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Separation and Detection of Phosphorylated and Nonphosphorylated Peptides in Liquid Chromatography−Mass Spectrometry Using Monolithic Columns and Acidic or Alkaline Mobile Phases

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Efficient chromatographic separation is a prerequisite for the sensitive analysis of complex peptide mixtures using liquid chromatography−mass spectrometry. This is especially true for the analysis of mixtures of unmodified and posttranslationally modified peptides, for example, phosphorylated peptides in the presence of their unmodified analogues. Applying monolithic capillary columns based on poly(styrene/divinylbenzene), the influence of acidic eluents based on trifluoroacetic and heptafluorobutyric acid as well as an alkaline eluent based on triethylamine-acetic acid (pH 9.2) on the separation of synthetic phosphopeptides was evaluated. Heptafluorobutyric acid offered the longest retention times and highest selectivities and, hence, the most effective separation. Application of the alkaline eluent in conjunction with detection in negative ion mode electrospray ionization mass spectrometry, on the other hand, allowed the detection of phosphorylated peptides with significantly lower limits of detection, as compared to acidic eluents in combination with detection in positive ion mode. Pairs of phosphorylated and nonphosphorylated synthetic peptides, ranging from 7- to 16-mers, as well as phosphorylated peptides form a tryptic protein digest could be separated both at acidic and alkaline pH. Utilizing a 60 × 0.20-mm-i.d. capillary column, the limit of detection in negative ion detection mode for a 4-fold phosphorylated peptide in a *β*-casein digest was 10 fmol. Together with the capability for fast separation of protein digests, monolithic columns, thus, facilitate the effective and sensitive analysis of this important posttranslational modification.

The modification of proteins by phosphorylation plays an important role in a number of biochemical processes in almost all classes of organisms, ranging from bacteria to humans. This reversible posttranslational modification is involved in the regulation of enzyme activities and metabolic pathways, in processes of signal transduction, the control of gene transcription and translation, and in the differentiation and proliferation of cells. In eukaryotic organisms, phosphorylation occurs mainly at the hydroxyl groups of serine, threonine, or tyrosine residues; in prokaryotic organisms the side chains of aspartate, glutamate, arginine, lysine, histidine, and cysteine were also found to be phosphorylated. About 25−30% of the proteins present in eukaryotic cells are estimated to be phosphorylated at some point in their life cycle, which makes the characterization of phosphorylation an important issue in protein and proteomic analysis.[1]

A number of methods, including radioactive ^{32}P-labeling and detection, staining techniques, immunological methods, and Edman degradation, have been developed and applied for the analysis of protein phosphorylation.[2,3] Due to its inherent sensitivity and the possibility to obtain additional data about the location of phosphorylation within a protein, mass spectrometry (MS) and tandem mass spectrometry (MS/MS) have become powerful tools for the analysis of phosphorylation. The most frequently used techniques are electrospray ionization- (ESI) and matrix-assisted laser desorption/ionization- (MALDI) MS(/MS). In addition to the measurement of the mass difference between nonphosphorylated and phosphorylated peptides of ∼80 Da, the analysis of specific fragmentation patterns obtained by collision-induced decomposition (CID),[4−7] or metastable decay[8,9] allows the unambiguous identification of phosphorylation sites.

In view of the high complexity of biological mixtures, for example, complete proteomes, and the fact that these mixtures are too complex to be analyzed simultaneously by mass spec-

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trometry, the combination of separation techniques with mass spectrometric methods is indispensable. Although the analysis of intact proteins embraces both primary amino acid sequence and posttranslational modifications,[10] investigation at the level of peptide fragments is more established in terms of separation and characterization by MS and MS/MS,[11] however, at the risk of not detecting the peptide containing the modification. The most popular method for the chromatographic separation of peptides is reversed-phase high-performance liquid chromatography (RP-HPLC) applying hydrophobic stationary phases and gradients of acetonitrile in aqueous solutions as eluents.[12] Separations can be performed at both acidic[13] and alkaline pH,[14] and ion-pairing additives such as trifluoroacetic acid (TFA),[15,16] heptafluorobutyric acid (HFBA),[17] or triethylamine/acetic acid (TEA/HOAc)[18] have been utilized to enhance electrostatic interaction of the peptides with the stationary phase.

Macroporous butyl or octadecyl silica stationary phases have been used for the separation of nonphosphorylated peptides from their phosphorylated analogues.[19−23] Hoffmann and co-workers reported that the separation efficiency increased when heptafluorobutyric acid was used as ion pairing reagent instead of TFA.[22] The addition of 0.1−1% phosphoric acid to the mobile phase significantly enhanced the detectability of phosphorylated peptides in HPLC/ESI-MS experiments.[24] Beck and co-workers reported the separation of phosphorylated peptides using alkaline mobile phases, thus increasing the detectability of phosphopeptides by negative-mode ESI-MS as well as an increased sensitivity of phosphospecific fragments in MS/MS experiments.[25]

Nonporous microparticles or monolithic stationary phases based on poly(styrene−divinylbenzene) polymer (PS-DVB) have been shown to represent a real alternative to conventional porous stationary phases based on silica gel for the highly efficient separation of peptide mixtures.[26−28] In contrast to silica-based stationary phases, they are highly stable, even during extended use at elevated temperature,[16,27] high pH, or both.[18] The influence of acidic and alkaline conditions on both separation efficiency and mass spectrometric detectability in positive and negative ion detection modes was recently investigated, showing that separation at high pH and on-line detection of the peptides in negative mode ESI represent an attractive alternative to peptide analysis by RP-HPLC at acidic pH hyphenated to positive-mode ESI-MS.[18]

In this paper, we focus on the applicability of PS-DVB-based monolithic columns to the separation of serine-, threonine-, and tyrosine-phosphorylated and nonphosphorylated peptides. Separations at both low and high pH using TFA, HFBA, and TEA/HOAc as additives are compared. The separation properties are tested for synthetic phosphorylated peptides and their nonphosphorylated counterparts. Furthermore, the methods are evaluated for phosphoprotein analysis by the analysis of a tryptic digest of β-casein, which contains five phosphorylation sites. Factors including separation efficiency and influence on the limits of detection applying on-line HPLC/ESI-MS are critically evaluated.

EXPERIMENTAL SECTION

Chemicals. Fmoc-protected amino acids and chemicals for peptide synthesis were purchased from Novabiochem (Schwalbach/Ts., Germany). Trifluoroacetic acid (TFA, for protein sequence analysis), heptafluorobutyric acid (HFBA, analytical reagent grade), and triethylamine (TEA, analytical reagent grade) were purchased from Aldrich or Fluka (Taufkirchen, Germany). Acetonitrile (HPLC gradient grade) Riedel deHaen (Seelze, Germany). Water was purified by a Purelab Ultra System water deionization system (Elga, Siershahn, Germany). Trypsin (sequencing grade, modified) was obtained from Promega (Madison, WI); β-casein (bovine) and alkaline phosphatase (porcine intestinal mucosa, type IV) were purchased from Sigma (Taufkirchen, Germany).

Peptide Synthesis and Purification. Peptides were synthesized on preloaded HMP resins (*p*-alcoxybenzyl alcohol with trityl linkers, PepChem, Tübingen, Germany) by automated standard Fmoc methodology on an Applied Biosystems ABI 433 peptide synthesizer applying HBTU (*O*-benzotriazole-*N*,*N*-*N*′-*N*′-tetramethyluronium hexafluorophosphate) activation. Phosphorylated serine, threonine, or tyrosine residues were incorporated by use of the building blocks Fmoc-Ser(PO(*O*-benzyl)(OH))−OH, Fmoc-Thr(PO(*O*-benzyl)OH))−OH or Fmoc-Tyr(PO(OH)$_2$)−OH, respectively. Peptides were purified by preparative RP-HPLC (Kromasil C_{18}, 5 μm, 100 Å) and lyophylized from water (three times) prior to use. Sequences and monoisotopic masses of the synthetic peptides are summarized in Table 1.

Protein Digestion and Dephosphorylation. Bovine β-casein (20 pmol/μL) was digested in 40 mM NH_4HCO_3 (pH 8) with modified porcine trypsin using a trypsin-to-protein ratio of 1:50 (w:w) for 12 h at 37 °C. Dephosphorylation of the β-casein-derived peptides was performed by addition of one unit of alkaline phosphatase per mole peptides to the digest and incubation for 1 h at 37 °C.

High-Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry. Monolithic PS-DVB capillary columns (60 × 0.20-mm-i.d.) were prepared according to the

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Table 1. Sequences and Retention Times t_R of Synthetic Peptides

peptide	sequence[a]	monoisotopic mass	t_R[b] TFA	t_R[b] HFBA	t_R[b] TEA/HOAc
I	ESLSS SEE	866.4	1.70	4.00	1.10
p-I5	ESLS**pS** SEE	946.3	1.70	1.80	0.75
p-I6	ESLSS **pS**EE	946.3	1.70	1.90	0.75
II	EAQAA T AAQAK	1058.5	1.00	5.05	0.80
p-II	EAQAA **pT** AAQAK	1138.5	0.90	1.55	0.40
III	EAQAA Y AAQAK	1120.5	3.80	7.50	2.30
p-III	EAQAA **pY** AAQAK	1200.5	1.30	3.90	1.00
IV	GNAAA AKKGS EQESV K	1573.8	1.50	7.90	0.50
p-IV	GNAAA AKKG**pS** EQESV K	1653.8	1.50	6.50	0.50
IV-M	**Myr**-GNAAA AKKGS EQESV K	1784.0	19.00	21.10	18.20
p-IV-M	**Myr**-GNAAA AKKG**pS** EQESV K	1864.0	19.30	20.90	18.40
V	LRRAS LG	771.5	3.50	9.20	2.90
p−V	LRRA**pS** LG	851.4	3.00	7.60	2.30
VI	LRRAY LG	847.5	6.50	11.10	6.40
p-VI	LRRA**pY** LG	927.4	5.70	8.90	5.30
VII	PVRRR SGANY RAY	1564.8	8.00	13.30	8.20
p-VII	PVRRR **pS**GANY RAY	1644.8	7.80	11.10	7.60
VIII	EAQRD SHLGP C	1211.5	10.00	13.50	8.10
p-VIII	EAQRD **pS**HLGP C	1291.5	6.90	12.10	7.20
IX	TRRTP DYFL	1167.6	12.10	15.05	8.80
p-IX	TRRTP D**pY**FL	1247.5	10.70	12.80	8.00

[a] Boldface residues (pS, pT, pY) indicate: phosphoserine, -threonine, or -tyrosine. [b] 200−500 fmol of the peptides was injected; elution was performed by application of a linear gradient from 0% A to 37.5% B in 25 min. Eluent systems as defined in the Experimental Section.

published protocol.[29] Monolithic PS-DVB capillary columns (50 × 0.20-mm-i.d.) are available from LC-Packings-A Dionex Company (Monoliths, Amsterdam, The Netherlands). The Ultimate capillary HPLC system with a Famos autosampler (LC-Packings) equipped with a 0.5-μL sample loop was used for HPLC/ESI-MS(/MS) experiments. ESI-MS and ESI-MS/MS were performed with a quadrupole ion trap mass spectrometer (Esquire HCT, Bruker Daltonics, Bremen, Germany) equipped with a pneumatically assisted electrospray ion source. The metal needle in the electrospray source was replaced by a 90-μm-o.d./20-μm-i.d. fused-silica capillary, which was grounded and connected to the capillary column by means of a stainless steel microtight union (Fritz Gyger AG Swiss, Gwatt-Thun, Switzerland). A needle voltage of −3.5 kV for positive ion detection mode and +3.5 kV for negative ion detection mode was applied.

For the analysis of the synthetic peptides, a 25-min linear gradient from 0 to 37.5% solvent B with a flow rate of 2 μL/min was applied. Three sets of experiments using different mobile phases were performed: (i) solvent A, water/0.05% TFA; solvent B, acetonitrile/0.05% TFA; (ii) solvent A, water/0.05% HFBA; solvent B, acetonitrile/0.05% HFBA; and (iii) solvent A, water/1.0% triethylamine, titrated to pH 9.2 with acetic acid (1.0% TEA/HOAc, pH 9.2); solvent B, acetonitrile/1.0% triethylamine, pH 9.2; adjustment of the eluent pH was performed on the neat aqueous solution (eluent A) by titration with acetic acid. Subsequently, the same amount of additives (triethylamine/acetic acid) was dissolved in acetonitrile for the preparation of solvent B. Peptides were injected as solutions in water with concentrations of 200−500 fmol/μL.

For the analysis of the protein digests, a linear gradient from 0% solvent A to 40% solvent B in 20 min was applied. Solvents A and B were as defined for the synthetic peptides. Digest solutions with concentrations of 0.02, 0.2, 2, and 20 pmol/μL were injected for all eluent systems tested. To prevent carryover of samples, the LC system was extensively washed between the runs.

RESULTS AND DISCUSSION

Influence of Mobile Phase Composition on the Separation of Phosphopeptides from Their Nonphosphorylated Analogues. The sequences of the synthetic peptides used in this study (Table 1) were derived from either consensus motifs of different protein kinases (protein kinase A and protein kinase CK II, peptides I, IV−IX) or artificial sequences (peptides II and III). The lengths of the peptides were in the range between 7 (peptide V) and 16 (peptide IV) amino acids, thus reflecting the common situation occurring in proteolytic digests of proteins. Additionally, a peptide N-terminally acylated by myristic acid, a C14:0 fatty acid (peptide IV-M), derived from the N terminus of cAMP-dependent protein kinase was investigated to study the separation behavior of strongly hydrophobic peptides.

Phosphorylation sites in naturally occurring proteins are rarely found to be completely modified.[30] Thus, in a cell, usually both nonphosphorylated and phosphorylated species can be found, which can, in principle, be distinguished due to their mass difference. On the other hand, coeluting peptides competitively ionize in the electrospray ionization process, frequently resulting in weak signals and poor detectabilities of the less abundant species, most often the phosphorylated peptide. Consequently, we investigated the separation behavior of pairs of phosphorylated and nonphosphorylated peptides on monolithic PS-DVB capillary columns to characterize their separability by HPLC. Elution was accomplished with two classes of eluent systems: (i) acidic eluents containing either TFA or HFBA as ion pairing reagents and (ii) an alkaline eluent (pH 9.2) based on triethylamine/acetic acid. To compare the separation behavior of peptides and phospho-

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peptides in the different eluents, the duration and steepness of the acetonitrile gradient were kept constant.

Table 1 contains the retention times that were measured with the phosphorylated and nonphosphorylated peptides I−IX in the three eluent systems. It is seen that retention times varied over a broad range of 0.4 (unretained) to 21.1 min. The peak widths at half-height of the eluting peptides were in the range of 0.08−0.13 min, and a difference in retention times of 0.2−0.25 min was usually sufficient for the resolution to baseline of two adjacent chromatographic peaks. Strongest retention was generally observed with the eluent containing HFBA, followed by TFA and TEA/HOAc. Since the nonphosphorylated peptides are ordered in Table 1 by increasing retention time in the HFBA eluent, it becomes clear that there is no correlation between length of the peptide and overall retention. The same holds true also for the other two investigated eluent systems. Phosphorylated peptides eluted before their nonphosphorylated analogues with the exception of the strongly hydrophobic peptides IV-M/p-IV-M, showing a reversed elution order in the TFA and TEA/HOAc eluents. This indicates that hydrophobicity and not analyte charge is the primary criterion determining retention in the three eluent systems. If charge were the property mainly responsible for retention, then the phosphorylated peptide carrying two more negative charges should be more strongly retained at pH 9.2 in the TEA/HOAc eluent.

Figure 1 presents a comparison of the separations of the phosphorylated-nonphosphorylated pairs of the peptides IV, IV-M, and V using the eluents containing TFA, HFBA, and TEA/HOAc. With TFA, the peptides IV and V showed only weak retention in the TFA eluent and were not satisfactorily separated (Figure 1a). The same was observed for the phosphorylated and nonphosphorylated peptides I and II, which did not have enough retention in the TFA eluent to be adequately separated, whereas the peptides VI−IX were sufficiently retained to allow the separation of phosphorylated and nonphosphorylated species (Table 1). Acylation of peptide IV with a long-chain fatty acid increased the retention time from 1.5 to 19.0 min and enabled at least a partial separation of the phosphorylated from the nonphosphorylated peptide, with the nonphosphorylated peptide eluting before the phosphorylated form. The same behavior of these peptides was observed earlier upon separation on a silica-based C_{18} column.[31] Concluding, eluents containing TFA as ion-pair reagent cannot be recommended as a generally applicable mobile phase for the separation of phosphorylated and nonphosphorylated peptides using PS-DVB monoliths.

Strongest retention and highest separation selectivity was observed with an eluent containing HFBA as ion-pairing reagent (Figure 1b). All six peptides of the mixture were completely separated. The resolution values between the phosphorylated peptides IV, V, and IV-M were 5.3, 5,3, and 1.4, respectively, which demonstrates that the separation system is highly selective for the separation of phosphorylated from nonphosphorylated analogues. This behavior has been described earlier for the separation of phosphopeptides on silica-based C_{18} stationary phases using TFA- and HFBA-based mobile phases.[22] As opposed to the separation in the TFA eluent, the selectivity for the strongly

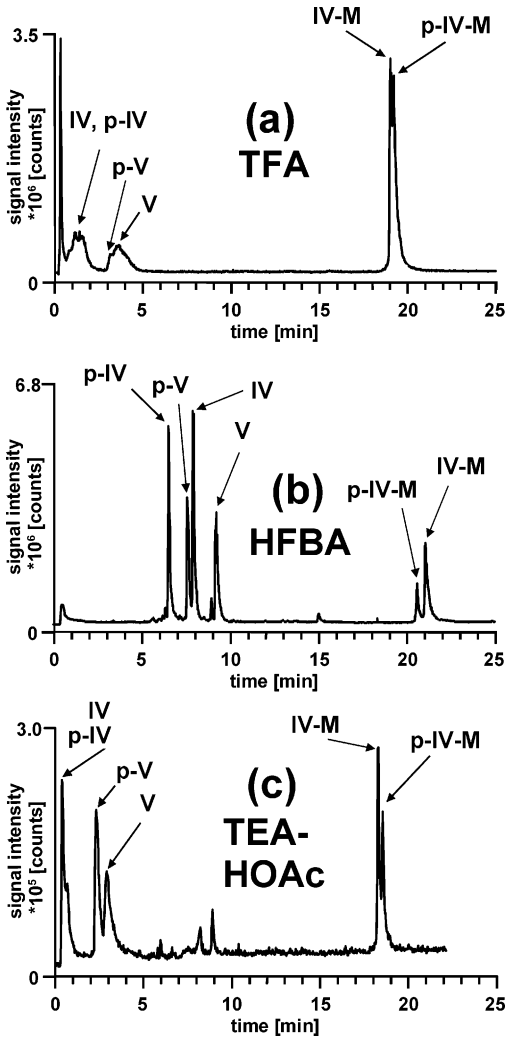

Figure 1. Reconstructed total ion current chromatogram of the separation of phosphorylated and nonphosphorylated peptides with eluents containing TFA, HFBA, and TEA/HOAc as ion-pairing reagents. Column, PS-DVB monolith, 60 × 0.2-mm i.d.; mobile phase, 25-min linear gradient of 0−37.5% acetonitrile in (a) 0.05% aqueous TFA, (b) 0.05% aqueous HFBA, and (c) 1% TEA/HOAc, pH 9.2; flow rate, 2 μL/min; detection, ESI-MS in (a), (b) positive ion detection mode and (c) negative ion detection mode; sample, peptides V/p-V, IV/p-IV, and IV-M/p-IV-M, 250 fmol. Peak identification in Table 1.

hydrophobic peptides IV-M and p-IV-M was high enough to facilitate baseline resolution in the eluent containing HFBA, but the resolution was lower than that between all other pairs of peptides. Note that the order of elution of the phospho- and the nonphosphopeptide was reversed compared to the elution with TFA. Presumably, the presence of the long nonpolar hydrocarbon chain diminishes the effect of the polar phosphoryl group. Upon changing the eluent from acidic with a negatively charged pairing ion to alkaline with a positively charged ion, we initially expected a reversal in the elution order of phosphorylated and nonphosphorylated peptides. However, the chromatogram depicted in Figure 1c clearly shows that the phosphorylated peptides still eluted before the nonphosphorylated. Compared to separations with HFBA as additive, both overall retention times and selectivities were smaller with the TEA/HOAc eluent. The peptides V and p-V coeluted in the hold-up volume and the retention of II and p-II was also insufficient to allow separation.

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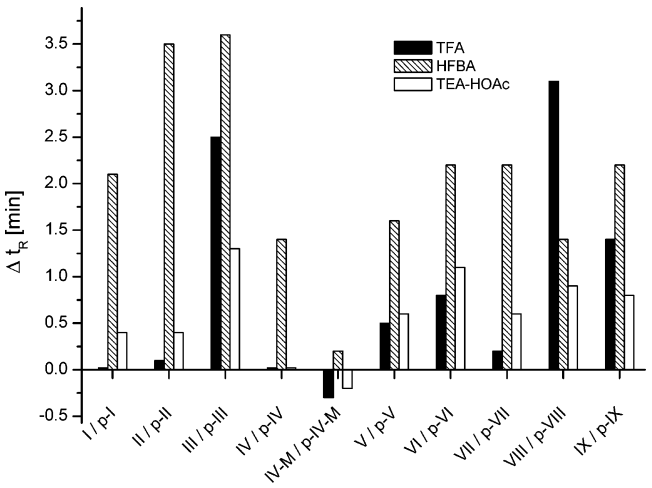

Figure 2. Differences in retention times (Δt_R) between nonphosphorylated and phosphorylated peptides. Experimental conditions as in Figure 1.

A comparison of the differences in retention times between phosphorylated and nonphosphorylated peptides using the three eluents is illustrated in Figure 2. The highest selectivities were normally obtained with HFBA as additive. Only the peptide-pairs IV-M/p-IV-M and VIII/p-VIII were better separated with TFA. With the alkaline eluent, the selectivity was mostly between that obtained with HFBA and TFA, but it was sufficient in all cases except for the peptides IV/p-IV and II/p-II to facilitate baseline separation of phosphorylated from nonphosphorylated species. From the differences in retention times of peptides phosphorylated at serine, threonine, or tyrosine, it can be recognized that there does not seem to be a correlation between the type of amino acid and the selectivity of the chromatographic separation with all solvent additives.

The basis for retention of peptides on monolithic PS-DVB stationary phases is both solvophobic and electrostatic interactions mediated upon adsorption of the charged mobile phase additives at the nonpolar stationary phase.[32] Phosphorylation of the uncharged hydroxyl functions at the side chains of serine, threonine, and tyrosine causes three major modifications of physicochemical parameters at the phosphorylation site: (i) the phosphoryl group is more bulky than the proton of the hydroxyl group; (ii) the hydroxyl group is a hydrogen bond donor, whereas the phosphoryl group is an acceptor for two hydrogen bonds; (iii) the phosphoryl group possesses two acidic functions, with $pK_{a1} < 2$ and $pK_{a2} = 5.9$ (values for phosphoserine in tetrapeptides),[20] leading to a net charge of the phosphoryl group of -1 at pH values in acidic eluents (pH = 2.1 for TFA, pH = 2.4 for HFBA) and -2 in the alkaline eluent (pH = 9.2 for TEA/HOAc).

The latter two modifications are responsible for a more hydrophilic character of the phosphopeptides, as compared with the nonphosphorylated species, which generally leads to less retention in RP-HPLC, as also observed in our experiments. Separation of phosphorylated from nonphosphorylated analogues with the acidic additives is also supported by reduced retention due to additional electrostatic repulsion between the negatively charged phosphoryl group and the negatively charged trifluoroacetate or heptafluorobutyrate ions adsorbed at the stationary phase. In separations performed at high pH, the situation is quite different. The two negative charges of the phosphoryl group contribute to additional electrostatic interactions, with the positively charged ion pairing reagent adsorbed at the stationary phase. Nevertheless, they are not strong enough to reverse the retention order of nonphosphorylated before phosphorylated peptides. Consequently, the selectivity to distinguish between phosphorylated and nonphosphorylated peptides is not as high at high pH as compared to low pH.

Influence of Eluent Systems on the Mass Spectrometric Detection of the Phosphopeptides. The on-line interfacing of RP-HPLC and ESI-MS provides a very powerful tool for the analysis of protein digests. For tryptic peptides usually carrying at least two positive charges (one at the amino terminus and another at the carboxy-terminal lysine or arginine), separation at low pH and detection by positive-mode ESI-MS have evolved as the methods of choice. Nevertheless, detection in negative ion mode has been shown to be advantageous, especially for acidic or phosphorylated peptides.[25] Detailed studies have shown that the composition of the mobile phase and the polarity of detection have a profound effect both on the chromatographic separation and on the mass spectrometric detection performance.[16,18] While separation efficiency and peak capacity have been found to be equivalent in separations of tryptic peptides carried out at acidic and alkaline pH, the limits of detection were about three times higher with detection in negative ion detection mode, as compared to positive ion detection mode.

To study the effect of mobile phase composition on mass spectrometric performance with phosphorylated and nonphosphorylated peptides, signal-to-noise ratios under different elution conditions were compared. The signal-to-noise ratios in the reconstructed total ion current chromatograms depended considerably on peptide sequence, the presence of modifications, and the nature of the ion pairing reagent. The peptides IV-M and p-IV-M were detected with similar signal-to-noise ratios (S/N = 305 and 264, respectively) with the TFA eluent and detection in positive ion mode (Figure 1a). Using HFBA as mobile phase additive, the signal-to-noise ratio for the nonphosphorylated peptide was significantly higher than that for the phosphorylated species (190 and 93, respectively, Figure 1b). The signal-to-noise ratio of the phosphorylated species (S/N = 23) chromatographed at alkaline pH and monitored in negative ion detection mode was less than that for the nonphosphorylated counterpart (S/N = 36) (see Figure 1b and c). Finally, the unmodified and modified peptides V and p-V showed similar signal-to-noise ratios upon elution with the HFBA-containing eluent (positive ion detection mode, S/N = 280 and 315, respectively, Figure 1b), but the signal for the phosphorylated species (S/N = 28) was significantly more intense, as compared to the nonphosphorylated peptide (S/N = 15) at alkaline pH with detection in the negative ion detection mode (Figure 1c). These findings suggest that there is no clear correlation between eluent pH, ion detection polarity, and the relative detectabilities of phosphorylated and nonphosphorylated peptides by RP-HPLC/ESI-MS.

The factors contributing to the detectability of peptides in ESI-MS are manifold. The composition of the eluent, the coelution of different analytes, the pH of the solvent, and the sequence of the

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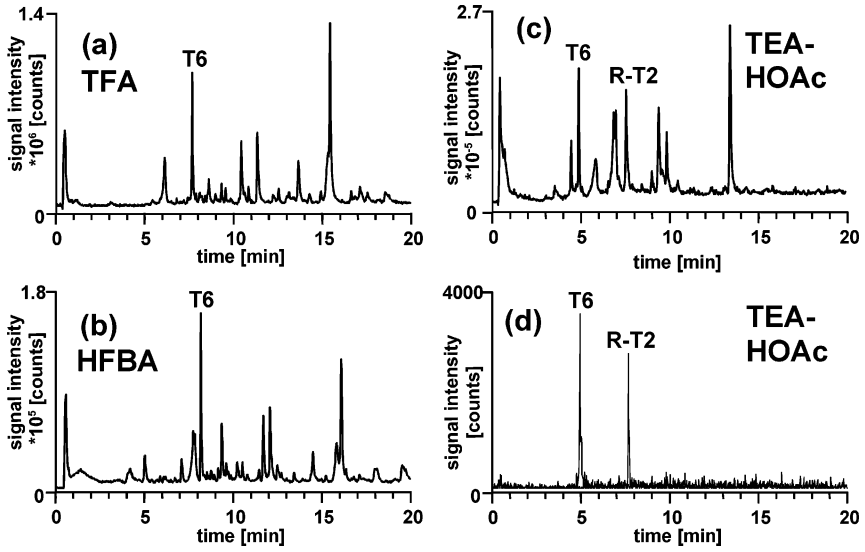

Figure 3. HPLC/ESI-MS analysis of a tryptic digest of β-casein. Eluent systems and ESI-MS ion detection modes: (a) total ion current chromatogram (TIC) 0.05% TFA/positive; (b) TIC, 0.05% HFBA/positive; and (c) TIC, 1.0% TEA/HOAc, pH 9.2/negative. Sample: 100-fmol digest (a−c). (d) Extracted ion chromatogram from *m*/*z* 1028.9−1029.9 (T6) and *m*/*z* 1039.1−1040.1 (R-T2); 1.0% TEA/HOAc, pH 9.2/negative, Sample: 10-fmol digest. Other experimental conditions as in Figure 1.

peptides (including modifications) are all known to influence the ionization behavior and, thus, the sensitivity. Further, the pH of the eluent also has an influence on the charge state of the analytes, which has to be taken into account for the accomplishment of MS/MS experiments. For example, the nonphosphorylated peptide VI showed a strong $[M - H]^-$ signal in negative ion detection mode but a weak $[M - 2H]^{2-}$ signal during elution with the alkaline mobile phase; whereas the ratio between $[M - 2H]^{2-}$ and $[M - H]^-$ was balanced with its phosphorylated analogue (p-VI). This behavior has been observed for several peptides in this study. The formation of higher charge states is beneficial for the detection either of sequence specific or phosphorylation specific ions generated by collision induced decomposition. The occurrence of stronger $[M - 2H]^{2-}$ ions upon elution with basic eluents may support the formation of $m/z = 97$ ($H_2PO_4^-$) and $m/z = 79$ (PO_3^-) fragment ions in negative ion detection mode.[25] It is noteworthy to mention that the use of TFA as an ion pairing reagent causes problems due to generation of the $C_2F_3O^-$ fragment, which also delivers a signal at $m/z = 97$. On the other hand, scans for the phosphopeptide specific neutral loss of H_3PO_4 (−98 Da) and HPO_3 (−80 Da) were most effective for 2-fold positively charged precursor ions.[5]

Analysis of Phosphorylated Peptides in a Tryptic Protein Digest. To confirm the results obtained with the synthetic peptides, a tryptic digest of bovine β-casein was investigated. The sequence of β-casein features five potential phosphorylation sites, one of which is located in the tryptic peptide T6 (FQ**S**EEQQQT-EDELQDK, monoisotopic mass of the monophosphorylated peptide 2060.8), whereas the other four are located in the peptide T2 (ELEELNVGPEIVE**SLSSS**EESITR, monoisotopic mass of the tetraphosphorylated peptide 2965.2). The latter sequence is usually found only in the incompletely cleaved peptide R-T2 containing an additional arginine residue (RELEELNVGPEIVE**SLSSS**-EESITR, monoisotopic mass of tetraphosphorylated peptide 3121.2).[33−35]

In this study, the separation of the digest was performed with a 200-μm-i.d. PS-DVB monolithic column using a gradient of 0−40% acetonitrile in aqueous TFA, HFBA, or TEA/HOAc solution in 20 min. The reconstructed total ion current chromatograms illustrated in Figure 3a−c clearly demonstrate that all three mobile phases are suitable for the separation of the tryptic peptides of β-casein. The monophosphorylated peptide T6 was readily detectable with all three mobile phases down to an amount of 10 fmol in both positive and negative ion detection mode (Table 2). Independent of the ion detection mode and the eluent system used, the doubly charged ions were the most abundant species detected at $m/z = 1031.4$ (positive ion detection mode) and $m/z = 1029.4$ (negative ion detection mode), respectively. The presence of the phosphoryl group in the peptide T6 was confirmed by dephosphorylation with alkaline phosphatase and reanalysis of the dephosphorylated digest (Table 2). The retention behavior of the phosphorylated and the nonphosphorylated species followed the observations described for the synthetic peptides, which means that the phosphorylated species eluted before the nonphosphorylated peptide and that the retention times were shortest at alkaline pH and longest upon application of the HFBA-based mobile phase.

From literature, it is known that the tetraphosphorylated peptide T2 or R-T2 is rather difficult to detect in a tryptic digest of β-casein.[33−35] Using a 150- or 360-μm-i.d. capillary column packed with 5-μm C_{18}-bonded silica particles, the addition of 0.1−1% phosphoric acid to the eluent to enable the detection of 50 fmol of the phosphorylated peptide T6 has been described, whereas the tetraphosphorylated peptide R-T2 could only be detected at the 50-pmol level.[24] In our experiments, R-T2 could be readily detected in positive ion detection mode upon elution with the TFA-based mobile phase down to an amount of 10 pmol (Table 2). For 1.0-pmol injections of the tryptic digest, the tetraphosphorylated peptide R-T2 could be observed upon elution neither with TFA- nor with HFBA-based mobile phases. The elution order

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Table 2. Identification of Phosphopeptides from Different Amounts of a β-Casein Digest upon Elution with Different Mobile Phases

			T6[a]		R-T2[a]	
eluent	ion detection mode	amt injected	phosphorylated, min	dephosphorylated,[b] min	phosphorylated, min	dephosphorylated,[b] min
TFA	positive	10 pmol	7.9	n.m.[c]	11.3	n.m.
		1.0 pmol	7.9	8.7	n.d.	14.35
		100 fmol	7.9	8.7	n.d.	14.25
		10 fmol	7.9	n.m.	n.d.	n.m.
HFBA	positive	10 pmol	n.m	n.m.	n.m.	n.m
		1.0 pmol	8.2	8.8	n.d.	n.d.
		100 fmol	8.2	n.m.	n.d.	n.m.
		10 fmol	8.2	n.m.	n.d.	n.m.
TEA/HOAc	positive	10 pmol	n.m.	n.m.	n.m.	n.m.
		1.0 pmol	4.9	n.m.	n.d.	n.m.
		100 fmol	5.0	n.m.	n.d.	n.m.
		10 fmol	4.9	n.m.	n.d.	n.m.
TEA/HOAc	negative	10 pmol	n.m.	n.m.	n.m.	n.m.
		1.0 pmol	4.8	5.0	7.6	9.4
		100 fmol	4.9	n.m.	7.6	n.m.
		10 fmol	4.9	n.m.	7.6	n.m.

[a] T6, FQ**S**EEQQQTEDELQDK, R-T2, RELEELNVGPEIVE**SLSSS**EESITR, serines in boldface may be phosphorylated or nonphosphorylated. [b] Retention times of dephosphorylated peptides were obtained after treatment with alkaline phosphatase. [c] n.m., not measured; n.d., not detectable.

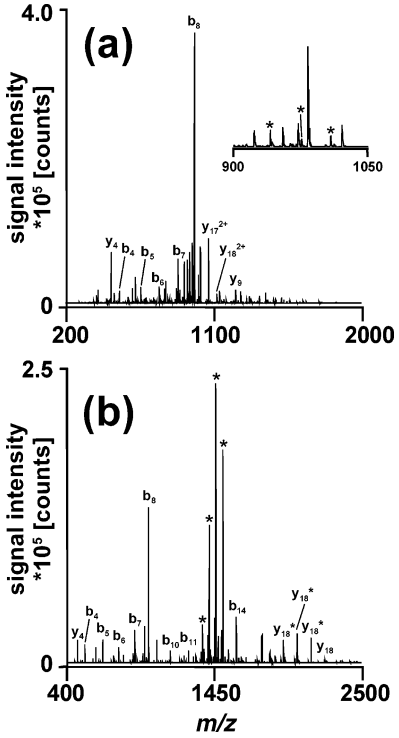

Figure 4. MS/MS spectra of the tetraphosphorylated peptide R-T2 in the tryptic digest of β-casein (10 pmol) separated with the TFA-based eluent. Precursor ion (a) m/z = 1039.4, triply charged R-T2; (b) m/z = 1562.0, doubly charged R-T2. An asterisk (*) indicates signals originating from neutral loss of phosphoric acid.

of the phosphorylated and the nonphosphorylated species again corresponded to the behavior described above. We observed both the doubly (m/z = 1562.0) and the triply charged (m/z = 1041.4) ions. The identity of the tetraphosphorylated peptide was confirmed by dephosphorylation with alkaline phosphatase (Table 2) as well as by MS/MS experiments (Figure 4). Using the doubly or triply charged species as precursor ions (Figure 4), the most abundant signals were assigned to sequence-specific ions as well as the neutral loss of H_3PO_4, leading to losses of 49 Da (doubly charged precursor, Figure 4b) and 33 Da (triply charged precursor, Figure 4a), respectively, for each phosphoryl group.

It has been reported that the application of alkaline pH can enhance the detectability of phosphorylated peptides by ESI-MS in the negative ion detection mode.[36−39] This effect was utilized by Beck and co-workers, who applied an alkaline eluent (pH 10.5) in combination with detection in the negative ion detection mode. Under these conditions, both the mono- and the tetraphosphorylated peptide could be detected in a 50-pmol sample of the digest using a 300-μm-i.d. C_{18} column.[25] In separations performed with the PS-DVB monoliths at alkaline pH and monitored in the positive ion detection mode, we did not observe any signal for the peptide R-T2 up to an amount of 1 pmol, whereas the same amount of peptide T6 could be readily observed. The situation changed when the negative ion detection mode was applied. Under these conditions, the signals of both the mono- and the tetraphosphorylated peptides delivered highly abundant signals in the reconstructed total ion current chromatogram down to an amount of 100 fmol (Figure 3c). In the extracted ion chromatogram, both phosphorylated peptides could clearly be assigned in a run with 10-fmol digest (Figure 3d). For the monophosphorylated peptide, this value is about a factor 5, for the tetraphosphorylated peptide a factor ∼5000 times lower than the lowest values reported so far in the literature.[24]

The gain in sensitivity in the positive ion detection mode upon use of the organic monolithic stationary phase instead of silica-based stationary phases presumably is a consequence of the total absence of silanol groups, which were already earlier identified as a potential reason for a decrease of detection sensitivity of multiphosphorylated peptides.[24] With silica-based stationary phases,

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the unwanted interactions between the silanol groups and the phosphopeptides could be alleviated through the addition of higher amounts of phosphoric acid, resulting in improved chromatographic peak shapes, however at the cost of a considerable decrease in the ionization efficiency in the electrospray process due to competitive ionization.[24] Therefore, the use of low concentrations of TFA as additive in combination with the monolithic stationary phase, which is devoid of any silanol groups, proves to be very advantageous in terms of both separation efficiency and mass spectrometric detectability in positive ion detection mode. Furthermore, the detectability of phosphopeptides in negative ion detection mode is tremendously increased as compared to the measurement applying acidic eluents in combination with positive ion detection mode as a consequence of the application of basic eluents, which effects practically complete dissociation of the phosphoryl group and, thus, maximizes the number of negative charges in the analytes.

CONCLUSIONS

Phosphorylated peptides can be separated effectively from their nonphosphorylated analogues in PS-DVB-based monolithic capillary columns. The choice of the ion pairing reagent in the mobile phase influences the selectivity of the separation as well as the total retention times of the peptides. Between the two acidic ion pairing additives TFA and HFBA, the latter performed significantly better in terms of retentivity and selectivity, facilitating the retention and resolution of even very small and hydrophilic phosphorylated and nonphosphorylated peptides. Using acidic eluents, mass spectrometric detection in positive ion mode is recommended. The use of an alkaline TEA/HOAc eluent in conjunction with detection in negative ion mode leads to a drastic improvement in the detection limits for a multiphosphorylated peptide from a β-casein digest. Furthermore, under these conditions, the detection of phosphospecific fragments ($m/z = 79, 97$) is possible upon collisionally induced decomposition. The parallel use of acidic eluent/positive ion detection mode and alkaline eluent/negative ion detection mode seems to be a viable combination to optimize the results of protein identification and characterization. Together with the capability for fast separations of protein digests and an increased base stability, as compared to silica-based columns, PS-DVB-based monolithic columns thus provide a valuable tool for the analysis of this important post-translational modification in protein and proteome analysis.

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