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Analysis of the phosphoamino acid content of phosphoproteins[1]

P.A. de Witte[a,*], J.F. Cuveele[a], W.J. Merlevede[b], P.M. Agostinis[b]

[a]*Laboratorium voor Farmaceutische Biologie en Fytofarmacologie, Faculteit Farmaceutische Wetenschappen, Van Evenstraat 4, B-3000 Leuven, Belgium*

[b]*Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit te Leuven, Herestraat 49, B-3000 Leuven, Belgium*

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Abstract

A method has been developed for the analysis of phosphoserine, phosphothreonine and phosphotyrosine in ^{32}P-phosphoprotein hydrolysates. The hydrolysates are treated with dabsyl reagent (28.8 mM) for 10 min at 70°C. After a clean-up using a disposable C18 column, the covalently modified phosphoamino acids are separated on silica TLC aluminum sheets using a one-dimensional solvent system. The method is straightforward and permits the simultaneous analysis of numerous samples. Very clean chromatograms are obtained enabling the unambiguous identification of the well separated dabsylated phosphoamino acids with autoradiography. The phosphoamino acids can be quantified by simply cutting out the relevant spots from the aluminum sheets followed by ^{32}P-quantification using liquid scintillation spectrometry.

Keywords: Dabsyl; Derivatization; Kinase; Phosphoamino acid; Phosphorylation; Protein

1. Introduction

Reversible protein phosphorylation is a key mechanism in the regulation of critical cellular functions such as cell proliferation, differentiation and transformation [1]. A multitude of protein kinases participate in the control of the phosphorylation level of cellular proteins. The phosphorylation of cellular proteins involves seryl to an overwhelming extent (90%) and threonyl residues to a lesser extent (10%) and is brought about by protein Ser/Thr kinases [2,3]. Another family of enzymes, the protein tyrosine kinases (PTK), phosphorylate specifically tyrosyl residues in proteins [4]. Although the phosphotyrosine level in the cell is nearly undetectable ($< 0.05\%$), protein tyrosyl phosphorylation has been clearly implicated in cell transformation and an increased amount of P-Tyr in proteins is a marker for human malignant cells. Indeed, most oncoproteins so far discovered exhibit tyrosine kinase activity [5].

Because the phosphoester bonds of hydroxyamino acids are partially stable to limited acid hydrolysis, hydrolytic release of *O*-phosphoserine, *O*-phosphothreonine and *O*-phosphotyrosine from phosphoproteins in the presence of a strong

* Corresponding author. Tel.: (+32) 16-32-34-33; fax: (+32) 16-32-34-60.

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acid (6 N HCl) has been used for their analysis. In order to correctly quantify all phosphoamino acids, a highly sensitive and selective method is required. Basically, phosphoamino acid analysis can be performed by 1D and 2D cellulose electrophoresis [6–8], HPLC analysis [9–11] with post-column [12–14] or pre-column derivatization [15–17] and capillary electrophoresis [18] of chemically stable derivatized phosphoamino acids [18]. Unfortunately, all these procedures are lengthy or do not permit the analysis of numerous samples.

Recently, a new method was described for the quantification of phosphoserine, phosphothreonine and phosphotyrosine as dabsyl derivatives in acid-hydrolyzed extracts of ^{32}P-labeled cells [19]. The complete separation of dabsylated phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) without interference of contaminants originating from hydrolyzed cell extracts, makes the procedure very convenient for routine analysis of the in-vivo phosphorylation state of cultured cells. Here, it is shown that a simplified method based on the same principle is also well suited for the quantification of the three phosphorylated hydroxyamino acids derived from enzymatic in-vitro phosphorylation of proteins.

2. Experimental

2.1. Materials and methods

Dabs-Cl (4-*N*,*N*-dimethylaminoazobenzene-4′-sulfonyl chloride) was purchased from Pierce (Rockford, IL) and used after recrystallization according to Ref. [20]. Phosphoamino acids (*O*-phospho-L-threonine, *O*-phospho-L-tyrosine, *O*-phospho-L-serine), polylysine (average molecular mass 46kDa) and ATP (diNa) were from Sigma (St. Louis, MO). [γ-^{32}P]ATP (10 mCi ml^{-1}) was supplied by Amersham (Amersham, UK). c-Fgr [21] (sp. act. 0.1 U mg^{-1}) was a generous gift from Dr. Donella-Deana (University of Padova). Casein kinases CK-2 (sp. act. 1 U mg^{-1} using casein as the substrate) and CK-1 (sp. act. 0.4 U mg^{-1}) were prepared from porcine spleen as described in Ref. [22]. Whole casein was prepared according to Ref. [23]. The peptides were synthesized with a peptide synthesizer (Milligen 9050, Millipore) using the 9-fluorenylmethoxycarbonyl (Fmoc) mode and purified using reverse-phase HPLC on a Delta-Pack C18 column from Millipore (Marlborough, MA). C18 disposable columns (100 mg sorbent, size 1 ml) were purchased from Varian (Harbor City, CA). Elution of columns was performed at 1–2 ml min^{-1}. TLC was performed on precoated silica gel 60 F_{254}/aluminum sheets (0.2 mm) (Merck, Darmstadt, Germany) under room saturation conditions at room temperature. All solvents and other chemicals used were supplied by Merck or Janssen Chimica (Beerse, Belgium) and were of analytical grade. Water was bidistilled before use.

2.2. Preparation of specifically ^{32}P-labeled substrates

The ^{32}P-Ser-containing substrate was obtained by phosphorylating whole casein with porcine spleen CK-1, as described previously [23]. Briefly, casein (100 μg) was incubated with CK-1 (100 ng) in the presence of Tris–HCl (50 mM, pH 7.5)–$MgCl_2$ (12 mM)–NaCl (100 mM)–[γ-^{32}P]ATP (250 μM, specific activity 2000 cpm pmol^{-1}) (100 μl) at 30°C. After incubation (2 h) the reaction was stopped by adding cold trichloracetic acid (TCA) (10%, w/v, final concentration). Subsequently, ^{32}P-labeled casein was pelleted and the free [γ-^{32}P]ATP in the supernatant completely removed by successive washings with TCA (10%, w/v). Finally, residual TCA was removed by two washes with ethanol.

The ^{32}P-Thr-containing substrate was obtained by phosphorylating the synthetic peptide RRREEETEEE (100 μg) with porcine spleen CK-2 (200 ng) under the same conditions described for CK-1. The phosphorylation reaction was stopped by adding acetic acid (30%, w/v, final concentration), and the ^{32}P-labeled peptide was separated from the [γ-^{32}P]ATP with Dowex 1-X8 anion exchange resin (1 ml) (Biorad, Richmond, CA) equilibrated in acetic acid (30%, w/v) according to Ref. [24].

The ^{32}P-Thr-containing substrate was prepared by phosphorylating the synthetic peptide EKIGEGTYGVVFK by rat spleen c-Fgr tyrosine

kinase as described in Ref. [25]. Briefly, the peptide (50 μg) was phosphorylated by c-Fgr (50 ng) in a medium containing Tris–HCl (50 nM, pH 7.5)–$MgCl_2$ (10 mM)–polylysine (30 μg ml^{-1})–[γ-^{32}P]ATP (250 μM, specific activity 2000 cpm $pmol^{-1}$) (100 μl) at 30°C. After incubation (3 h) the reaction was stopped by adding acetic acid (30%, w/v, final concentration) and the free [γ-^{32}P]ATP separated as described for the CK-2 substrate phosphorylation.

2.3. Dabsyl-phosphoamino acid analysis

The ^{32}P-phosphorylated protein and peptides were vacuum dried, dissolved in HCl (2 ml, 6 N) and hydrolyzed in ampoules (2 ml) for 1.5 h at 110°C according to Ref. [8]. After evaporation of the solvent, the hydrolysate residue was spiked with cold phosphoamino acids (1 μg each), dissolved in borax derivatization buffer (100 μl, pH 9.2) (boric acid (0.2 M)–sodium carbonate (0.2 M) (63:37, v/v)) and quantitatively transferred to a microtube (1.7 ml) with screwcap. Freshly prepared dabsyl reagent (200 μl, 28.8 mM in acetone) was then added, after which the microtube was tightly closed and the mixture heated (10 min, 70°C) with intermittent shaking.

Prior to use, a C18 disposable column was conditioned with methanol (3 ml) and formate buffer (4 ml, 0.02 M, pH 3.5). The derivatized sample was diluted in formate buffer (2 ml) and applied to the C18 column, after which the column was washed with formate buffer (1 ml) and dried by applying a vacuum for 15 min. Subsequently, the column was washed with a freshly prepared mixture of ethyl acetate–methyl ethyl ketone–ammonia (1 M) (10:10:0.5, v/v/v) (3 ml) and dried for 10 s. The dabsylated phosphoamino acids were then eluted with ammonia (10 M)–acetonitrile–water (10:70:20, v/v/v) (0.5 ml). The latter fraction was dried in a vacuum concentrator.

The residue was dissolved in ammonia (10 M)–acetonitrile–water (10:70:20, v/v/v) (50 μl), and applied as a 5–7 mm line to a TLC sheet under a stream of air. In a separate lane a standard solution (10 μl) of dabsylated phosphoamino acids was applied. Approximately 15 samples could be analyzed simultaneously. 1D development (16–17 cm) was carried out with a mixture of isobutanol–acetonitrile–acetic acid–water (33:46:8:13, v/v/v/v) at room temperature. Afterwards the sheets were dried and sprayed with a solution of sulfuric acid–ethanol (3:97, v/v). The red-colored dabsylated phosphoamino acids were located by means of comparison with the standards. After autoradiography, the phosphoamino acid derivatives were cut out of the aluminum sheets and transferred to vials for ^{32}P-quantification by liquid scintillation spectrometry.

2.4. Preparation of dabsylated phosphoamino acid standards

A mixture of the phosphoamino acids (150 μg each) in borax derivatization buffer (200 μl) was treated with dabsyl reagent (400 μl) as described. After cooling, the microtube was opened, 1 ml formate buffer (0.02 M, pH 3.5) was added and the whole sample applied to a conditioned C18 column. The column was washed as described above, until discoloration of the elute. The dabsylated phosphoamino acids were then eluted with ammonia (10 M)–acetonitrile–water (10:70:20, v/v/v). The latter fraction was vacuum dried, the residue dissolved in ammonia (10 M)–acetonitrile–water (10:70:20, v/v/v) (15 ml) and stored at −20°C.

3. Results and discussion

Dabsylation is an efficient chromophoric labeling method yielding derivatives of primary and secondary amino groups, thiols imidazoles and hydroxyls [26,27] and has been extensively used for qualitative and quantitative identification of amino acids [20,26–28] and phosphoamino acids [17,18]. Usually the dabsyl reagent is used at concentrations of up to 10 mM. However, as demonstrated before [19], higher concentrations of dabsyl reagent are required in order to obtain substantial derivatization (70–90%) of the three phosphoamino acids. Since the excess reagent in the present method interfered dramatically with the subsequent chromatographic analysis (results

not shown), the reaction could not be applied directly to the TLC sheets. Therefore, a clean-up step using a disposable C18 column was necessary to prepare the derivatized sample for TLC analysis of the dabsyl-phosphoamino acids. Besides the excess reagent, the ethylacetate–methyl ethyl ketone–ammonia wash also removed dabsyl-derivatized amino acids.

In the present paper, dabsylation was merely used to render the phosphoamino acids more lipophilic, and this greatly increased the flexibility in finding suitable TLC solvent systems to separate them adequately. The result of a dabsyl-phosphoamino acid analysis of a specifically ^{32}P-labeled phosphopeptide is shown in Fig. 1. Dabsylated P-Ser, P-Thr and P-Tyr are well separated in one run (16–17 cm) with R_f values of 0.16, 0.19 and 0.35 respectively (Fig. 1A). The corresponding autoradiogram is shown in Fig. 1B. The dabsyl-phosphoamino acid analysis of the protein- and peptide-containing ^{32}P-Ser and ^{32}P-Tyr gave similar results.

Quantification of the radiolabeled spots separated by TLC demonstrated that 80–90% of the radioactivity present in the lanes was present as dabsyl-phosphoamino acid. The repeatability of the method for each of the phosphoamino acids was determined by replicate analysis ($n = 7$) of specifically labeled acid-hydrolyzed proteins and peptides over a period of 2 days. The RSD values obtained were 2.9%, 3% and 8.6% for the determination of ^{32}P-Ser, ^{32}P-Thr and ^{32}P-Tyr respectively. The linearity was not assessed since all hydrolyzed samples were routinely spiked with non-radioactive phosphoamino acids. The amount of each of the phosphoamino acids used (1 μg) represents a large excess in comparison with the radiolabeled phosphoamino acids released during hydrolysis. The validation of the derivatization of the phosphoamino acids with dabsyl reagent has been published before by this group [19].

These results prove that the dabsylation of phosphoamino acids followed by TLC analysis is very useful to analyse the phosphoamino content of ^{32}P-phosphoprotein hydrolysates. The method is straighforward and permits the simultaneous analysis of numerous samples. Very clean chromatograms are obtained enabling the unambiguous identification of the well separated dabsylated phosphoamino acids with autoradiography.

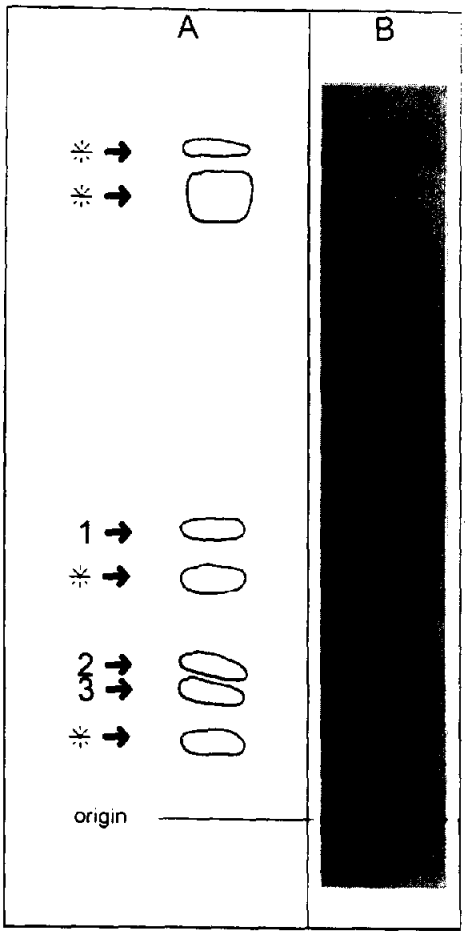

Fig. 1. (A) Schematic presentation of a TLC separation of dabsylated phosphoamino acids. A phosphopeptide, specifically ^{32}P-labeled at the threonyl residue, was processed as described in Section 2.3. After spraying the TLC sheet with sulfuric acid–ethanol (3:97, v/v), the dabsyl derivatives turned red: (1), dabsyl-P-Tyr; (2) dabsyl-P-Thr; (3), dabsyl-P-Ser. Some compounds (*) represent dabsyl reagent and some unknown nonradioactive dabsyl derivatives. (B) Corresponding autoradiogram.

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