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Protein Phosphorylation

Chemoenzymatic Enrichment of Phosphotyrosine-Containing Peptides**

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Protein phosphorylation on serine, threonine, and tyrosine residues is a dynamic post-translational modification that is involved in virtually all biological processes, including cell growth, proliferation, and differentiation. Many serious diseases such as cancer and diabetes are associated with dysregulation of this event, indicating its critical roles in multiple cellular-signaling pathways. Thus, improved methods with which to detect and analyze phosphorylation sites have always been sought to help us understand this important modification.[1]

Although there are a variety of methods available, mass spectrometry (MS) has recently become the primary choice for the study of protein phosphorylation owing to its high sensitivity and high throughput.[2] Proteins are digested to peptides, which are then analyzed with MS to identify phosphorylated residues. However, it remains technically challenging for such efforts because of intrinsic difficulties associated with protein-phosphorylation research.[3] For example, the stoichiometric ratio of phosphorylation is often low so that it requires enrichment of phosphorylated peptides from a large excess of unphosphorylated background peptides. The signal of phosphorylated peptides in MS is usually suppressed owing to the negative charge of the phosphate group. In addition, the assignment of phosphorylation sites can be ambiguous, which is caused by the poor quality of the fragmentation of phosphorylated peptides. As a result, it is highly desirable that new techniques can be developed to address these issues.

Because the phosphate group from phosphoserine (pSer) and phosphothreonine (pThr) can be cleaved through a β-elimination mechanism to produce an orthogonal double bond under basic conditions, which can further react with a thiol-containing compound, peptides bearing these two phosphorylated residues can be modified with a biotin[4] or other tags[5] for affinity enrichment. The removal of the phosphate group from peptides also improves their ionization and fragmentation in MS, thus enhancing the detection limit and simplifying de nova sequencing. Unfortunately, phosphotyrosine (pTyr) is stable under the same conditions, making this approach useless for the study of protein tyrosine phosphorylation. Therefore, current approaches to study tyrosine phosphorylation generally rely on immunoaffinity enrichment of pTyr-containing tryptic peptides with immobilized anti-pTyr antibodies, which are then sequenced directly by liquid chromatography (LC)-MS/MS to identify the sites of phosphorylation.[6] Even though these methods have been demonstrated with success, the low sensitivity of phosphorylated peptides in MS has not been solved, making it a limiting factor because protein tyrosine phosphorylation is a modification with a low abundancy that only counts for 0.05–0.5 % of total phosphorylation.[7]

Herein, we reported a novel chemoenzymatic method based on tyrosinase that could modify the pTyr residues with a biotin tag containing a cleavable linker, allowing their enrichment based on the biotin–avidin system and removing the signal-suppressing phosphate group simultaneously.

Tyrosinases are a family of enzymes that widely exist in bacteria, fungi, plants, and animals and that are involved in melanin synthesis.[8] These enzymes are copper-containing proteins that use molecular oxygen to catalyze the reaction of tyrosine to *ortho*-quinone through an *ortho*-dihydroxyl phenylalanine intermediate. *ortho*-Quinone is a highly active molecule that can easily react with other species, such as thiol-containing compounds.[9] Tyrosinase, however, can not oxidize the tyrosine residues whose phenolic group is masked by post-translational modification including phosphorylation and sulfation. Herein, we show that one could take advantage of this property of the enzyme to enrich pTyr-containing peptides from a large excess of unmodified peptides.

Scheme 1 shows the involvement of guanidination of the C-terminal lysine of tryptic peptides with methylisourea, followed by the acetylation of nonphosphorylated tyrosines with a mixture of *N*-acetylimidazole (NAI) and acetyl *N*-hydroxysuccinimide ester (Ac-NHS). Under these conditions, the C-terminal lysine would be transformed to homoarginine, which could significantly improve its sensitivity in MS detection. The N-terminal α-amine group would be acetylated as well. Next, the phosphate group on pTyr residues could be cleaved with alkaline phosphatase. Finally, in the presence of *N*-biotinyl-4-amino-2-methylbutan-2-yl 2-mercaptoethylcarbamate (BAC), which is a bifunctional molecule containing both a biotin and a thiol group joined by an acid-labile linker, the newly exposed phenolic group would be oxidized by tyrosinase to an *ortho*-quinone, which would be expected to undergo rapid and efficient Michael addition to form a thiol adduct. If successful, this method would result in only peptides containing pTyr residues coupling with the biotin tag, allowing them to be enriched by binding with avidin–agarose. The enriched peptides could then be released from the resin and treated with trifluoroacetic acid (TFA) to

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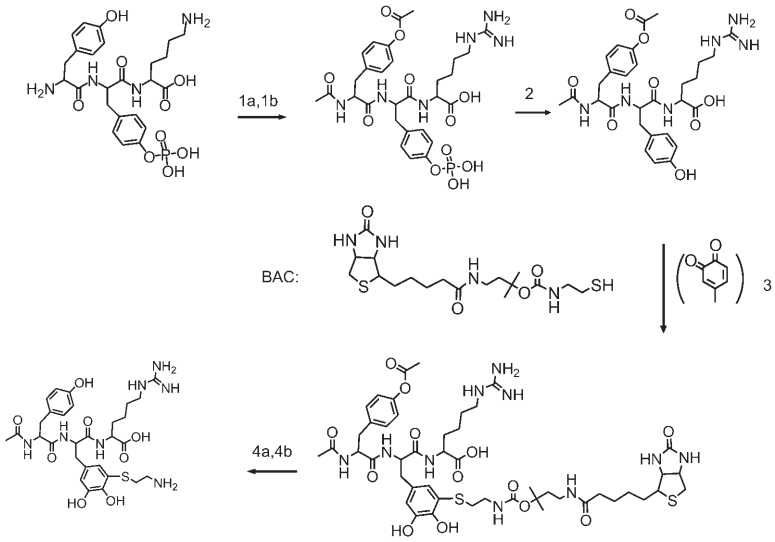

Scheme 1. Tyrosinase-assisted assay for pTyr-containing peptides. Conditions: 1a) methylisourea, 1b) *N*-acetylimidazole and acetyl *N*-hydroxysuccinimide ester; 2) alkaline phosphatase; 3) tyrosinase in the presence of BAC; 4a) removal of excess BAC reagent and avidin enrichment, 4b) TFA.

remove the bulky biotin tag, which would also remove acetyl groups on the nonphosphorylated tyrosine residues.

As the guanidination of the C-terminal lysine,[10] the acetylation of tyrosine[11] (see also the Supporting Information), and the acetylation of the N-terminal amine are all well-established chemical reactions that can go to completion rapidly and cleanly, the key step in this method is the oxidation of tyrosine to *ortho*-quinone by tyrosinase. It is essential to prove that tyrosinase can transform tyrosine residues embedded in any peptide sequence into a quinone adduct completely and specifically in the presence of thiol-containing compounds. This includes two requirements: 1) other naturally occurring amino acids, except cysteine, which is usually alkylated in proteomic assays, should not interfere with this enzymatic reaction; 2) different sequences of pTyr-containing peptides should only have a minimum effect on the activity of tyrosinase. We therefore did a series of experiments to test the properties of tyrosinase. First, we prepared a peptide (pep-1: H-FNMGpYKWDHSYR-OH, calculated *m*/*z* 1682.7) by standard solid-phase peptide synthesis, which contained all active functional groups existing in 20 naturally occurring amino acids except cysteine. We then incubated this peptide with tyrosinase and a large excess of cysteine. Under these conditions, pep-1 was fully converted into the peptide–cysteine adduct (pep-2: H-FNMGpYKWDHSUR-OH, U = quinone–thiol adduct, calculated *m*/*z* 1817.7) quickly, indicating that tyrosinase could completely oxidize tyrosine residues from peptide scaffolds without modifying other amino acids such as methionine, tryptophan, and pTyr. To examine if tyrosinase can oxidize various peptides with different sequences, we developed a high-throughput, fluorescence-quench-based activity assay for tyrosinase (see the Supporting Information). After measuring the activity of tyrosinase on several peptide substrates with diverse sequences, we concluded that tyrosinase could catalyze a broad spectrum of tyrosine-containing peptides, suggesting that this approach would potentially be able to label any pTyr-containing peptides from a proteolytic peptide mixture.

To demonstrate whether or not this approach could enrich pTyr-containing peptides from a large excess of nonphosphorylated peptide mixture, we decided to test the idea with a tryptic *E. coli* lysate spiked with a synthetic pTyr-containing peptide (pep-3: H-FENIVSGpYAHK-OH, calculated *m*/*z* 1529.6). As this *E. coli* lysate was pretreated with alkaline phosphatase prior to trypsin digestion, any peptides other than the anticipated synthetic peptide after the enrichment step would indicate nonspecific contamination. This was a complex biological system, yet it was also simple enough for us to evaluate and optimize our protocols. We then followed our procedure to treat this mixture and obtained a strong peak in the mass spectrum whose *m*/*z* ratio matched that of the modified peptide (Figure 1a–b; pep-4: Ac-FENIVSGUYAHX-OH, Ac = acetyl; U = quinone–thiol adduct; X = homoarginine; calculated *m*/*z* 1624.7), indicating efficient enrichment of pTyr-containing peptides and removal of the nonspecific

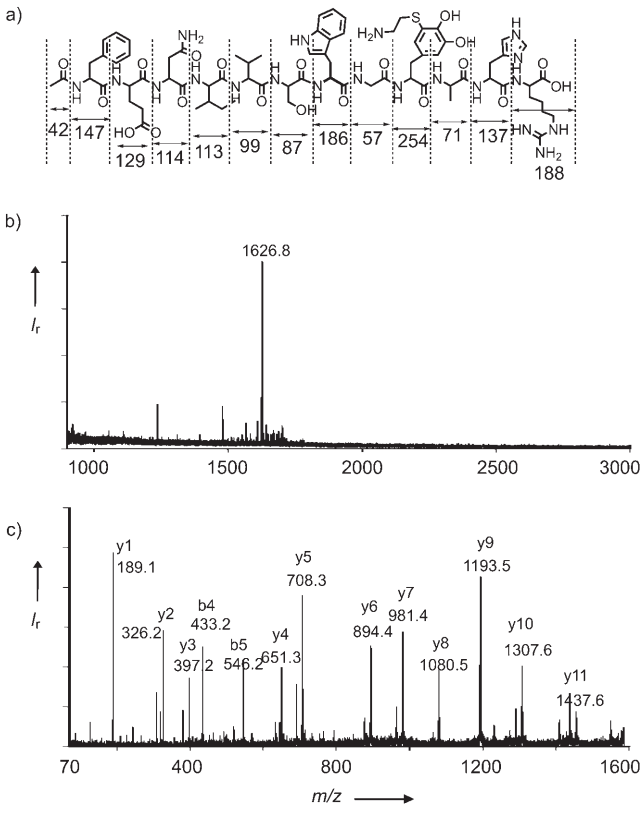

Figure 1. a) The structure of pep-4, which is the modified form of pep-3. The numbers indicate the *m*/*z* ratio for that particular segment. b) MS of pep-4 after our chemoenzymatic treatment. c) MS fragmentation of pep-4. All y series fragments and selected b series fragments are labeled. Note: b and y are two series of ions obtained after fragmentation of a peptide in MS/MS. I_r = % relative intensity.

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background. To compare the performance of our method with immunoaffinity purification, we followed a literature protocol to purify pep-3 with an anti-phosphotyrosine antibody[12] from the same sample and run a MS assay. Even though this antibody-enriched phosphorylated peptide (pep-3) also gave good signals, the signal-to-noise ratio of pep-3 was less than that of pep-4, implying that our method would potentially be more sensitive (see the Supporting Information). Finally, we were also able to cleanly assign the sequence of pep-4 after its fragmentation in MS/MS (Figure 1c).

This tyrosinase-assisted method for enrichment of pTyr-containing peptides from proteolytic peptide mixtures would represent a promising alternative to immunoaffinity purification by using anti-pTyr antibodies. Despite our approach involving multiple steps, the removal of negatively charged phosphate groups, in combination with the guanidination of the C-terminal lysine, could potentially enhance the sensitivity of detection and allow the unambiguous assignment of phosphorylation sites on peptides. In addition, this approach would enable us to incorporate various isotopic tags onto pTyr residues for the quantification of tyrosine phosphorylation from multiple samples.[13] We are currently optimizing our protocol to further improve the detection limit of our method.

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