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Electrophoretic Gel Band Handling Procedures

Cutting of 1- and 2-Dimensional Gel Spots

All gel bands were cut within 24 hours of receipt from collaborators. If intact, colloidal coomassie brilliant blue (CBB, EMD Biosciences, San Diego, CA) stained two-dimensional polyacrylamide gels were received from collaborators, the protein-containing spots were excised using a modified plastic pipette tip. The bottom of a 1-200 μ L pipette tip (Fisher) was cut off such that its opening was approximately 1.5 mm in diameter. This tool was then used to excise the spots from the gel. If a particular gel spot was larger than 1.5 mm, its center was excised. Intact, CBB-stained one-dimensional gel bands were cut from the gel with a razor blade. If two bands ran together, a region containing both bands was carved out, and that gel piece was sliced down the middle along the long axis (the 1-D gel bands tended to be rectangular in shape) through the top face

(as seen if the gel was lying flat on a table). The bands were then chopped into cubes one to two mm in length. Two or three of these smaller pieces were used for a single analysis. All gels were kept hydrated with distilled water to prevent shrinkage during cutting. Excised spots and bands were placed in 650 μ L polypropylene centrifuge tubes (Midwest Scientific, Valley Park, MO) and stored dry at -20 $^{\circ}$ C prior to destaining.

Destaining of Gel Pieces

Coomassie Brilliant Blue is a negatively charged dye that can interfere with trypsin digestion as well as MALDI or ESI. One- and two-dimensional gel pieces were destained using a procedure similar to that described by Fountoulakis and Langen.¹⁻³ Briefly, gel pieces were suspended in 100 μ L of 100 mM (7.8 g/L) ammonium bicarbonate (Fisher), 50% v/v acetonitrile (ACN) and shaken at 190 rpm on a rotary shaker table for 20 minutes. The supernatant was discarded, and the process repeated. The samples were then shaken twice in 100 μ L of distilled water for 15 minutes. Again, the supernatants were discarded. 100 μ L of pure ACN were added and the gel pieces were allowed to stand for five minutes. The solvent was then discarded and the gel pieces were dried for 15 minutes in a vacuum centrifuge (Jouan Inc., Winchester, VA). Trypsin digestion immediately followed destaining.

Reduction of Disulfide Bonds and Alkylation of Cysteine

Residues

Protein tertiary structure can deny a proteolytic enzyme's access to hydrolyzable residues, preventing complete digestion. In order to combat this, disulfide bonds are commonly reduced. The free cysteines must be alkylated with either iodoacetamide or iodoacetic acid to prevent the formation of new, random disulfide bonds. Iodoacetamide is often preferred over iodoacetic acid since it does not introduce a negatively charged group into a protein. The iodoacetamide procedure that follows worked well, thus there was no need to attempt alkylation with iodoacetic acid. The proteins from two-dimensional gels were reduced and alkylated between the two electrophoretic separations.² Those proteins that were not reduced and alkylated prior to receipt were handled by the following procedure¹. Proteins entrained in gels were reduced and alkylated by rehydrating a destained, dried gel band with 40 μ L of aqueous 10 mM (1.543 g/L) dithiothreitol (Aldrich) in 100 mM NH_4HCO_3 . The solution was incubated at 56 $^\circ\text{C}$ for 45 minutes to denature the proteins and reduce any disulfide bonds. The free cysteines were alkylated by addition of 40 μ L of 55 mM (10 g/L) iodoacetamide (Aldrich) and incubation in the dark for 30 minutes. The reagents were removed and the gel bands were washed first with 100 μ L of water, then 100 μ L of acetonitrile. The bands were then dried in a vacuum centrifuge for at least 15 minutes prior to digestion.

Proteins in solution were reduced and alkylated in a similar manner except that the volumes of reagents were significantly reduced (often 5 or 10 μ L

of each). The reagents were removed from the proteins by C₄ microscale solid phase extraction. Alternatively, the reagents were left in place during digestion. Since most liquid phase enzymatic digestions were only four to six hours in length, the alkylation of peptide N-termini as reported by Boja and Fales⁴ was not observed. After digestion, the peptides were purified by C₁₈ micro-SPE.

Trypsin Digestion

The proteins contained in 2-D gel spots were digested by adding 15 µL of 10 mM (0.78 g/L) ammonium bicarbonate containing 16.67 mg/L TPCK-treated, aseptically filled, bovine trypsin (Sigma T8802). The solution was incubated overnight (12-16 hours) at 37 °C in a dry-block heater. During the course of the digestion, the water often distilled into the caps of the tubes as the caps were exposed to 20 °C air while the bottom of the tube was held at 37 °C. Distillation could cause the gel band at the bottom of the tube to dry out, stopping the enzymatic digestion. This process was drastically slowed when the 650 µL centrifuge tubes were placed in holes intended for 1.6 mL tubes and a sheet of aluminum foil placed over the tops of the tubes. The foil was held in contact with the warming block with a weight.

One-dimensional gel bands were digested by adding 18 µL of 10 mM ammonium bicarbonate containing 13.89 mg/L methylated^{5, 6}, TPCK-treated, porcine trypsin (Promega, Madison, WI or Sigma T6567) and 2.78 mg/L TPCK-treated bovine trypsin and 20 µL of pure water. This mixture was incubated overnight as described for the 2D gel spots.

The reason for two recipes is to exercise some control over the presence of trypsin autolysis peaks. Trypsin autolysis peaks make excellent internal calibrants (see MALDI procedure section and Unmatched Masses Chapter), and internal calibration provides the highest mass accuracy. However, if the peaks from autolysis are too intense, those due to tryptic peptides from the protein in the gel band can be obscured. Methylated trypsin is created by reductive methylation of all lysine residues in the trypsin. Dimethylated lysine residues are not cleaved by trypsin, thus autolysis is significantly reduced. The methylated trypsin is approximately 100 times more expensive than the non-methylated trypsin, and should be used sparingly. Also, even when methylated trypsin is used, a small amount of non-methylated trypsin may be added to ensure the presence of some internal calibrants

Peptide Extraction

The trypsin digests were stopped by addition of 100 μ L of 0.1% v/v trifluoroacetic acid (TFA) (EMD Chemicals). The gel pieces were sonicated for 20 minutes in this solution. The supernatant from each tube was decanted and saved in a separate 650 μ L centrifuge tube. The gel pieces were then sonicated for 20 minutes in 100 μ L of 30% v/v ACN in water. The supernatant from each tube was decanted and combined with the supernatant from the previous step. Finally, the gel pieces were sonicated for 20 minutes in 100 μ L of 60% v/v ACN in water; the supernatant from this step was combined with those from the

previous two. The combined supernatants were then dried in a vacuum centrifuge and the peptides were resuspended in 8 μL of water.

Guanidination Procedures

O-methyl isourea Protocol

Peptides were guanidinated as described by Beardsley and Reilly.⁷ Briefly, 4 μL of peptide extract was combined with 1.5 μL of 8 molal (1.016 μL water added to each mg of solute) O-methyl isourea hemisulfate (Acros Organic, Pittsburgh, PA) and 5.5 μL of 7 M NH_3 . Seven-molar ammonia was manufactured by adding 4.48 mL of concentrated ammonia solution (28 wt. % NH_3 , Aldrich, Milwaukee, WI) to a 10 ml centrifuge tube and adding water to a total volume of 10 mL. The peptide-containing solution was incubated for 20 minutes at 65 $^\circ\text{C}$. The ammonia was removed by five minutes of vacuum centrifugation (the sample was not allowed to dry completely). Six μL of 5% v/v TFA was added to acidify the mixture. The peptides were then extracted using microliter-scale C_{18} solid phase extraction (μSPE). The net result was a mass increase of 42.0218 Da per lysine residue due to the net addition of CH_2N_2 . The chemical reaction is shown in Scheme 1.

S-methyl isothiurea Protocol

Peptides were guanidinated with S-methyl isourea as described by Beardsley et al.⁸ Four μL of peptide extract were combined with 4 μL of 1 molar (139.2 g/L) S-methylisothiurea hemisulfate (Sigma) in 6 wt. % ammonia. The

mixture was incubated for one hour at 65 °C, and the ammonia removed by vacuum centrifugation as described above. Six μL of 5% v/v TFA were added to the peptides, and they were extracted using μSPE . The net addition to peptide masses was identical to that observed when O-methylisourea was used.

The S-methylisourea guanidination procedure was developed to obviate the need for μSPE after derivatization. Sufficiently concentrated samples do not need to be purified after they have been guanidinated by S-methylisourea due to the lower concentrations of all reactants. However, most gel band samples are quite dilute. It was my experience that the S-methylisourea procedure offered no advantage over the one with O-methylisourea. Gel band samples guanidinated by either procedure required μSPE afterwards in order to see any peptides other than trypsin autolysis. Furthermore, unlike O-methylisourea, S-methylisourea is not commercially available, is water sensitive, and has a foul stench.

Microliter-Scale SPE Peptide Purification

The μSPE cartridges (see above for cartridge manufacturing instructions) were used to purify picomolar quantities of peptides in a manner similar to that described by Kopaciewicz.⁹ For optimum results, the sample solution should contain little or no organic solvent, and be at an acidic pH (pH < 3). Often a volume of 5% v/v TFA in water equal to the volume of the sample solution was added to ensure acidity. Because only 8 to 10 μL of liquid were aspirated with each stroke, the sample volume was limited to 30 μL or less to ensure that the vast majority of the peptides were presented to the C_{18} material. An SPE tip was placed on a 2-20 μL pipette set to 12 μL . Since the tips must be wetted with an

organic-containing solution, 50% v/v ACN in water was aspirated and then dispensed to waste two times. The tips were equilibrated by aspirating twice with a 0.1% v/v aqueous TFA solution. The sample solution was then aspirated and dispensed (back into its original tube) at least eight times, allowing the peptides to adsorb to the stationary phase. The peptides were desalted by aspirating 0.1% v/v TFA solution and dispensing it to waste. The TFA solution was aspirated again, but not dispensed. Using a different pipette, 2 μ L of 50% v/v ACN with 0.1% v/v TFA in water were dispensed into a new 650 μ L centrifuge tube. The TFA solution in the SPE tip was then dispensed to waste; the peptides were eluted from the tip by aspirating and dispensing the ACN/TFA solution (into the same tube) five times.

Microliter-Scale SPE Peptide Purification (Elution Into MALDI Matrix)

The sample is handled as described in the microliter-scale SPE peptide purification procedure, except that the 50% v/v ACN/0.1% v/v TFA in water solution was replaced with a MALDI matrix solution (see MALDI-TOF procedures section). The matrix solution, containing both MALDI matrix and eluted peptides, was deposited directly onto a MALDI target. This procedure ensured maximum sensitivity because the peptides and matrix are already mixed (the MALDI spotting procedures require two to ten fold dilution of the sample). This procedure is not appropriate when further chemistry and/or sample handling steps are to be performed after μ SPE because it adds 50 to 150 mM MALDI

matrix to the peptide solution. The presence of the matrix may interfere with subsequent chemical steps and MALDI matrices can be difficult to remove from peptide solutions.

Phosphopeptide-Specific Procedures

Phosphopeptide Isolation by IMAC Using ZipTip_{MC}[®]

Phosphopeptides were isolated using ZipTip_{MC}[®] micro-SPE cartridges according to the manufacturer's instructions. Briefly, samples containing phosphopeptides were suspended in 50 mM (10.66 g/L) aqueous 2-(N-morpholino)-ethanesulfonic acid (Sigma) adjusted to a pH of 5.5 using ammonium hydroxide, 30% v/v ACN (MES buffer). A new ZipTip_{MC}[®] (Millipore, Billerica, MA), containing iminodiacetic acid-derivatized resin, was placed onto a 2-20 μ L pipette (set to 12 μ L). A solution of 50% v/v ACN in water was aspirated and dispensed to waste three times. A solution of 0.2 M FeCl₃•6H₂O (54.06 g/L, Fisher) in 10 mM HCl (made by diluting 828 μ L of 36.5 wt. % HCl (Fisher) to 100 mL with water) was then aspirated and dispensed to waste ten times (solutions of 0.2 M Ga(NO₃)₃•xH₂O (51.15 g/L, Aldrich) or 0.2 M CuSO₄ (31.92 g/L, Fisher) in water may be used in place of the acidified FeCl₃). The Fe³⁺ solution gave the most consistent results, and thus was used for all experiments described in this document. Twelve μ L of water were then aspirated and dispensed to waste three times. This tip was subsequently rinsed three times with 0.1% v/v acetic acid (Aldrich), 10% v/v ACN in water and three times with MES buffer. The sample solution was then aspirated and dispensed (into the same tube) eight

times. Following adsorption of the sample peptides, the tip was rinsed three times with MES buffer and three times 1.0% v/v aqueous acetic acid in 30% v/v ACN. The tip was rinsed twice with water, and then 12 μL of water were aspirated but not dispensed. Using a different pipette, 2 μL of 2% v/v (~0.3 M) aqueous ammonia were placed into a new 650 μL centrifuge tube. The water in the sample-containing tip was then dispensed to waste and the phosphopeptides were eluted by aspirating and dispensing the ammonia solution eight times. The eluted phosphopeptides were analyzed by MALDI-TOF mass spectrometry using the overlay method described elsewhere in the chapter. This procedure proved difficult to integrate with β -elimination procedures. Furthermore, complete elution of the phosphopeptides from ZipTip_{MC} with ammonia has been shown to be nearly impossible.¹⁰

Phosphopeptide Isolation by IMAC Using Fe(III)-NTA Agarose Beads¹¹

The Fe(III)-NTA beads were prepared by placing one volume (usually 50 μL) of Ni-NTA (nickel(II) charged nitrilotriacetic acid-derivatized Sepharose CL-6B (cross-linked 6% agarose) 45-165 μm diameter beads) bead suspension (Qiagen Inc., Valencia, CA) into a clean 650 μL centrifuge tube. All subsequent liquid-handling operations involving separation of the beads from the suspending solution were performed using a 1-200 μL gel-loader tip (VWR International, West Chester, PA) that had been crimped at the end with a hemostat. The tip was crimped to prevent the beads from being sucked into the tip. The beads

were washed three times with three volumes of distilled water and three times with three volumes of 100 mM (37.22 g/l) (ethylenedinitrilo)tetraacetic acid, disodium salt, dihydrate ($\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$) (Sigma). The beads were then washed three times with one volume of 100 mM (27.03 g/l) iron(III) chloride hexahydrate in 100 mM acetic acid (made by adding 5.72 μL of glacial acetic acid (Fisher) to 994 μL of distilled water), and rinsed three times with three volumes of 100 mM acetic acid. The beads were suspended in one volume of 100 mM acetic acid and kept at 4 $^\circ\text{C}$ prior to use. The bead suspension was stable for several weeks when stored in this manner.

Phosphopeptides were captured from a tryptic digest solution by combining one volume (usually 5 μL) of tryptic digest solution (which should contain approximately 10 mM ammonium bicarbonate) with one volume of Fe(III)-NTA/100 mM acetic acid suspension in a 650 μL centrifuge tube. The mixture was shaken for 15 minutes on a rotary shaker table. The phosphopeptide-depleted supernatant was usually discarded. The beads were then washed three times with ten volumes of 100 mM acetic acid and three times with ten volumes of water. The phosphopeptides were eluted either by addition of a base or by β -elimination/Michael addition. In the addition of base elution procedure, two volumes of 2% v/v ammonia were added to the beads. The supernatant was decanted after five minutes and saved. MALDI spots were then prepared using the overlayer method. The procedure listed above was designed to use a β -elimination step to elute the phosphopeptides from the agarose beads. Furthermore, it allows complete flexibility in terms of the volume of the

stationary phase used. If a large sample must be purified, more Fe(III)-NTA beads are used.

The β -elimination/Michael addition elution procedure¹¹ started with the addition of one volume of freshly prepared 100 mM (31.55 g/L) barium hydroxide octahydrate (Aldrich) solution and 0.4 volumes of freshly prepared 50 mM (5.68 g/L) cysteamine hydrogen chloride ($\text{H}_2\text{NCH}_2\text{CH}_2\text{SH}\cdot\text{HCl}$) (Aldrich) to one volume of phosphopeptide-laden Fe(III)-NTA beads. The suspension was incubated for two hours at 37 °C, and the solution was agitated by tapping the bottom of the tube lightly every 30 minutes during the reaction. One volume of 100 mM (13.21 g/L) ammonium sulfate (Fisher) was added to stop the reaction (by neutralizing some of the base and precipitating the Ba^{2+}). The suspension was centrifuged at 13,000g for five minutes, and the supernatant was decanted and saved for further analysis. The phosphopeptides were separated from the ammonium hydroxide and cysteamine by microliter-scale C_{18} SPE after acidification with TFA. The chemical steps of this procedure are shown in Scheme 2.

The β -elimination/Michael addition procedure quantitatively converted phosphoserine (residue formula $\text{C}_3\text{H}_6\text{NO}_5\text{P}$, residue mass = 166.9984 Da) into S-(2-aminoethyl)cysteine (residue formula = $\text{C}_5\text{H}_{10}\text{N}_2\text{OS}$, residue mass = 146.0514 Da) and phosphothreonine (residue formula $\text{C}_4\text{H}_8\text{NO}_5\text{P}$, residue mass = 181.0140 Da) into β -methyl-S-(2-aminoethyl)cysteine (residue formula = $\text{C}_6\text{H}_{12}\text{N}_2\text{OS}$, residue mass = 160.0670 Da)¹². The residue formula is defined as the formula of the entity that is added when an amino acid is included in a polypeptide chain. The formula differs from that of the free amino acid by the

loss of H₂O during peptide bond formation. The residue mass is defined as the mass added to a polypeptide chain by incorporation of a particular amino acid and also accounts for the loss of H₂O upon peptide bond formation. The observed mass shift of a phosphorylated residue due to β -elimination/Michael addition was -20.9467 Da. The mass difference between an unmodified serine or threonine and its cysteamine-modified version was +59.0194 Da. Automated data interpretation routines, such as ThermoFinnigan's Sequest¹³ and Micromass's Protein Lynx Global Server can be programmed to search for phosphopeptides after β -elimination/Michael addition as Ser or Thr residues with a 59.1094 Da mass increase.

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