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Phosphoproteomics: Recent advances in analytical techniques

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Abstract- Post-genomic biology seeks identification and quantification of multiple proteins from complex mixtures and the research is still on. Despite recent progress in high-throughput proteomics, proteomic analysis of post-translationally modified [PTM] proteins remains particularly challenging. Several strategies for isolating phosphoproteins are explored herein. Quantification of phosphoproteins seems to be a novel solution to identify the underlying disease mechanisms, mostly cancer.
Keywords- phosphorylation, enrichment, chemical modification, SILAC, mass spectrometry, HILIC

Introduction

Phosphoproteomics is a branch of proteomics that focuses on deriving a comprehensive view of the extent & dynamics of protein phosphorylation by way of identifying & characterizing proteins that contain a phosphate group as a post-translational modification. The addition of phosphate is a key reversible modification catalyzed by protein kinases, and is known as 'phosphorylation'. Protein kinases account for 1.7% of the human genome and 40% of all the proteins may be assumed to be phosphorylated at any given time. Interestingly, the first evidence of Ser/Thr/Tyr protein phosphorylation in a member of the "third domain of life" was reported in the extreme halophilic archaeon *Halobacterium salinarum* in 1980, using ^{32}P radiolabeling. This was followed by discovering the existence of protein phosphorylation in the extreme acidothermophile *Sulfolobus acidocaldarius* [1, 16, 28, 39]. Traditional biochemical and genetic analyses of phosphoproteins, and of the kinases and phosphatases that modify them, have provided a barrage of information about signaling pathways. Since these methods focus on one protein at a time, they are not readily amenable to understand the complexity of protein phosphorylation or how individual phosphoproteins function in the context of signaling networks. Recent advancements in analytical technology, particularly mass spectrometry, coupled with availability of biological databases have fuelled the active study of many phosphoproteins and phosphorylation sites at once [28]. In sharp contrast to conventional expression profiling studies, phosphoproteomics potentially provides two additional layers of information. First, it provides clues on what protein or pathway might be activated [because a change in the phosphorylation status of proteins almost always reflects a change in protein activity]. Second, it indicates what proteins might be potential drug targets [because phosphoproteomics focuses on proteins that have kinase activities or are substrates of kinases] [21].

Techniques

Of late, there is an increasing interest in phosphoproteomics as reflected by a considerable number of works describing various strategies, including the use of different isotopic technologies [e.g., isotope-coded affinity tag, stable isotope labeling by amino acids in cell culture], protein separation techniques [e.g., gel-based versus liquid chromatography-based methods], and chemical modifications [e.g., β-elimination and esterification] to identify phosphoproteins and/or phosphorylation sites in a global fashion [21, 32]. Conventional phosphoproteomic analysis used radioactive labeling, followed by two-dimensional gel electrophoresis, phosphopeptide mapping by thin-layer chromatography and electrophoresis, or sequencing by Edman degradation [30, 34]. In due course, they laid more emphasis on site-directed mutagenesis, mass spectrometry and enriching phosphoproteins. Emergent technologies like microarrays and fluorescence-based single-cell analysis are characterized by high sensitivity and high throughput, but require prior knowledge of particular phosphoprotein targets. Mass spectrometry-based methods offer rapid, selective and sensitive analysis and discovery of new phosphoproteins.

Enriching Phosphoproteins & Phosphopeptides

Since many phosphoproteins [notably signaling intermediates] are low-abundance proteins phosphorylated at sub-stoichiometric levels, several enrichment strategies have been developed in consonance with mass spectrometry [33, 45]. The commonly used enrichment techniques are immobilized metal affinity chromatography [IMAC], phosphoprotein isotope-coded affinity tag [PhIAT] and phosphoprotein isotope-coded solid-phase tag [PhIST]. IMAC has undergone incremental improvements like the replacement of traditional metal chelating resins with cellulose powder, silica monolithic supports or poly [glycidyl methacrylate/divinylbenzene] [GMD] derivatized with imino-diacetic acid and bound Fe [III] as a material [4]. The use of aluminium hydroxide or addition of 40% 1, 1, 1, 3, 3, 3-hexafluoroisopropanol to buffers have improved

the overall efficiency of IMAC [38]. Nowadays, phosphopeptide enrichment is a highly accelerated process, based on the availability of several commercial kits like PhosphoProtein Purification Kit [Qiagen], Pro-Q Diamond Phosphoprotein enrichment kit [Invitrogen / Molecular Probes] and BD Phosphoprotein enrichment kit [BD Biosciences] [10, 13, 15, 28]. Sequential elution from IMAC [SIMAC] is also being used to sequentially separate monophosphorylated and multiphosphorylated peptides from complex biological samples [46]. Current techniques for peptide enrichment are mainly based on various flavours of metal affinity chromatography. Non-specific binding by acidic proteins and peptides, and favoured enrichment of poly-phosphorylated species tend to plague this system. Chemical modification is considered necessary to bypass this deficiency [43].

Chemical Modification

Chemical modification of phosphorylated residues has so far proven the most successful phosphoproteomic strategy, and entails two approaches. In the first approach, base-mediated β-elimination of phosphate from phospho-Ser or -Thr residues permits subsequent addition of biotinylated probes at pre-phosphorylated sites. Though not applicable to tyrosine phosphorylation, this method has seen some success for the global analysis of phosphorylated proteins from *Arabidopsis*, but it still suffers from a low but significant background of β-elimination from glycosylated and free Ser residues [25, 31, 43]. In the second reported approach, a short series of reactions based on reversible phosphoramidate chemistry was applied to introduce a thiol group at phosphorylated sites for using them as a handle to introduce secondary labels. This method has recently been elaborated for the identification of phosphorylated proteins in *Drosophila melanogaster* Kc167 cells and for use in general chemical phosphoproteomics. In addition to these two methods, some progress has resulted in the analysis of phosphorylated proteins tagged metabolically with a γ-thiophosphate ATP analogue [6, 7, 37, 43].

Stable Isotope Labeling by Amino Acids in Cell Culture [SILAC]

Separation of identical biochemical species by mixing cellular lysates derived from isotopically distinct media sources forms the basis of metabolic labeling. SILAC is a mass spectrometry-based, metabolic labeling technique that paved way for the identification and quantification of complex protein mixtures. It differs from other metabolic labeling strategies since only a specific amino acid is isotopically labeled. It has been purportedly effective for characterization of cellular signaling and protein-protein interactions of receptor tyrosine kinases. The technology has also been successfully applied to the study of epidermal growth factor [EGF] and fibroblast growth factor [FGF] signaling. SILAC is advantageous because it removes false positives, reveals large-scale kinetics of proteomes and uncovers important points along signaling pathways directly [34, 38]. In a typical SILAC experiment, cells representing two different biological conditions are grown in media supplemented with 'light' or 'heavy' isotope-containing amino acids. Metabolic incorporation of labeled amino acids into all proteins from cells of one population, and subsequent combination of labeled and unlabeled samples in equal ratios, enables quantification of proteins from the two samples based on the intensities of the light and heavy peptides. Within the same mass spectrometric experiment, tandem mass spectrometry [MS/MS] can be carried out to obtain sequence information for protein identification. Thus, targeted proteomic comparisons can be achieved in a high-throughput way. With evolution, SILAC has emerged as a very powerful method to study cell signaling, protein-protein interactions and regulation of gene expression [17, 24, 48].

Mass Spectrometry

Any mass spectrometer has three quintessential parts: an ionization source, a mass analyzer and a detector. Electron spray ionization [ESI], matrix-assisted laser desorption/ionization [MALDI] and/or surface-enhanced laser desorption / ionization [SELDI] have been used for phosphoproteomic analysis of the adipose secretome. Time-of-flight [TOF] and quadrupole TOF [QTOF] instruments, as also ion traps and linear quadrupole ion trap-Fourier transforms [LTQ-FTs] have been constructively used. Sustained efforts are on to improve four aspects of mass spectrometry-based phosphoproteomic analyses, namely improved accuracy and speed, improved separation of complex mixtures, computational refinement of the signals obtained, & integrating the flood of results in intelligent databases [9, 29, 40]. Protein characterization by mass spectrometry is usually done by the "bottom-up" or "top-down" approach. However, characterization of phosphoproteins uses the "bottom-up" approach, wherein protease digestion is followed by qualitative and/or quantitative analysis by mass spectrometry [MS]. Electron capture dissociation [ECD] and electron transfer dissociation [ETD] are instrumental in fragmenting the peptide backbone leaving the phosphoserine/phosphothreonine intact. They have also been used constructively in "top-down" experiments to sequence phosphoproteins. ETD identifies 60% more phosphopeptides than collision-induced dissociation [CID]. However, a combination of ETD & CID can effectively be used for a more comprehensive analysis. Till

date, ECD has been exclusively coupled with Fourier transform ion cyclotron resonance mass spectrometry [FTICR-MS], the most expensive MS instrumentation available [12, 27, 42]. Developments in MS have frequently been plagued by inconsistent reproducibility arising from automated selection of precursor ions for fragmentation, identification and quantification. On one hand, false positives are avoided by high stringency criteria based on MS/MS data, while, on the other hand, these criteria induce many false negatives. A new MS-based strategy, based on multiple reaction monitoring of stable isotope-labeled peptides, was used to quantify temporal phosphorylation profiles of 222 tyrosine phosphorylated peptides following pervanadate or EGF treatment. 88% of the signaling nodes were reproducibly quantified, as against 34% in conventional information-dependent analysis [3, 23].

Phosphoprotein Profiling by Pa-GeLC-MS/MS

This involved a combination of phosphoprotein affinity chromatography, denaturing gel electrophoresis, tandem mass spectrometry, and informatics analysis. Native phosphoproteins were selectively isolated from a G2/M phase yeast whole cell extract using a phosphoprotein affinity matrix – Pro-Q Diamond resin [Molecular Probes – Invitrogen]. This was followed by 1-D PAGE separation, proteolysis and ESI LC-MS/MS [PA-GeLC-MS/MS], and generated 131 proteins. The Pro-Q eluate was separated into two fractions by size [less than 100 kDa & greater than 100 kDa] before PAGE and ESI LC-MS/MS analyses. The component proteins were then queried against databases to reveal the correlation between protein-protein interactions & protein phosphorylation [15]. In another study, a multidimensional chromatography technology combining IMAC, hydrophilic interaction chromatography [HILIC] and reversed-phase LC was used to analyze DNA damage in the yeast *Saccharomyces cerevisiae*. 8764 resultant phosphopeptides were obtained from 2278 phosphoproteins, proving sufficient in-depth phosphoproteome analysis. A recent study of HILIC, reversed phase-high performance liquid chromatography [RP-HPLC] and strong cation exchange [SCX] chromatography revealed that HILIC could ensure better orthogonal separation for unphosphorylated peptides. As suited to phosphoproteomic analyses, HILIC columns prefer to retain hydrophilic molecules, thereby reducing sample complexity [2, 26, 36].

Current Research

Of late, enrichment of phosphotyrosine-containing proteins via monoclonal antiphosphotyrosine antibodies has been a notable recent success. Experimental data from liquid chromatography-based mass spectrometry analysis is being used to generate phosphorylation site-specific antibodies that could be used as reagents to develop antibody array/chip or for immunohistochemistry on tissue microarrays [TMA] in molecular pathology [5, 21]. One very recent and interesting application of these phosphorylation site–specific antibodies was in single cell profiling of signaling networks using multivariable flow cytometry. The human embryonic stem cell phosphoproteome was recently revealed by electron transfer dissociation tandem mass spectrometry. This was done by IMAC enrichment followed by analysis via nanoflow reversed-phase LC-MS/MS on a spectrometer that had been modified to perform ETD. Several phosphorylation site repositories curated by researchers are popping up every now and then, thereby accelerating phosphopeptide identification & discovery. The PHOSIDA database contains 8969 human phosphorylated sites, gathered from collective literature reports. The NetworKIN database integrates consensus substrate motifs with context modeling to improve prediction of cellular kinase-substrate reactions. It can also be used to study phosphorylation-modulated interaction networks at the global and the molecular level [22, 41]. To assist in LC-MS/MS analysis, the PhosphoPIC [PhosphoPeptide Identification & Compilation] software has been created to perform a variety of functions including automated selection and compilation of phosphopeptide identifications from multiple MS levels [MS^n]. It has also been used for the estimation of dataset false discovery rate [FDR], and application of appropriate cross-correlation [XCorr] filters. CID studies have revealed that a LTQ-Orbitrap LC-MS/MS platform can identify approximately 3 times more phosphopeptides than Q-TOF LC-MS/MS instrumentation. This may be attributed to the versatility of the Orbitrap, which features high resolution [up to 150,000], high mass accuracy [2-5 ppm], a mass-to-charge range of 6000, and a dynamic range greater than that of a low resolution instrument [3, 8, 47]. The use of titanium dioxide [TiO_2], zirconium dioxide [ZrO_2] and niobium pentoxide [Nb_2O_5] has led to greater selectivity in enriching phosphopeptides. Miniaturized technologies like capillary and microfluidic platforms have greatly sped up bioanalytical and biological applications, with added sensitivity and throughput-related demands [11, 18, 38]. Global quantification of phosphoproteins has become much easier with iTRAQ & AQUA. iTRAQ is an *in vitro* labeling procedure and can be applied to clinical samples such as serum, urine and tumor tissues. AQUA provides for absolute quantification of phosphoproteins, unlike the relative level of quantification produced by SILAC or iTRAQ [12]. Another phosphoproteomic approach corresponds to the identification of several

extracellular signal-regulated kinase [ERK] mitogen-activated protein [MAP] kinase substrates, using a combination of steroid receptor fusion system, IMAC, 2D differential gel electrophoresis [2D-DIGE] & phosphomotif-specific antibodies. The phosphorylation of various nucleoporins by ERK could herald novel theories in the regulation of the nucleocytoplasmic transport [14].

Technical Challenges

Low stoichiometry has proved a major impediment in global phosphoproteomic analyses [35]. A prime challenge is the cost-effective production of large quantities of a wide range of highly purified and phosphorylation site–specific antibodies. High-density antibody array construction is a tough task, & requires large amount of protein samples for analysis. Also, a considerable amount of technical inertia has to be overcome before clinically designing and applying a high-throughput antibody/protein chip. Routine clinical application of phosphoproteomics could be realistically achieved using the Aptamer technology and semi-high throughput TMAs. Currently, efforts are on to make TMAs from frozen tissue blocks, so that protein antigens can be better preserved than on paraffin blocks [21]. The presence of single- or double-stranded nucleic acids in IMAC beads to a loss of approximately 50% of phosphopeptides during enrichment. Acetonitrile precipitation was a preferential strategy to remove nucleic acids from protein samples prior to IMAC [20]. Phosphoprotein profiling serves well to improve the statistical significance of protein identification. Also, the integrity and conformational stability of intact proteins makes them a feasible fodder for polyacrylamide gel electrophoresis [PAGE] separations. However, phosphopeptide enrichment could suffer a setback if phosphorylation sites are misidentified. Co-purification of associated, non-phosphorylated peptides is also a deviating factor [15].

Applications

Recent developments in chromatographic and mass spectrometric techniques have spurred phosphoproteomics to move beyond mere experiments, to a stage where complex biological issues can be resolved [35]. The next big thing could be a combination of phosphoproteomics with enzyme-substrate engineering. This involves mutating the ATP-binding site of a specific kinase such that it will additionally accept a bulky ATP analogue. If a chemical tag is incorporated into this analogue at the gamma phosphate the protein substrates of the kinase will be tagged at the site of modification, enabling their enrichment and identification. This technology would be a potent tool for the analysis of kinase networks because there is no other *de novo* method available to determine the targets of a single kinase against its homologues without resorting to pleiotropic kinase knockouts [43]. Tyrosine phosphorylation prominently features in cancer biology, since they serve as attractive drug targets and molecular cancer markers. As such, the tumour phosphoproteome is a rich mine of potential biomarkers. The SILAC method has unveiled the role of the spleen tyrosine kinase Syk in phosphorylating E-cadherin, for proper localization of p120-catenin at adherens junctions. Subsequent identification of the biochemical pathways regulated by Syk in cancer cells could offer valuable insights into tumor formation and progression. Targeting tyrosine kinases like the receptors Met and EphA2, the cytoplasmic tyrosine kinase Fak, and pseudo-tyrosine kinase SgK223 may be of significant therapeutic value in human colorectal carcinoma [CRC] [17, 19, 21, 44].

Conclusion

Developments in the field of phosphoproteomics have been fuelled by the need to simultaneously monitor many different phosphoproteins within the signaling networks that coordinate responses to changes in the cellular environment. Phosphotyrosine-directed signaling analyses are increasingly being used to provide robust measurements for functional biological interpretation of drug action on signaling and phenotypic outcomes. While phosphoproteomics will greatly expand our knowledge about the numbers and types of phosphoproteins, its greatest promise is the rapid analysis of entire phosphorylation based signaling networks. The enormity of the protein phosphorylation profile has created many a challenge for future researchers. Creating a phosphoprotein-specific antibody chip may bode well for cancer studies in the time to come.

Abbreviations

post-translationally modified [PTM], immobilized metal affinity chromatography [IMAC], phosphoprotein isotope-coded affinity tag [PhIAT], phosphoprotein isotope-coded solid-phase tag [PhIST], poly [glycidyl methacrylate/divinylbenzene] [GMD], sequential elution from IMAC [SIMAC], stable isotope labeling by amino acids in cell culture [SILAC], epidermal growth factor [EGF], fibroblast growth factor [FGF], Electron spray ionization [ESI], matrix-assisted laser desorption/ionization [MALDI], surface-enhanced laser desorption/ionization [SELDI], time-of-flight [TOF], quadrupole TOF [QTOF], linear quadrupole ion trap-Fourier transforms [LTQ-FTs], mass spectrometry [MS], Electron capture dissociation [ECD], electron transfer dissociation [ETD], collision-induced dissociation [CID], Fourier transform ion cyclotron resonance mass

spectrometry [FTICR-MS], hydrophilic interaction chromatography [HILIC], reversed phase-high performance liquid chromatography [RP-HPLC], strong cation exchange chromatography [SCX], tissue microarrays [TMA], PhosphoPeptide Identification & Compilation [PhosphoPIC], false discovery rate [FDR], titanium dioxide [TiO_2], zirconium dioxide [ZrO_2], niobium pentoxide [Nb_2O_5], extracellular signal-regulated kinase [ERK], mitogen-activated protein [MAP], 2D differential gel electrophoresis [2D-DIGE], polyacrylamide gel electrophoresis [PAGE], colorectal carcinoma [CRC]

References

[1] Aivaliotis M., Macek B., et al. (2009) *PLoS ONE*, 4(3), e4777.

[2] Albuquerque C.P., Smolka M.B., et al. (2008) *Mol Cell Proteomics*, 7(7), 1389-1396.

[3] Alcolea M.P., Kleiner O. and Cutillas P.R. (2009) *J Proteome Res.*, 8(8), 3808-3815.

[4] Aprilita N.H., Huck C.W., et al. (2005) *J Proteome Res.*, 4(6), 2312-2319.

[5] Ashman K. and Villar E.L. (2009) *Clin Transl Oncol.*, 11(6), 356-362.

[6] Blethrow J.D., Glavy J.S., et al. (2008) *PNAS*, 105(5), 1442-1447.

[7] Bodenmiller B., Malmstrom J., et al. (2007) *Molecular Systems Biology*, 3, 139.

[8] Boersema P.J., Mohammed S. and Heck A.J. (2009) *J Mass Spectrom.*, 44(6), 861-878.

[9] Chen X. and Hess S. (2008) *Expert Rev Proteomics*, 5(8), 827-839.

[10] Diu X., Yang F., et al. (2008) *J Proteome Res.*, 7(6), 2195-2203.

[11] Ficarro S.B., Parikh J.R., et al. (2008) *Anal Chem.*, 80(12), 4606-4613.

[12] Gafken P.R. and Lampe P.D. (2006) *Cell Commun Adhes.*, 13(5-6), 249-262.

[13] Gunawardena H.P., Emory J.F. and McLuckey S.A. (2006) *Anal Chem.*, 78(11), 3788-3793.

[14] Kosako H., Yamaguchi N., et al. (2009) *Nat Struct Mol Biol.*, 16(10), 1026-1035.

[15] Kristjansdottir K., Wolfgeher D., et al. (2008) *J Proteome Res.*, 7(7), 2812-2824.

[16] Lachmann A. and Ma'ayan A. (2009) *Bioinformatics*, 25(5), 684-686.

[17] Larive R.M., Urbach S., et al. (2009) *Oncogene*, 28(24), 2337-2347.

[18] Lazar I.M. (2009) *Electrophoresis*, 30(1), 262-275.

[19] Leroy C., Fialin C., et al. (2009) *Cancer Res.*, 69(6), 2279-2286.

[20] Li Y., Luo Y., et al. (2009) *Mol Biotechnol.*, 43(1), 59-66.

[21] Lim Y.P. (2005) *Clin Cancer Res.*, 11(9), 3163-3169.

[22] Linding R., Jensen L.J., et al. (2008) *Nucleic Acids Research*, 36, D695-D699.

[23] Liu T., Belov M.E., et al. (2007) *Chem Rev.*, 107(8), 3621-3653.

[24] Mann M. (2006) *Nat Rev Mol Cell Biol.*, 7(12), 952-958.

[25] McLachlin D.T. and Chait B.T. (2003) *Anal Chem*, 75(24), 6826-6836.

[26] McNulty D.E. and Annan R.S. (2008) *Mol Cell Proteomics*, 7(5), 971-980.

[27] Molina H., Horn D.M., et al. (2007) *PNAS*, 104(7), 2199-2204.

[28] Mumby M. and Brekken D. (2005) *Genome Biology*, 6, 230.

[29] Nita-Lazar A., Saito-Benz H. and White F.M. (2008) *Proteomics*, 8(21), 4433-4443.

[30] Nousiainen M., Sillje H.H.W., et al. (2006) *PNAS*, 103(14), 5391-5396.

[31] Nuhse T.S., Stensballe A., et al. (2004) *The Plant Cell*, 16, 2394-2405.

[32] Paradela A. and Albar J.P. (2008) *J Proteome Res.*, 7(5), 1809-1818.

[33] Pocsfalvi G. (2009) *Methods Enzymol.*, 457, 81-96.

[34] Reinders J. and Sickmann A. (2005) *Proteomics*, 5, 4052-4061.

[35] Rogers L.D. and Foster L.J. (2009) *Mol Biosyst.*, 5(10), 1122-1129.

[36] Saleem R.A. and Aitchison J.D. (2009) *J Vis Exp.*, 12(32), 1474.

[37] Salih E. (2005) *Mass Spectrom Rev.*, 24(6), 828-846.

[38] Smith J.C. and Figeys D. (2008) *Biochem Cell Biol.*, 86, 137-148.

[39] Soufi B., Gnad F., et al. (2008) *Proteomics*, 8(17), 3486-3493.

[40] Spahn C.M.T., Lehrach H. and Jungblut P.R. (2004) *Genome Biology*, 5, 322.

[41] Swaney D.L., Wenger C.D., et al. (2009) *PNAS*, 106(4), 995-1000.

[42] Sweet S.M.M., Bailey C.M., et al. (2009) *Mol. Cell Proteomics*, 8(5), 904-912.

[43] Tate E.W. (2008) *J Chem Biol.*, 1, 17-26.

[44] Tedford N.C., Hall A.B., et al. (2009) *Proteomics*, 9(6), 1469-1487.

[45] Thingholm T.E., Jensen O.N. and Larsen M.R. (2009) *Proteomics*, 9(6), 1451-1486.

[46] Thingholm T.E., Jensen O.N., et al. (2008) *Mol Cell Proteomics*, 7(4), 661-671.

[47] Yates J.R., Ruse C.I. and Nakrochevsky A. (2009) *Annu Rev Biomed Eng.*, 11, 49-79.

[48] Zhang G., Spellman D.S., et al. (2006) *J Proteome Res.*, 5(3), 581-588.

Table 1- Methods for enriching phosphoproteins & phosphopeptides

Method	Comments
Chemical Modification	
Affinity Tagging	Phosphorylated amino acids are derivatized by β-elimination or carbodiimide to introduce tags; β-elimination strategy is limited to P-Ser and P-Thr, and also occurs with O-glycosylated residues.
Bio-orthogonal Affinity Purification	Analog-specific kinases are used selectively to phosphorylate substrates *in vitro* or *in vivo*; the phosphate analogs are then derivatized to generate a hapten for immunoprecipitation. This method requires expression and/or isolation of an engineered kinase.
Fluorous Affinity Tagging	Perfluoroalkyl groups are selectively coupled to P-Ser and P-Thr using β-elimination, modified peptides are enriched with fluorous-functionalized stationary phase. This method is highly selective for derivatized peptides.
Phosphospecific Proteolysis	Chemical modification of P-Ser and P-Thr introduces lysine analogs and cleavage by lysine-specific proteases. This method allows direct identification of phosphorylation site without sequencing the phosphopeptide.
Thiophosphorylation & Affinity Tagging	Proteins are phosphorylated with ATPγS; thiophosphates are alkylated to form linkages with biotin or solid supports. This method requires *in vitro* phosphorylation and most kinases utilize ATPγS poorly.
Direct Enrichment	
Anti-phosphotyrosine Antibodies	Anti-P-Tyr antibodies have proven very useful for the enrichment of P-Tyr-containing proteins; they have been used alone or in combination with IMAC. They have been used to enrich P-Tyr peptides.
Anti-phosphoserine & Anti-phosphothreonine Antibodies	Anti-P-Ser and anti-P-Thr antibodies have been used; but currently are less useful than anti-P-Tyr antibodies.
Cation Exchange	Strong cation exchange chromatography has been used for the large-scale identification of phosphorylation sites. This method selects for peptides phosphorylated on a single residue.
Immobilized Metal Affinity Chromatography [IMAC]	Introduction of an esterification step greatly enhances the selectivity of this method, which is very useful, has been widely used and can be automated.

