ELSEVIER

Journal of Chromatography A, 853 (1999) 225–235

JOURNAL OF CHROMATOGRAPHY A

Phosphopeptide analysis by on-line immobilized metal-ion affinity chromatography–capillary electrophoresis–electrospray ionization mass spectrometry

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Abstract

The analysis of large phosphoproteins by mass spectrometry is a particular challenge, in many cases, because of the small proportion of phosphopeptides in the presence of a large number of non-phosphorylated peptides. In addition, phosphopeptides are generally available in dilute solutions. Thus, methods to specifically identify phosphopeptides at low concentrations are important. In this work, on-line Fe(III) immobilized metal-ion affinity chromatography (IMAC)–CE–electrospray ionization MS was developed and applied to sub-pmol analysis of phosphopeptides. Phosphopeptides bind Fe(III) with high selectivity. The IMAC resin is packed directly at the head of the CE column. After the phosphopeptides are bonded to the resin and washed, they are eluted at high pH and separated by CE. This method has several advantages: (1) selective retention and pre-concentration of phosphopeptides on an Fe(III)-IMAC resin; (2) a pre-wash of the sample to remove salts and buffers that are not suited for CE separation or ESI operation; (3) facile fabrication with common tools and chemicals (less than 10 min); (4) adaptation to commercial CE instruments without any modifications. The applications of IMAC–CE–MS are demonstrated by the analysis of phosphopeptide mixtures and a phosphoprotein digest.

Keywords: Immobilized metal affinity chromatography–CE–ESI-MS; Phosphopeptides; Peptides; Phosphoproteins; Proteins

1. Introduction

The study of protein phosphorylation has grown exponentially in recent years as researchers from various disciplines have come to realize that key cellular functions are regulated by protein phosphorylation and dephosphorylation [1–5]. Protein phosphorylation reactions have been clearly established as major components of metabolic regulation and signal transduction pathways [3–5]. To understand more about the function of protein phosphorylation and dephosphorylation, it is necessary to identify the specific amino acid residues that become phosphorylated. Currently, the most common method used for the analysis of protein phosphorylation relies largely on two-dimensional (2D) phosphopeptide mapping of isolated proteins which have been phosphorylated with a radiolabel either via in vitro kinase reactions, or in vivo following metabolic labeling of cells [6–11]. However, in addition to the precautions required when working with radioactivity, this method may suffer from incomplete incorporation of ^{32}P.

Mass spectrometry (MS) has revolutionized biological molecular analysis over the past decade [12,13]. Recently, there has been a significant in-

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00481-1

crease in the development of MS-based methods for the analysis of protein phosphorylation. These include electrospray ionization (ESI) MS [14,15] and matrix-assisted laser desorption (MALDI) time-of-flight (TOF) MS [16–19]; however, the analysis of large phosphoproteins by mass spectrometry is still a particular challenge, primarily due to the often low stoichiometry of phosphorylation observed on peptides of interest in the presence of a large number of non-phosphorylated peptides. Therefore, a method to specifically identify phosphopeptides is valuable. Immobilized metal-ion affinity chromatography (IMAC) has been shown to be useful for this purpose since immobilized Fe(III) ions selectively retain and pre-concentrate phosphorylated proteins and peptides [20–26]. However, results from this laboratory and others show that direct coupling of Fe(III)-IMAC columns with MS detection results in the simultaneous detection of all phosphopeptide species eluted from the column, with often inadequate detection limits. The optimal conditions for the elution of phosphopeptides from the IMAC column and their detection by ESI-MS are not compatible leading to the lower detection limits. For analysis of low amounts of material, on-line methods are desired because sample handling can be minimized. Recently, Watts, et al. reported the analysis of ZAP-70 phosphorylation sites by on-line IMAC–HPLC–ESI-MS [27]. They linked a microIMAC column and microbore HPLC in series by a switching valve. A detection limit of at least 250 fmol for a synthetic phosphopeptide standard was achieved using this system. In addition, off-line IMAC was reported from Aebersold's laboratory [28]. The phosphopeptides eluted from IMAC were manually collected before analysis using solid-phase extraction (SPE)–CE–MS–MS.

Capillary electrophoresis (CE) is the premier analytical separation method for biological compounds such as peptides, proteins, and polynucleotides due to its high separation efficiency and high resolution [29]. However, the small capillaries used in CE separations accommodate only small volumes of sample. As a consequence, concentration limits of detection (cLODs) are poor. In order to overcome the poor cLODs, several analyte concentration techniques have been developed including combining capillary isotachophoresis (cITP) with CE [30], transient isotachophoresis (tITP) in a single capillary [31,32], analyte stacking [33,34], and field amplification [35]. Such electrophoretic techniques have extended the applicability of CE for the analysis of dilute analyte solutions. More recently, the technique of analyte concentration using a bed of reversed-phase packing (e.g. C_{18}) [36–39] or a membrane [40–42] with reversed-phase properties have been reported. By use of this technique, a sample can be pre-cleaned on-line, therefore reducing sample handling and improving analyte recovery. With this technique, in-vivo derived metabolites, peptides, and proteins have been successfully analyzed [43].

Since Fe(III)-IMAC can selectively retain, and therefore pre-concentrate phosphorylated proteins and peptides, the advantages of integration of IMAC with CE–ESI-MS are clear. In this work, on-line IMAC–CE–ESI-MS was developed and applied to sub-pmol analysis of phosphopeptides. The utility of IMAC–CE–ESI-MS is demonstrated by the analysis of standard phosphopeptide mixtures and a phosphoprotein digest (β-casein).

2. Experimental

2.1. Chemicals

All chemicals were used without further purification. The High-Performance Liquid Chromatography Peptide Standard (Sigma, St. Louis, MO, USA) [composed of approximately 0.125 mg GY (M_r 238.2) and 0.5 mg each of VYV (379.5), methionine enkephalin (573.7), leucine enkephalin (555.6), and angiotensin II (1046.2)] was dissolved in 1 ml 0.1% acetic acid solution. Modified-trypsin was purchased from Promega (Madison, WI, USA). Methanol was purchased from B-J Baxter (Muskegon, MI, USA). Ferric chloride hexahydrate, ammonium hydroxide, and aminopropylsilane were purchased from Aldrich (Milwaukee, WI, USA). Acetic acid glacial was purchased from Fisher Chemical (Fair Lawn, NJ, USA). Zwittergent 3-16 detergent was purchased from Calibiochem-Novabiochem (La Jolla, CA, USA). Four phosphotyosine peptides (Table 1) were used as standards.

Tryptic digestion of β-casein (10 μ*M*) containing

Table 1
Phosphopeptide sequences

Molecular mass	Sequence
457.43	PpYV
703.65	GVpYAASG (Fragment of viral protein P85gag-fes)
1126.18	DRVpYIHPF (Human angiotensin II)
1586.71	TFLPVPEpYINOQSV (Primary autophosphorylation site of EGF receptor kinase)

five phosphoserine residues was performed in 100 m*M* NH_4HCO_3 by using an enzyme/substrate ratio of 1:50 (w/w). The digest was carried out for 12 h at 37°C.

2.2. IMAC–CE column

The in-capillary electrode sheathless interface was prepared according to the procedure previously reported [44]. A 75 cm×75 μm I.D. 150 μm O.D. capillary (Polymicro Technology, Phoenix, AZ, USA) derivatized with aminopropylsilane (APS) was employed for CE separation.

The IMAC column was made by packing fused-silica capillaries with Poros metal chelating beads (thereafter called beads) (particle size 20 μm, PerSeptive Biosystems, Cambridge, MA, USA). Prior to packing the capillary with beads, the beads were activated by loading Fe^{3+} ions as follows: (1) beads were mixed with 100 m*M* $FeCl_3$ in 1 *M* sodium acetate solution in a ratio of 1: 3 (v/v), centrifuged, and the top liquid layer was discarded; (2) Milli-Q water (Millipore, Bedford, MA, USA) was added to wash the beads until the top liquid layer turns colorless.

The inlet-end of a 75 cm×75 μm I.D. 150 μm

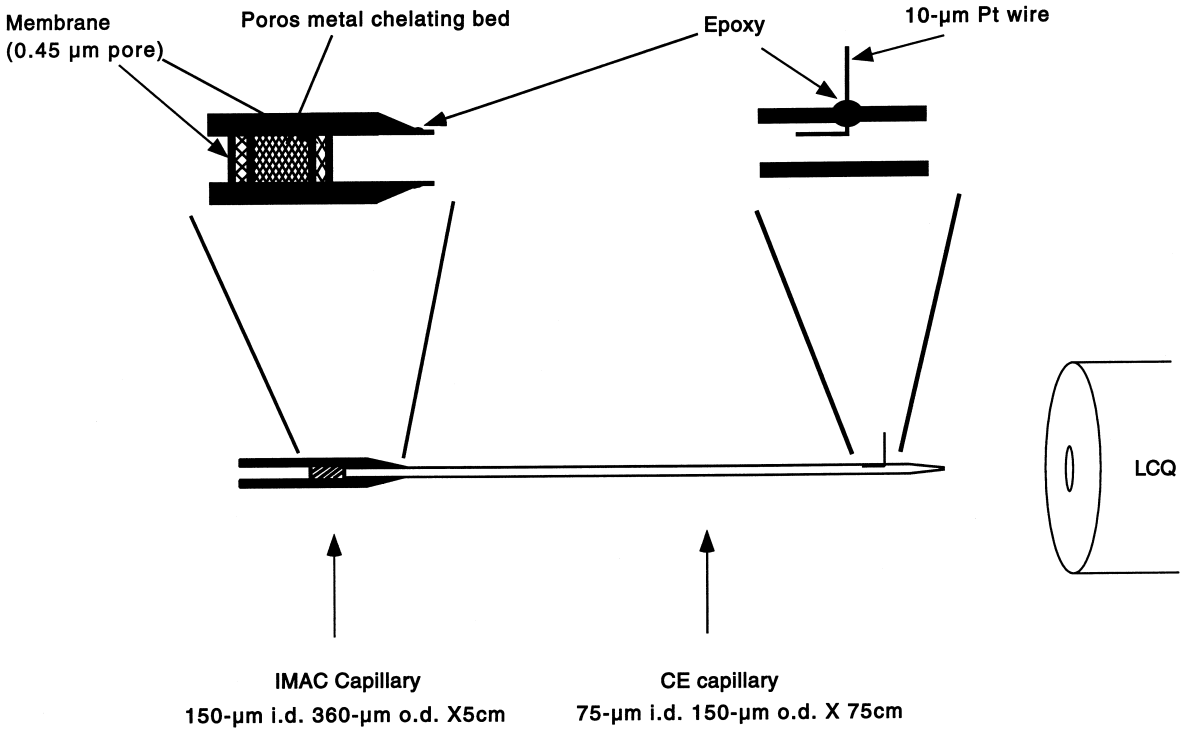

Fig. 1. A schematic of the on-line IMAC–CE–ESI-MS–(MS) apparatus.

O.D. APS treated fused-silica capillary (CE capillary) was inserted 1 cm into a 5 cm×150 μm I.D.×360 μm O.D. piece of fused-silica capillary (IMAC capillary), and two capillaries were connected with 5 min fast drying epoxy (Devcon Consumer Products, Danvers, MA, USA) (see Fig. 1). Monitored under a 40× magnification stereo microscope (Edmund Scientific, Barrington, NJ, USA), a small piece of Durapore [hydrophilic poly-(vinylidene difluoride) (PVDF)] low-protein-binding membrane (0.45 μm pore size, Millipore, Bedford, MA, USA) was manually put into the IMAC capillary and firmly pushed with a piece of 75 μm I.D. 150 μm O.D. capillary to the end which served as a frit. Then Fe^{3+}-activated Poros beads were pulled into the capillary by applying a pulse of vacuum on the opposite end of the capillary (ESI end). A bed of approximately 0.2 to 1.0 cm of beads was packed at the inlet end of CE capillary (30–180 nl). Finally, another piece of filter paper used as the terminating frit was put into the capillary. Total IMAC–CE assembly time (not including the fabrication of in-capillary electrode) is less than 10 min. An IMAC–CE capillary may accommodate 50 or more injections without significant deterioration of performance. When deterioration is noted, the CE portion of the column is retained and a new IMAC portion is constructed (as above).

The following buffers were used for IMAC–CE–ESI-MS operation:

Buffer 1 (CE running buffer): 0.1% acetic acid with 10% methanol.

Buffer 2: 0.02% acetic acid with 80% methanol.

Buffer 3 (eluting buffer): 30% methanol, 0.1% ammonium acetate adjusted to pH 9.6 with ammonium hydroxide.

Fig. 2 gives a summary of the IMAC–CE–ESI-MS–(MS) operation. CE and ESI conditions are discussed later.

2.3. CE/MS

A P/ACE System 2050 (Beckman Instruments, Fullerton, CA, USA) CE instrument operating with Beckman P/ACE Station software was employed in this study. There is no need to modify the instrument when using the IMAC–CE capillary. Buffer selection is controlled via software.

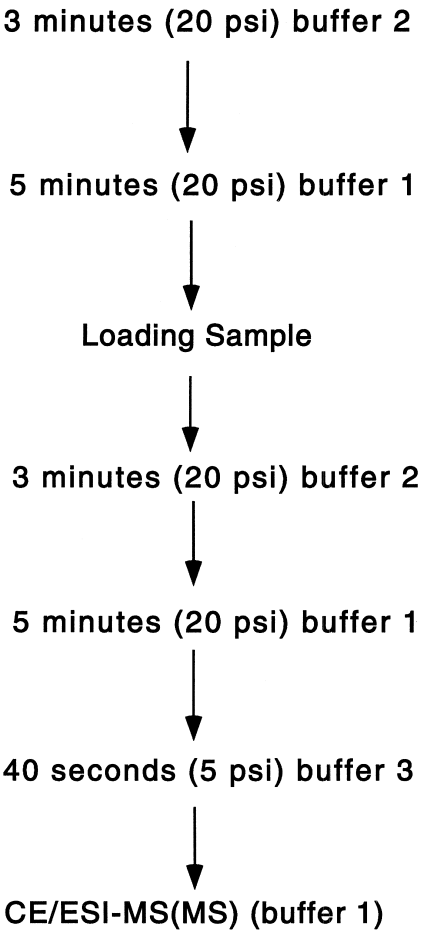

Fig. 2. Summary of the IMAC–CE–ESI-MS–(MS) operation.

An ion trap mass spectrometer (LCQ; Finnigan MAT, San Jose, CA, USA) was employed for these experiments. The CE capillary tip (see sheathless interface above) was positioned approximately 5 mm directly in front of the heated capillary of the Finnigan electrospray source. The electrospray voltage was ''on'' during sample loading and washing but no data were collected because the peptide concentration was normally below the detection limit. Data acquisition was initiated by a contact closure on the P/ACE instrument. The trap was run with automatic gain control (AGC) for all experiments to maintain the number of ions present in the trap at a constant, pre-selected value. The target number of ions was set to 5×10^7 in MS mode and 5×10^6 for MS–MS mode. In MS mode, the trap was

scanned from m/z 450–1500. Ions above a set intensity threshold were selected automatically for MS–MS. The peptides were fragmented with a relative collision energy of 35.

3. Results and discussions

To investigate the viability of on-line IMAC–CE–ESI-MS, a mixture of synthetic peptides was analyzed. Four phosphotyrosine-containing peptides, 500 fmol/μl each of a tripeptide (PpYV), a fragment of viral protein P85gag-fes (GVpYAASG), human angiotensin II (DRVpYIHPF), and the primary autophosphorylation site of EGF receptor kinase (TFLPVPEpYINQSV), were mixed with the Sigma HPLC peptide standard (390 fmol/μl of methionine enkephalin, 450 fmol/μl of leucine enkephalin, and 240 fmol/μl of angiotensin II). Approximately, a 5 μl of sample was loaded onto the column using high-pressure injection. CE separation was carried out at field strength of −133 V/cm with 5 p.s.i. back pressure applied to the capillary inlet (1 p.s.i.= 6894.76 Pa). The ESI voltage was set at +2.1 kV. Fig. 3 shows the selective ion electropherograms for this mixture. The phosphopeptides were effectively retained while the non-phosphopeptides were washed out with buffer. No phosphopeptides were observed in the flow-through (data not shown). Notably, the small phosphorylated tripeptide, which would not be effectively retainded by C_{18} resin in an HPLC separation, generated a strong, well defined peak (Fig. 3A). Doubly-charged molecular ions were formed for phosphopeptides DRVpYIHPF and TFLPVPEpYINQSV (m/z 563.8 and 794.4, respectively). Singly-charged molecular ions were formed for PpYV and GVpYAASG (m/z 458.4 and 704.6, respectively).

While this technique significantly enhances cLODs, the CE performance is compromised. The use of C_{18} or membrane often results in reduced analyte resolution, broader peaks, and substantial component tailing [45]. However, the data-dependent scan mode of MS makes this problem less significant. Even when two or more compounds show

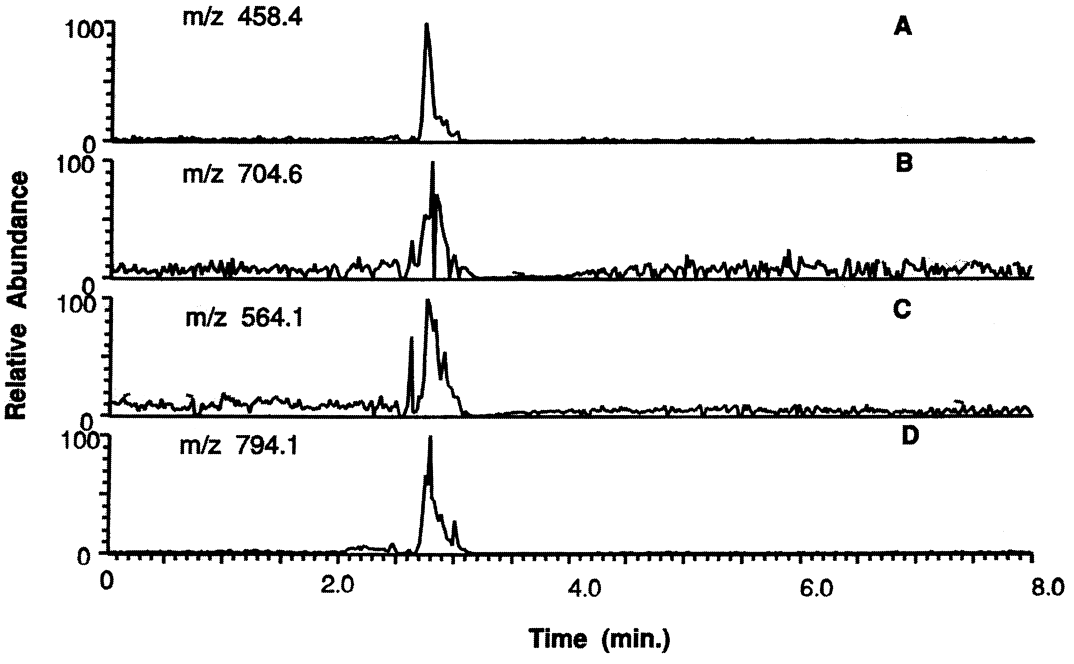

Fig. 3. Selected ion electropherograms for 5 μl injections of a standard peptide mixture. (A) Tripeptide (PpYV); (B) a fragment of viral protein P85gag-fes (GVpYAASG); (C) human angiotensin II (DRVpYIHPF); (D) the primary autophosphorylation site of EGF receptor kinase (TFLPVPEpYINQSV).

partial or complete co-elution, the MS–MS spectrum of each compound is still achievable.

The detection limits for IMAC–CE–ESI-MS were studied by analyzing the same standard peptide solution with serial dilutions. Fig. 4 shows the selected ion electropherograms of human angiotensin II under different concentrations. An 8 μl sample was loaded into the IMAC column (0.2 cm of beads). CE separation was run under field strength of −133 v/cm with 5 p.s.i. back pressure applied to the capillary inlet. The ESI voltage was set at +2.1 kV. Fig. 4C shows the signal for the injection 28 fmol of human angiotensin II. The signal to noise ratio is approximately 4 to 1, therefore, the detection limit of this system for human angiotensin II is approximately 28 fmol under the experimental conditions employed.

The application of on-line IMAC–CE–ESI-MS–MS to a more complex mixture of peptides was studied with a tryptic digest of the phosphoprotein β-casein. This digest yields 16 different peptides, two of which contain phosphoserine residues: the quadruply phosphorylated T1-2 (4P) and the single phosphorylated T6 (1P) as shown in Table 2. In previous work, the HPLC of β-casein digest, highly phosphorylated peptides, T1-2(4P), was detected in

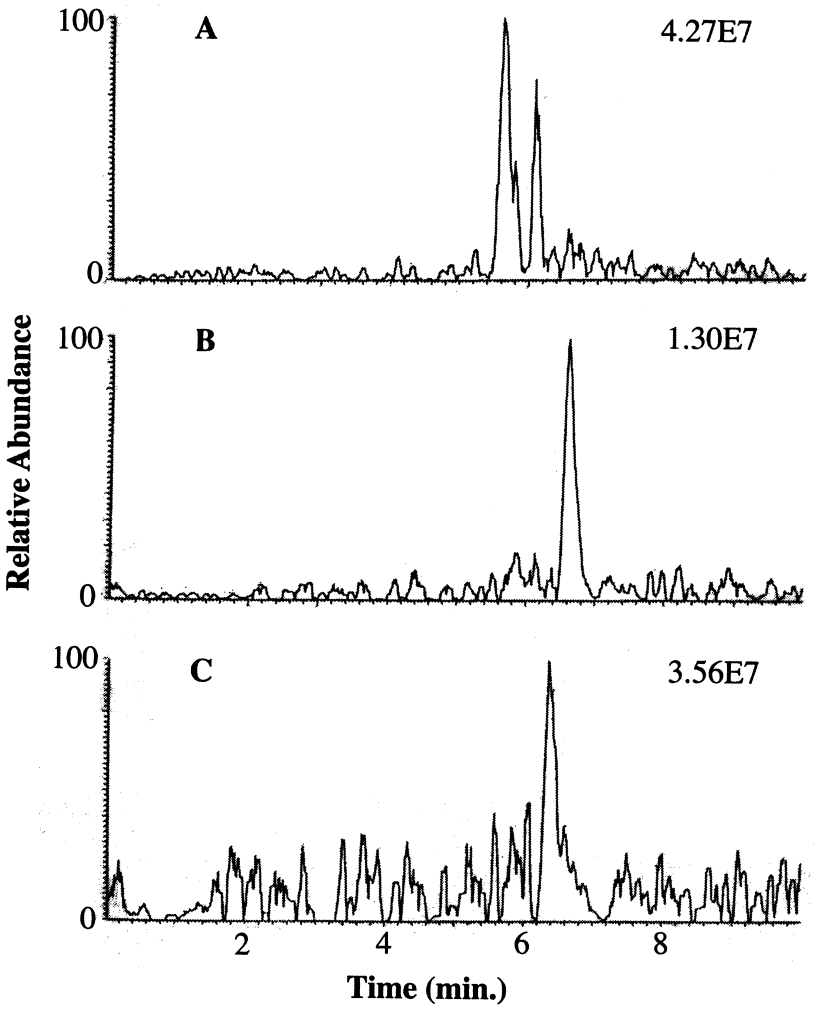

Fig. 4. Selected ion electropherograms for 8 μl injections of a standard peptide mixture at different dilutions. (A) 350 fmol/μl; (B) 35 fmol/μl; (C) 3.5 fmol/μl.

Table 2
Sequences of phosphopeptides in the β-casein tryptic digest

Label	From	To	M_r	Sequence
T1-2(4P)	1	25	3122.95	RELEELNVPGEIVEpSLpSpSpSEESITR
T6(1P)	33	48	2061.98	FQpSEEQQQTEDELQDK

very low abundance or not at all [26]. Fig. 5 shows the total ion electropherogram (TIE) and selected ion electropherograms of the β-casein digest by IMAC–CE–ESI-MS–MS. A 12 μl aliquot of a 20 fmol/μl tryptic digest β-casein (240 fmol each peptide) was loaded on the IMAC column. The CE separation was performed at −267 V/cm with 5 p.s.i. at the injection end for the first 20 min, then the pressure increased to 20 p.s.i.. From Fig. 5, the two phosphoserine peptides were effectively retained and separated by IMAC–CE–ESI-MS–MS. Non-phosphopeptide T12 was also retained by Fe^{3+} IMAC, possibly because it contains three histidine residues. However, T12 does not interfere with the separation and detection of the two phosphopeptides. Once the phosphopeptides are identified by mass, the precise location of the phosphate(s) within the peptide can be determined by tandem mass spectrometry (MS–MS). Fig. 6 gives the MS–MS spectrum of T6 (1P). In agreement with previous findings [46], the dominant ion correspond to loss 98 u (H_3PO_4) (m/z 982.7) and but a large series of b-type and y-type ions are

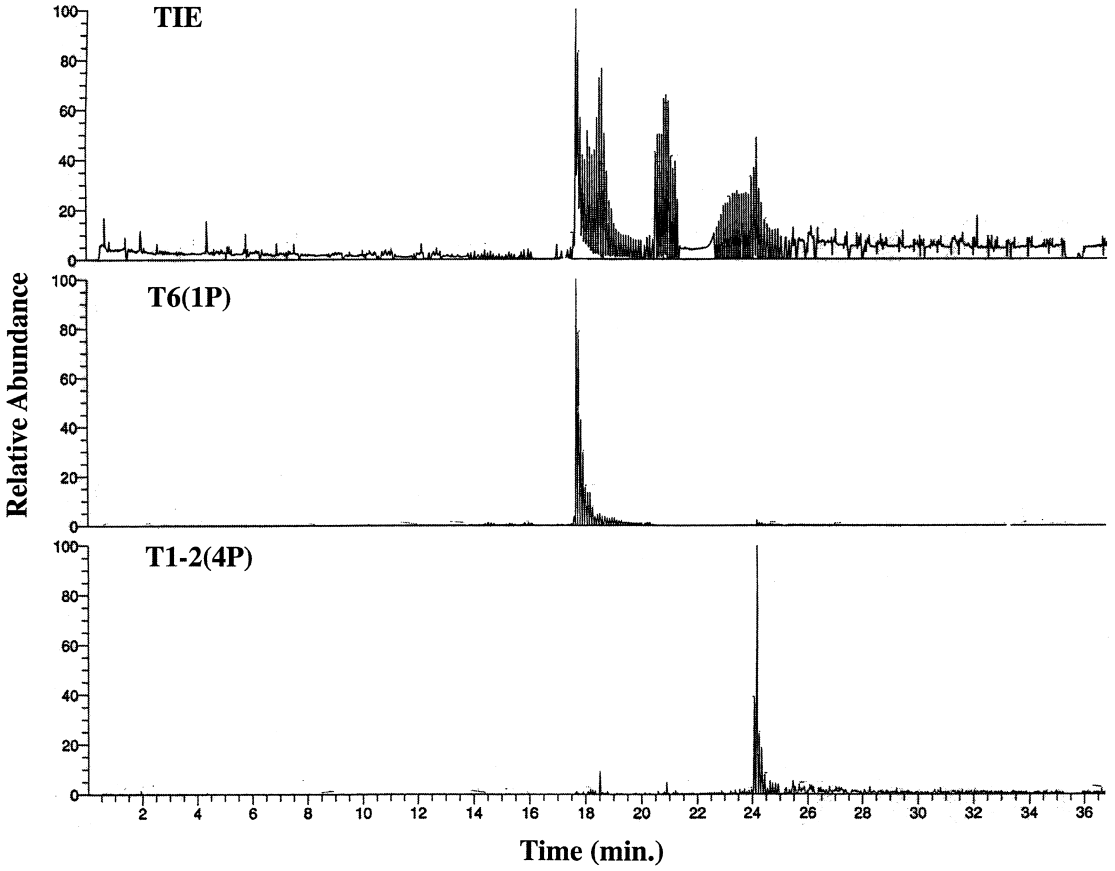

Fig. 5. Total ion electropherogram and selected ion electropherograms of 12 μl injections of 20 fmol/μl tryptic digest β-casein.

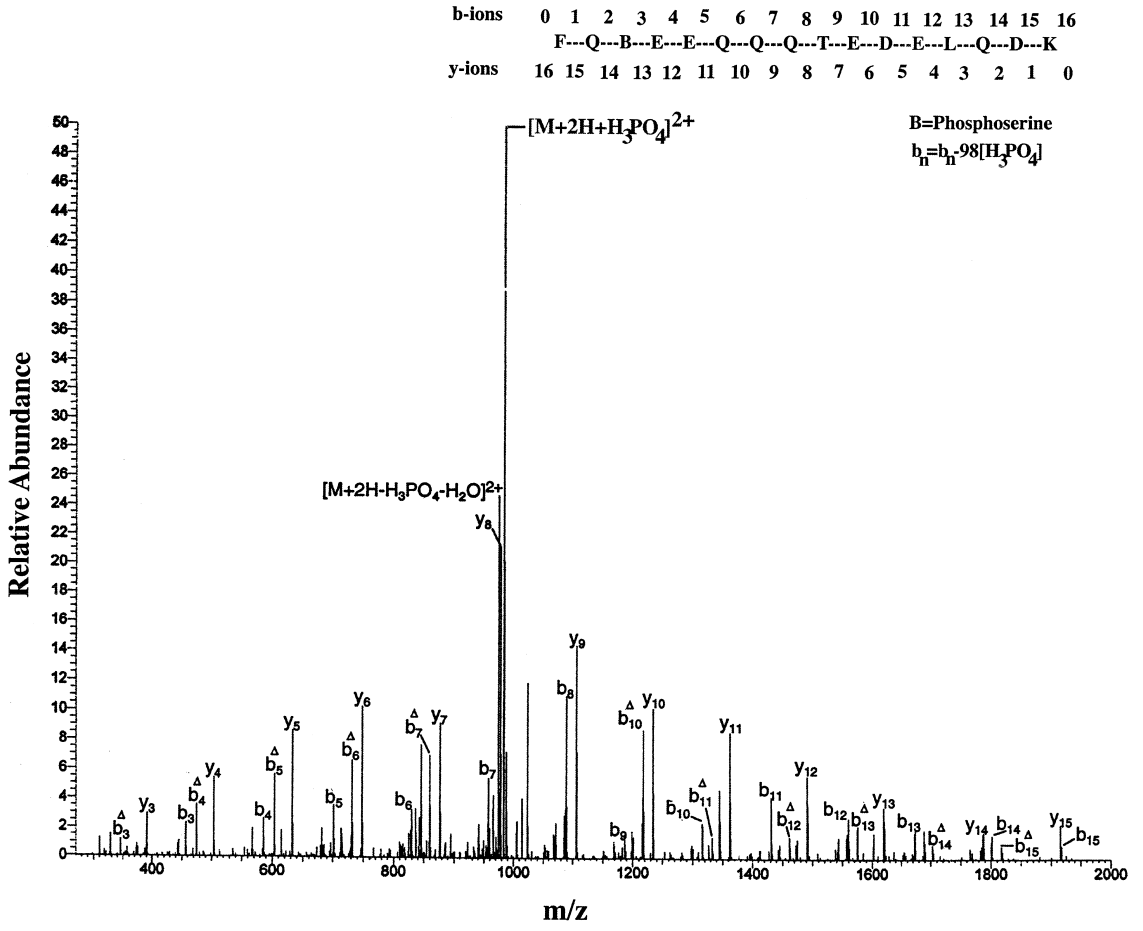

Fig. 6. MS–MS spectrum of T6(1P) of 12 μl injections of 20 fmol/μl tryptic digest β-casein.

also observed. The mass difference (168 u) between y_{13} and y_{14} corresponds to the molecular mass of phosphoserine and immediately locates the phosphorylation site.

Successful analysis of 10 μl of a 2 fmol/μl tryptic digest β-casein (20 fmol injected) was also achieved by IMAC–CE–ESI-MS–MS (data not shown). The β-casein digest described above demonstrates excellent sensitivity of IMAC–CE–ESI-MS. However, these results were obtained from dilutions of a large-scale digest and, as such, were relatively clean and free of salt or detergent and displayed high peptide recovery. In many instances, such as tryptic peptide solutions derived from in-gel digestions of proteins separated by 2D gel electrophoresis, higher concentration of salts and often contaminants are found, and peptide recovery is frequently poor. We and others have shown improved peptide recovery by the addition of detergent to the digestion buffer [47]. Therefore, we sought to demonstrate the analysis of phosphopeptides in the presence of detergent. Fig. 7 shows the selective ion electropherograms of 100 fmol/μl tryptic digests of β-casein with two different detergents. Approximately 10 μl sample was injected for each experiment. CE separation was carried out using a field strength of −267 V/cm with 5 P.S.I. backing pressure applied to the capillary inlet. The ESI voltage was set at +2.1. Fig. 7A corresponds to the normal detergent-free digest solution, Fig. 7B contains 0.5% zwittergent detergent (M_r 391.6), and Fig. 7C contains 0.1% sodium dodecylsulfate (SDS) in the solution. The peptide

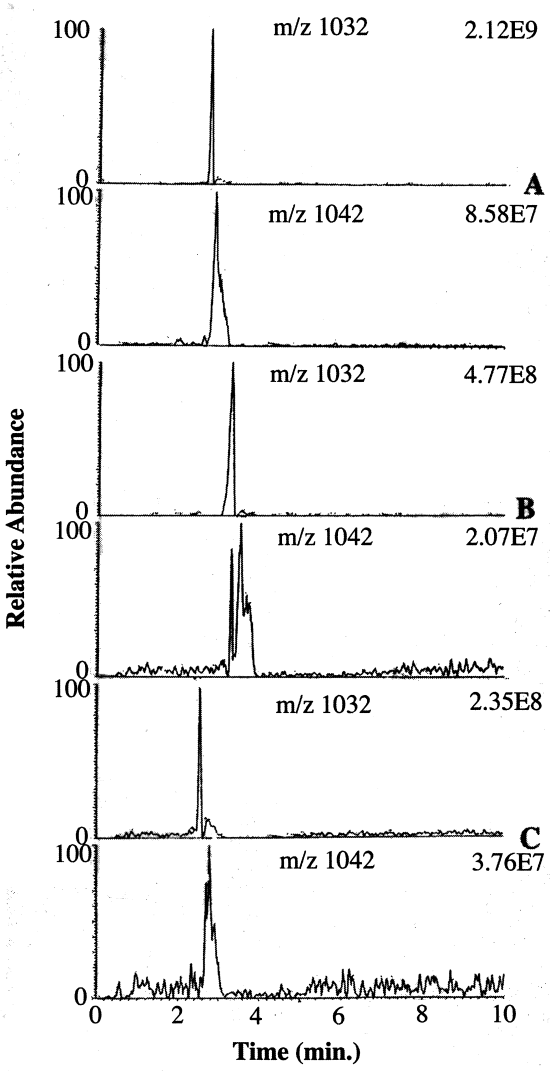

Fig. 7. Selected ion electropherograms for 10 μl injections of 100 fmol/μl tryptic digest of β-casein with different detergents. (A) Detergent free; (B) 0.1% SDS; (C) 0.5% zwittergent.

signal was still observed at high level detergent concentrations that would otherwise completely eliminate the electrospray signal. The mass spectra demonstrate that the 0.1% SDS in the solution did not show any significant background noise compared with the 0.5% zwittergent detergent which generated the m/z 783 background peak (zwittergent dimer). Fig. 8 shows the MS–MS of T1-2(4P) fragment of Fig. 7B. Loss of 98, 196, and 294 Da corresponding to one, two, and three H_3PO_4 groups (m/z 1008.8, 976.1, and 943.4, respectively) are strong fragments as well as b_8 and y_{17}^{+2} (cleavage at proline). Few other fragments were also observed.

The results described above demonstrated excellent sensitivity for the phosphopeptide analysis using IMAC–CE–ESI-MS. This work, however, was done with a large scale tryptic digest (10 pmol/μl). Analysis of in-gel tryptic digest of low-pmole levels of proteins is much more challenging and useful for real world samples. Experiments with phosphopeptides generated from low pmole in-gel protein digests are currently in progress. In addition, by collecting the non-phosphorylated peptides which are not bound to the IMAC resin and analyzing them using HPLC–ESI-MS and/or CE–ESI-MS, it should be possible to obtain an indication of the stoichiometry (relative levels of phosphorylation at each site). Knowledge of each phosphorylation site allows one to search specifically for its non-phosphorylated counterpart. Such studies are also underway.

4. Conclusions

In proteome-wide identification of proteins, the possibility to selectively enrich proteins with specific functional groups at low concentration level would be invaluable for the analysis of post-translational modifications at high sensitivity. This report demonstrates a fast, simple, and sensitive on-line Fe^{3+} IMAC–CE–ESI-MS–MS method for the determination of phosphopeptides. This method can selectively retain, pre-concentrate, and pre-wash the sample. In addition, it has high tolerance for detergents and can be applied to a wide range of samples at low concentration (demonstrated for $3.5 \cdot 10^{-9}$ M). In addition, facile fabrication with common tools and chemicals (less than 10 min) and easy adaptation to commercial CE instruments without any modifications make it a choice for the phosphopeptide analysis in laboratories.

Acknowledgements

The authors thank Dr. David Arnott for assistance with the mass spectrometry and Dr. Richard Vandlen for helpful discussions about IMAC.

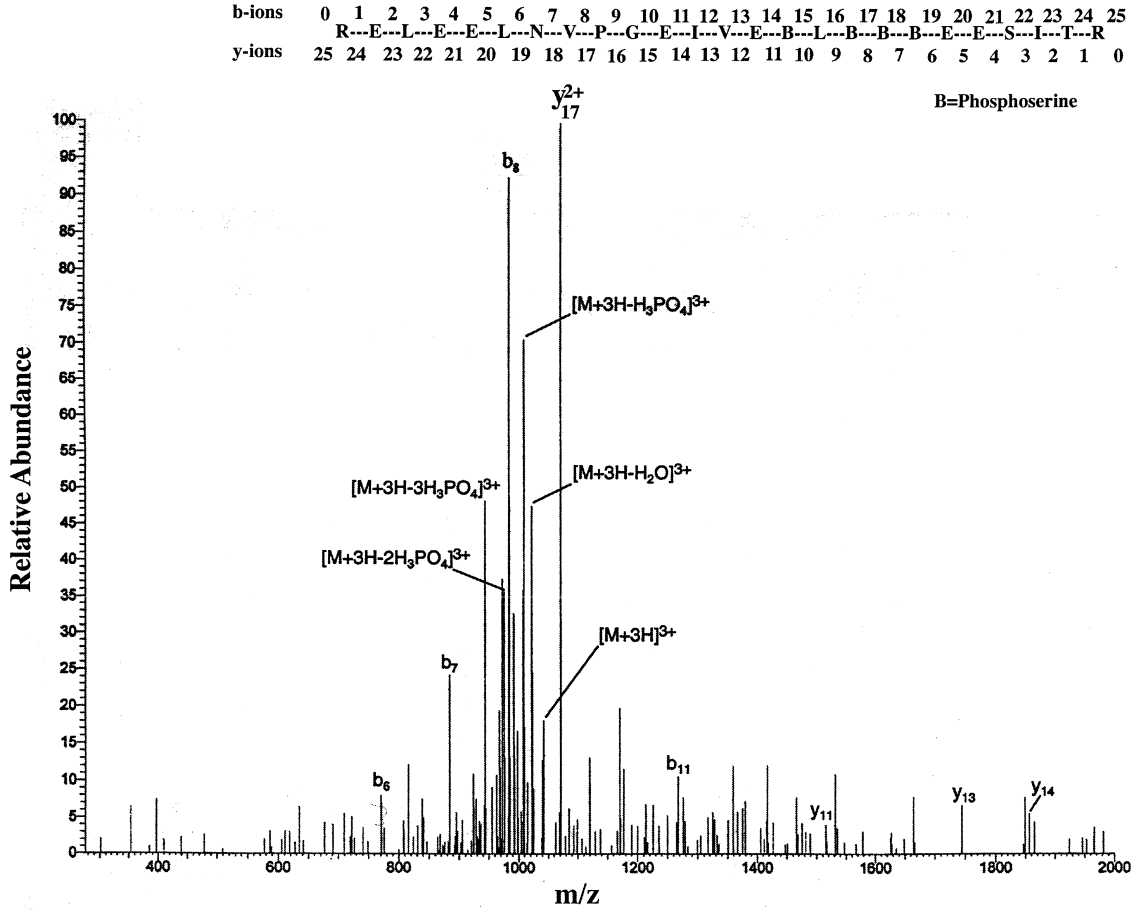

Fig. 8. MS–MS spectrum of T1-4(4P) of Fig. 7B.

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