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Absolute and Accurate Quantification of Protein Phosphorylation by Using an Elemental Phosphorus Standard and Element Mass Spectrometry**

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Protein phosphorylation is one of the most important post-translational modifications that is critically involved in many significant cellular processes.[1] It is estimated that one third of all proteins in eukaryotic cells are phosphorylated at any given time.[2] Moreover, a single protein can be phosphorylated and dephosphorylated by different kinases and phosphatases, respectively, on different sites at different times. These phosphorylation variations, that is, resulting from a targeted perturbation, can only be detectable if quantitative information is available. Unfortunately, quantification of protein phosphorylation is a very challenging task that is often hampered by low relative amounts of phosphoproteins and a lack of adequate analytical methods.[3] ESI and MALDI molecular mass spectrometry have been successful in identifying and measuring relative changes in quantity of a particular (phospho)protein.[4] Element mass spectrometry (inductively coupled plasma, ICPMS) has also been reported to compute protein phosphorylation stoichiometry by using the relative measurement $^{31}P/^{34}S$ and the protein sequence information obtained by ESIMS.[5,6] As this latter approach requires the presence of S-containing residues (cysteine or methionine), it mostly provides the phosphorylation degree of the whole protein studied. Furthermore, sample-preparation steps, such as reduction and alkylation, may strongly affect the P/S ratio obtained, leading to biased protein phosphorylation results.

Absolute quantification of phosphoproteins at given phosphorylation sites is much less commonly addressed, and so far reported methods require chemical synthesis (preferably with incorporated stable isotopes) of each individual phosphopeptide, which must already be known.[7,8] In fact, the main limiting factor to obtain absolute and reliable phosphorylation quantifications is the lack of the phosphopeptide and phosphoprotein standards required. Interestingly, the elemental response by ICPMS, when operated under certain conditions, could be directly proportional to the absolute amount of the element introduced (P in this case).[9] Therefore, in contrast to molecular MS techniques, the signal is independent of the species and sample matrix. However, a problem arises when ICPMS is used as an elemental detector in reversed-phase gradients in which the organic content (mostly acetonitrile) of the mobile phase strongly influences the ionization efficiency in the plasma, even at capillary ($4\ \mu L\ min^{-1}$)[10] and nano ($300\ nL\ min^{-1}$)[11] flow rates.

Herein, we describe the addition of a postcolumn sheath flow with a constant acetonitrile content that is able to buffer gradient composition changes. This leads to a constant ^{31}P sensitivity along the μHPLC–ICPMS gradient, which is required to separate the different tryptic phosphopeptides originally present in the sample digest and the spiked P-containing standard. We then investigated the accuracy and precision that is attainable by using commercially available phosphopeptides. Moreover, the potential applicability of this novel method of absolute quantification has been evaluated in β-casein as a model protein.

Figure 1a demonstrates the profound effect of the amount of acetonitrile introduced into the plasma ICP on the ^{31}P ionization efficiency even at the capillary flow rates used (3–$9\ \mu L\ min^{-1}$). This effect results in very different detector responses depending on the retention time of the P species that elutes from the column. This therefore prevents the use of an elemental P-containing standard for the quantification of the different phosphopeptides that are separated by the reversed-phase gradient used. The addition of a postcolumn sheath flow (with a constant organic content, 40% acetonitrile, $5.5\ \mu L\ min^{-1}$) ensures that ICPMS ^{31}P sensitivity remains constant during gradient elution (see the dotted line in Figure 1a). In fact, the relative standard deviation (RSD) of the ^{31}P signal across the acetonitrile gradient was less than 3%. In these conditions, the ^{31}P signal obtained should be directly proportional to the mass of ^{31}P present in the compound (species) and completely independent of its chemical structure.

As inorganic P salts would elute in the void volume of the column, a more-complex molecule certified in its P content, bis(4-nitrophenyl) phosphate (BNPP), was selected for use as a standard in the intended absolute phosphopeptide quantification. It is worth stressing that BNPP has been used before in phosphoproteomics studies with ICPMS for optimization purposes.[9,12] In our work, BNPP is used as a reference material to directly convert the ICPMS signal into absolute mass (pmol) for each phosphopeptide separated in the μHPLC chromatogram (a similar approach[13] has been recently published for quantification of DNA adducts).

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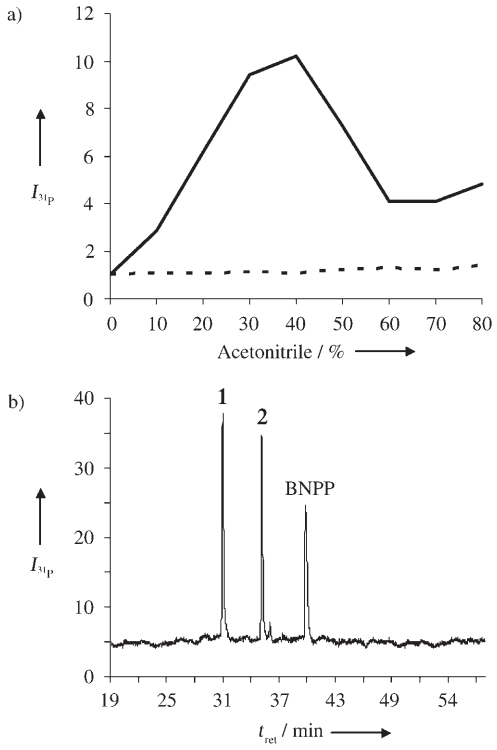

Figure 1. a) Effect of increasing acetonitrile content on the ^{31}P sensitivity without (bold line) and with (dotted line) postcolumn sheath flow. b) μHPLC–ICPMS chromatogram obtained under the optimized conditions for a mixture containing two phosphopeptide standards (**1** and **2**; 8.1 pmol) and BNPP (8.1 pmol). Gradient: 0–4.5 min 0% eluent B (isocratic), 4.5–39.5 min 0–80% eluent B (lineal) and 39.5–44.5 min 80–0% eluent B (lineal). $I_{^{31}P}$ = intensity of the ^{31}P signal, t_{ret} = retention time.

The performance of the setup was initially tested with two phosphopeptide standards. Figure 1b shows the μHPLC–ICPMS chromatogram obtained for a mixture containing P (8.1 pmol) for both model phosphopeptides (**1** and **2**) and the same molar amount of BNPP. Then, the sensitivity factor obtained for BNPP (peak area units per pmol of ^{31}P injected) was used to quantify the molar amount of P present in the two phosphopeptides. Results obtained were (8.4 ± 0.3) and (7.9 ± 0.3) pmol, which translate into absolute errors as low as $+4\%$ and -2%, respectively. Note that when postcolumn sheath flow was not used to buffer gradient acetonitrile changes, errors for phosphopeptide quantification increased up to -57% and -31%, respectively. Analytical figures of merit comprise a wide dynamic range (covering more than two orders of magnitude as can be seen in Figure S1 in the Supporting Information), an adequate detection limit of 110 fmol for a singly phosphorylated peptide, and excellent precision (3–6% RSD depending on the P concentration level).

As a proof of concept, the approach developed was applied to the quantification of the phosphopeptides present in a tryptic digest of β-casein from bovine milk. The total P content was first determined by continuous capillary flow ICPMS and was found to be (4.76 ± 0.11) P/protein, (pmol, $n = 3$), which agreed well with the composition provided by the manufacturer (4–5 P/protein). The use of microcentrifugation with a 3-KDa cut-off filter in the original sample allowed us to demonstrate that more than 99.9% of this P was protein bound. Finally, P recovery from the reversed-phase column was also calculated, after collection of the complete eluate for a total P determination, to be $(91 \pm 5)\%$ ($n = 3$). To our knowledge, this is the first time that recoveries of casein phosphopeptides from HPLC columns have been quantitatively studied. This fact is especially important for proteins with large and very hydrophobic peptides that can be permanently retained in reversed-phase columns, as is the case for casein proteins.[9]

The β-casein tryptic digest was then spiked with BNPP and analyzed by μHPLC–ICPMS in a single chromatographic run. The elution of the phosphopeptides was recognized selectively by ^{31}P detection (Figure 2). As expected, BNPP eluted close to the elution window of the phosphopeptides, assuring the same ICPMS sensitivity under optimized conditions. As can be observed, two main peaks were detected accounting for more than 90% of the P present and a group of very minor peaks is apparent between them. Table 1 shows the amount of P (pmol) determined per pmol of protein digested for each independent P-containing peak detected. Because the phosphopeptides are quantified in absolute terms, we are able to express them as pmol per pmol of protein, per mL of tryptic digest, or per g wet tissue weight (depending on the type of sample). It is worth stressing that precision associated with the absolute mass of P quantified for each peak was below 2.5% RSD. The total quantified amount of P was (4.57 ± 0.05) P/protein (pmol, $n = 3$). Taking into consideration the total P content for β-casein and its measured recovery from the column, the expected amount of P bound to protein to be quantified should be (4.3 ± 0.3) P/protein (pmol). This value is statistically indistinguishable from the experimental value obtained, therefore confirming the quantitative character and robustness of the proposed methodology.

The coupling of μHPLC with ESIMS allowed the unambiguous elucidation of the amino acid sequences

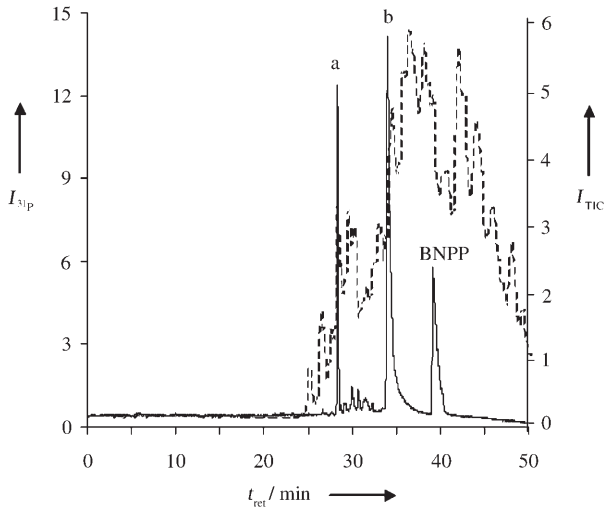

Figure 2. Tryptic digest of β-casein. μHPLC chromatogram with ICPMS ^{31}P (solid line) and ESIMS (dotted line) detection. Amino acid sequences for peak a and peak b are given in Table 1.

Table 1: Quantification of phosphopeptides detected in the tryptic digest of the β-casein.[a]

Peak	P/protein [pmol][b]	Sequence[c]	pPeptide/protein [pmol]
a	0.99 ± 0.02	FQpSEEQQQTEDELQDK	0.99 ± 0.02
b	3.20 ± 0.05	RELEELNVPGEIVEpSLpSpSpSEESITR	0.80 ± 0.01
rest	0.38 ± 0.01	–-	–-
total	4.57 ± 0.05		

[a] Corresponds to the μHPLC–ICPMS chromatogram shown in Figure 2 (solid line). [b] Uncertainty corresponds to one standard deviation ($n = 3$). [c] pS corresponds to phosphorylated serine.

(Table 1) of the phosphopeptides quantified by ICPMS. This information could be used to translate the mass of P obtained in absolute values (pmol) for each phosphopeptide separated in the ICPMS chromatogram. The total ion count (TIC) of the ESIMS chromatogram obtained is also shown in Figure 2. As expected, this chromatogram is much more complicated than the ICPMS profile. This fact stresses the capability of the μHPLC–ICPMS coupling for a fast "screening" of any phosphopeptides present in the sample. TOFMS and MS–MS spectra obtained at the retention time of the two main P-containing peaks determined by ICPMS (28.3 and 34.2 min, see Figures S2 and S3 in the Supporting Information) provided the identity of the phosphopeptides quantified by ICPMS. It is interesting to note that the combination of the quantitative ICPMS and qualitative ESIMS information gave a complete picture of the stoichiometry of the different phosphorylation sites (Table 1) involved in β-casein.

In summary, we believe that this strategy is superior to other methods for absolute phosphopeptide quantification that requires synthesis of every individual phosphopeptide. In our method, the simple addition of a commercially available P standard to the sample and a postcolumn sheath flow are able to provide reliable and simultaneous quantitative results for all individual phosphopeptides present (corresponding to the different phosphorylation sites), regardless if they were initially expected or not. Moreover, the high precision and accuracy attained here make this approach ideal for the discrimination between very small temporal changes in phosphorylation levels (proteomics kinetic studies). In light of our results, it seems that μHPLC–ICPMS in conjunction with adequate P-containing standards could also become a powerful tool to optimize and validate procedures for phosphorylation determination and be particularly valuable for common sample-preparation steps (e.g. preconcentration using IMAC). Such high-quality absolute quantitative data that are directly traceable to a standard certified in its P content should be not only stable over time but also robust enough to allow data comparison between different laboratories. This new approach could be invaluable to address quality assurance requirements and protocols urgently needed in quantitative (phospho)proteomics.[14,15]

Experimental Section

In terms of quality assurance, BNPP was purchased as a highly pure chemical reagent (purity checked by HPLC and then by titration versus NaOH: 99.3 and 99.2%, respectively). Moreover, the direct comparison of the phosphorus signal for a certified element salt (Na_2HPO_4, purity > 99.5%) and BNPP by using isocratic ICPMS confirmed and validated the total phosphorus content of the latter. Amino acid sequences of the standard phosphopeptides were TSTEPQpYQPGENL (peptide **1**) and *N*-acetyl-DpYVPML-NH_2 (peptide **2**).

Total phosphorus content was determined by standard additions to the samples and direct infusion to the ICPMS with the syringe pump (flow rate: 5 μL min^{-1}). Samples were diluted 1:4 with a 10% acetonitrile solution containing 100 ng Ge mL^{-1} to monitor the flow system stability.

Tryptic digest of β-casein (35 μL, 1.6 mg mL^{-1}) was spiked with BNPP (5 μL, 20 μg P mL^{-1}) and 1 μL of the resulting mixture was injected into the μHPLC system. Eluent A: 98.8% water, 1% acetonitrile, 0.2% formic acid. Eluent B: 99.8% acetonitrile, 0.2% formic acid. Gradient: 0–6.5 min 0% eluent B (isocratic), 6.5-40.5 min 0–55% eluent B (linear), 40.5–48.5 min 55–95% eluent B (linear), 48.5–53.5 min 95% eluent B (isocratic), and 53.5–55.5 min 95–0% eluent B (linear). μHPLC column and sheath-flow rates were 3.5 and 5.5 μL min^{-1}, respectively. Column: 300 μm × 15 cm packed with Zorbax SB C18, 5 μm, 80 Å.

Element mass spectrometry was performed by using a quadrupole ICPMS (Agilent 7500c, Yokogawa Analytical Systems, Tokio, Japan) equipped with a collision cell (2.5 mL He min^{-1}) for interference attenuation in the specific detection of ^{31}P. The capillary column was directly connected to the ICPMS by means of a microflow total-consumption nebulizer (DS-5 nebulizer, CETAC, Omaha, USA). For the μHPLC–ESIMS analysis a tandem mass spectrometer, Q-TOF type (QSTAR XL, Applied Biosystems MDS, Langen, Germany) was operated in the postive-ion mode.

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