is the longest established and most widely, used phosphoenrichment technique. IMAC has allowed the identification of phosphorylation sites in individual proteins (e.g., Michel et al., 1988; Neville et al., 1997; Fuglsang et al., 1999) as well as in complex mixtures (Vener et al., 2001; Ficarro et al., 2002; Nühse et al., 2003; Salomon et al., 2003; Olsen et al., 2006).

The mechanism of phosphoamino acid binding to chelated Fe^{3+} is a combination of ion exchange, i.e., binding of the negatively charged phosphate groups to the positive metal ion, and a more specific coordination of phosphate in the metal ligand sphere (Holmes and Schiller, 1997). It is easy to see why highly acidic peptides can bind to IMAC material as contaminants—the ion exchange function applies to them just as to phosphopeptides. Typical chromatographic conditions for IMAC make it more specific for phosphorylated peptides, and at least for some types of samples, the degree of purity is sufficient for large-scale mass spectrometric analysis (Nühse et al., 2003). For more complex sample analysis such as for total lysates or total cellular tyrosine phosphorylation, methyl esterification of carboxyl groups provides remarkable selectivity and efficiency in phosphopeptide isolation (Ficarro et al., 2002; Salomon et al., 2003).

To increase the number of phosphorylation sites discovered, very complex samples may be fractionated at the peptide or protein level prior to IMAC and LC-MS. In the case of phosphoproteins, one-dimensional gel electrophoresis or affinity purification may be helpful. For phosphopeptides, anion- or cation-exchange chromatography is useful. Both have a double effect of "deconvoluting" the sample as well as enriching phosphopeptides to some extent over the bulk of unmodified peptides. In cation exchange chromatography, many phosphopeptides bind weakly and elute very early or do not bind at all, while many unphosphorylated peptides bind well. Although not as selective as IMAC, this reasonably selective enrichment in the early eluting fractions may be sufficient for analysis without IMAC, especially at low pH (Beausoleil et al., 2004). In anion exchange chromatography, the bulk of phosphopeptides binds better than other peptides and is eluted over a wider range of salt concentrations (Nühse et al., 2003). This orthogonal subfractionation of phosphopeptides may provide added benefits over cation exchange chromatography, where the interesting fraction elutes over a very narrow salt range.

Critical Parameters and Troubleshooting

The most typically encountered problems are too many unphosphorylated peptides in the IMAC eluates, too few phosphopeptides, or both.

Following Basic Protocol 1, an abundance of non-phosphopeptides means that the IMAC procedure was not specific enough. If spin columns are used for IMAC, it is critical to resuspend the resin well in the various wash buffers and to not simply add the buffers onto the column. To improve specificity, additional wash steps can be introduced but need to be balanced against the phosphopeptide yield—their affinity to the medium is not high enough to withstand extensive washing. Alternatively, different suppliers of IMAC resin can be tested. Similar sets of peptides have been successfully identified with both Sigma's Phos-Select and Pierce's gallium-loaded IMAC spin columns, but results may vary depending on the sample. If acidic peptides are the major contaminating non-phosphopeptides, methyl ester derivitization

of peptides is recommended (see Basic Protocol 2).

There are many possible reasons for finding too few phosphopeptides. The in vivo phosphorylation status of proteins needs to be preserved by harvesting cells rapidly and either isolating proteins under denaturing conditions until digesting with trypsin, or by adding sufficient amounts of phosphatase inhibitors to the native extract. It may be worth increasing inhibitor concentrations above levels published in the literature, and in some cases a substantially higher yield of phosphopeptides can be achieved by adding calyculin A (5 to 20 nM) as a phosphatase inhibitor even during the tryptic digestion. Although performing the protein digestion overnight is the most convenient way, it can be detrimental for smaller amounts of protein to be left at 37°C for too long; a few hours may be sufficient. The addition of RapiGest detergent (Waters) speeds up tryptic digestion and is, in the authors' experience, compatible with IMAC, provided acid hydrolysis of the detergent is performed according to the manufacturer's instructions and the digest is cleaned up by cation exchange separation.

If too few phosphopeptides are recovered from IMAC columns following Basic Protocol 1, the incubation time of the sample with the resin may be increased, washes shortened, and elution times prolonged, although the manufacturers' protocols are generally optimized.

In Basic Protocols 2 and 3, phosphopeptide yield is fully optimized by direct elution of phosphopeptides from the reversed-phase desalt column to the IMAC column to the reversed-phase precolumn and gradient elution into the mass spectrometer via the analytical column. In this method, the peptide never encounters plastic once the phosphopeptides are enriched providing an ideal yield in their separation.

After elution of phosphopeptides from the beads in Basic Protocol 1, it is crucial to bear in mind that even with a very good yield, the phosphopeptide solution is dilute and total amounts are low. Any delay before the analysis should be kept to a minimum; ideally, IMAC purification is scheduled to be directly followed by mass spectrometric analysis. If necessary, isolated phosphopeptides can be frozen at −80°C if they are not too dilute, but freezing total (or fractionated) protein digests is always preferable. Freeze-dried samples should not be refrozen; they are very hygroscopic and degrade when wet.

Finally, it is self-evident that high-sensitivity sequencing of phosphopeptides requires optimal calibration and sensitivity of the mass spectrometer. Optimal sensitivity is achieved through minimization of the electrospray flow rate (<20 nl/min) and electrospray emitter tip diameter.

Anticipated Results

The number of sequenced phosphopeptides depends on the amount and complexity of the sample, the detected abundance of phosphopeptides in the sample (which in turn depends on the natural abundance of the phosphoproteins and on each phosphopeptide's unique and unpredictable ionization efficiency) and the sensitivity and speed of the mass spectrometer. Following Basic Protocol 1, several tens to >100 phosphopeptide sequences can be identified from an enriched sample analyzed in a single LC-MS run, and ~100 to 200 with manual prefractionation of the sample. With a larger input of sample, more extensive fractionation and possibly multiple repeated LC-MS runs for each IMAC-enriched sample, this figure can be increased to several thousand identified peptides. With most types of samples, ~70% to 90% pure phosphopeptides have been obtained using samples prefractionated by ion-exchange chromatography.

With Basic Protocols 2 and 3, hundreds to thousands of phosphopeptides are identified routinely depending on the nature of the sample and the amount of prefractionation of proteins and peptides performed prior to IMAC.

If the isolated phosphopeptides are analyzed on an ion trap mass spectrometer, serine- and threonine-phosphorylated peptides typically display a prominent neutral loss, i.e., an abundant fragment ion corresponding to loss of phosphoric acid (−98 Da for +1; −49 *m/z* for +2; −32.7 Da for +3 precursor ions) that often completely dominates the spectrum and reduces the quantity and quality of useful b and y ion peaks. Rapidly evolving mass spectrometry methodologies such as MS^3 (Olsen and Mann, 2004), pseudo MS^3 (Schroeder et al., 2004), and ETD (Syka et al., 2004) aim to overcome this significant problem.

It is important to note that tyrosine phosphorylation is substantially less frequent than serine or threonine phosphorylation. Without enrichment of phosphotyrosine-containing proteins or peptides by immunoprecipitation, peptides containing this residue will be outnumbered several hundred fold in

the protein digest by serine- and threonine-phosphorylated peptides.

Time Considerations

The entire procedure from sample preparation to mass spectrometric analysis will take between 2 and 3 days; there are several steps where samples can be frozen before continuing. Protein extracts are prepared on day 1 and will require between a few hours and a full day, depending on whether subcellular fractionation and, optionally, immunoprecipitation is performed. In almost all cases, the digestion is performed overnight. On the second day, the lyophilization before ion exchange chromatography (see Basic Protocol 1) requires several hours, and for ion exchange fractions, >0.5 ml, the lyophilization of these fractions will preferably be done overnight again. On the third day, the IMAC purification will take ~2 hr for up to twelve separate samples. For Basic Protocol 2, peptide methylation or peptide IP takes 1 day and the entire procedure of desalting the peptides, IMAC enrichment, and reversed-phase elution into the instrument can be accomplished in 3 hr.

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