Phosphorylation site mapping

Despite progress made in instrumentation and techniques, locating the site of phosphorylation in cold samples is still a significant challenge. According to the recent survey from ABRF (Association of Biomolecular and Resource Facilities), only 3/67 (4%) facilities could locate the correct sites of phosphopeptide at 1 pmol level. To facilitate LC-MS/MS characterization of phosphorylation site, we used immobilized metal ion affinity chromatography (IMAC) step after methyl ester derivatization to enrich the signal of calmodulin kinase phosphopeptides similar as described by Ficarro (1) . This enables us to detect phosphopeptides with higher sensitivity.

The IMAC column (0.10 mm ID ×0.24 mm OD, 8 cm) was constructed in-house using POROS 20 MC (Applied Biosystems, CA). Columns were activated with 0.2 M $FeCl_3$ and equilibrated with 10 column volumes of 25:74:1 acetonitrile:water:acetic acid (pH3.0). The tryptic camodulin kinase peptides after methyl-esterification were applied to the IMAC column through a homebuilt pressure cell. Non-specific binding peptides were washed with acetonitrile:water:acetic acid (25:74:1, pH3.0) containing 100 mM NaCl. Phosphopeptides were eluted with 50 mM Na_2HPO_4 (pH9.0). The peptide mixture was acidified and analyzed by Q-Tof 2 LC-MS/MS. The Q-Tof 2 mass spectrometer was operated in a data dependent acquisition mode whereby following the interrogation of MS data, ions were selected for MS/MS analysis based on their intensity and charge state. Collision energies were chosen automatically based on the m/z and charge-state of the selected precursor ions. All MS/MS spectra recorded on tryptic phosphopeptides derived from the protein were searched against the NR database by using the MASCOT Algorithm. Search parameters included a differential modification of +80 Da (presence or absence of phosphate) on serine, threonine and static modification of +14 Da (methyl groups) on aspartic acid, glutamic acid, and the C-terminal of each peptide.

Fig 1 shows the chromatograms of calmodulin kinase peptides with and without IMAC pretreatment. Without IMAC capture, all peptides were loaded onto the mass spectrometer and a complicated spectrum was obtained. A neutral lost search to identify phosphopeptides was unproductive because of co-elution of many species with similar m/z. With IMAC capture, the non-phosphopeptides were washed away and a much cleaner chromatogram was obtained, allowing straightforward identification of phosphopeptides. Fig 2 is an MS/MS spectrum for phosphopeptide GAILTpTMLATR.

1. Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, et al. 2002. *Nat Biotechnol* 20: 301-5

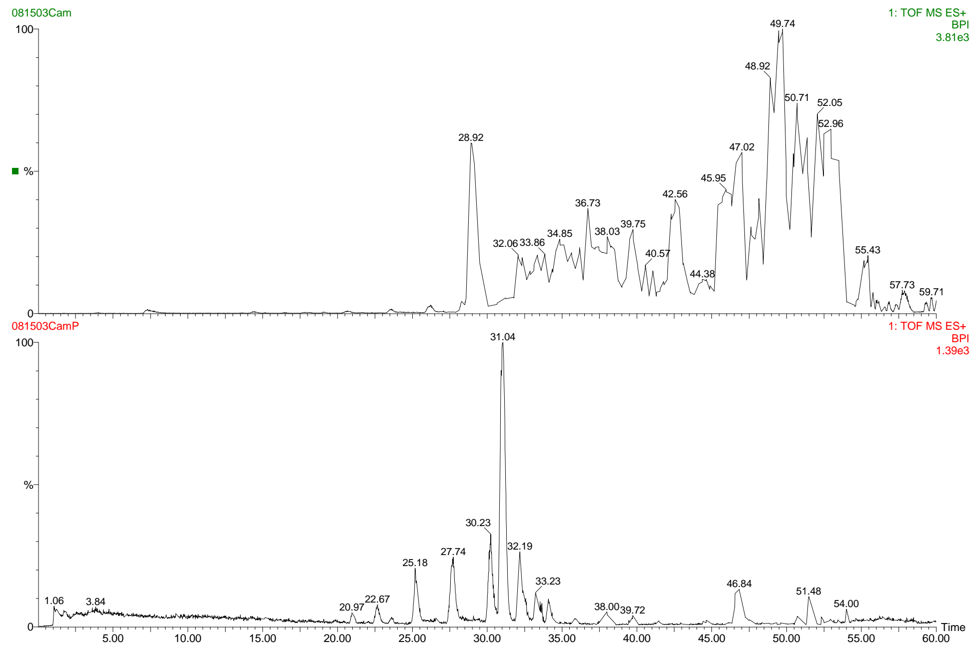

Fig 1. Base peak chromatography of calmodulin kinase peptides before (top panel) and after (bottom panel) Fe^{3+} IMAC capture. The analytical column was 75 µm ID ×110 mm customer-packed with BetaBasic C18 resin (ThermoHypersil Keystone). The solvent was A, 3% CH_3CN+ 0.4% acetic acid+0.005% heptafluorobutyric acid, and B, 90% CH_3CN+0.4% acetic acid+0.005%heptafluorobutyric acid. Peptide mixture was separated by the application of a series of mobile phase B gradients (1 to 10%B in 5 min, 10 to 15%B in 25 min, 15 to 20% B in15min, 20 to 45%B in 10min, 45 to 80% B in 5 min)

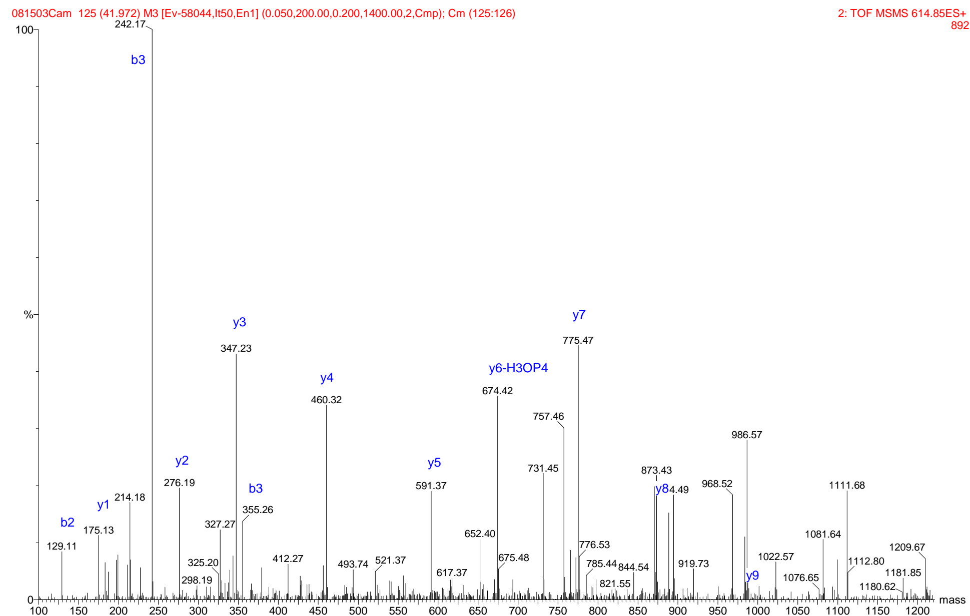

Fig 2. Calmodulin kinase phosphopeptide identified by CID fragmentation. The doubly charged precursor of m/z = 614.85 was identified as phosphorylated calmodulin kinase GAILTpTMLATR. The phosphorylation site is identified as Thr-307 by the loss of H_3PO_4 from y_6 fragment after β –elimination.