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# An integrated procedure of selective injection, sample stacking and fractionation of phosphopeptides for MALDI MS analysis

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**Abstract**

Protein phosphorylation is one of the most important post-translational modifications (PTM), however, the detection of phosphorylation in proteins using mass spectrometry (MS) remains challenging. This is because many phosphorylated proteins are only present in low abundance, and the ionization of the phosphorylated components in MS is very inefficient compared to the non-phosphorylated counterparts. Recently, we have reported a selective injection technique that can separate phosphopeptides from non-phosphorylated peptides due to the differences in their isoelectric points (p*I*) [1]. Phosphorylated peptides from α-casein were clearly observed at low femtomole level using MALDI MS. In this work, further developments on selective injection of phosphopeptides are presented to enhance its capability in handling higher sample complexity. The approach is to integrate selective injection with a sample stacking technique used in capillary electrophoresis to enrich the sample concentration, followed by electrophoresis to fractionate the components in preparation for MALDI MS analysis. The effectiveness of the selective injection and stacking was evaluated quantitatively using a synthetic phosphopeptide as sample, with an enrichment factor of up to 600 being recorded. Next, a tryptic digest of α-casein was used to evaluate the separation and fractionation of peptides for MALDI MS analysis. The elution order of phosphopeptides essentially followed the order of decreasing number of phosphates on the peptides. Finally, to illustrate the applicability, the integrated procedure was applied to evaluate the phosphorylation of a highly phosphorylated protein, osteopontin. Up to 41 phosphopeptides were observed, which allowed us to examine the phosphorylation of all 29 possible sites previously reported [2]. A high level of heterogeneity in the phosphorylation of OPN was evident by the multiple-forms of variable phosphorylation detected for a large number of peptides.


*Keywords:* Protein phosphorylation; Phosphopeptide purification; Peptide enrichment; Sample stacking; Capillary electrophoresis; MALDI MS

## 1. Introduction

The reversible phosphorylation of proteins regulated by kinases and phosphatases plays an important role in many cellular events including metabolism, transcription, translation and apoptosis [3,4]. Over 30% of the total human proteins, including many kinases, are reported to be phosphorylated. Since phosphorylation occurs at different levels and multiple sites, each event of phosphorylation may greatly affect a protein's activities and interactions with other molecules [5,6]. Abnormal regulation of phosphorylation has been linked to many severe diseases such as cancer, diabetes and rheumatoid arthritis [7]. Many new therapeutic developments are now targeting at the control of phosphorylation, and hence, sensitive techniques that can effectively identify the phosphorylation status on minute quantities of sample are becoming very important.

Mass spectrometry (MS) is one of the most convenient techniques commonly used for identifying phosphorylation in proteins [8,9]. Nevertheless, the sensitivity of phosphopeptide detection by MS is considered low due to the poor ionization efficiency caused by the extremely acidic phosphates [10]. One way to overcome this problem is to perform chemical derivatization such as β-elimination to remove the phosphate and form dehydroalanine, or Michael addition to form a lysine analogue [11,12]. While effective in signal enhancement, these methods suffer from side reactions and sample loss,

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and thus many low-abundance phosphopeptides remain undetectable. The more common solution is to isolate and enrich phosphopeptides, mainly by preferential sorption. Examples include immobilized metal affinity chromatography [13–15], bio-affinity based anchors [16], phospho-amino acid-specific antibodies [17], reverse phase liquid chromatography [18,19], ion exchange chromatography [20,21] and the newly developed titanium oxide [22,23], zirconium dioxide [24] and graphite powder columns [25]. Many of these sorption-based methods have been proven to greatly improve the MS detection of phosphopeptides. Nevertheless, there exists the risk of sample loss due to irreversible sorption, which is particularly problematic for components at ultra-low concentration and/or those with multiple phosphorylated domains that are most strongly retained [19]. For this reason, non-sorptive approaches of phosphopeptide isolation, such as capillary electrophoresis (CE), represent a logical alternative.

CE separates charged molecules based on their electrophoretic mobilities, which are determined mainly by their charge-to-size ratios. Since phosphorylation can significantly alter the charge and thus the mobility of a peptide [26], separation of the phosphorylated form from the non-phosphorylated counterpart can be performed by CE [27–29]. Building on this idea, we have previously reported the "selective injection" of phosphopeptides [1,30]. This technique is based on the fact that many phosphorylated peptides have isoelectric points (p*I*) below 4, while on average the majority of the non-phosphorylated peptides have p*I* above 5. When the sample pH is adjusted to acidic conditions (below pH 5), phosphorylated peptides would have a much higher likelihood of possessing a negative net charge, and thus can be selectively introduced into a capillary by a positive potential difference. However, to achieve this separation, the electroosmotic flow (EOF) would have to be greatly suppressed, as it could otherwise override the electrophoretic migrations of peptides and prevent their selective injection. Low nanomolar phosphopeptides had been successfully isolated by this method from the non-phosphorylated counterpart even when the concentration of the latter is at 1 million times higher [1]. Together with nanolitre–volume sample spotting, attomole-level detection limit was reported using MALDI MS. Nevertheless, this approach has two main drawbacks. Firstly, the selectively injected components remain similar in concentration as in the original mixture; i.e., no enrichment was achieved. This is inferior to the chromatographic-based isolation where sample concentration is achieved by using an elution volume smaller than the loading volume. Secondly, when a large number of phosphopeptides are present in the sample, all of them will be simultaneously injected, yet the subsequent MS analysis may not be capable of adequately detecting all components.

To address the first drawback, sample preconcentration by CE is proposed. Various CE-based techniques have been reported to concentrate analytes ranging from small organic molecules to peptides and even whole proteins with micro- or submicroliter sample volumes. Among these, sample stacking [31] is one of the most compatible methods with MS, as it does not involve the use of incompatible reagents; e.g., the surfactants in micellar electrokinetic chromatography [32] or the ampholytes in capillary isoelectric focusing [33–35], which typically require pre-MS cleanup [36–38]. Generally, sample enrichment by stacking results from a sudden reduction of the analyte mobility within the capillary. The mobility reduction can be induced by a pH change in a discontinuous buffer system [39–41], or more commonly by a change (increase) in conductivity which is referred to as field-amplified sample stacking [31,42,43]. This change in conductivity can simply be achieved by introducing a low conductivity zone, often a plug of water, at the injection end of the capillary. Ions will migrate faster within this zone due to the higher field, and they will slow down (or stack) as they cross the boundary to the buffer in the capillary. Applications of such sample stacking have been reported for peptides [43] and phosphopeptides [44]. Additionally, ions will continue to migrate after crossing the water-buffer boundary and develop a separation based on their differential mobilities. Such CE separation can be used to address the aforementioned second drawback in resolving a mixture of selectively injected phosphopeptides prior to MS.

The focus of this study is on the integration of the previously developed selective injection of phosphopeptides with sample stacking and separation, followed by fractionation via spotting for MALDI MS analyses. A number of experimental conditions, such as buffer concentration and length of the water plug, are carefully examined, as they are found to play very important roles in the effectiveness of stacking. The benefit of performing an integrated procedure of isolation, enrichment and fractionation is illustrated by the analyses of two highly phosphorylated proteins, α-casein and osteopontin. These two proteins, respectively, have up to 22 and 29 reported phosphorylation sites [2,22,45]. Their digests contain numerous peptides with multiple phosphorylated domains, for which the non-sorptive approaches of phosphopeptide isolation by CE is most advantageous.

## 2. Experimental section

### 2.1. Chemicals and reagents

Zwitterionic surfactant, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, $C_{14}$), was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Reagent grade phosphoric acid, ammonium hydroxide, sodium hydroxide, acetic acid and trifluoroacetic acid were purchased from EM Science (Gibbstown, NJ, USA). Mesityl oxide, citric acid, ammonium bicarbonate, calcium chloride, 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid were obtained from Sigma–Aldrich (Markham, ON, Canada). Buffers were prepared by adjusting the pH of acetic acid (20–100 mM) with ammonium hydroxide. HPLC grade acetone and ethanol (100%) were from Fisher Scientific Ltd. (Nepean, ON, Canada). Deionized water (Millipore, MA, USA) was used to prepare all solutions.

### 2.2. Preparation of phosphopeptides

Endoproteinase Asp-N and α-casein were purchased from Sigma–Aldrich. Modified sequence grade trypsin was from