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Isolation of phosphopeptides using solid phase enrichment

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Abstract—Protein phosphorylations are post-translational protein modifications that are crucial for intercellular signal transduction and the regulation of many cellular events. We describe herein a novel technology utilizing α-diazo functionalized solid phase resins to isolate phosphorylated peptides from non-phosphorylated substrates.

The reversible process of phosphorylation/dephosphorylation of proteins is a post-translational protein modification that is crucial for intercellular signal transduction. Protein phosphorylations are widely recognized as critical events in the regulation of division, gene expression and metabolism.[1–4] It is estimated that more than one-third of all proteins can be modified by phosphorylation in mammalian cells and up to 2% of the genes in a vertebrate genome encode either protein kinases or phosphatases.[2,5] Deregulation of the signal transduction cascade upsets this well-balanced system and has been implicated in diseases such as cancer,[6,7] type II diabetes,[8,9] cystic fibrosis,[10] Alzheimer's disease,[11–14] and many more.[15] Even though the human genome map presents invaluable insight into the structure and sequence of our genes, it offers limited insight into these critical post-translational protein modifications. Unfortunately, proteomic techniques relevant to the elucidation of signal transduction have been lacking in development in comparison to genomic technologies.[16–18] In light of the role of protein phosphorylations in cellular deregulation, a universal phospho-enrichment technique would be of great value to improved strategies for drug design and target validation.[15,19]

Phosphoproteomic research is focused the identification of the phosphorylation states of proteins and of the specific phosphorylation site of proteins. The first step in the mapping of the phosphorylation sites in proteins generally requires the digestion of phosphoproteins or protein complexes into an intricate mixture of its corresponding smaller peptide fragments. Despite several recent advances in tandem mass spectrometry and Edman degradation, the characterization of a complex pool of phosphorylated and non-phosphorylated peptides is still a very tedious or sometimes impossible task.[20,21] The most common technique currently used to enrich the phosphorylated substrates from the peptide pool is by the use of immobilized metal affinity chromatography (IMAC).[22] Due to the non-covalent binding of the substrate to the column, loss of phosphopeptides, difficulties in eluding multiple phosphorylated peptides and high background from non-phosphorylated peptides has limited this approach.[23] Recently, intense research has been focused on the chemical identification of phosphorylation sites. Despite the power of this approach, current methods are still limited to the identification of serine and threonine residues.[24–27]

We report herein a general method for the isolation of phosphorylated peptides from peptide mixtures using α-diazo-substituted resins. The solid phase enrichment procedure is capable of isolating phosphorylated serine, threonine, or tyrosine peptides from a crude peptide mixture. The α-diazo substituted resins covalently bind to the phosphate moiety of the phosphorylated peptide substrate, which allows for the selective isolation and characterization of the phosphorylated substrate after a simple filtration. The α-diazo functionalized resins were prepared from the commercially available Wang resins (Novabiochem). Esterification of the solid phase supported benzyl alcohol with the Fmoc-protected glycine utilizing 2,6-dichlorobenzoyl chloride, followed by deprotection and oxidation, provided the α-diazo substituted

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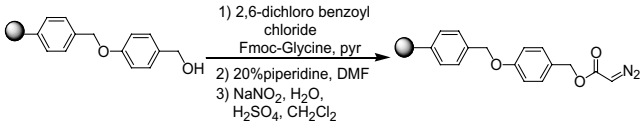
Wang resin according to well-established procedures (Scheme 1).[28,29]

The oxidation of the glycine residue was readily followed by FT-IR, resulting in the appearance of the characteristic diazo signal at 2108 cm^{-1}.[29,30]

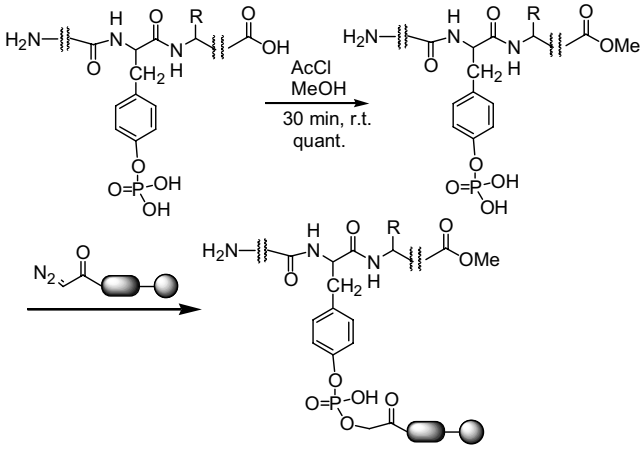
In order to ensure selective binding of phosphorylated peptides to the α-diazo resin all carboxylic acids present in the peptide coagulate must be blocked. This was accomplished by esterification of the crude peptide mixture using acetyl chloride and methanol (Scheme 2). The in situ generation of the anhydrous methanolic hydrochloric acid solution (2 N) quantitatively and selectively esterified the carboxylic acid residues and not the phosphate group within 30 min.[31]

The efficacy of the methylation procedure was evaluated on a known peptide mixture containing the tyrosine phosphorylated peptide angiotensin II [tyr(OPO_3H_2)], and the non-phosphorylated peptides, neurotensin, fibrinopeptide B and angiotensin I (Mixture I, Table 1).

The peptide mixture I was methylated for 30 min with anhydrous methanolic hydrochloric acid (2 N) generated



Scheme 1. Synthesis of α-diazo resin.



Scheme 2. General scheme for the solid phase isolation of phosphorylated peptides via a covalent phosphate–resin bond.

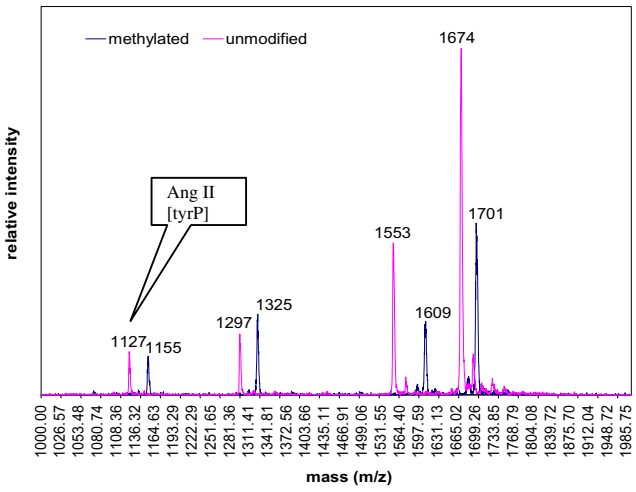


Figure 1. Methylation of peptide mixture.

in situ from acetylchloride in methanol and an aliquot of mixture I was subjected to MALDI-tof. Comparison of the mass spectra of the mixture before and after the esterification indicates that this treatment quantitatively esterified all of the carboxylic acids, and none of the phosphate moieties present in mixture I (Fig. 1). Mass increases of 14 units per carboxylic acid corresponded to all of the carboxylic acids found in each of the peptide substrates.

Exposure of an excess of the α-diazo substituted resin (10 mg) to 500 fmol of the methylated mixture I in 500 μL DMF overnight, resulted in the formation of a covalent phosphopeptide–resin bond (Scheme 2). The unbound peptides were washed away with DMF and methanol and the covalently bound phosphopeptides were subsequently liberated from the resin. Cleavage of the phosphorylated substrate from the resin with 90% TFA for 2 h provided the dimethylated angiotensin II tyrosine phosphate as the sole product (data not shown, m/z 1155). Liberation of the product with 10% NH_4OH for 30 min resulted in the recovery of the fully hydrolyzed angiotensin II tyrosine phosphate after evaporation of the solvent (Fig. 2). The liberated peptides collected in the eluant were identified by MALDI-tof (Fig. 2, m/z 1127 corresponds to angiotensin II tyrosine phosphate, m/z 1141 corresponds to the partial hydrolysis of the diester angiotensin II to the monomethyl ester).

In conclusion, we have illustrated a novel technique for the isolation of phosphorylated peptides from a peptide mixture. This chemical-based enrichment approach allows for the rapid isolation of phosphorylated substrates

Table 1. Sequence and mass of peptide mixture I

Peptide	Sequence	Average mass	Methylated
Ang II [tyrP]:	D-R-V-Y(**P**)-I-H-P-F-OH	1126	1154
Ang I:	D-R-V-Y-I-H-P-F-H-L-OH	1296	1324
FibB:	p-E-G-V-N-D-N-E-E-G-F-F-S-A-R-OH	1552	1608
Neur:	pyr-L-Y-E-N-L-P-R-R-P-Y-I-L-OH	1673	1701

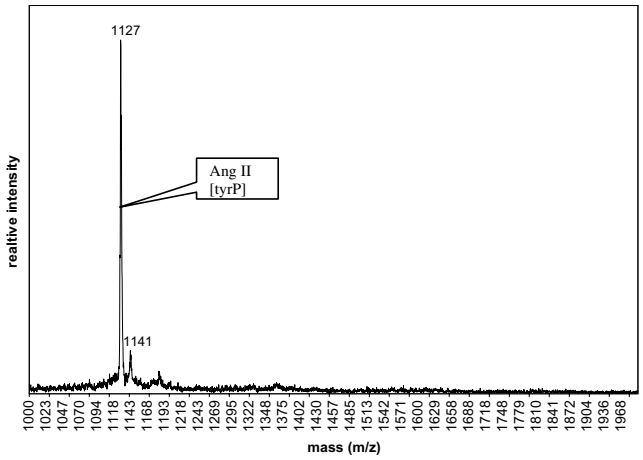


Figure 2. Solid phase enrichment of angiotensin II [tyr(OPO_3H_2)].

from crude peptide mixtures or protein extracts. Further studies of this methodology on a wide variety of phosphorylated protein digests of crude cell extracts and immunoprecipitates are currently under investigation in these laboratories and will be reported in due course.

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