

Phosphoamino Acid Analysis of ^{32}P -Labeled Proteins in SDS Gels

Buffers Needed:

50 mM Ammonium Carbonate, pH 8.0

Concentrated HCl

Electrophoresis Buffer: 189 ml water:10 ml glacial acetic acid:1ml pyridine, pH 3.5

Electrophoresis Standards: The standard mixture is made of equal parts of 1mg/ ml each of phosphoserine, phosphothreonine and phosphotyrosine in electrophoresis buffer

0.2% Ninhydrin: in acetone

Procedure:

Extraction of Peptides from Gel Slices

1. Expose wet ^{32}P -labeled gel to X-ray film to locate phosphorylated bands.
2. Excise appropriate gel slices and place in a capped test tube. Incubate the gel slice for 1 hr in dH_2O with shaking to remove the SDS.
3. Equilibrate in 50 mM ammonium carbonate, pH 8. for 1 hour.
4. Remove the old buffer and replace with 0.5-1 ml ammonium carbonate containing 25 $\mu\text{g}/\text{ml}$ trypsin (from 1 mg / ml stock 50 μl aliquots frozen at -70°C)
5. Incubate 5 hr with shaking at 37°C . Remove the supernatant and save. Add a fresh aliquot of trypsin in ammonium carbonate at one-half the original concentration, ie 12.5 $\mu\text{g}/\text{ml}$.
6. Incubate overnight at 37°C .
7. In the morning, collect the second supernatant and combine with the first. Count the combined supernatants and the gel slice by Cherenkov counting. At least 75-80% of the total counts (ie. supernatant plus gel slice) should be present in the supernatant.
8. Remove the ammonium carbonate by multiple rounds of lyophilization, adding dH_2O each time to take up the lyophilized pellet.

Amino Acid Hydrolysis

Do the hydrolysis in a screw top Eppendorf tube.

1. Add 125 μl of H_2O + 125 μl conc HCl to the lyophilized sample.
2. Place the tube in a heating block with some ethylene glycol to conduct heat from the block to the tube. Incubate 1hr at 110°C .
3. At the end of the hydrolysis reaction, spin the liquid to the bottom of tube. Remove cap and speed vac to near dryness.
4. Remove the sample and finish the removal of water and HCl by lyophilization. Multiple lyophilizations may be required. Add 250 μl H_2O each time until sample can be completely dried.

Electrophoresis

1. When the sample is dry, add 7.5 μl electrophoresis buffer and 7.5 μl of the phosphoamino acid standard mixture.

2. Spot the sample on cellulose plates, applying 0.5 to 1.0 μ l per application. Allow spot to dry between applications of sample to keep the area of the spot as small as possible.
3. Spot standard lanes with 15 μ l of the standard mixture.
4. Spot a solution of bromphenol blue along side the standards as a measure of the rate of electrophoresis.
5. Set up the flatbed electrophoresis apparatus and fill the side chambers with 50 ml electrophoresis buffer.
6. Cool the bed by pumping ice water through the appropriate inlet
7. Place paper wicks (3 layers of 3 MM paper-9 x 23 cm) on opposite sides of the bed with ends dipping into buffer chambers
8. Lightly wet the plate by spraying with electrophoresis buffer. Place the plate on the flatbed apparatus and place the wicks on each side of the plate. The origin of the plate should be on the negative side of the flatbed. Cover and let the chamber equilibrate for 2 min. If the bromphenol blue begins to migrate, turn the power supply on immediately.)
9. Electrophorese at 500 V until the bromphenol blue is about 5 cm from the end of the plate. Current will vary from 15 mAmp to 4 or 5 mAmp..
10. Remove the plate and dry in an oven. When completely dry, spray the plate with 0.2% ninhydrin in acetone. Incubate 2-3 minutes in 100° C oven to visualize phosphoamino acid standards.
11. Expose plate to X-ray film. Labeled phosphoamino acids are identified by co-migration with the phosphoamino acid standards.