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Identification of phosphoproteins and determination of phosphorylation sites by zirconium dioxide enrichment and SELDI-MS/MS

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Reversible protein phosphorylation mediated by protein kinases and phosphatases is the most studied post-translational modification. Efficient characterization of phosphoproteomes is hampered by (1) low stoechiometry, (2) the dynamic nature of the phosphorylation process and (3) the difficulties of mass spectrometry to identify phosphoproteins from complex mixtures and to determine their sites of phosphorylation. Combination of the phosphopeptide enrichment method with MALDI-TOFMS, or alternatively, with HPLC-ESI-MS/MS and MS^3 analysis was shown to be a step forward for the successful application of MS in the study of protein phosphorylation. In our study we used phosphopeptide enrichment performed in a simple single-tube experiment using zirconium dioxide (ZrO_2). A simple protein mixture containing precipitated bovine milk caseins was enzymatically digested and the mixture of tryptic fragments was analysed before and after enrichment using nanoflow HPLC-ESI-MS/MS and surface-enhanced laser desorption/ionization (SELDI)-MS/MS on QqTOF instruments to compare the efficiency of the two methods in the determination of phosphorylation sites. Both approaches confirm the high selectivity obtained by the use of batch-wise, ZrO_2-based protocol using di-ammonium phosphate as the eluting buffer. More phosphorylation sites (five for β-casein and three for α_{S1}-casein) were characterized by SELDI-MS/MS than by nanoflow HPLC-ESI-MS/MS. Therefore, ZrO_2-based phosphopeptide enrichment combined with SELDI-MS/MS is an attractive alternative to previously reported approaches for the study of protein phosphorylation in mixtures of low complexity with the advance of fast *in situ* peptide purification. The method was limited to successful analysis of high-abundance proteins. Only one phosphorylation site was determined for the minor casein component α_{S2}-casein by ESI-MS/MS and none for κ-casein. Therefore an improvement in enrichment efficiency, especially for successful phosphoproteomic applications, is needed.

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KEYWORDS: bovine milk caseins; phosphopeptide enrichment; ZrO_2; SELDI-MS/MS; nano-HPLC-ESI-MS/MS

INTRODUCTION

Reversible post-translational addition/removal of phosphate groups to/from the side chains of specific amino acid residues (serine, threonine and tyrosine) of proteins is involved in the regulation of several biological processes, such as signal transduction, gene expression, cell cycle and apoptosis.[1,2] Because of the central role of protein phosphorylation in cellular regulation, there is an increasing interest in the development of experimental methods for the qualitative and quantitative analysis of phosphoproteins and for the determination of phosphorylation site(s).[3–7] Despite of the fact that one-third of the proteins in eukaryotic cells are thought to be phosphorylated at some point in their life cycle, only a low percentage of the intracellular proteins is phosphorylated at a given time. Phosphorylation/dephosphorylation rate can also vary during cell cycle, and therefore unsynchronized cells may contain proteins phosphorylated to various degrees. Characterization of phosphoproteins in complex biological mixtures therefore is, indeed, very difficult.

A great variety of analytical techniques are used for the analysis of phosphoproteins. Traditional methods use autoradiography of ^{32}P radiolabeled samples to visualize phosphorylated proteins combined with Edman degradation to determine protein identity.[6] Classical, *in vitro* phosphorylation studies using radiolabeling, added ATP and protein kinases are being gradually replaced by the recently developed gel-based[8,9] and in-solution methods[10] applying

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mass spectrometry-based strategies for the analysis of naturally occurring phosphoproteins. Owing to the big success and the central role of MS in proteomics, there was a great expectation in its similar application in phosphoproteomics. Efficient analysis of phosphoproteins by mass spectrometry, however, is hindered by the low ionization efficiency of phosphopeptides compared to non-phosphorylated ones, by the prompt neutral loss of phosphoric acid during analysis and by the insufficient dynamic concentration range of MS for the study of low-abundant phosphopeptides in complex mixtures. Reduction of sample complexity by chromatographic separation and/or enrichment of sample in phosphopeptides prior the MS analysis is necessary in order to improve the analytical capability of MS-based approaches for the investigation of complex biological samples.[3,11] For example, separation by strong cation exchange (SCX) followed by reversed-phase liquid chromatography and electrospray ionization tandem mass spectrometry (RP-LC-ESI-MS/MS) has been successfully applied for the determination of more than 2000 phosphorylation sites in the nuclear fraction of HeLa cells.[10] Recently a number of works have shown that affinity enrichment of phosphorylated peptides prior RP-HPLC-MS analysis can also greatly increase the detection efficiency of phosphoproteins.[3,11–17] Immunoaffinity separation,[7,18] chemical modification of the phosphate moieties by affinity tags,[3,11] immobilized metal affinity chromatography (IMAC),[19–21] ion exchange chromatography[10,22] and enrichment using metal oxides[12–16,23–27] are the most frequently used enrichment methods in post-translational phosphorylation studies. The selectivity of the phosphopeptide enrichment using titanium dioxide (TiO_2) has been demonstrated by the detection of phosphopeptides derived from enzymatic digestion of a mixture of known protein(s) with single-stage MALDI mass spectrometry.[24] Single-stage MS, however, is limited to the detection of post-translational modification. On the other hand, tandem mass spectrometry (MS/MS) can be applied for the determination of the sites of modification as well. Various scanning techniques are available for such experiments depending on ionization mode (ESI *vs* MALDI) and mass analysers.[28] Besides the most widely used product ion scan, precursor ion scanning in negative ion mode for the phosphate-derived anion m/z 79 on a triple-quadrupole instrument was also shown to be useful.[29] Alternatively, the neutral loss of phosphoric acid can be monitored in positive ion mode both using in-source or collision cell fragmentation. A combination of scanning techniques using a hybrid triple-quadrupole linear ion trap mass spectrometer can also be of value for identifying phosphorylation.[30] Besides ESI-MS/MS, MALDI MS/MS has also been proved to be useful for the analysis of phosphorylation event using a quadrupole time-of-flight (QqTOF) instrument in combination with off-line HPLC,[12,31] immunoprecipitation[18] and methyl esterification.[32]

Here we report a new method for the identification of phosphoproteins from mixtures and for the determination of their phosphorylation sites. The method is based on the combination of selective phosphopeptide enrichment with (1) a rapid 'on-chip' purification and (2) a direct mass spectrometric analysis using a QqTOF instrument equipped with a surface-enhanced laser desorption/ionization (SELDI) interface. Single-stage SELDI-TOF MS is widely applied for protein expression profiling from clinical and biological samples as well as in biomarker research.[33] Employing this type of source on a QqTOF analyser, similar to MALDI, allows high-resolution peptide sequencing in the MS/MS mode. Peptides up to ~3.5 kDa are selected by quadrupole 1 (Q) and transmitted to quadrupole 2 (q) for collision induced dissociation (CID) fragmentation to generate sequence information for protein identification and for detection of post-translational modifications. The additional advantage of the 'on-target' peptide purification using the SELDI chip is the enrichment of the target peptides and the reduction of the time spent per sample analysis. In our work the efficiency of SELDI-MS/MS method was compared to that of nanoflow HPLC-ESI-MS/MS previously reported to be efficient in the characterization of phosphoproteins.[13–16] Casein (CN), the major protein fraction of bovine milk, was studied without separation. CNs consist of four protein families α_{s1}-CN (up to 40% of CN fraction), α_{s2}-CN (up to 10%), β-CN (up to 45%) and κ-Casein (up to 5%).[34] They are highly phosphorylated by the mammary gland casein kinase and their phosphorylation pattern has been the basis of numerous studies.[35–37] Casein phosphopeptides are considered as bioactive peptides because they play an important role in the calcium bioavailability.[38] Here, the precipitated caseins were enzymatically digested by trypsin and loaded on zirconium dioxide chromatographic media to be enriched using a simple batch-wise experiment.[27] Samples before and after enrichment were analysed both by SELDI-MS and nanoflow HPLC-ESI-MS. CID product ion scanning was used to obtain peptide sequence information and to determine site of phosphorylation in both cases. The principal aim of the study was to explore the analytical capability of SELDI-MS/MS combined with phosphopeptide enrichment using ZrO_2 for the study of protein phosphorylation.

EXPERIMENTAL

Materials

Acetic acid was from Baker (Phillipsburg, NJ, USA), and sodium acetate and dichloromethane were from Carlo Erba (Milan, Italy). Ammonium hydrogen carbonate (NH_4HCO_3), di-ammonium phosphate (DAP), trypsin, acetonitrile, adrenocorticotropic hormone (ACTH) fragment 18–39 and trifluoroacetic acid (TFA) were from Sigma (St. Luis, MO, USA). α-Cyano-4-hydroxycinnamic acid (HCCA) was from Bruker Daltonics (Madison, WI, USA). Zirconium(IV) dioxide powder, purity better than 99%, from Reanal (Budapest, Hungary) was used. Formic acid (HCOOH) was from Riedel-de Haen (Seelze, Germany).

Precipitation of caseins and tryptic digestion

Bovine milk caseins were obtained by isoelectric precipitation (at pH 4.6) from whole cow's milk. Three hundred microlitres of 10% (v/v) acetic acid was added to 3 ml of milk sample, mixed and incubated at 40 °C for 5 min. Three-hundred and thirty microlitres of 1 M sodium acetate and 1 ml of dichloromethane was added to the mixture. The

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sample was mixed and centrifuged at 5000 rpm for 10 min. After centrifugation, three phases could be observed (from bottom to top): dichloromethane containing fat, precipitated caseins, aqueous phase containing mainly whey proteins. Casein fraction was washed two times with cold TFA 0.1% in order to remove traces of serum proteins co-precipitated with caseins. The sample was dissolved in 100 mM NH_4HCO_3 at a concentration of 100 mg/ml. Enzymatic digestion was performed at an enzyme/substrate ratio 1:100 by trypsin at 37 °C for 5 h. Digestion was stopped by acidifying the solution with TFA to 0.1% (v/v) final concentrations.

Phosphopeptide enrichment using ZrO_2

Casein tryptic digest (1 mg/ml) was enriched in phosphopeptides using a single-tube, batch-wise method (Fig. 1). ZrO_2 particles (4.5 mg) were placed into an eppendorf tube and washed three times with 100 µl 80% acetonitrile in 0.1% (v/v) TFA. The slurry was centrifuged on a benchtop centrifuge and the solvent was removed. Particles were conditioned by 100 µl 0.1% TFA. The sample (20 µg/mg ZrO_2) was added and incubated at room temperature under gentle shaking for 30 min and centrifuged, and the supernatant was removed. Non-phosphorylated peptides were eluted by 10 µl 80% acetonitrile in 0.1% TFA. The particles were washed five times by 200 µl 80% acetonitrile in 0.1% TFA. Bound phosphopeptides were eluted by adding 20 µl of di-ammonium phosphate 100 mM (pH 9). After centrifugation, the supernatant (containing the bound phosphopeptides) was removed and acidified by 2 µl TFA 10% (v/v).

Nanoflow HPLC-ESI-MS/MS analysis

CN digest before and after enrichment was analysed using a hybrid QqTOF instrument, QTOF-Micro (Waters, Milford, MA, USA), equipped with a nanoflow electrospray ion source. A distal-coated silica capillary, with 360 µm outer diameter (o.d.) and 20 µm inner diameter (i.d.) and tip i.d. 10 µm (New Objective, Woburn, MA, USA), was used as the nanoflow tip. Samples (1 µl) were loaded, purified and concentrated on a pre-column, PepMap, C18, 5 mm length, 300 Å, (LCPackings, Sunnyvale, CA, USA), using a CapLC micro-HPLC (Waters, Milford, MA USA) at 20 µl/min flow rate. A capillary column, PepMap, C18, 15 cm length, 75 µm i.d., 300 Å (LCPackings, Sunnyvale, CA, USA), was used for peptide separation with a flow rate split to approximately 200 nl/min using solvents A: 2% acetonitrile, 0.1% HCOOH and B: 95% acetonitrile, 0.1% HCOOH. Separation was performed using linear gradients 2–60% B in 30 min, 60–95% B in 2 min. CID experiments were performed in the data directed analysis (DDA; product ion survey) mode using the MassLynx 4.0 software. Single-stage survey spectrum was recorded for 1 s. The two most abundant ions present in the survey spectrum were automatically mass-selected and fragmented by CID. Argon was used as the collision gas. The peak list was generated by Protein Lynx Global Server 2.1 (Waters, Milford, MA, USA). Database search was performed by the Mascot program (www.matrixscience.com) using the following criteria: database: Swiss-Prot (release 51.4 containing 252 616 entries) type of search: MS/MS Ion Search, Taxonomy: mammalia enzyme: trypsin or none (as specified), variable modifications: oxidation on methionine, phosphorylation on serine, threonine and tyrosine; phosphorylation with prompt loss of phosphate on serine and threonine residues (P + PL); mass values: monoisotopic, parent tolerance: 0.1 Da, MS/MS tolerance: 150 ppm and number of maximum missed cleavages: 2. Phosphorylation sites assigned by the Mascot program were manually validated.

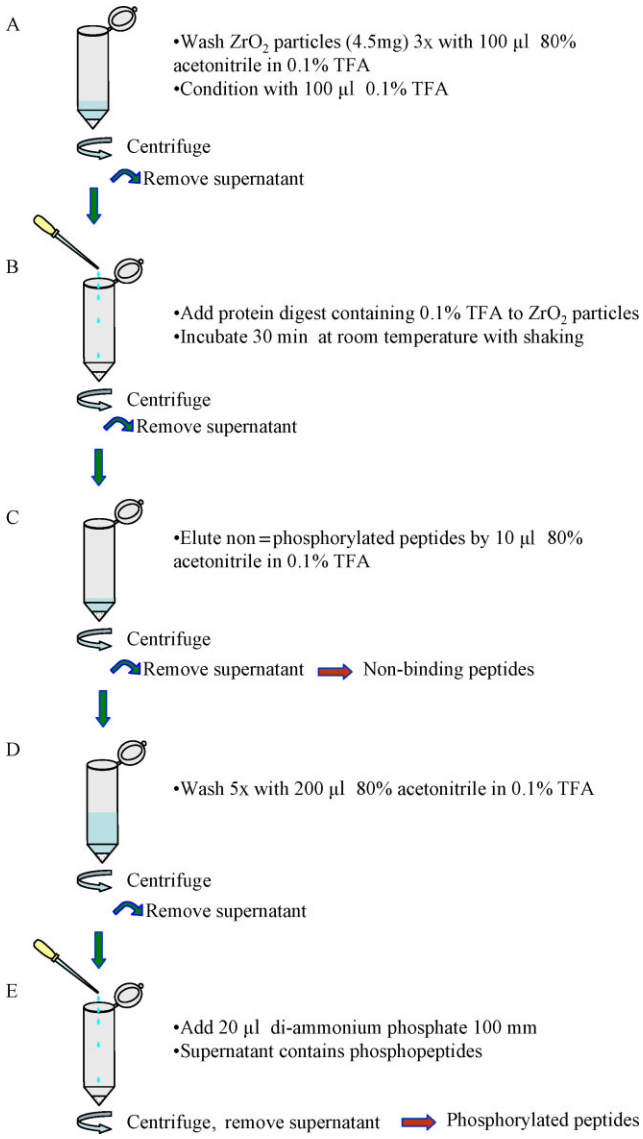

Figure 1. Schematics of single-tube, solid-phase phosphopeptide enrichment by ZrO_2 particles. The method is based on five simple steps: (A) conditioning, (B) sample loading, (C) elution of non-phosphorylated peptides, (D) washing and (E) elution of phosphopeptides.

SELDI-MS and MS/MS analysis

A 1 µl sample was spotted on the normal phase ProteinChip, NP20 (Ciphergen, Fremont, CA), dried and washed with ice cold TFA 0.1% in order to remove salts. The matrix solution, HCCA, 2 mg/ml in 50% acetonitrile:0.1% (v/v) TFA (1:1), was added, and the sample was analysed on QqTOF type hybrid mass spectrometer, QStar-Pulsar (Applied Biosystems, Foster City, CA, USA) equipped with

a SELDI ion source (Ciphergen, Fremont, CA) and controlled by a PS1000 interface. Nitrogen laser was used for ionization (VSL-337 ND-S, Laser Science, Franklin, MA, USA). Spectra were acquired in positive ion mode, in the range m/z 300–3000. The instrument was calibrated using selected fragment ions of ACTH. The most abundant ions observed in the SELDI single-stage mass spectra of the sample were manually selected for CID experiment. Argon was used as the collision gas. Peak lists were generated by the Analyst QS (version 1.1) program, which were used for database search in the Mascot program (www.matrixscience.com). Criteria for database searches were as follows: Type of search: MS/MS Ion Search, Database: Swiss-Prot (release 51.4 containing 252 616 entries), Taxonomy: Mammalia, enzyme: Trypsin, maximum number missed cleavages: 2, variable modifications: oxidation on methionine, phosphorylation on serine, threonine and tyrosine, phosphorylation with prompt loss of phosphate on serine and threonine residues (P + PL), mass values: monoisotopic, parent tolerance: 100 ppm, MS/MS tolerance: 0.08 Da.

MS/MS search not resulting in protein identification with the above search parameters were resubmitted without the specification of enzyme (no enzyme). Phosphorylation sites assigned by Mascot program were manually validated.

RESULTS

Total tryptic digest of bovine milk caseins was analysed by SELDI and nanoflow HPLC-ESI mass spectrometry before and after affinity enrichment in order to explore and compare the capability of the two methods in the characterization of phosphorylation sites. Table 1 shows the expected tryptic peptides derived from the theoretical tryptic digestion of the four different caseins. Considering the main genetic variants of caseins (i.e. α_{S1}-CN B-8P, α-$_{S2}$-CN A-11P, β-CN A^2-5P and κ-CN A-1P), there are 25 serine residues known to be phosphorylated, resulting in 8 theoretically expected tryptic phosphopeptides in the MW range 500–3000 Da.

In this work, ZrO_2 was used for the solid-phase affinity purification of phosphopeptides. The enrichment method which consists of five simple steps: (1) conditioning, (2) sample load, (3) elution of non-phosphorylated peptides, (4) washing and (5) elution of phosphorylated peptides, was performed in a single-tube experiment (Fig. 1). Different eluting conditions reported in the literature[24,26,27] were tried in order to optimize the separation procedure for the recovery of phosphorylated peptides from the ZrO_2 particles (data not shown). Best results were obtained by using phosphate as a strong Lewis-base anion and ammonium as buffer counterion (100 mM di-ammonium phosphate at pH 9) for the elution of phosphopeptides.

Nanoflow HPLC-ESI-MS/MS for the determination phosphorylation sites of caseins

On the basis of the m/z values measured in the single-stage survey scans, 28 peptides matching to the theoretical expected tryptic peptides (Table 1) were detected in the casein tryptic peptides mixture without enrichment. CID experiments performed in the DDA mode on 27 selected ions account for 12, 5, 9 and 2 peptides for α_{S1}-CN (sequence coverage 52.8%), α_{S2}-CN (18.9%), β-CN (39.7%) and κ-CN (11.0%), respectively (Fig. 2 and Supplementary Table

α_{S1}-CN, sequence coverage: 52.8%

```
  1 MKLLILTCLV AVALARPKHP IKHQGLPQEV LNENLLRFFV APFPEVFGKE
 51 KVNELSKDIG SESTEDQAME DIKQMEAESI SSSEEIVPNS VEQKHIQKED
101 VPSERYLGYL EQLLRLKKYK VPQLEIVPNS AEERLHSMKE GIHAQQKEPM
151 IGVNQELAYF YPELFRQFYQ LDAYPSGAWY YVPLGTQYTD APSFSDIPNP
201 IGSENSEKTT MPLW
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α_{S2}-CN, sequence coverage: 18.9%

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  1 MKFFIFTCLL AVALAKNTME HVSSSEESII SQETYKQEKN MAINPSKENL
 51 CSTFCKEVVR NANEEEYSIG SSSEESAEVA TEEVKITVDD KHYQKALNEI
101 NQFYQKFPQY LQYLYQGPIV LNPWDQVKRN AVPITPTLNR EQLSTSEENS
151 KKTVDMESTE VFTKKTKLTE EEKNRLNFLK KISQRYQKFA LPQYLKTVYQ
201 HQKAMKPWIQ PKTKVIPYVR YL
```

β-CN, sequence coverage: 39.7%

```
  1 MKVLILACLV ALALARELEE LNVPGEIVES LSSSEESITR INKKIEKFQS
 51 EEQQQTEDEL QDKIHPFAQT QSLVYPFPGP IPNSLPQNIP PLTQTPVVVP
101 PFLQPEVMGV SKVKEAMAPK HKEMPFPKYP VEPFTESQSL TLTDVENLHL
151 PLPLLQSWMH QPHQPLPPTV MFPPQSVLSL SQSKVLPVPQ KAVPYPQRDM
201 PIQAFLLYQE PVLGPVRGPF PIIV
```

κ-CN, sequence coverage: 11.0%

```
  1 MMKSFFLVVT ILALTLPFLG AQEQNQEQPI RCEKDERFFS DKIAKYIPIQ
 51 YVLSRYPSYG LNYYQQKPVA LINNQFLPYP YYAKPAAVRS PAQILQWQVL
101 SNTVPAKSCQ AQPTTMARHP HPHLSFMAIP PKKNQDKTEI PTINTIASGE
151 PTSTPTTEAV ESTVATLEDS PEVIESPPEI NTVQVTSTAV
```

Figure 2. Amino acid sequence and sequence coverage based on peptide mass fingerprint and obtained by nano-HPLC-ESI-MS on caseins α_{S1}-CN, α_{S2}-CN, β-CN and κ-CN. (Signal peptides were considered for the calculation of sequence coverage).

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Table 1. Theoretical tryptic peptide fragments of the main genetic variants of bovine milk caseins, α_{S1}-CN B-8P, α-$_{S2}$-CN A-11P, β-CN A^2-5P and κ-CN A-1P (P is for phosphate group), in the MW range 500–3000 Da

Protein	From	To	Sequence	m/z (MH^+)	Modification
α_{S1}-CN	3	18	LLILTCLVAVALARPK	1694.08	–
	23	37	HQGLPQEVLNENLLR	1759.95	–
	38	49	FFVAPFPEVFGK	1384.73	–
	52	57	VNELSK	689.38	–
	58	73	DIGSESTEDQAMEDIK	1927.69	pSer61, 63
	74	94	QMEAESISSSEEIVPNSVEQK	2720.91	pSer79, 81, 82, 83, 90
	95	98	HIQK	525.31	–
	99	105	EDVPSER	831.38	–
	106	115	YLGYLEQLLR	1267.71	–
	121	134	VPQLEIVPNSAEER	1660.79	pSer130
	135	139	LHSMK	615.33	–
	140	147	EGIHAQQK	910.47	–
	148	166	EPMIGVNQELAYFYPELFR	2316.14	–
	209	214	TTMPLW	748.37	–
α_{S2}-CN	3	16	FFIFTCLLAVALAK	1556.89	–
	17	36	NTMEHVSSSEESIISQETYK	2618.91	pSer23, 24, 25, 31
	40	47	NMAINPSK	874.45	–
	48	56	ENLCSTFCK	1044.45	–
	57	60	EVVR	502.30	–
	86	91	ITVDDK	690.37	–
	92	95	HYQK	575.29	–
	96	106	ALNEINQFYQK	1367.70	–
	107	128	FPQYLQYLYQGPIVLNPWDQVK	2709.41	–
	130	140	NAVPITPTLNR	1195.68	–
	141	151	EQLSTSEENSK	1411.50	pSer144, 146
	153	164	TVDMESTEVFTK	1466.61	pSer158
	168	173	LTEEEK	748.37	–
	176	180	LNFLK	634.39	–
	182	185	ISQR	503.29	–
	189	196	FALPQYLK	979.56	–
	197	203	TVYQHQK	903.47	–
	204	212	AMKPWIQPK	1098.61	–
	215	220	VIPYVR	746.46	–
β-CN	3	16	VLILACLVALALAR	1438.92	–
	17	40	ELEELNVPGEIVESLSSSEESITR	2966.17	pSer30, 32, 33, 34
	48	63	FQSEEQQQTEDELQDK	2061.83	pSer50
	115	120	EAMAPK	646.32	–
	123	128	EMPFPK	748.37	–
	185	191	VLPVPQK	780.50	–
	192	198	AVPYPQR	830.45	–
	199	217	DMPIQAFLLYQEPVLGPVR	2186.17	–
	218	224	GPFPIIV	742.45	–
κ-CN	38	42	FFSDK	643.31	–
	46	55	YIPIQYVLSR	1251.71	–
	90	107	SPAQILQWQVLSNTVPAK	1980.09	–
	108	118	SCQAQPTTMAR	1193.54	–
	119	132	HPHPHLSFMAIPPK	1608.85	–
	134	137	NQDK	504.24	–

S1(a)). Sequence coverage shows that the in-solution proteomic approach gives a relatively good characterization of protein mixture of moderate complexity. Without phosphopeptide enrichment, only 3 (Table 2) out of the 25 expected phosphorylation sites (Table 1) could be assigned by this method: ^{130}pSer of α_{S1}-CN represented by two tryptic peptides, 119–124 (MH^{2+} m/z 830.94) and 121–134 (MH^{3+} m/z 651.36), ^{158}pSer of α_{S2}-CN represented by tryptic peptide

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153–164 (MH^{2+} m/z 733.84) and ^{50}pSer of β-CN based on the sequencing of doubly charged (MH^{2+} m/z 1031.47) peptide fragment 48–63.

After enrichment using ZrO_2, seven phosphopeptides and four non-phosphopeptides were detected, indicating that the complexity of the mixture is reduced a great deal and also that the mixture was selectively enriched in phosphopeptides (Table 2, Supplementary Table S1(b)). In the case of α_{S1}-CN, phosphorylation of ^{130}Ser residue was confirmed. Moreover, two additional phosphorylation sites of α_{S1}-CN (^{61}pSer and ^{63}pSer) were identified by sequencing the peptide 58–73 (MW 1767.84 Da). Two isoforms of peptide 58–73 were detected: one of them with ^{61}Ser phosphorylated (^{58}DIGpSESTEDQAMEDIK^{73}, MH^{2+} m/z 924.36) and the other one with two phosphoserines (^{58}DIGpSEpSTEDQAMEDIK^{73}, MH^{2+} m/z 964.35). The peptide 74–94 of α_{S1}-CN containing five expected phosphoserines was not detected.

One phosphorylation site (^{158}pSer) was determined for α_{S2}-CN (fragment 153–164, oxidized ^{156}Met, MH^{2+} m/z 741.80) and one for β-CN (^{50}pSer, fragment 48–63, MH^{2+} m/z 1031.42 and MH^{3+} m/z 687.94), while no phosphorylated peptide of κ-CN could be identified.

Using a similar approach (i.e. TiO_2 enrichment in combination with nanoflow HPLC-ESI/MS/MS) for the study of a mixture containing seven proteins present in equimolar ratio and containing three casein standards (α_{S1}-CN, α_{S2}-CN and β-CN), Larsen *et al*. has identified seven casein tryptic phosphopeptides (i.e. 121–134, 119–134, 52–73 of α_{S1}-CN; two isoforms of 141–51 and 153–164 of α_{S2}-CN; and 33–48 of β-CN) and their corresponding ten phosphorylation sites.[24] The main difference between the two experiments is that in our work not a standard protein mixture but a biological sample (milk precipitate) containing the individual proteins in natural concentration ratio was investigated. Similar to our experiments, Ellegard *et al*. has analysed casein tryptic phosphopeptides from cow's milk and enriched by a process-scale anion-exchange chromatography and separated by RP-HPLC into 13 fractions. Using *N*-terminal sequencing and MALDI-MS performed off-line on the RP-HPLC separated fractions, nine phosphopeptides could be detected (58–73 of α_{S1}-CN; 16–36, 17–36, two isoforms of 61–85 of α_{S2}-CN; two isoforms of 16–40, 45–63, 48–63 of β-CN).[38] Successful determination of the site of phosphorylation, however, in this case was limited to a few cases by the low sensitivity of *N*-terminal sequencing.

Table 2. Phosphopeptides of bovine milk caseins characterized using nanoflow HPLC-ESI-MS/MS data directed acquisition (survey scan) and SELDI-MS/MS

Protein	From	To	Sequence	m/z	$\Delta m/z$	Charge	Miss. cleav.	Modification	Score	Sample
HPLC-ESI-MS/MS										
α_{S1}-CN	58	73	K.DIGSESTEDQAMEDIK.Q	964.34	0.00	2	0	pSer61,63	32	*a.e.*
α_{S1}-CN	58	73	K.DIGSESTEDQAMEDIK.Q	924.36	−0.01	2	0	pSer61	47	*a.e*
α_{S1}-CN	119	134	K.YKVPQLEIVPNSAEER.L	651.35	0.09	3	1	pSer130	49/38	*b.e./a.e.*
α_{S1}-CN	121	134	K.VPQLEIVPNSAEER.L	830.94	0.07	2	0	pSer130	35/73	*b.e./a.e.*
α_{S2}-CN	153	164	K.TVDMESTEVFTK.K	733.84	0.06	2	0	pSer158	75	*b.e.*
α_{S2}-CN	153	164	K.TVDMESTEVFTK.K	741.80	−0.01	2	0	pSer158, oxMet156	21	*a.e*
β-CN	48	63	K.FQSEEQQQTEDELQDK.I	1031.46	0.10	2	0	pSer50	31/65	*b.e./a.e.*
β-CN	48	63	K.FQSEEQQQTEDELQDK.I	687.94	−0.01	3	0	pSer50	18	*a.e*
SELDI-MS/MS										
α_{S1}-CN	58	73	K.DIGSESTEDQAMEDIK.Q	1927.68	0.01	1	0	pSer61,63	56	*a.e.*
α_{S1}-CN	119	134	K.YKVPQLEIVPNSAEER.L	1853.89	0.07	1	1	PL Ser130	76/58	*b.e./a.e.*
α_{S1}-CN	119	134	K.YKVPQLEIVPNSAEER.L	1951.89	0.05	1	1	pSer130	72/55	*b.e./a.e.*
α_{S1}-CN	121	134	K.VPQLEIVPNSAEER.L	1562.79	0.02	1	0	PL Ser130	109/61	*b.e./a.e.*
α_{S1}-CN	121	134	K.VPQLEIVPNSAEER.L	1660.79	0.01	1	0	pSer130	46/77	*b.e./a.e.*
α_{S1}-CN	128	134	V.PNSAEER.L	882.29	0.03	1	n.s.	pSer130	26	*a.e.*
β-CN	16	40	A.RELEELNVPGEIVESL-SSSEESITR.I	2731.29	0.06	1	n.s.	PL Ser30, 32,33,34	82	*a.e.*
β-CN	16	40	A.RELEELNVPGEIVESL-SSSEESITR.I	2828.27	0.05	1	n.s.	pSer30, PL Ser32,33,34	55	*a.e.*
β-CN	16	40	A.RELEELNVPGEIVESL-SSSEESITR.I	2926.19	0.11	1	n.s.	pSer30,32, PL Ser33,34	53	*a.e.*
β-CN	48	63	K.FQSEEQQQTEDELQDK.I	1963.79	0.05	1	0	PL Ser50	78	*a.e.*
β-CN	48	63	K.FQSEEQQQTEDELQDK.I	2061.79	0.03	1	0	pSer50	62/70	*b.e./a.e.*

m/z (Th) are values measured in survey scan.

$\Delta m/z$ are differences between measured and calculated m/z values.

Score refers to Mascot MS/MS score.

Sample refers to sample before enrichment = *b.e.* and after enrichment = *a.e.* procedure.

n.s. indicates non-specific cleavage.

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α_{S1}–CN, sequence coverage: 38.8%

```
  1 MKLLILTCLV AVALARPKHP IKHQGLPQEV LNENLLRFFV APFPEVFGKE
 51 KVNELSKDIG SESTEDQAME DIKQMEAESI SSSEEIVPNS VEQKHIQKED
101 VPSERYLGYL EQLLRLKKYK VPQLEIVPNS AEERLHSMKE GIHAQQKEPM
151 IGVNQELAYF YPELFRQFYQ LDAYPSGAWY YVPLGTQYTD APSFSDIPNP
201 IGSENSEKTT MPLW
```

α_{S2}-CN, sequence coverage: 15.3%

```
  1 MKFFIFTCLL AVALAKNTME HVSSSEESII SQETYKQEKN MAINPSKENL
 51 CSTFCKEVVR NANEEEYSIG SSSEESAEVA TEEVKITVDD KHYQKALNEI
101 NQFYQKFPQY LQYLYQGPIV LNPWDQVKRN AVPITPTLNR EQLSTSEENS
151 KKTVDMESTE VFTKKTKLTE EEKNRLNFLK KISQRYQKFA LPQYLKTVYQ
201 HQKAMKPWIQ PKTKVIPYVR YL
```

β-CN, sequence coverage: 43.7%

```
  1 MKVLILACLV ALALARELEE LNVPGEIVES LSSSEESITR INKKIEKFQS
 51 EEQQQTEDEL QDKIHPFAQT QSLVYPFPGP IPNSLPQNIP PLTQTPVVVP
101 PFLQPEVMGV SKVKEAMAPK HKEMPFPKYP VEPFTESQSL TLTDVENLHL
151 PLPLLQSWMH QPHQPLPPTV MFPPQSVLSL SQSKVLPVPQ KAVPYPQRDM
201 PIQAFLLYQE PVLGPVRGPF PIIV
```

κ-CN, sequence coverage: 8.9%

```
  1 MMKSFFLVVT ILALTLPFLG AQEQNQEQPI RCEKDERFFS DKIAKYIPIQ
 51 YVLSRYPSYG LNYYQQKPVA LINNQFLPYP YYAKPAAVRS PAQILQWQVL
101 SNTVPAKSCQ AQPTTMARHP HPHLSFMAIP PKKNQDKTEI PTINTIASGE
151 PTSTPTTEAV ESTVATLEDS PEVIESPPEI NTVQVTSTAV
```

Figure 3. Amino acid sequence and sequence coverage based on peptide mass fingerprint and obtained by SELDI-MS on caseins α_{S1}-CN, α_{S2}-CN, β-CN and κ-CN. (Signal peptides were considered for the calculation of sequence coverage).

SELDI-MS/MS for the determination phosphorylation sites of caseins

Total tryptic digest of caseins was analysed also by SELDI mass spectrometry after desalting the sample on the surface of the normal phase protein chip (NP-20). Single-stage SELDI mass spectrum (Fig. 4(a)) yields intense peaks corresponding to the protonated molecular ions of a number of expected tryptic peptides (Table 1). On the basis of peptide mass fingerprints sequence coverage, 38.8, 15.3, 43.7 and 8.9% were obtained for α_{S1}-CN, α_{S2}-CN, β-CN and κ-CN, respectively (Fig. 3). CID MS/MS spectra taken on the most abundant ions led to the identification of 15, 4, 7 and 2 peptides for α_{S1}-CN, α_{S2}-CN, β-CN and κ-CN, respectively (Supplementary Table S2(a)). Only two phosphorylation sites could be assigned without enrichment: ^{130}Ser of α_{S1}-CN and ^{50}Ser of β-CN.

Figure 4(b) shows the SELDI single-stage mass spectrum of casein after ZrO_2 affinity enrichment. Among the 19 most abundant precursor ions selected for MS/MS experiment, 11 were identified as phosphopeptides or peptides with the prompt loss of phosphoric acid (Table 2, Supplementary Table S2(b)). Interestingly, in the SELDI mass spectrum molecular ions of peptides deriving from the prompt in-source loss of the phosphoric acid (P + PL) were also observed. This is not surprising because a hot matrix (HCCA) was used. Cleavage of the phosphate group through the neutral loss of a phosphoric acid results in the formation of a dehydroalanine residue, corresponding to a 97.977 Th mass difference compared to the phosphorylated peptide. In the case of α_{S1}-CN, three of the eight expected phosphorylation sites could be determined: fragments 119–134 (Fig. 5) and 121–134 account for pSer130 (also identified without enrichment). These peptides were detected both as phosphorylated (Fig. 5(a)) and non-phosphorylated forms, together with the corresponding peaks showing the loss of phosphoric acid (Fig. 5(b)). In addition, precursor ion with m/z 882.32 was sequenced and identified as phosphopeptide fragment 128–134 from α_{S1}-CN. This peptide was generated from a non-specific cleavage between residues 127V and 128P. (Chemical instability of XP peptide bonds is known from the literature). Two additional phosphorylation sites of α_{S1}-CN, Ser61 and Ser63 were revealed by sequencing precursor ion MH^+ m/z 1927.69. The phosphorylated peptide 74–94 of α_{S1}-CN containing five phospho-serine (MH^+ m/z 2722.41) was not identified by this method.

In the case of β-CN, SELDI-MS/MS sequencing confirms all the five known phosphorylation sites (Ser30, Ser32, Ser33, Ser34, and Ser50) distributed in two peptides, ^{16}RELEELNVPGEIVEpSLpSpSpSEESITR^{39} and ^{48}FQpSEEQQQTEDELQDK^{63}. Phosphorylation on Ser50 was confirmed by the identification of the phosphorylated tryptic peptide 48–63 together with P + PL form ($MH+$ m/z 2061.82 and 1963.84, respectively). Four phosphorylated residues (Ser30, 32, 33 and 34) were present in peptide ^{16}RELEELNVPGEIVESLSSSEESITR^{40} generated from an unspecific cleavage between residues A15 and R16. We detected various forms of this peptide: the peak corresponding to the loss of all four phosphate groups (4Phospho + PL, MH^+ m/z 2731.35) together with the partial losses of phosphate moieties (2Phospho and 2Phospho + PL, MH^+ m/z 2926.19 and 1Phospho and 3Phospho +

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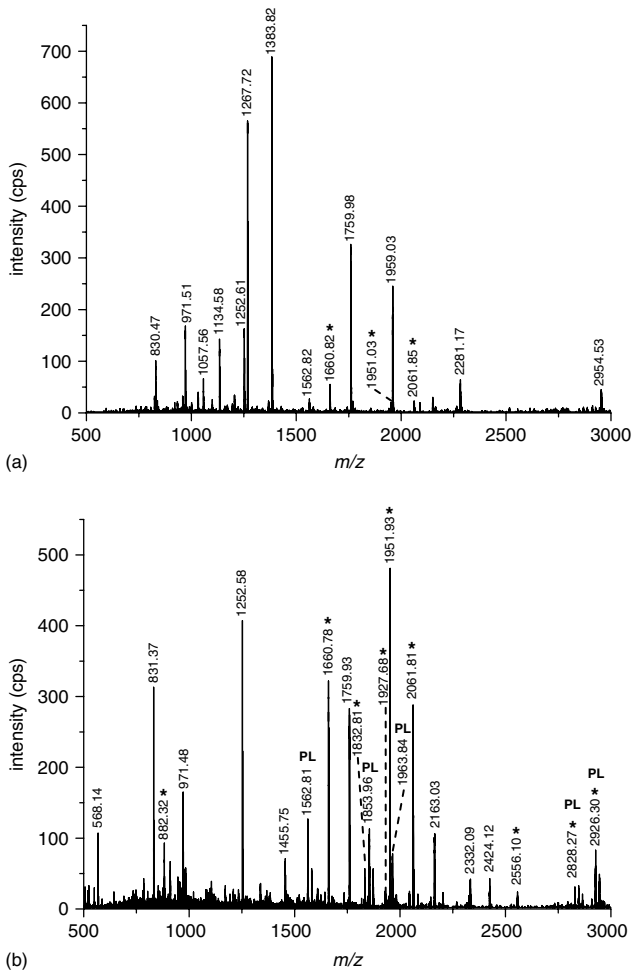

Figure 4. SELDI single-stage mass spectra of tryptic digest of bovine caseins (a) before and (b) after affinity enrichment using ZrO_2.

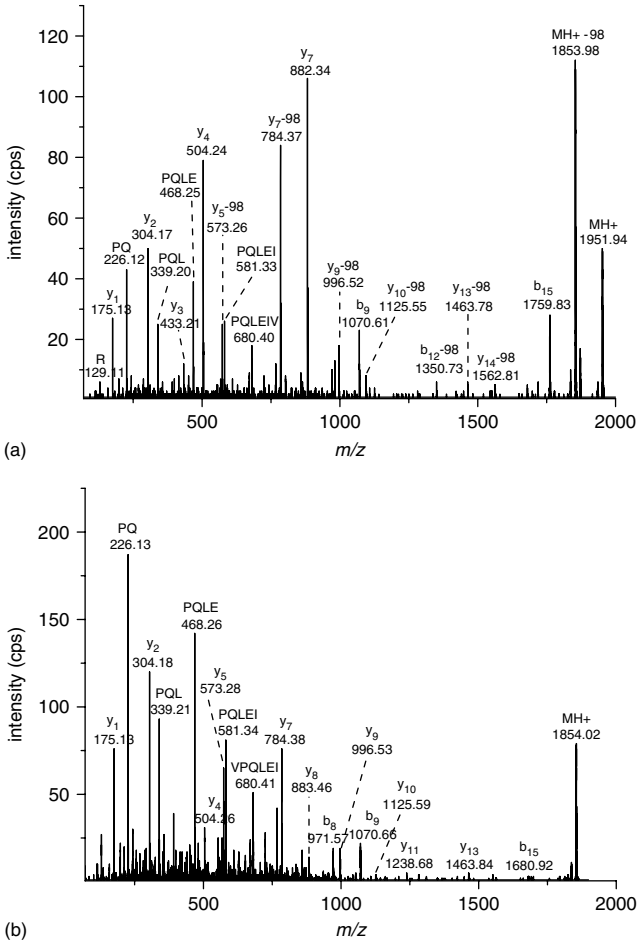

Figure 5. SELDI-MS/MS spectra of (a) phosphopeptide fragment 119–134, MH^+ 1951.94 and (b) peptide, MH^+ 1854.02 corresponding to the same fragment 119–134 after an in-source neutral phosphoric acid loss (Phospho + PL) resulting dehydroalanine at position 130.

PL MH^+ m/z 2828.27). By this method no phosphopeptides were detected for the two minor casein components, κ-CN and α_{S2}-CN. These proteins are present in low concentrations compared to α_{S1}- and β-caseins and are characterized by one major and several minor components with complex post-translational phosphorylation pattern and intra- and intermolecular disulfide bridges.[34] In addition, in the case of κ-CN the only expected phosphorylation site (Ser148) is located in a part of the protein where the number of tryptic cleavage sites is limited, resulting in a theoretical tryptic peptide containing the phosphate group with high mass (above 5000 Da) which cannot be detected using SELDI ionization on a QqTOF instrument.

DISCUSSION

Titanium dioxide-based enrichment coupled on-line to HPLC-ESI-MS/MS[13–16] or off-line to MALDI-MS[23,24] has been employed in a number of works for the selective isolation and analysis of phosphopeptides. Recently, Kweon *et al*. has shown that zirconium dioxide also has similar properties but higher selectivity compared to TiO_2 for the isolation of singly phosphorylated peptides.[26] Owing to the anion-exchange properties and extreme chemical, mechanical and thermal stability, ZrO_2 is often used as stationary phase in liquid chromatography. In this work, efficient enrichment in phosphopeptides was achieved by the use of zirconium dioxide and DAP as eluting buffer as was demonstrated both by nanoflow HPLC-ESI- and SELDI-MS/MS experiments. Using the same casein tryptic digest from bovine milk, the two techniques resulted in similar protein sequence coverage. By SELDI-MS/MS a larger number of phosphorylation sites were identified compared to nanoflow HPLC-ESI-MS/MS. Our results demonstrate that SELDI-MS/MS used in combination with ZrO_2-based enrichment method is a promising approach for the detection of phosphopeptides as well as for the identification of phosphorylation sites in simple mixtures. It is important to note that most of the peptides analysed originated form non-specific cleavages (Supplementary Tables S1 and S2). This could be explained by the fact that raw milk contains proteases[40] (plasmin, plasminogen, acid milk proteases, aminopeptidase, etc.) which can yield the formation of non-tryptic peptides, and bioactive peptides[39] as well. Some of these peptides naturally present in milk may co-purify with the caseins. Additional peptides may be formed by the low chymotryptic activity of trypsin.[41] Cleavage of the

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acid-sensitive Xxx-Pro peptide bonds can also occur during the protein isolation and digestion processes. In the case of SELDI, we observed abundant ions corresponding to the loss of phosphoric acid from phosphopeptides as well. This can be explained by the higher energy deposition by laser desorption/ionization, leading to the cleavage of phosphate group during ionization. Such a side reaction was not observed in the case of electrospray ionization.

The possibility to make fast *in situ* purifications makes SELDI-MS/MS an attractive alternative to previously described MALDI- or ESI-based approaches for the study of phosphoproteins. However, determination of phosphorylation sites of low-abundant proteins present in complex mixture, which is the task in phophoproteomics applications, still remains challenging.

Supplementary material

Supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1076-5174/suppmat/.

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