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Rapid separation of phosphopeptides by microchip electrophoresis–electrospray ionization mass spectrometry

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ABSTRACT

Protein phosphorylation is a significant biological process, but separation of phosphorylated peptide isomers is often challenging for many analytical techniques. We developed a microchip electrophoresis (MCE) method for rapid separation of phosphopeptides with on-chip electrospray ionization (ESI) facilitating online sample introduction to the mass spectrometer (MS). With the method, two monophosphorylated positional isomers of insulin receptor peptide (IR1A and IR1B) and a triply phosphorylated insulin receptor peptide (IR3), all with the same amino acid sequence, were separated from the nonphosphorylated peptide (IR0) in less than one minute. For efficient separation of the positional peptide isomers from each other derivatization with 9-fluorenylmethyl reagents (either chloroformate, Fmoc-Cl, or *N*-succinimidyl carbonate, Fmoc-OSu) was required before the analysis. The derivatization improved not only the separation of the monophosphorylated positional peptide isomers in MCE, but also identification of the phosphorylation site based on MS/MS.

1. Introduction

Protein phosphorylation plays an important role in diverse biological processes, including cell proliferation, differentiation, and apoptosis. In addition, abnormal phosphorylation has been associated with many diseases (e.g., cancer, diabetes) [1]. Thus, identification of a protein's phosphorylation site (phosphorylated amino acid residue) and state (number of phosphorylated residues) is highly important for our understanding of disease progression.

Phosphopeptides are usually characterized by mass spectrometry (MS) after proteolytic digestion of the protein(s). Phosphorylated peptides are present in only low amounts, however, and their ionization efficiency in ESI is poor compared with that of nonphosphorylated peptides [2,3]. This makes the analysis challenging, and often a phosphopeptide enrichment step is needed before MS analysis. Common enrichment techniques are immobilized metal affinity chromatography (IMAC) [4] and titanium dioxide (TiO_2) chromatography [5,6]. In addition to detection sensitivity, separation of positional phosphopeptide isomers (same amino acid sequence, same number of phosphorylated residues but, at different sites) continues to be challenging for liquid-phase separations. These isomers form when the peptide has multiple phosphorylation sites but the phosphorylation is incomplete. Identification of the isomers by MS alone is not possible because the m/z values are identical. Even identifications based on MS/MS fragmentation may be problematic if the phosphorylation sites are at successive amino acids and isomers have different abundances. Capillary electrophoresis (CE) techniques, including microchip-based CE separations, have high application potential in analysis of phosphopeptides (and peptides in general) [7–13]. Separation of positional phosphopeptide isomers has been achieved by capillary zone electrophoresis [10,12] and micellar electrokinetic chromatography [13], but the separation times required are relatively long (several tens of minutes) and the separation conditions are often not compatible with MS detection. In addition to electrophoretic techniques, hydrophilic interaction liquid chromatography (HILIC) [14] and (ultra high performance) reversed phase liquid chromatography have also been used for effective separation of peptide isomers (also those bearing post-translational modifications other than phosphorylation) [15], but also in this case, the separation times are relatively long. Moreover, for unambiguous identification of positional phosphopeptide

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isomers by liquid chromatography, coupling to an orthogonal separation based on ion mobility spectrometry (IMS) is often required before MS detection [16]. In some cases, IMS separation in the gas phase alone has enabled identification of localization variants of modified peptides [17–22]. However, there is a clear need for analytical techniques that enable rapid liquid-phase separation of (positional) phosphopeptide isomers before MS detection to allow determination of a protein's phosphorylation profile. During the past decade, the microchip technologies have emerged as a powerful combination for bioanalysis thanks to the high speed of microchip electrophoresis (MCE, separation times typically below one minute) combined with the good selectivity of the MS detection [23].

In this study, we demonstrate rapid (<1 min) liquid-phase separation of phosphopeptide isomers by microchip electrophoresis–electrospray ionization mass spectrometry (MCE–ESI/MS). The microfabricated ESI emitter is monolithically integrated with the upstream separation channel, enabling straightforward, nanospray-like sample ionization and introduction into the MS. The separation channel allows rapid separation of phosphorylated peptides with different phosphorylation states. In addition, to facilitate the separation of positional phosphopeptide isomers, we developed a derivatization procedure involving rapid reaction of the free amino residues of the peptides with 9-fluorenylmethyl reagents (either chloroformate, Fmoc-Cl, or *N*-succinimidyl carbonate, Fmoc-OSu). The Fmoc derivatization not only improved the resolution of positional isomers in MCE, but also facilitated identification of the phosphorylation site on the MS.

2. Experimental

2.1. Materials and reagents

Methanol, acetonitrile, ammonium acetate, acetic acid, formic acid, 9-fluorenylmethyl chloroformate (Fmoc-Cl), and 9-fluorenylmethyl *N*-succinimidyl carbonate (Fmoc-OSu) were from Sigma–Aldrich (Steinheim, Germany). Sodium hydroxide and boric acid were purchased from Riedel-de Haën (Seelze, Germany). Nonphosphorylated insulin receptor peptide (IR0; TRDIYETDYYRK, amino acids 1142–1153 of insulin receptor, M_r = 1621.77), monophosphorylated insulin receptor peptides (IR1A and IR1B; TRDIpYETDYYRK and TRDIYETDpYYRK, tyrosine 1146 and 1150 phosphorylated, respectively, M = 1701.74), and triply phosphorylated insulin receptor peptide (IR3; TRDIpYETDpYpYRK, tyrosines 1146, 1150, and 1151 phosphorylated, M = 1861.67) were from AnaSpec (Fremont, CA, USA). All reagents and solvents were of analytical or HPLC grade. Water was purified with a Milli-Q water purification system (Millipore, Molsheim, France).

A 100 mM stock solution of ammonium acetate was prepared in Milli-Q water and diluted with Milli-Q water and methanol so that the final ion concentration of the background electrolyte (BGE) varied between 20 and 40 mM and the final organic solvent composition between 40 and 60% (v/v). No further pH adjustment of the BGE was made. Borate buffer (18 mM) was prepared in Milli-Q water and adjusted to pH 10 with concentrated sodium hydroxide. Peptide stock solutions (1 mM) were prepared in Milli-Q water. Further dilutions were made with water–methanol 50:50 (v/v) solution containing 20 mM ammonium acetate (pH 7.3).

2.2. Derivatization of monophosphopeptides with 9-fluorenylmethyl reagents

Two different procedures were studied for derivatization of the monophosphorylated peptide isomers (IR1A and IR1B). In the first procedure, Fmoc-Cl was used as the derivatization reagent in stoichiometric ratio or in 5-fold or 10-fold molar excess relative to the free amino residues of the peptide. Reactions were performed in triplicate and initiated by mixing 20 μL of Fmoc-Cl (in ACN) with 10 μL of 18 mM sodium borate (pH 10.0) and 10 μL of peptide stock solution (1 mM in Milli-Q water). The mixtures were vortexed for one minute and the reactions were left to proceed at room temperature for 2, 5, or 10 min. The second procedure used 10-fold molar excess of Fmoc-OSu (instead of Fmoc-Cl) as the derivatization reagent. The reactions were initiated by mixing 10 μL of Fmoc-OSu (in ACN) with 10 μL of 18 mM sodium borate (pH 10.0) and 20 μL of peptide stock solution (1 mM in Milli-Q water) and, left to proceed at room temperature overnight. After the specified time, the reaction mixtures were diluted 1:1 (v/v) with the BGE (water/methanol 60:40, v/v, containing 30 mM ammonium acetate, pH 7.2) for analysis by microchip capillary electrophoresis or 1:100 with water/methanol (50:50, v/v) containing 0.1% (v/v) formic acid for analysis by direct infusion ESI.

2.3. Microchip capillary electrophoresis

The microchips were fabricated of SU-8 epoxypolymer by photolithography and adhesive bonding techniques as previously described [24,25]. The microchips comprised a 25 mm-long separation channel (50 μm × 50 μm, $w \times h$) intersected by a 10 mm-long injection channel (simple cross) and a 12 mm-long auxiliary channel (100 μm × 50 μm, $w \times h$) providing sheath liquid to the integrated ESI emitter tip (Fig. 1). The effective separation length between the injection cross and the sheath liquid interface was 20 mm. Before use, polydimethylsiloxane (PDMS) sheets with 2 mm inlet holes were placed on top of the SU-8 chip to avoid sample spreading on the relatively hydrophilic SU-8, and the chip was briefly rinsed with the BGE.

Samples were injected electrokinetically in pinched injection mode (20 s for native and 60 s for derivatized peptides). An electric field of 800–1000 V/cm was applied between sample inlet and sample waste. Also, a small focusing potential was applied to the buffer inlet to prevent sample leakage into the separation channel. During injection, the sheath liquid inlet was left floating and thus no spray was produced.

Electrophoretic separation was performed under electroosmotic flow in cathodic direction at electric field strengths of 150–750 V/cm. Water/methanol solutions containing 40–60% (v/v) methanol and 20–40 mM ammonium acetate were used as the BGE. In all cases, small push-back voltages were applied to the sample inlet and sample waste during separation. The ESI voltage applied to the sheath liquid inlet also served as the counter voltage for the MCE separation. An external power supply (Micralyne, Edmonton, Canada) was used to apply the voltages through platinum wires that were placed on the microchip inlets.

2.4. Mass spectrometry

The SU-8 microchips were coupled to an Agilent 6330 ion trap mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an xyz-alignment stage (to replace the standard ion source) and CCD cameras. The mass spectrometer was operated in positive ion mode with a capillary voltage of −1.6 kV, end-plate offset of −500 V, and a trap drive value of 71.8. Nitrogen (AGA Ltd., Espoo, Finland) was used as the drying gas (4.0 L/min, +70 °C) and data was acquired over a mass range of m/z 100–2000 with a maximum accumulation time of 100 ms. DataAnalysis software was used for acquisition and data processing.

The microchip was placed on the xyz-alignment stage in front of the MS inlet and an ESI voltage of 2.0–3.2 kV was applied to the sheath liquid inlet. This voltage also served as the counter