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Enrichment Strategies for Identification and Characterization of Phosphoproteome

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Abstract: Phosphorylation upon protein is well known to a key regulator that implicates in modulating many cellular processes like growth, migration, and differentiation. Up to date, grafting of multidimensional separation techniques onto advanced mass spectrometry (MS) has emerged as a promising tool for figuring out the biological functions of phosphorylation in a cell. However, advanced MS-based phosphoproteomics is still challenging, due to its intrinsic issues, i.e., low stoichiometry, less susceptibility in positive ion mode, and low abundance in biological sample. To overcome these bottlenecks, diverse techniques (*e.g.*, SCX, HILIC, ERLIC, IMAC, TiO_2, etc.) are continuously developed for on-/off-line enrichment of phosphorylated protein (or peptide) from biological samples, thereby helping qualitative/quantitative determination of phosphorylated protein and its phosphorylated sites. In this review, we introduce to the overall views of enrichment tools that are universally used to selectively isolate targeted phosphorylated protein (or peptide) from ordinary ones before MS-based phospoproteomic analysis.

Keywords: phosphoproteomics, fractionation, enrichment, nLC-ESI-MS/MS

Introduction

Reversible protein phosphorylation occurring on serine, threonine and tyrosine in eukaryotes, one of post-translati-onal modifications (PTMs), plays a crucial role in regulation of the many cellular processes including signal transduction, differentiation, transcription and translation.[1-4] Aberrant phosphorylation in cell signaling pathway can lead to mutation, overexpression or abnormal operation of regular enzymes such as protein kinases and phosphatases.[5,6] Hence, the global profiling and quantification of phosphoproteins and their phosphorylated sites are of importance for understanding the biological pathway related with the key biological processes in a cell.[7-9]

Liquid chromatography-mass spectrometry (LC-MS) have been reported for analyzing the phosphoproteins and their phosphorylation sites because of the high sensitivity, precision and accuracy.[10,11] Especially, tandem mass spectrometry (MS/MS) has served for information associated with the presence of phosphorylation events and their localization on phosphoproteins. However, the detection of phosphoproteins by MS might be difficult because phosphorylation of proteins is a transient modification, phosphoproteins within cells are relatively low stoichiometry in comparison to non-phosphorylated counterparts, and phosphate group causes by ionization suppression in MS analyses.[12,13] Thus, the fractionation and enrichment strategies prior to MS analysis are necessary for reducing the above mentioned challenges.

A variety of enrichment and fractionation methods in phosphoproteomics have been reported including the enrichment with immobilized metal affinity chromatography (IMAC)[6,14-16] and metal oxide affinity chromatography (MOAC) as well as titanium dioxide (TiO_2),[17-25] and fractionation with strong cation exchange (SCX),[26,27] hydrophilic interaction chromatography (HILIC),[28-30] and electrostatic repulsion-hydrophilic interaction chromatography (ERLIC).[31-33] More recently, the two sequential steps of preparation methods are commonly used in large scale phosphoproteomics. A fractionation step performs to reduce sample complexity, and followed by an enrichment for increasing the number of identified phosphopeptides and their phosphorylation sites, such as SCX-TiO_2,[34-36] SCX-IMAC,[37,38] ERLIC-IMAC[39] and IMAC-TiO_2.[40,41] Overall workflow of phosphoproteomic analysis using fractionation and enrichment are illustrated at Figure 1.

MS/MS-based experiments of phosphoprotein is accomplished with the fragmentation techniques, such as

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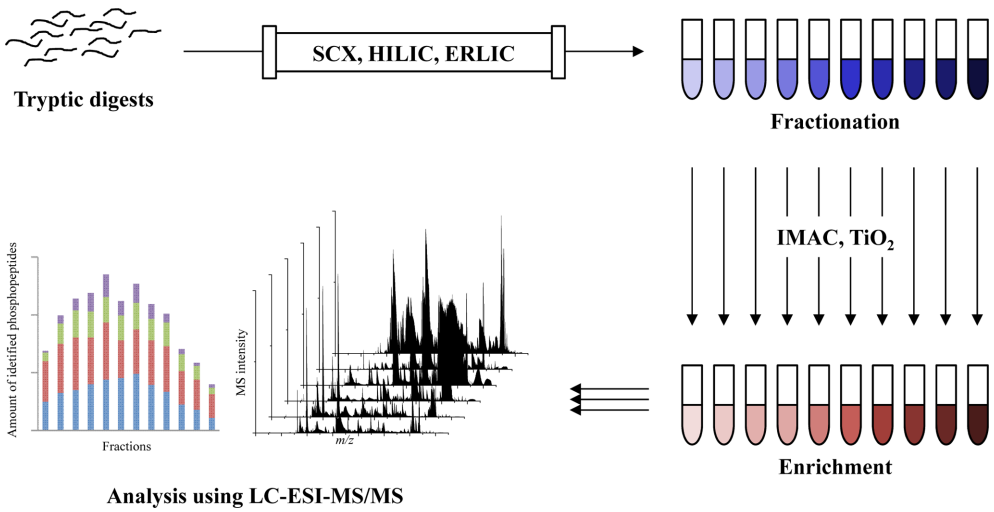

Figure 1. Systemic illustration for isolation of phosphopeptides from tryptic digests using fractionation and enrichment with LC-ESI-MS/MS.

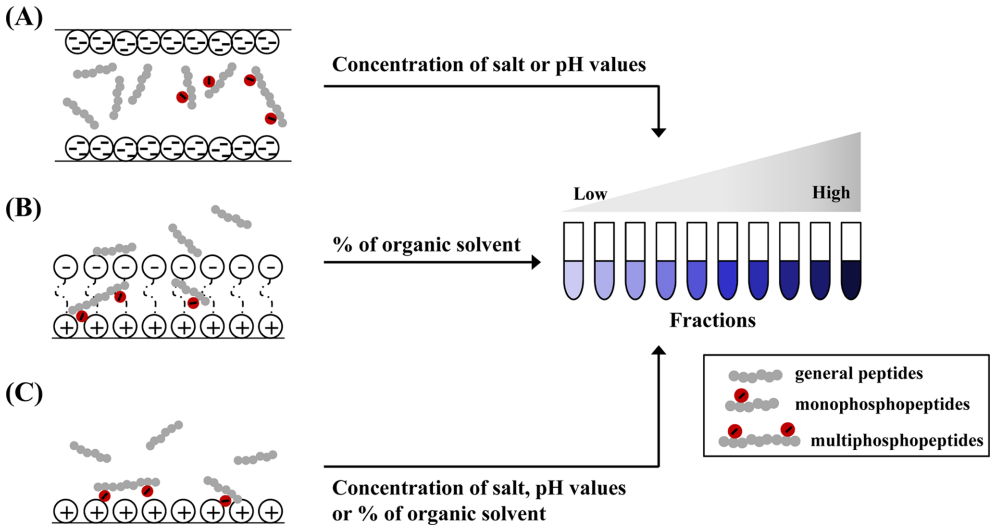

Figure 2. Phosphopeptide fractionation strategies. Mono- and multi-phosphopeptides are separated from by (A) strong cation exchange (SCX), (B) hydrophilic interaction liquid chromatography (HILIC), and (C) electrostatic repulsion hydrophilic interaction chromatography (ERLIC) according to the concentration of salts, pH values of eluted solution, or percentage of organic solvent.

collision induced dissociation (CID),[42-44] electron capture dissociation (ECD),[45] and electron transfer dissociation (ETD).[46] Phosphoproteins consisting of phospho-serine and phospho-threonine residues in MS/MS analysis with CID are often produced the significant neutral loss of phosphate group, and these phenomenon affects to fragmentation for identification of phosphoproteins. On the other hand, phosphoprotein analysis by ECD or ETD prevents to cleave the unstable phosphate group, and offers the unambiguous information of phosphorylation site locations.[47]

This article provides an overview of preparative and analytical strategies using LC-MS/MS for identification and characterization of phosphoproteins and their phosphorylation sites localizations. Furthermore, we reviewed on the fragmentation reactions of phosphoproteome by MS/MS in understanding biological network pathway.

Sample fractionations

Strong cation exchange (SCX)

SCX chromatography is a universal fractionation technique for phosphoprotein (or phosphopeptide).[48,49] Proteins or peptides are retained on a column that contains a hydrophilic, anionic resin through the affinity to positively charged groups on amino acids (Figure 2-A). The main principle of SCX is the relationship between

SCX matrix and oppositely charged proteins (or peptides) from biological sample according to their net surface charge and *pI* value. Because general peptides can change their charge depending on the pH of environment, phosphoproteins or phosphopeptides are weakly captured by the SCX column matrix, compare to general ones. All peptides will bind to a cation exchanger at low pH condition, and bound peptides are predominantly separated by a stepwise elution with increasing ionic strength and/or increasing pH value.[28] Through the above process, the peptides obtained from complex biological sample are divided to 6~12 fractions. Phosphoprotein (or phosphpeptides) bound to SCX matrix are eluted in the early fractions, and peptides except for phosphorylated counterparts are eluted gradually on high salt or pH conditions.

Hydrophilic interaction chromatography (HILIC)

HILIC for separating the proteins and peptides rely on the partition of analytes between the used solvents and solid resins.[29] In HILIC, the hydrophilic peptides take to remain into chromatography column, the hydrophobic peptides eluted at early step in contrast with reverse phase chromatography. HILIC has been reported frequently in recent years in phosphoproteomic studies, because highly hydrophilic solutes as phosphorylated ones in comparison with common peptides are retained on the column for the longest time.[28-30] In HILIC, hydrophilic components acquired from biological specimens are typically bound onto HILIC column matrix when used with below 10 % aqueous. To isolate the phosphopeptides from ordinary ones, all peptides are fractionated by predominantly increasing the aqueous mobile phase up to 40% (Figure 2-B). Fractionations using HILIC facilitates the identification and characterization of phosphopeptides and number of phosphorylation sites, when combined with either IMAC or TiO_2. Zappacosta and co-worker provided a collaboration of HILIC and IMAC for identification of more than 20,000 phosphopeptide from rat liver.[30] Most non-phosphopeptides from rat liver were removed in early steps (fraction 1~15), followed by the elution of mono and multiphosphopeptides by stepwise. Accordingly, HILIC is an alternative tool for phosphoprotein or phosphopeptide fractionation, and thus can be coupled online to MS analysis.

Electrostatic repulsion-hydrophilic interaction liquid chromatography (ERLIC)

ERLIC chromatography, one of phosphopeptide fractionations, was first introduced by Alpert in 2008.[31] This technique is modified from HILIC approach and can be accomplished isocratically.[32] ERLIC matrix containing weak anion exchange and hydrophilic interactions allows for the isolation of phosphopeptides from other ones in proteolytic digests. Many phosphorylated proteins and peptides are more hydrophilic in comparison with their unmodified counterparts, can be separated with ERLIC (Figure 2-C). Separation of ERLIC is influences by pH, salt concentration, count-ion, a percent of organic solvent in mobile phase, etc.[32,33] ERLIC matrix prior to sample loading are acidified with a combination of organic acids with high percent of acetonitrile to remain the phosphopeptides from tryptic digests, and gradually fractionated by increasing concentration of salts. The eluted phosphopeptides using ERLIC protocol is recently applied in combination with reverse phase, TiO_2,[50] SCX,[32,33] and IMAC.[39] Loroch, S. and co-worker demonstrated that HeLa cells digests were sequentially separated to 21 fractions using ERLIC combined with SCX, total 8998 phosphorylation sites from 3013 phosphoproteins are detected.[32] Early elutions (fraction 1-5) among 21 fractions are detected with mono-phosphopeptides, whereas the pooled middle fractions (fraction 10-15) are confirmed with multi-phosphopeptides.

Phosphopeptide enrichments

Immobilized metal ion affinity chromatography (IMAC)

IMAC is a widely used technique to enrich phosphopeptides prior to MS analysis. Positively charged metal ions, such as Fe^{3+},[16,47] Zn^{2+},[15] Ga^{3+},[51] La^{3+},[52] and Ti^{4+},[38,47] are chelated to nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) resin and interacted with negatively charged phosphate groups (Figure 3A). One of the limitations in the IMAC technique is its partial non-specific binding of acidic peptides containing glutamic acid or aspartic acid residues when used for phosphopeptide enrichment, thereby introducing the inefficiency of IMAC. The pH values in IMAC specificity are an important factor, because the acid dissociation constant (pK_a) of phosphate group at serine/threonine (pKa 2.1) and tyrosin residues (pK_a 1.0) is lower than other acidic amino acid including glutamic acid (pKa 3.65) and aspartic acid (pKa 4.25).[53-55] To minimize these issues, loading and binding buffers during phosphopeptide enrichment with IMAC were often used to pH 2-2.5 containing with organic acid [*e.g.,* trifluoroacetic acid (TFA), acetic acid, hydrochloric acid and formic acid].[50] A variety of organic acids have been

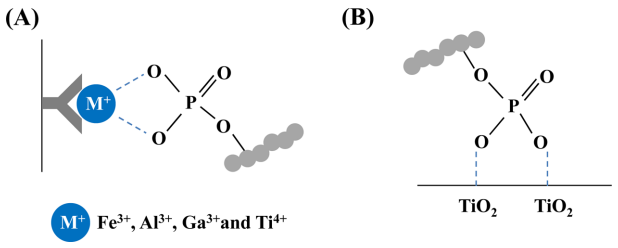

Figure 3. Typical enrichment methods for phosphopeptides. (A) Immobilized metal ion affinity chromatography (IMAC) and (B) titanium dioxide (TiO_2) able for the specific enrichment of phosphopeptides from ordinary peptides

used for high selectivity of IMAC, their influence resulted as follows: TFA > hydrochloric acid > formic acid > acetic acid.[53] On the contrary, the elution of phosphopeptides bound on IMAC was performed at strong basic conditions containing ammonium hydroxide, urea, or tris-buffer. Although IMAC is a friendly used approach for enrichment of phosphopeptides, further optimization of loading and eluting buffers requires as an essential part.

Titanium dioxide (TiO_2)

The TiO_2-based strategy, one of metal oxide affinity chromatography (MOAC), is the robustness, simplicity, and high efficiency for enrichment of phosphorylated proteins or peptides from complex biological sample (Figure 3B). TiO_2 techniques are usually performed using self-packed or commercial products with microcolumn,[17,56] or beads.[20,25,40] To improve the reproducibility of TiO_2 for phosphopeptide enrichment, Tape and co-worker described that the automatic phosphopeptide enrichment using magnetic TiO_2 beads with IMAC.[40] The automated enrichment system with magnetic TiO_2 beads is achieved by controlling 96-well plates at the eight carousel positions. Phosphopeptides from tryptic digests are more identified with magnetic beads and automatic system, compare to manual enrichment.

Selectivity of TiO_2 resin is influenced by the pH value of loading and eluting buffers similar to IMAC approach. To enhance the enrichment efficiency of TiO_2 resin, the loading buffer was acidified by organic acids (*e. g.,* TFA, acetic acid, and formic acid).[57,58] However, acidification of buffers in phosphopeptide enrichment was not enough to diminish the binding of nonphosphopeptide.[59] In many reports, acidic loading buffers on TiO_2 have been tested and optimized with the modifiers including 2, 5-dihydroxybenzoic acid (DHB), malic acid, lactic acid, and other acidic reagents to enhance the enrichment efficiency of phosphopeptide.[17-21] Capacity of acidic modifier in loading buffer for removing non-specific binding decreased as follows: DHB = salicylic acid = phthalic acid > benzoic acid = cyclohexanecarboxylic acid > phosphoric acid > TFA > acetic acid.[53] In recently, specificity of TiO_2 chromatography was optimized by adding glycerol[60] or citric acid.[61] On the other hand, the phosphopeptides bound onto TiO_2 resins was released by diverse amines and salts (*e. g.,* ammonium bicarbonate, ammonium hydroxide, and pyrrolidine). Due to many singly and multiply charged phosphopeptides exist in biological sample, the release of phosphorylated ones carried out by stepwise elution with two elution buffers having the different pH values. The first elution was performed by weakly basic conditions, subsequently eluted by adding strongly basic buffers.

In addition, on-line TiO_2-based approach reported for the reproducibility, use-to-easy, and high-throughput in phosphoproteomics, unlike the abovementioned off-line approaches.[55,62,63] Pinkse and co-worker demonstrated that online phosphopeptide enrichment with TiO_2 resin is automatically carried out using 'sandwich precolumns' made up of reverse phase (RP)-TiO_2-RP materials.[55] All peptides obtained from tryptic digests are remained in first RP precolumn at 3 μL/min. During an initial H_2O/ACN gradient at low flow rate (~100 nL/min), non-phosphopeptides and phosphopeptides are transferred to analytical column and TiO_2 precolumn. The phosphopeptides binding on TiO_2 resins are released by injecting the elution buffer at high pH value and concentration of salts in a second H_2O/ACN gradient. Briefly, pH value of buffer solution, concentration of salts, and flow rate are the crucial parameters for phosphopeptide enrichment using online RP-TiO_2-RP system. The above processes on TiO_2 protocol are beneficial for increasing the probability of phosphopeptide identification.

Phosphopeptide entichment using β-elimination reaction

Affinity purification in cooperation with β-elimination of phosphopeptides is helpful for isolating phosphopeptide by modifying phosphate groups.[64-66] McLachlin and co-worker introduced the improved chemical modification-based phosphopeptide enrichment strategy.[65] β-Elimination of phosphopeptide can be achieved with the solution made up of 25 mM *N*-hydroxysuccinmide and 0.5 M *N,N*-dimethylaminopropyl ethyl carbodiimide HCl, after condensation reaction using *t*-butyl-dicarbonate (tBoc). Then the resulting solutions are reacted utilizing ethanedithiol for improving the affinity with solid resins. Addition of EDTA during β-elimination reaction reduces the side effects. The treated phosphopeptides are reacted with the activated thiol affinity resins for isolating phosphopeptides from ordinary ones. Phosphopeptides enriched through affinity purification are separated from solid phase with TFA. The chemical modification-based affinity purification is the useful tool for enriching phosphopeptides from sample complex, and offers unambiguous information for phosphorylation site location.

Combination with fractionation and enrichment

In phosphoproteomics, the two sequential steps with fractionation and enrichment methods are, in some cases, used to more identify phosphopeptides and their phosphorylation sites up to date. As shown in Table 1, the starting materials of biological samples for phosphopeptide enrichment are used at approximately 0.1~3 mg, and the maximum amount is 15 mg of cell lysates. Most combinational strategies achieve utilizing two preparation methods, and present the synergy effect for identifying phosphopeptides and their phosphorylated sites than each approach. Zhou and co-worker introduced 3D-cooperative strategies with SCX, Ti^{4+}- IMAC and HILIC.[28] Tryptic digests are fractionated *via* SCX collecting 22 fractions, and followed by desalting with C18 cartridge. Then, the

Table 1. The recent trends of fractionation and enrichment in phosphoproteomics

no.	sample source	starting material (ug)	fractionation & enrichment	no. of identified phosphopeptides	no. of identified phosphorylation sites	efficiency (phosphopeptide/ug)	Ref.
1	HeLa cells	100	TiO_2	3627	4166	36.27	[41]
			Ti^{4+}-IMAC	3968	4354	39.68	
2	HeLa cells	800	ERLIC, SCX	8087	8998	10.11	[32]
3	HeLa cells	1250	SCX, Ti^{4+}-IMAC, HILIC	20788	16740	16.63	[28]
	K562			28993	23196	23.19	
4	HeLa cells	1000	SCX, TiO_2	9483	15713	9.48	[61]
5	MCF-10A	3000	SCX, IMAC	8969	6337	2.99	[17]
6	pPC3 prostate cancer cells	1000	HILIC, TiO_2	6841	-	6.84	[60]
7	hepatocellular carcinoma	250	SCX, Ti^{4+}-IMAC	-	6260	-	[38]
8	NH-3T3 cells	3000	SCX, TiO_2	6215	6827	2.07	[26]
			High pH RP, TiO_2	17566	27712	5.86	

eluted peptides are reconstituted with Ti^{4+}-IMAC loading buffer after (80% ACN/6% TFA) dried under vacuum condition. The Ti^{4+}-IMAC Gel-loader tips were packed with a C8 plug and Ti^{4+}-IMAC beads. The prepared samples are applied to the Ti^{4+}-IMAC tip conditioned with loading buffer. Subsequently, the retained phosphopeptides in Ti^{4+}-IMAC beads are eluted by adding the elution buffer, and resulting phosphopeptides are additionally isolated by HILIC fractionation. The 3D strategies are remarkable for isolating the phosphopeptides from general counterparts, and identified 20788 and 28993 phosphopeptides for HeLa cells and K562 cells, respectively. Consequentially, the appropriate combination of pretreatments can help to acquire the significant information of phosphorylated peptides and its localizations related cell signaling pathways such as the protein ubiquitination pathway and the ERK/MAPK signaling pathway.

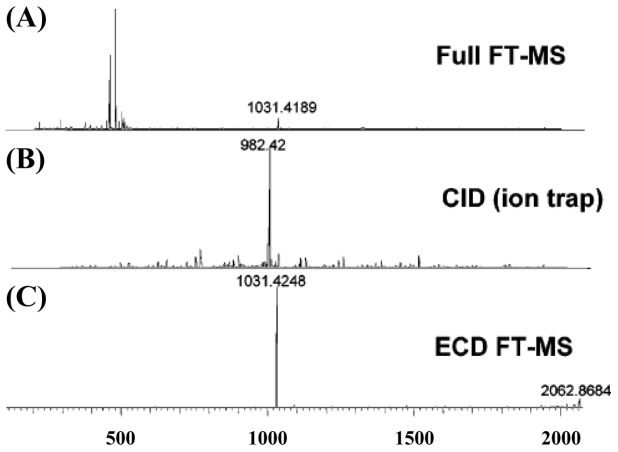

Figure 4. MS scan and tandem mass spectra of FQpSEEQQQTEDELQDK (+2, *m/z* 1031.4181) obtained from β-casein. (A) A full FT-MS scan (retention time 23.7 min.), (B) CID MS/MS scan of *m/z* 1031.4 analyzed using the linear ion trap, and (C) ECD MS/MS scan of *m/z* 1031.4. The reference reproduced from Sweet et al. (2006).[62]

Mass spectrometry analysis of phosphopeptides

Phosphopeptides enriched from tryptic digests are introduced into MS *via* electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI), and precursor ions of phosphopeptides is sequenced by MS/MS analysis.[47,62] The MS/MS techniques extensively used in phosphoproteomics are CID, ECD, and ETD.[45,46,67] In CID-MS/MS experiments, the peptide is slowly generated the precursor ion and fragment through lowest energy pathway, it is cleaved the peptide bond from N-terminus (b-type ions) or C-terminus (y-type ions).[45] Peptides having phosphoserine or phosphothreonine residues usually produce to both phosphopeptide backbone fragments and neutral loss of phosphoric acid (H_3PO_4) during CID fragmentation. Although neutral loss of H_3PO_4 indicates a presence of phosphorylation in peptide sequence, it prevents for identification and quantification of phosphopeptide and their phosphorylated locations with accuracy.[43,44] While ECD is trapped a low energy electron by a multiply charged precursor cation,[45] and ETD is transferred electrons from anion to a multiply charged precursor cation.[47] Both lead to dissociate the peptide bond, resulting in the production of c- and z-type ions. The fragmentation by ECD or ETD is adequate for phosphoproteome analysis because of the wide cleavage of peptide backbone without labile PTM as phosphorylation.[68] Sweet and co-worker demonstrated the availability of neutral loss triggered ECD for identifying and charactering phosphopeptides from β-casein as phosphoprotein standard.[45] The mass spectrum of β-casein phosphopeptides obtained from first MS scan event exhibit FQpSEEQQQ-TEDELQDK (*m/z* 1031.4) corresponding to the doubly

protonated ion (Figure 4A). The detected phosphopeptide ion is consequently introduced into CID MS/MS, and measured at m/z 982.4 with a neutral loss of 98 Da (H_3PO_4) (Figure 4B). Finally, precursor ion (*m/z* 1031.4) is analyzed with ECD MS/MS. Both precursor ion and charged-reduced species $[M+2H]^{+\bullet}$ are observed at 1031.4 and 2062.8684, respectively (Figure 4C). Consequentially, the dissociation methods described above can offer the complementary information that the existence of phosphorylation in peptide, identification and quantification of phosphopeptides, and specific phosphorylation site locations.

Conclusion

We have introduced the overview of diverse fractionation and enrichment strategies for isolation of phosphoproteins and phosphopeptides from complex cellular components.

The cited phosphopeptide fractionating and enriching protocols are respectively relied on ion strength and chelating reaction between their matrix and phosphopeptides, and contributing to discovery of low abundant phosphopeptide functions and early diagnosis of diverse diseases. Additionally, the combination of phosphopeptide enrichment with sample fractionation prior to MS analysis is of key importance to overcome the existing challenges and to enable the identification of more phosphopeptides and phosphorylation site localizations.

The entire above-mentioned fractionation and enrichment approaches are well appropriated in phosphoproteomics, and two or more parallel strategies can be got the new information for understanding the function of phosphorylations in cellular network, and thus can help to identify novel phosphoproteins (or phosphopeptides) involved in therapeutic mechanisms from diseases.

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