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High-performance thin-layer chromatography as a fast screening tool for phosphorylated peptides

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ABSTRACT

This study aimed at developing a rapid chromatographic assay to monitor phosphorylation sites in peptides. For the analysis of nociceptive signal transduction pathways, the detection of phosphorylated proteins/peptides plays a fundamental role. To get further insights in the phosphorylation mechanism of protein kinase C-ε (PKC-ε) and protein kinase A (PKA), potential targets were divided into subsections resulting in peptides that contain only one possible phospho-binding site. The use of high-performance thin-layer chromatography (HPTLC) offers the possibility of a high throughput of samples and the advantage of a quick sample clean-up. A combined strategy of an effect-directed overlay procedure on the TLC plate using specific antibodies (immunostaining, HPTLC-IS) as well as a parallel, direct mass spectrometric methodology by HPTLC-MALDI-TOF-MS was developed. With regard to HPTLC-IS, validation of the data exhibited a lower limit of detection than the traditionally used protein derivatization reagent fluorescamine. Besides the identification of the phosphorylated peptides, a semi-quantitative estimation can be performed with HPTLC-IS.

1. Introduction

The phosphorylation of proteins is one of the most important post translational modifications (PTM) as it is a key regulator of many signaling pathways. For example, the development of pain and its appearance rests upon integration of various signals in nociceptive neurons, but is still insufficiently elucidated on molecular level. Certain pathways have been already enlightened, but the mode of action still remains unclear. In this context, PTMs of proteins and peptides play a key role in the pathway dynamics and their corresponding crosstalks. Phosphorylations are of dominant importance because a change of substrate specificity, besides enzymatic activity can influence the signal transduction cascade [1–8].

Nowadays, PTM analysis is primarily performed using liquid chromatography–mass spectrometry (LC–MS). However, samples have to be purified and concentrated prior to analysis [9,10]. Many isolation strategies for identifying PTM are already established, but lead to different yields of phosphopeptides [11]. Additionally, the subsequent analysis and the further sequencing of phosphopeptides using MS^n experiments is not a trivial task, as often a low ionization efficiency and labile phosphate residues remain challenging [12].

To simply monitor the phosphorylation status of known peptides, a separation using high-performance thin-layer chromatography (HPTLC) might be a fast and cost-efficient alternative. With regard to the analysis of phosphopeptides, HPTLC provides several advantages: (I) up to 40 samples can be analyzed simultaneously. On that score, the application of HPTLC would also allow monitoring the progress of the reaction (*e.g.*, determination of the activity of kinases) [13]. (II) Moreover, an effect-directed analysis performed on the HPTLC plate (*e.g.*, by immunostaining) can complement the investigation. (III) Analysis can be accomplished without any laborious clean-up or concentrating steps. (IV) Additionally, HPTLC offers a significant number of hyphenation possibilities with various mass spectrometric techniques such as a direct coupling of HPTLC to MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight), HPTLC-ESI-MS also in combination with a previous desorption (DESI-MS) [14–17]. In this way, phosphorylated peptides can be identified easily and modifications

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Table 1
Sequences and selected properties of the peptides investigated.

Origin	Synonym	Sequence	Mass (Da)	Hydro-phobicity	IEP	R_f value
CREB-S133-p	C1	HQKRREILpSRRPSYRKILNDLGKPIPNPLLGLDST	4160.3			0.22
CREB-S133	C2	HQKRREILSRRPSYRKILNDLGKPIPNPLLGLDST	4080.3	−0.963	10.95	0.22
PKA pseudosubstrate	C3	HQRREILLRRPAYRKILNDLGKPIPNPLLGLST	3847.2	−0.579	11.45	0.22
PKC-ε substrate-p	E1	HERMRPRKRQGpSVRRRV	2283.2			0.12
PKC-ε substrate	E2	HERMRPRKRQGSVRRRV	2203.3	−2.241	12.40	0.12
PKC-ε pseudosubstrate	E3	HERMRPRKRQGAVRRRV	2187.3	−2.088	12.40	0.12
PKC-ε peptide-p; centric	K1	HERMRPRKRQGpSVRRRVGKPIPNPLLGLDST	3686.0			0.29
PKC-ε peptide	K2	HERMRPRKRQGSVRRRVGKPIPNPLLGLDST	3606.0	−1.297	12.13	0.29

of peptides can be investigated in detail, as well. Some publications report the use of a combination of planar electrophoretic separation with traditional thin-layer chromatography resulting in a two dimensional partition of (phospho) peptides [18–20]. In these cases, the detection was realized using ^{32}P radioactive labeling or a phospho-specific dye, Pro-Q Diamond. The subsequent analysis of the peptides was either done by extracting the peptides of interest, prior to MS analysis or further investigations, as Edman degradation for example, or *via* directly coupled TLC-MALDI-TOF-MS analyzes.

The background of the present study was the need to identify the occurrence of phosphorylations when using protein kinase C-ε (PKC-ε) and protein kinase A (PKA), as these play a key role for evaluating the nociceptive transduction cascades. It is important to know to which extent the corresponding peptides are phosphorylated. For this purpose, selected, commercially available phosphorylated peptides were analyzed.

The aim of this study was to develop a HPTLC methodology as separation and clean-up technology for analyzing phosphorylated peptides being involved in nociceptive transduction cascades. For gaining a highly specific detection of the phosphorylation, a TLC overlay technique with antibodies was established. In parallel, the direct mass spectrometric detection from the TLC should enable the structural identification of the peptides.

2. Material and methods

2.1. Peptides and kinases

The designation of the protein and the peptide sequences are shown in Table 1. Peptides analyzed were acquired in phosphorylated ('p-') and non-phosphorylated form. All peptides were purchased from Peps 4LS GmbH, Heidelberg, Germany. The investigated kinases PKA and PKC-ε were either expressed and purified in *Escherichia coli* with a pET15b-mCα vector or purchased from Biaffin GmbH & Co., KG, Kassel, Germany.

Initially, the peptides were dissolved in PBS buffer containing 137 mM sodium chloride, 1.47 M potassium dihydrogen orthophosphate, 2.7 mM potassium chloride and 7.8 mM sodium hydrogen phosphate dihydrate with a pH value of 7.2 (according to [21] and modified slightly). Moreover, mixtures containing the several peptides were prepared: sequentially related peptides with and without phosphorylation were applied (*e.g.*, peptides E1 and E2). The chromatographic system was primarily established and evaluated using the single peptides (see Table 1). Further, peptides of different size/origin were mixed for an improvement of the chromatographic separation. Single (model) peptides as well as peptide mixtures were also used for the mass spectrometric characterization as well as for developing the HPTLC detection with the antibodies.

2.2. Chemicals

1-Butanol, ammonium dihydrogen phosphate, potassium dihydrogen orthophosphate, sodium hydrogen phosphate dihydrate, potassium chloride, magnesium chloride, bovine serum albumin and aluminum-backed as well as glass-backed cellulose HPTLC plates were obtained from Merck KGaA, Darmstadt, Germany. Acetic acid, sodium chloride, fluorescamine, acetone and acetonitrile were purchased from VWR International GmbH, Darmstadt, Germany. Trifluoroacetic acid (TFA) was purchased from AppliChem GmbH, Darmstadt, Germany. Tween 20® and Tris–HCl were obtained from Carl Roth GmbH & Co., KG , Karlsruhe, Germany and 2,5-dihydroxybenzoic acid (DHB) and β-lactoglobulin (βLG) were purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. Adenosine triphosphate (ATP) was obtained from Roche Diagnostics Corp., Indiana, USA. All solvents were of HPLC grade, otherwise, ACS grade was used. Water was double distilled (ddH_2O).

For the detection of the different peptides, anti-V5-tag antibodies as well as anti-p-PKA and p-PKC-ε antibodies have been used as primary antibodies. The rabbit monoclonal p-PKA substrate antibody (100G7E) and rabbit monoclonal p-PKC-ε antibody were purchased from Cell Signaling Technology Inc., Cambridge, UK. The rabbit monoclonal anti-V5-tag antibody was purchased from Bethyl Laboratories Inc., Montgomery, USA. Visualization was carried out by a polyclonal, Alexa Flour® 555 conjugated secondary antibody (goat anti-rabbit IgG) obtained from Life Technologies Corp., Carlsbad, USA.

2.3. High-performance thin-layer chromatography (HPTLC)

A defined sample volume was applied as 6 mm bands onto the HPTLC plates using an HPTLC autosampler (ATS4, CAMAG AG, Muttenz, Switzerland) or a micropipette, alternatively. Application by micropipette yielded spots with around 5 mm per diameter. Separation was carried out in a twin-through chamber with a mobile phase containing 1-butanol/acetic acid/ddH_2O (2.75/1/2; v/v/v) optimized after van der Geer et al. [20]. Chromatography was performed on 10 × 10 cm cellulose HPTLC aluminum foils or glass plates (Merck KGaA, Darmstadt, Germany) up to a migration distance of 70 mm. Afterwards the remaining solvent(s) were evaporated from the plate overnight (12 h) at room temperature.

Post-chromatographic derivatization was performed by dipping the plate in a protein-specific staining solution (fluorescamine, 0.05% in acetone) for 2 s. Subsequently, evaporation was carried out for 10 min at room temperature. Detection was performed with a photodocumentation system (TLC visualizer, CAMAG AG, Muttenz, Switzerland) at ultraviolet light (UV 366 nm).

2.4. High-performance thin-layer chromatography matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (HPTLC-MALDI-TOF-MS)

For HPTLC-MALDI-TOF-MS detection, analytes were separated on 7.5 × 5.0 cm cellulose HPTLC aluminum foils and developed as described in Section 2.3. Chromatography was done in duplicate to obtain two identical plates (so called 'twin-plates'). One plate was dipped into fluorescamine staining solution; the other