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Phosphopeptide Mapping by Alkaline Phosphatase Treatment and MALDI-TOF Mass Spectrometry

Phosphopeptide mapping procedure: Dissolve the dried peptide mixture in 10 ml of 50% ACN. Dilute 1 ml Roach alkaline phosphatase (20U / ml) in 99 ml of 50 mM NH4HCO3. Mix 2 ml of the digested sample and 5 ml of diluted AP in a thin wall microfuge tube (PCR tube) and spin briefly. Incubate the tube at 37 oC for 30 min. Then dry the reaction mixture in a SpeedVac. Add 1 ml DHB working solution and 1 ml 50% ACN. After a pulse spin, load 1 ml of the mix on MALDI probe. This will be the de-phosphorylated sample. The same procedure is used for the control sample, except that the diluted alkaline phosphatase is inactivated by heating at 95 oC for 5 minutes before mixing with the protein digest.

MALDI-TOF spectra for each sample are taken in similar conditions. Spectra from both de-phosphorylated and control samples are displayed in two different colors, (e.g. one red and one green) to aid in the identification of the phosphopeptides. The candidate phosphopeptides are detected by visual inspection of the spectra. A candidate phosphopeptide should meet the following criteria: (1) this peak is present in control sample but is totally absent in the de-phosphorylated sample. This peak is assigned to a candidate phosphopeptide; (2) 80 Da (or N x 80 Da, N = 1, 2, 3...) lower than this candidate phosphopeptide peak, there must be another peak which is present in the de-phosphorylated sample but absent or with lower intensity in the control sample. This peak will be assigned to the potential de-phosphorylated peptide; (3) the candidate peptide should be able to assign to the protein sequence within the mass accuracy. The possible number of phosphate groups (N) should be small than the total number of Ser, Thr and Tyr present in the peptide.

It is important to use two or more digestions with different proteolytic enzymes to achieve significant sequence coverage. Trypsin and Asp-N are the most commonly used enzymes. Trypsin digestion often results in good sequence coverage and MS/MS spectra of tryptic peptides are often easier to interpret than Asp-N peptides for the determination of the phosphorylation sites by LC-MS/MS in the next step. Asp-N cleavages at different amino acid residues from trypsin so that Asp-N digestion often leads to sequence coverage of regions that are absent in the trypsin digestion. It should be noted that Asp-N can also cleavage at N-terminal of Glu in addition to Asp under the in gel digestion condition. Glu-C is the enzyme of choice for in solution digestion, but it does not work well for in gel digestion.

We like to take two MALDI-TOF spectra for each digested sample. One at lower laser energy to optimize for good mass resolution, and the other at higher laser energy to measure weak peaks, despite of that strong peaks are saturated. We will discuss this issue in detail later in this chapter.

Issues of concern for phosphopeptide mapping. The cleanness of the sample can significantly impact phosphopeptide mapping, especially for low stoichiometric phosphorylation. The ability to detect low stoichiometric phosphorylation depends critically on signal-to-noise ratio. High noise level caused by salt and other contaminants makes the detection of a weaker peak more difficult or impossible. In our experience, samples prepared according to the in-gel digestion procedure described here are generally clean enough to satisfy the requirement. It is often necessary to use ZipTip C18 (from Millipore) or packed C18 micro-columns to clean up samples that are digested in solution. Differential losses of peptides may occur. Such sample loss is not a problem for protein identification, but it may become a real problem in mapping protein phosphorylation sites.

Laser energy in MALDI-TOF measurement is another critical parameter for phosphopeptide mapping, especially for detecting low stoichiometric phosphorylation. In routine MALDI-TOF measurements, laser energy is optimized to achieve the best resolution and mass accuracy. Unfortunately this may not be the best condition to detect phosphopeptides of low stoichiometry in most cases. Signal intensity from the phosphopeptide is often lower than that of most other peptides due to its low abundance, so disappearance of such a weak peak after phosphatase treatment is more difficult to detect by human eyes. To increase the sensitivity (or more accurately to increase the dynamic range of the mass spectrometer) of detecting phosphopeptide of low stoichiometry, it is often necessary to increase the laser power to a level that is 20% to 40% higher than the optimum laser energy for obtaining good mass resolution. Under such conditions, the strong peaks are saturated, but weaker peaks are easier to be recognized. The disappearance of the phosphopeptide of low stoichiometry becomes easier to detect. Saturation of strong peaks decreases mass resolution and mass accuracy. As a result, ambiguity in peak assignments may arise. This ambiguity will be resolved in the subsequent LC/MS/MS experiment. It is often better to record another spectrum with optimized laser energy for high resolution. The high-resolution spectrum is used for mass assignments, and the low-resolution spectra with higher laser power will be used to identify the phosphopeptides by comparing the spectra before and after phosphatase treatment.

Sequence coverage in phosphopeptide mapping: Sequence coverage is a general problem in all phosphopeptide-mapping procedures. Peptides resulted from a in-gel trypsin digest often cover less than 70% of the protein sequence, sometimes even lower when the amount of sample is less. The ability to recover peptides is dictated by the physical properties of the peptides (mainly solubility in the extraction solvent) that are derived from the enzymatic digestion, thus there is little room to play to improve the sequence coverage. To cover more sequences, it is often necessary to use Asp-N to digest another gel piece to recover Asp-N peptides. Using an alternative enzyme generates peptides of different physical property so that different sequences can be recovered. From our experience, it is possible to cover 50% to 70% of a protein sequence from a trypsin digestion with 1 picomole sample in a gel band. If another Asp-N digestion is carried out, the sequence coverage may be further increased to 80-90%. We suggest to use multiple digestions even the amount of sample is limited. We prefer to cut the gel band in half for two digestions if there is reasonable amount of protein present in the gel band.