Journal of Chromatography A, 1217 (2010) 8032–8040

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Iminodiacetic acid-modified magnetic poly(2-hydroxyethyl methacrylate)-based microspheres for phosphopeptide enrichment

Lenka Novotna[a,*], Tereza Emmerova[a,b], Daniel Horak[c], Zdenka Kucerova[a], Marie Ticha[a,b]

[a] Institute of Pathological Physiology and CEH, First Faculty of Medicine, Charles University in Prague, U Nemocnice 5, 128 53 Praha 2, Czech Republic
[b] Department of Biochemistry, Faculty of Science, Charles University in Prague, Albertov 2030, 128 40 Praha 2, Czech Republic
[c] Institute of Macromolecular Chemistry, AS CR, Heyrovsky Sq. 2, 162 06 Praha 6, Czech Republic

ARTICLE INFO

Article history:
Available online 16 September 2010

Keywords:
IMAC phosphopeptide separation
IDA-modified magnetic microspheres
Porcine pepsin A
Bovine α-casein
Bovine milk

ABSTRACT

Magnetic non-porous hydrophilic poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) microspheres prepared by the dispersion polymerization and modified with iminodiacetic acid (IDA) were employed for the IMAC separation of phosphopeptides. Fe^{3+} and Ga^{3+} ions immobilized on IDA-modified magnetic microspheres were used for the enrichment of phosphopeptides from the proteolytic digests of two model proteins differing in their physico-chemical properties and phosphate group content: porcine pepsin A and bovine α-casein. The optimum conditions for phosphopeptide adsorption and desorption in both cases were investigated and compared. The phosphopeptides separated from the proteolytic digests were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The ability of the prepared Fe^{3+}- and Ga^{3+}-IDA-modified magnetic microspheres to capture phosphopeptides from complex mixtures was shown on an example of bovine milk proteolytic digest.

1. Introduction

Post-translational modification of proteins is a fundamental regulatory mechanism, and characterization of protein modifications is primary task for understanding protein function. Protein phosphorylation is one of the most common of all protein modifications and has been found in nearly all cellular processes. Deregulation of protein kinases or phosphatases can result in oncogenesis. Identification of phosphorylation sites might be very important to understand disease states and from the pharmaceutical point of view [1–4]. MS analysis of the proteolytic digest of studied modified proteins represents one of the most powerful tools for the analysis of protein phosphorylation [5,6]. However, the results of analysis are largely influenced by low abundance of phosphorylated proteins, relatively low degree of phosphorylation and the reversible nature of the modification. It has been shown that pre-concentration or enrichment of phosphopeptides from a pool of non-phosphorylated peptides significantly improved phosphoprotein characterization.

IMAC originally introduced by Porath et al. [7] is one of the most frequently applied techniques used for this purpose nowadays [7–11]. Binding of non-phosphorylated peptides containing high numbers of acidic amino acid residues such as glutamic and aspartic acid represents the major limitation of this method [12–14]. Various immobilized metal ions besides Fe^{3+} have been examined, such as Ga^{3+}, Al^{3+}, or Zr^{4+}, for better selectivity and phosphopeptide recovery [15–18]. Results of the IMAC separation of phosphopeptides are influenced also by a nature of the used sorbent, coupled chelating compound, binding and elution conditions and significantly by the number and distribution of acidic residues and the phosphorylation sites of studied proteins.

The combination of magnetic and affinity separation has received considerable attention in recent years [19,20] and represents a perspective separation technique. In combination with MS analysis, magnetic bead-based fractionation of complex biological samples is a powerful tool in proteome profiling and can be used for biomarker discovery and identification [21–23]. An increasing attention to this approach has been also paid to magnetic-based IMAC separation applied for phosphoprotein and esp. for phosphopeptide enrichment [5,24–28].

In our previous investigation [28], we have used magnetic non-porous poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) [P(HEMA–GMA)] microspheres with hydrophilic properties containing coupled iminodiacetic acid (IDA) and immobilized metal ions for the separation of proteins exhibiting metal ion binding properties. The specific interaction of porcine pepsin A with Fe^{3+} ions immobilized to IDA-modified magnetic microspheres and human IgG binding to the same sorbent but with immobilized Ni^{2+} ions was studied.

* Corresponding author. Tel.: +420 224 965 910; fax: +420 224 912 834.
E-mail address: lenka.novotna@LF1.cuni.cz (L. Novotna).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.08.058

The aim of this study was to apply the magnetic non-porous P(HEMA–GMA) microspheres for the IMAC separation of phosphopeptides. Fe^{3+} and Ga^{3+} ions immobilized on IDA-modified magnetic microspheres were used for the enrichment of phosphopeptides from the proteolytic digests of two model proteins differing in their physico-chemical properties and phosphate group content: porcine pepsin A and bovine α-casein.

2. Materials and methods

2.1. Materials

2-Hydroxyethyl methacrylate (HEMA) and glycidyl methacrylate (GMA; both from Röhm GmbH, Germany) were vacuum-distilled before use. Dibenzoyl peroxide was purchased from Fluka and cellulose acetate butyrate (CAB; M_n = 100,000; 35/15 acetyl/butyryl) was obtained from Eastman (Kingsport, TN, USA). Diethyl ester of iminodiacetic acid was obtained according to the literature [28]. Porcine pepsin A, bovine α-casein, α-chymotrypsin from bovine pancreas, iron(III) chloride, gallium(III) chloride, diammonium hydrogenphosphate, acetonitrile, trifluoroacetic acid, phosphoric acid, and ammonium hydroxide solution were purchased from Sigma–Aldrich (Praha, Czech Republic). Trypsin gold, mass spectrometry grade, was obtained from Promega Corporation (Madison, WI, USA). Glycine was purchased from Serva Feinbiochemica (Heidelberg, Germany). Peptide calibration standard and 2,5-dihydroxybenzoic acid was obtained from Bruker Daltonics (Bremen, Germany). Dried low-fat bovine milk was obtained from PML (Novy Bydzov, Czech Republic). Unless otherwise stated, other chemicals were of analytical grade and were purchased from IPL (Uhersky Brod, Czech Republic). TopTip TiO_2 and TopTip ZrO_2 were from Glygen (Columbia, VA, USA).

2.2. Preparation of magnetic microspheres

The magnetic microspheres were prepared by the dispersion copolymerization of 2-hydroxyethyl methacrylate (HEMA) and glycidyl methacrylate (GMA) in a toluene/2-methylpropan-1-ol mixture in the presence of Fe_3O_4 [29]. Briefly, 38.2 g of toluene was added to 1 g of oleic acid-coated Fe_3O_4 nanoparticles, obtained by coprecipitation of $FeCl_3$ and $FeCl_2$ aqueous solutions by NH_4OH in the presence of oleic acid stabilizer [29], and sonicated for 20 min with a 4710 series ultrasonic homogenizer from Cole-Palmer Instruments (Vernon Hills, IL, United States) at 50% power. Then, a solution of 4 g of cellulose acetate butyrate (CAB) in 29.8 g of 2-methylpropan-1-ol was added, and after sonication, the flask containing the dispersion was mounted to a reactor; 12 g of the monomers (6 g HEMA and 6 g GMA) and 0.24 g of dibenzoyl peroxide were added, and the solution was deoxygenated by nitrogen purging for 10 min. The reaction was run at 70 °C with stirring (500 rpm) for 17 h. The resulting poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) [P(HEMA–GMA)] microspheres were purified by repeated magnetic separation from the toluene/2-methylpropan-1-ol mixture and decantation until excessive CAB was removed. Then, the product was dried from toluene.

2.2.1. Modification of magnetic P(HEMA–GMA) microspheres with iminodiacetic acid

Diethylester of iminodiacetic acid (IDA; 4.5 g) was reacted with a suspension of magnetic P(HEMA–GMA) microspheres (3 g) in methanol (24 mL) at 60 °C for 8 h under stirring (200 rpm). The microspheres were repeatedly washed with methanol (150 mL), methanol/water mixture (1/1, 2/1 and 4/1, v/v) and water. The ester groups were then hydrolyzed with 0.5 M NaOH aqueous solution (150 mL) at 60 °C for 7 h. Finally, the microspheres were transferred in H^+ form by treatment with 0.5 M HCl aqueous solution (50 mL), washed with water and freeze-dried.

2.3. Proteolytic digestion of phosphoproteins

2.3.1. Porcine pepsin A digestion with α-chymotrypsin

Porcine pepsin (1 mg), dissolved in 0.05 M ammonium bicarbonate solution (1 mL), was reduced by 0.2 M dithiothreitol solution (50 μL) in 0.1 M ammonium bicarbonate solution. The solution was heated up to 95 °C for 10 min. Then 1 M iodoacetamide solution in 0.1 M ammonium bicarbonate (40 μL) was added to the mixture cooled to room temperature and resulting solution was incubated for 45 min at room temperature in the darkness. The reaction was stopped by an addition of 0.2 M dithiothreitol solution (200 μL) in 0.1 M ammonium bicarbonate and incubation at 25 °C for 45 min. Afterwards, α-chymotrypsin solution in 0.05 M ammonium bicarbonate (1 mg/mL; 100 μL) was added (mass ratio of pepsin to α-chymotrypsin was 10:1) and the mixture was incubated at 37 °C for 18 h. The protein digestion was stopped by the acidification to pH 4 with concentrated trifluoroacetic acid.

2.3.2. Bovine α-casein digestion with trypsin

Bovine α-casein (0.25 mg) was dissolved in 25 mM ammonium bicarbonate solution (2.5 mL). After an addition of trypsin solution in 25 mM ammonium bicarbonate (0.2 mg/mL; 25 μL) (mass ratio of α-casein to trypsin was 50:1), the mixture was incubated at 37 °C for 18 h. The proteolytic digestion was stopped by acidification to pH 4 with concentrated trifluoroacetic acid.

2.3.3. Bovine milk digestion with trypsin

Dried bovine milk (2 mg corresponding to 0.68 mg of proteins), dissolved in 0.025 M ammonium bicarbonate solution (345 μL), was reduced by 0.2 M dithiothreitol solution (25 μL) in 0.025 M ammonium bicarbonate solution. The solution was heated up to 95 °C for 10 min. Then 1 M iodoacetamide solution in 0.025 M ammonium bicarbonate (20 μL) was added to the mixture cooled to room temperature and resulting solution was incubated for 45 min at room temperature in the darkness. The reaction was stopped by an addition of 0.2 M dithiothreitol solution (100 μL) in 0.025 M ammonium bicarbonate and incubation at 25 °C for 45 min. Afterwards, trypsin solution in 0.025 M ammonium bicarbonate (1 mg/mL; 10 μL) was added (mass ratio of proteins to trypsin was 68:1) and the mixture was incubated at 37 °C for 18 h. The protein digestion was stopped by the acidification to pH 4 with concentrated trifluoroacetic acid.

2.4. Phosphopeptide enrichment using IDA-modified magnetic P(HEMA–GMA) microspheres with immobilized Fe^{3+} or Ga^{3+} ions

2.4.1. Loading of metal ions to IDA-modified magnetic microspheres

IDA-modified magnetic hydrophilic P(HEMA–GMA) microspheres (50 mg) were suspended in water (1 mL) and stirred for 24 h. Then microspheres were washed with 0.05 M ethylenediaminetetraacetic acid disodium salt solution containing 1 M NaCl (5× 1 mL), water (5× 1 mL), and with 0.1 M $FeCl_3$ or $GaCl_3$ solution (5× 1 mL). Finally, microspheres with immobilized metal ions were washed with water (5× 1 mL), 1 M NaCl solution (5× 1 mL) and 0.05 M sodium acetate buffer, pH 4.0 (5× 1 mL) and stored in 0.05 M sodium acetate buffer, pH 4.0 at 4 °C.

2.4.2. Phosphopeptide enrichment from proteolytic digests of porcine pepsin A, bovine α-casein and bovine milk using IDA-modified magnetic microspheres with immobilized Fe^{3+} or Ga^{3+} ions

Fe^{3+}- or Ga^{3+}-IDA-modified magnetic microsphere suspension (50 mg/mL; 10 μL) was used in each experiment. Microspheres were subsequently washed with elution solution (5× 10 μL) and adsorption solution (5× 10 μL). The solution (10 μL) of proteolytically digested pepsin (0.71 mg/mL) or α-casein (0.10 mg/mL) or milk (1.36 mg/mL) was added and the suspension was stirred for 30 s. The microspheres were then washed with adsorption solution (5× 10 μL). In the case of adsorption solutions containing NaCl, microspheres with adsorbed peptides were washed first with solution containing NaCl and then with the same solution without NaCl. Adsorbed peptides were desorbed with elution solution (3× 5 μL) and subjected to MALDI-TOF MS analysis. Each experiment was performed 3–5 times. Following adsorption solutions were tested: acetic acid (0.1–6%, v/v), acetate buffer (0.025 M; pH 3.5–6.0), glycine buffer (0.025 M, pH 2.5–3.5) containing NaCl (0–1 M) and/or acetonitrile (0–30%, v/v). The following solutions were used for the elution of adsorbed peptides: ammonium hydroxide (5 mM), diammonium hydrogenphosphate (0.2–1 M), or phosphoric acid (0.1–10%, v/v) containing acetonitrile (0 or 50%, v/v).

2.5. Phosphopeptide enrichment from proteolytic digest of bovine α-casein using titanium dioxide or zirconium dioxide tips

Titanium dioxide (TopTip TiO_2) tip and zirconium dioxide (TopTip ZrO_2) tip were washed with 0.1 M glutamic acid in 0.1 M HCl and 50% ACN, and 6% acetic acid containing 0.5 M NaCl and 30% ACN, respectively (adsorption solution; 5× 20 μL). Then α-casein digest (10 μL; 0.10 mg/mL) was applied and tips were washed with the adsorption solution (5× 20 μL). In the case of zirconium dioxide, tip was then washed with 6% acetic acid. Adsorbed peptides were eluted with 0.2 M ammonium hydrogenphosphate, pH 7.9 (3× 5 μL) and subjected to MALDI-TOF MS analysis.

2.6. MALDI-TOF/TOF MS analysis and database searching

MALDI-TOF spectra were acquired on an Autoflex II TOF/TOF system (Bruker Daltonics, Bremen, Germany), using a nitrogen laser (337 nm) and the FlexControl software. The mass spectrometer was externally calibrated with the peptide calibration standard containing angiotensin II, angiotensin I, substance P, bombesin, ACTH (1–17), ACTH (18–39), and somatostatin 28.

Matrix solution (0.5 μL; 5 mg of 2,5-dihydroxybenzoic acid in 1 mL of 0.1% (v/v) trifluoroacetic acid containing 30% (v/v) acetonitrile and 1% (v/v) phosphoric acid) was deposited on MTP 384 massive target plate (Bruker Daltonics, Bremen, Germany) and allowed to air-dry at room temperature. Afterwards, non-desalted peptides eluted from IMAC magnetic microspheres (0.5 μL) were added and allowed to air-dry at room temperature. The plate was then inserted in the mass spectrometer (MS) and subjected to MS analysis.

Values of measured peptide ion *m*/*z* were compared with those obtained by theoretical cleavage of appropriate protein with trypsin in the following way. Pepsin and α-casein sequences were obtained from ExPASy Proteomics Server (Swiss Institute of Bioinformatics, Lausanne, Switzerland). mMass (version 2.4) was used to generated a list of peptides resulting from *in silico* proteolytic digestion of these sequences. The masses of these peptides were compared with the measured data to see any matches. Matched peptide sequences were confirmed by MS/MS analysis of appropriate peptide ions. Peptides from tandem mass spectra were identified using FlexAnalysis and BioTools (Bruker Daltonics, Bremen, Germany) with Mascot (Matrix Sciences, London, UK) as a search engine and Swiss-Prot (Swiss Institute of Bioinformatics, Lausanne, Switzerland) as a database. The peptide mass tolerance was set to 150 ppm, the tandem mass tolerance was set to 0.5 Da, and the missed cleavage was set to 3.

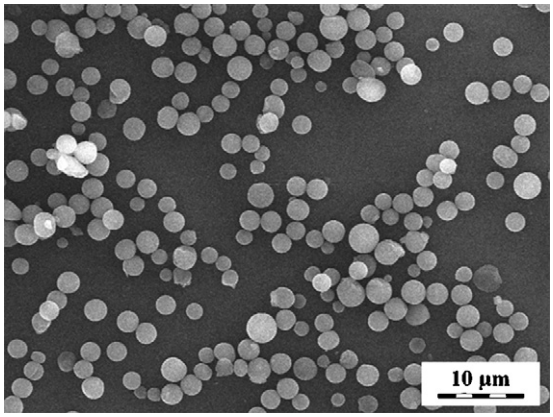

Fig. 1. Scanning electron micrograph of magnetic P(HEMA–GMA) microspheres.

3. Results

3.1. IDA-modified magnetic microspheres

Magnetic microspheres were obtained by the dispersion polymerization of HEMA and GMA in a toluene/2-methylpropan-1-ol mixture in the presence of Fe_3O_4 core nanoparticles. Dibenzoyl peroxide initiated the polymerization and cellulose acetate butyrate served as a steric stabilizer to prevent microsphere aggregation. Fe_3O_4 nanoparticles were obtained by the coprecipitation of $FeCl_3$ and $FeCl_2$ by NH_4OH and oleic acid was used to stabilize them in organic medium in which the monomers were dispersion polymerized. The resulting microspheres (Fig. 1) were 2.3 μm in diameter with a relatively narrow size distribution characterized by the polydispersity index (ratio of weight- to number-average particle diameter) = 1.1. Magnetic properties of the prepared microspheres were described in a detail previously [28,30,31]. The content of the reactive oxirane groups was 3.5 mmol/g as determined from the analysis of the peak area at 906 cm^{-1} in the IR spectrum (not shown). Amount of iron measured by atomic absorption spectroscopy was 6 wt.%. Resulting magnetic P(HEMA–GMA) microspheres were then reacted with diethylester of iminodiacetic acid in methanol at 60 °C and the attached diester was then hydrolyzed in alkaline medium. Iminodiacetic derivative of magnetic P(HEMA–GMA) microspheres contained 1.3 mmol COOH/g according to the titration. The prepared IDA-modified magnetic microspheres were loaded with Fe^{3+} or Ga^{3+} ions.

3.2. Phosphopeptide enrichment from α-casein and pepsin digests using IDA-modified magnetic P(HEMA–GMA) microspheres with immobilized Fe^{3+} or Ga^{3+}ions

The prepared IDA-modified magnetic P(HEMA–GMA) microspheres with immobilized Fe^{3+} or Ga^{3+} ions were used for the enrichment of phosphopeptides from proteolytic digests of two model phosphoproteins, bovine α-casein and porcine pepsin A, that differ in their physico-chemical properties and the number of bound phosphate groups. Porcine pepsin A is a protein with a low p*I* containing a high number of acidic amino acid residues [32] and one phosphoserine residue (pS 127) [33,34]. On the other hand, α-casein (composed of α-S1- and α-S2-forms) is a multiphosphorylated protein containing 8–10 (α-S1- form) and 10–13 (α-S2-form) phosphate groups [35,36]. This phosphoprotein yields

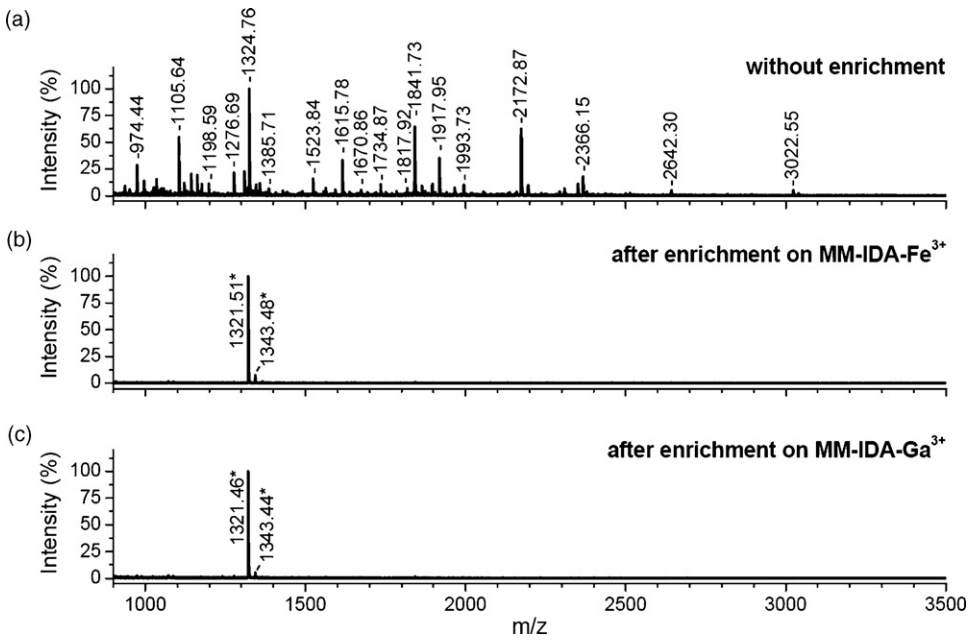

Fig. 2. MALDI-TOF MS analysis of porcine pepsin A digest before (a) and after phosphopeptide enrichment by MM-IDA-Fe^{3+} (b) or MM-IDA-Ga^{3+} (c). Phosphopeptide ions are marked with asterisk. MM – magnetic microspheres. Conditions for phosphopeptide enrichment: sample – porcine pepsin A digest, adsorption/washing solution – 3% acetic acid containing 0.5 M NaCl and 30% acetonitrile followed by 3% acetic acid in the case of MM-IDA-Fe^{3+} or 6% acetic acid containing 0.5 M NaCl and 30% acetonitrile followed by 6% acetic acid in the case of MM-IDA-Ga^{3+}; elution solution – 0.2 M diammonium hydrogenphosphate.

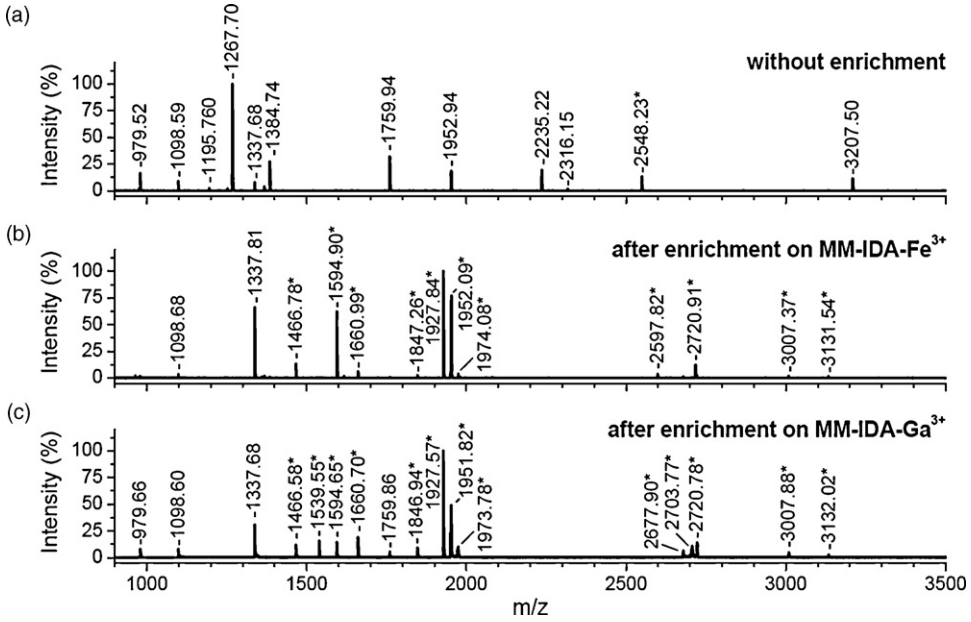

Fig. 3. MALDI-TOF MS analysis of bovine α-casein digest before (a) and after phosphopeptide enrichment by MM-IDA-Fe^{3+} (b) or MM-IDA-Ga^{3+} (c). Phosphopeptide ions are marked with asterisk. MM – magnetic microspheres. Conditions for phosphopeptide enrichment: sample – bovine α-casein digest, adsorption/washing solution – 6% acetic acid containing 0.5 M NaCl followed by 6% acetic acid; elution solution – 0.2 M diammonium hydrogenphosphate.

after the proteolytic digestion numerous phosphopeptides containing not only singly but also multiply phosphorylated peptides. Tryptic digest of α-casein and α-chymotryptic digest of porcine pepsin A were used to evaluate a phosphopeptide binding selectivity of prepared IDA-modified magnetic particles with immobilized Fe^{3+} or Ga^{3+} ions.

A representative MALDI-TOF mass spectrum of the complete un-treated tryptic α-casein digest and α-chymotryptic porcine pepsin A digest is shown in Figs. 2a and 3a. Prior enrichment, no phosphopeptide was detected in the case of porcine pepsin A digest (Fig. 2a) and only one phosphopeptide signal (*m*/*z* 2548, YKVPQLEIVPN**pS**AEERLHSMK, highlighted with an asterisk) was detected in the case of complete non-separated tryptic digest of α-casein (Fig. 3a).

Proteolytic digests of both studied proteins were separated using the prepared IDA-modified magnetic microspheres loaded with Fe^{3+} or Ga^{3+} ions. Optimum conditions for adsorption and recovery of a maximum number of phosphopeptides in the absence of non-phosphorylated peptides were investigated. The following adsorption/washing solutions were tested: 0.025 M acetate buffer, pH 3.5–6.0, 0.025 M glycine–HCl buffer, pH 2.5–3.5 or 0.1–6% (v/v) acetic acid. The best recovery of phosphopeptides from the tryp-

Table 1
Peptides enriched from tryptic digest of bovine α-casein by Fe^{3+}-IDA-magnetic microspheres.

Observed *m*/*z*	Theoretical *m*/*z*	Residues	Peptide sequences
1098.68	1098.61	α-S2/204–212	AMKPWIQPK
1337.81	1337.68	α-S1/95–105	HIQKEDVPSER
1466.78	1466.61	α-S2/153–164	TVDME**pS**TEVFTK
1594.90	1594.71	α-S2/153–165	TVDME**pS**TEVFTKK
1660.99	1660.79	α-S1/121–134	VPQLEIVPN**pS**AEER
1847.26	1847.73	α-S1/58–73	DIGSE**pS**TEDQAMEDIK
1927.84	1927.69	α-S1/58–73	DIG**pS**E**pS**TEDQAMEDIK
1952.09	1951.95	α-S1/119–134	YKVPQLEIVPN**pS**AEER
1974.08	1973.92	α-S1/119–134	YKVPQLEIVPN**pS**AEER[a]
2597.82	2598.06	α-S1/52–73	VNEL**pS**KDIGSE**pS**TEDQAMEDIK[b]
2720.91	2720.91	α-S1/74–94	QMEAE**pS**I**pSpSpS**EEIVPN**pS**VEQK
3007.37	3008.03	α-S2/61–85	NANEEEYSIG**pSpSpS**EE**pS**AEVATEEVK
3131.54	3131.28	α-S2/141–165	EQL**pS**T**pS**EENSKKTVDoxME**pS**TEVFTKK

oxM – oxidized methionine.
[a] Sodium adduct.
[b] Phosphopeptide *m*/*z* 2597 corresponds to phosphopeptide *m*/*z* 2677 in Table 2, however contains only one phosphate group. The second phosphate group was probably lost during the ionization process in mass spectrometer.

Table 2
Peptides enriched from tryptic digest of bovine α-casein by Ga^{3+}-IDA-magnetic microspheres.

Observed *m*/*z*	Theoretical *m*/*z*	Residues	Peptide sequences
979.66	979.56	α-S2/189–196	FALPQYLK
1098.60	1098.61	α-S2/204–212	AMKPWIQPK
1337.68	1337.68	α-S1/95–105	HIQKEDVPSER
1466.58	1466.61	α-S2/153–164	TVDME**pS**TEVFTK
1539.55	1539.60	α-S2/141–152	EQL**pS**T**pS**EENSKK
1594.65	1594.71	α-S2/153–165	TVDME**pS**TEVFTKK
1660.70	1660.79	α-S1/121–134	VPQLEIVPN**pS**AEER
1759.86	1759.94	α-S1/23–37	HQGLPQEVLNENLLR
1846.94	1847.73	α-S1/58–73	DIGSE**pS**TEDQAMEDIK
1927.57	1927.69	α-S1/58–73	DIG**pS**E**pS**TEDQAMEDIK
1951.82	1951.95	α-S1/119–134	YKVPQLEIVPN**pS**AEER
1973.78	1973.92	α-S1/119–134	YKVPQLEIVPN**pS**AEER[a]
2677.90	2678.02	α-S1/52–73	VNEL**pS**KDIG**pS**E**pS**TEDQAMEDIK
2703.77	2703.50	α-S1/114–135	LRLKKYKVPQLEIVPN**pS**AEERL
2720.78	2720.91	α-S1/74–94	QMEAE**pS**I**pSpSpS**EEIVPN**pS**VEQK
3007.88	3008.03	α-S2/61–85	NANEEEYSIG**pSpSpS**EE**pS**AEVATEEVK
3132.02	3131.28	α-S2/141–165	EQL**pS**T**pS**EENSKKTVDoxME**pS**TEVFTKK

oxM – oxidized methionine.
[a] Sodium adduct.

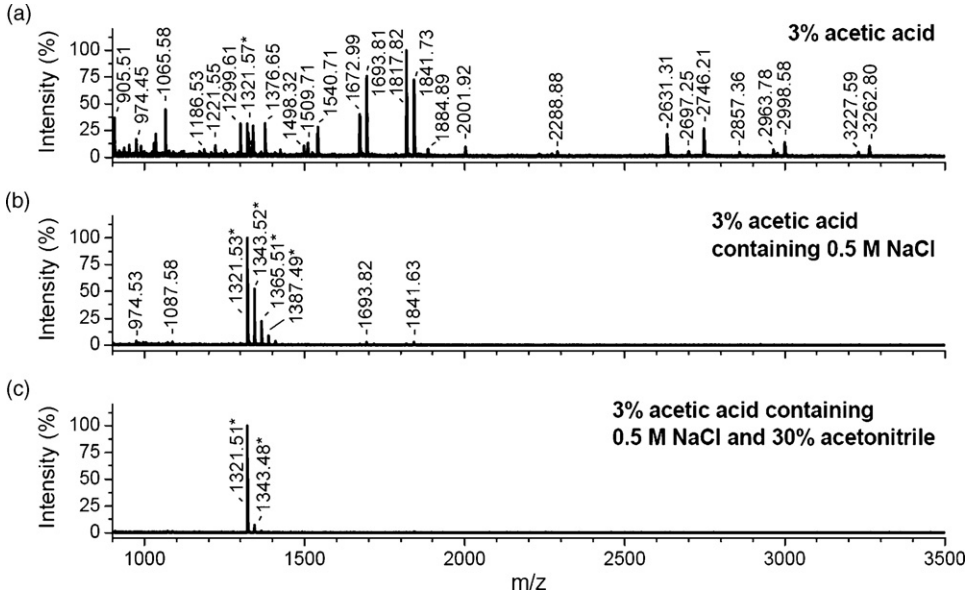

Fig. 4. MALDI-TOF MS analyses of porcine pepsin A digest after phosphopeptide enrichment by MM-IDA-Fe^{3+} performed under following adsorption and desorption conditions. Adsorption/washing solution – 3% acetic acid (a) or 3% acetic acid containing 0.5 M NaCl (b) or 3% acetic acid containing 0.5 M NaCl and 30% acetonitrile followed by 3% acetic acid (c). Elution solution – 0.2 M diammonium hydrogenphosphate. Phosphopeptide ions are marked with asterisk. MM – magnetic microspheres.

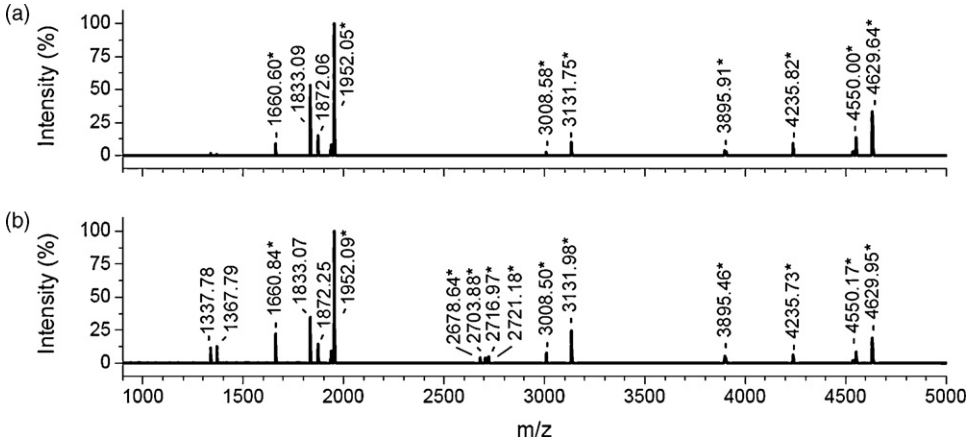

Fig. 5. MALDI-TOF MS analyses of bovine α-casein digest after phosphopeptide enrichment by TiO_2 (a) and ZrO_2 (b) tips. Phosphopeptide ions are marked with asterisk. Conditions for phosphopeptide enrichment: sample – bovine α-casein digest, adsorption/washing solution – 0.1 M glutamic acid containing 0.1 M HCl and 50% ACN (TiO_2) or 6% acetic acid containing 0.5 M NaCl and 30% acetonitrile followed by 6% acetic acid (ZrO_2); elution solution – 0.2 M diammonium hydrogenphosphate.

tic digest of α-casein was observed in the case of 3–6% acetic acid using both immobilized metal ions. The increased ionic strength of the adsorption/washing solution (0.5 M NaCl) significantly reduced the number of non-phosphorylated peptides adsorbed to both magnetic sorbents. Similar situation was observed in the case of a selective phosphopeptide recovery from α-chymotryptic digest of porcine pepsin A using both types of immobilized metal ions: the increase in ionic strength (0.5 M NaCl) of the adsorption/washing solution (3–6% acetic acid) significantly reduced the number of adsorbed non-phosphorylated peptides. Moreover, in the presence of acetonitrile in the adsorption/washing solution only phosphopeptide and no non-phosphorylated peptides were detected in the desorbed fraction (Fig. 4).

As far as the elution buffer is concerned, the highest number of phosphopeptides were recovered using 0.2 M $(NH_4)_2HPO_4$; not only singly phosphorylated but also multiply phosphorylated peptides were detected. Similar results were obtained using 1% (v/v) phosphoric acid solutions containing 0 or 50% (v/v) acetonitrile.

Results of MALDI-TOF MS analysis of α-casein phosphopeptides recovered from IDA-modified magnetic microspheres with immobilized Fe^{3+} or Ga^{3+} ions are shown in Fig. 3 and summarized in Tables 1 and 2. Out of 13 phosphopeptides detected in the eluates obtained from both IMAC sorbents, 6 were singly phosphorylated and 7 multiply phosphorylated peptides. Two known phosphopeptides of α-S2-casein with *m*/*z* 954 (40–47, NMAINP**pS**K) and *m*/*z* 2618 (17–36, NTMEHV**pSpSpS**EESII**pS**QETYK) were not detected. Only 2 (on immobilized Fe^{3+} ions) or 4 non-phosphorylated peptides (on immobilized Ga^{3+} ions) were found in the eluted fraction. The reduced number of non-phosphorylated peptides detected in eluates from microspheres with immobilized Fe^{3+} ions was however accompanied with a decrease of phosphopeptide recovery (*m*/*z* 1539 and 2703). Only one phosphopeptide *m*/*z* 2548 (α-S1-casein/119–139, 2 missed cleavages, YKVPQLEIVPN**pS**AEERLHSMK) was detected in the non-enriched proteolytic digest but its signal was not found in the samples enriched using both sorbents. Contrary to that, two shorter phosphopeptides *m*/*z* 1952 (α-S1-casein/119–134, 1 missed cleavage, YKVPQLEIVPN**pS**AEER) and *m*/*z* 1660 (α-S1-casein/121–134, 0 missed cleavage VPQLEIVPN**pS**AEER) were detected in enriched fractions (Tables 1 and 2, Fig. 3).

Fe^{3+} and Ga^{3+} ions immobilized on IDA-modified magnetic microspheres did not preferably enrich different classes of phosphopeptides even if the loaded sample amount was higher then the loading capacity of the microspheres used. The 15 times increase of the amount of applied α-casein digest (150 μL instead of 10 μL) had no effect on the phosphopeptide enrichment. The same set of phosphopeptides was detected by using given metal ions regardless the loaded sample amount.

Table 3
Peptides enriched from tryptic digest of bovine α-casein by TiO_2 and ZrO_2 tips.

Observed *m*/*z*		Theoretical *m*/*z*	Residues	Peptide sequences
TiO_2	ZrO_2			
–	1337.78	1337.68	α-S1/95–105	HIQKEDVPSER
–	1367.79	1367.70	α-S2/96–106	ALNEINQFYQK
1660.60	1660.84	1660.79	α-S1/121–134	VPQLEIVPN**pS**AEER
1833.09	1833.07		Not identified	
1872.06	1872.25	1871.99	α-S1/119–134	YKVPQLEIVPNSAEER
1952.05	1952.09	1951.95	α-S1/119–134	YKVPQLEIVPN**pS**AEER
–	2678.64	2678.02	α-S1/52–73	VNEL**pS**KDIG**pS**E**pS**TEDQAMEDIK
–	2703.88	2703.50	α-S1/114–135	LRLKKYKVPQLEIVPN**pS**AEERL
–	2716.97	2716.26	α-S2/130–152	NAVPITPTLNREQL**pS**T**pS**EENSKK
–	2721.18	2720.91	α-S1/74–94	QMEAE**pS**I**pSpSpS**EEIVPN**pS**VEQK
3008.58	3008.50	3008.03	α-S2/61–85	NANEEEYSIG**pSpSpS**EE**pS**AEVATEEVK
3131.75	3131.98	3131.28	α-S2/141–165	EQL**pS**T**pS**EENSKKTVDoxME**pS**TEVFTKK
3895.91	3895.46	3895.64	α-S1/74–105	QoxMEAESISSSEEIVPNSVEQKHIQKEDVPSER + **3 phosphate groups**[a]
4235.82	4235.73	4235.65	α-S2/61–95	NANEEEYSIG**pSpSpS**EE**pS**AEVATEEVKITVDDKHYQK
4550.00	4550.17	4549.62	α-S1/58–94	DIGSESTEDQAMEDIKQMEAESISSSEEIVPNSVEQK + **6 phosphate groups**[a]
4629.64	4629.95	4629.59	α-S1/58–94	DIG**pS**E**pS**TEDQAMEDIKQMEAE**pS**I**pSpSpS**EEIVPN**pS**VEQK

oxM – oxidized methionine.
[a] Exact position of phosphate groups was not identified.

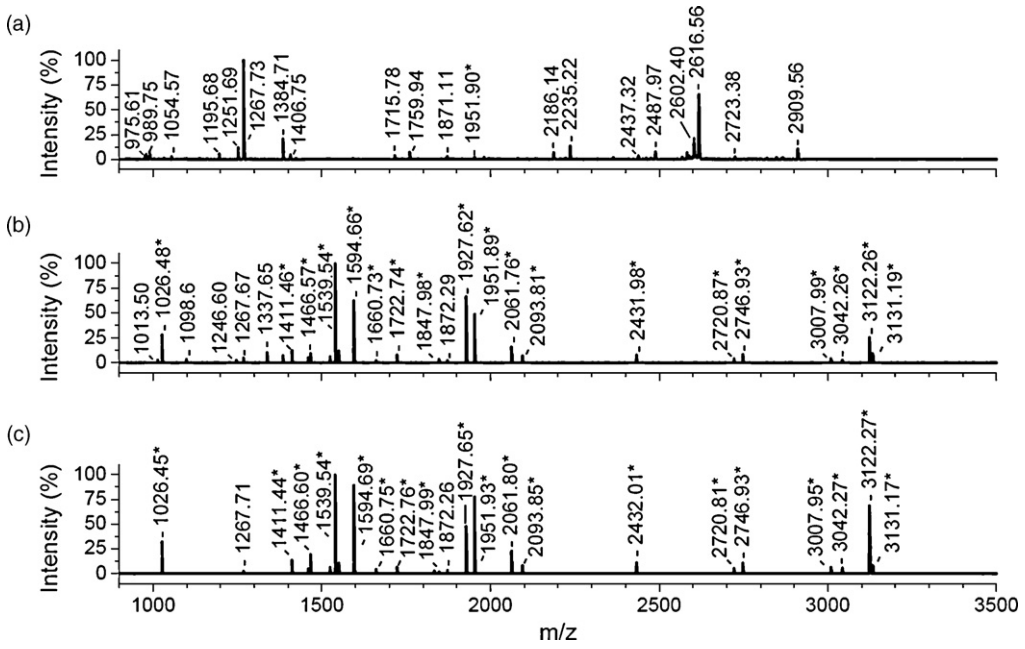

Fig. 6. MALDI-TOF MS analyses of bovine milk digest before (a) and after phosphopeptide enrichment by MM-IDA-Fe^{3+} (b) or MM-IDA-Ga^{3+} (c). Phosphopeptide ions are marked with asterisk. MM – magnetic microspheres. Conditions for phosphopeptide enrichment: sample – bovine milk digest, adsorption/washing solution – 6% acetic acid containing 0.5 M NaCl and then 6% acetic acid; elution solution – 0.2 M diammonium hydrogenphosphate.

MALDI-TOF MS analysis of porcine pepsin A peptides desorbed from IMAC magnetic microspheres containing both Fe^{3+} and Ga^{3+} ions showed the presence of only two peptide ions (Fig. 2). The predominant peptide ion (*m*/*z* 1321) was previously identified as peptide EAT**pS**QELSITY containing one phosphorylated serine residue [34]. This sequence corresponds to residues 124–134 of porcine pepsinogen A with the phosphorylated serine residue in the position 127. The second peptide ion (*m*/*z* 1343) represents adduct of the predominant peptide with sodium ion. No non-phosphorylated peptides were detected.

Results of the phosphopeptide enrichment from α-casein digest using Fe^{3+}- and Ga^{3+}- IDA-modified magnetic microspheres were

Table 4
Peptides enriched from bovine milk digest using Fe^{3+}- and Ga^{3+}-IDA-magnetic microspheres.

Observed *m*/*z*		Theoretical *m*/*z*	Residues	Peptide sequences
Fe^{3+}	Ga^{3+}			
1013.50	–	1013.52	β/121–128	HKEMPFPK
1026.48	1026.45	1026.49	α-S1/50–57	EKVNEL**pS**K
1098.6	–	1098.61	α-S2/204–212	AMKPWIQPK
1246.60	–	1246.64	α-S2/86–95	ITVDDKHYQK
1267.67	1267.71	1267.70	α-S1/106–115	YLGYLEQLL
1337.65	–	1337.68	α-S1/95–105	HIQKEDVPSER
1384.69	–	1384.73	α-S1/38–49	FFVAPFPEVFGK
1411.46	1411.44	1411.50	α-S2/141–151	EQL**pS**T**pS**EENSK
1459.06	1459.03	1459.63	α-S2/141–152	EQL**pS**TSEENSKK
1466.57	1466.60	1466.61	α-S2/153–164	TVDME**pS**TEVFTK
1524.63	1524.61		Not identified	
1539.54	1539.54	1539.60	α-S2/141–152	EQL**pS**T**pS**EENSKK
1550.55	1550.58		Not identified	
1594.66	1594.69	1594.71	α-S2/153–165	TVDME**pS**TEVFTKK
1660.73	1660.75	1660.79	α-S1/121–134	VPQLEIVPN**pS**AEER
1722.74	1722.76	1722.80	α-S2/152–165	KTVDME**pS**TEVFTKK
–	1832.80		Not identified	
1847.98	1847.99	1847.73	α-S1/58–73	DIGSE**pS**TEDQAMEDIK
1872.29	1872.26	1871.99	α-S1/119–134	YKVPQLEIVPNSAEER
1927.62	1927.65	1927.69	α-S1/58–73	DIG**pS**E**pS**TEDQAMEDIK
1951.89	1951.93	1951.95	α-S1/119–134	YKVPQLEIVPN**pS**AEER
2061.76	2061.80	2061.83	β/48–63	FQ**pS**EEQQQTEDELQDK
2093.81	2093.85	2093.89	α-S2/40–56	NMAINP**pS**KENLmCSTFmCK
2431.98	2432.01	2432.05	β/45–63	IEKFQ**pS**EEQQQTEDELQDK
2720.87	2720.81	2720.91	α-S1/74–94	QMEAE**pS**I**pS**p**S**p**S**EEIVPN**pS**VEQK
2746.93	2746.93	2747.00	α-S2/16–36	KNTMEHV**pS**p**S**p**S**EESII**pS**QETYK
3007.99	3007.95	3008.03	α-S2/61–85	NANEEEYSIG**pS**p**S**p**S**EE**pS**AEVATEEVK
3042.26	3042.27	3042.30	β/16–40	RELEELNVPGEIVESL**pS**p**S**p**S**EESITR
3122.26	3122.27	3122.27	β/16–40	RELEELNVPGEIVE**pS**L**pS**p**S**p**S**EESITR
3131.19	3131.17	3131.28	α-S2/141–165	EQL**pS**T**pS**EENSKKTVDoxME**pS**TEVFTKK

oxM – oxidized methionine; mC – carbamidomethyl cysteine; α-S1 – α-S1-casein; α-S2 – α-S2-casein; β – β-casein.

compared with those obtained using commercially available titanium or zirconium dioxide tips (TopTip TiO_2 or TopTip ZrO_2). As apparent from the obtained MS spectra (Fig. 5), different set and number of phosphopeptides were detected. Using TiO_2 and ZrO_2 (Fig. 5, Table 3), 8 and 12 phosphopeptides, respectively, and 1 and 3 non-phosphorylated peptides, respectively, were found. In contrast to Fe^{3+}- and Ga^{3+}-IDA-modified magnetic microspheres (Tables 1 and 2, Fig. 3), some more multiply phosphorylated peptides (*m*/*z* 3895, 4235, 4550, 4629) were obtained using TiO_2 and ZrO_2, but some singly- (*m*/*z* 1466, 1594, 1847) or doubly-phosphorylated peptides (*m*/*z* 1539, 1927) were not detected. Moreover, in the case of TiO_2, the reduced number of non-phosphorylated peptides was accompanied with the loss of some other phosphopeptides (*m*/*z* 2677, 2703, 2720).

3.3. Phosphopeptide enrichment from dried bovine milk using IDA-modified magnetic P(HEMA–GMA) microspheres with immobilized Fe^{3+} or Ga^{3+} ions

To evaluate the ability to capture the phosphopeptides from a complex sample, the prepared IDA-modified magnetic P(HEMA–GMA) microspheres with immobilized Fe^{3+} or Ga^{3+} ions were applied to trap phosphopeptides from tryptic digest of bovine milk. Results are shown in Fig. 6 and Table 4. Except for phosphopeptide *m*/*z* 1951, no other phosphopeptide derived from α-S1-, α-S2-, β-, and κ-casein was detected in original bovine milk digest. On the other hand, all phosphorylation sites of α-S1-, α-S2-, and β-casein were identified in 20 detected phosphopeptides obtained using Fe^{3+}- and Ga^{3+}-IDA-modified magnetic microspheres (Table 4).

4. Discussion

Magnetic microspheres represent a powerful purification tool for protein/peptide separation esp. in combination with high-sensitive mass spectrometric analysis. They serve not only as magnetic carriers for hydrolytic enzymes used in proteomic studies, but magnetic sorbents can be modified by various ligands and thus they can be applied for rapid and easy affinity separations [37]. Chelate-modified magnetic particles represent one of such examples [25–27,38,39].

Magnetic particles used for bioseparations consist besides of magnetic cores also of functional groups on a polymer shell of different nature, such as silica, agarose, cellulose or synthetic polymers including polystyrene, poly(methyl methacrylate) or poly(vinyl alcohol). They often suffer from drawback of non-specific protein adsorption. Magnetic microspheres based on poly(2-hydroxyethyl methacrylate) or poly(glycidyl methacrylate) have been already used in several bioapplications [40,41]. They are conveniently prepared by the dispersion polymerization that is a suitable technique to obtain particles in the micron size range with a narrow size distribution in a single step. Here, magnetic microspheres were synthesized from poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) [P(HEMA–GMA)]. While HEMA introduces hydrophilicity in the matrix (suppressing undesirable non-specific protein adsorption), GMA provides reactive oxirane groups available for easy modification with diethyl ester of iminodiacetic acid.

In the present study, magnetic non-porous P(HEMA–GMA) microspheres with hydrophilic properties modified with iminodiacetic acid (IDA) and immobilized Fe^{3+} or Ga^{3+} ions were used for IMAC separation of phosphopeptides from proteolytic digests of two model phosphoproteins: bovine α-casein and porcine pepsin A. Previously, Fe^{3+} ions immobilized on these IDA-modified magnetic microspheres were used for the specific binding of porcine pepsin A and the participation of phosphate groups in the pepsin interaction was shown [28].

Here, we have chosen two different phosphoproteins differing in the number of phosphate groups in the protein molecule. Porcine pepsin A contains only one phosphate group while α-casein belongs to multiply phosphorylated phosphoprotein yielding after proteolytic digestion multiply phosphorylated phosphopeptides. Besides that, these two phosphoproteins differ in their physico-chemical properties. Porcine pepsin A contains a relatively high number of acidic amino acid residues that are known to limit phosphopeptide separation procedures [10,42,43].

Recovery of phosphopeptides from IMAC sorbents and the selectivity of this separation process depend on several factors: properties of the polymer matrix, chelating agent and immobilized metal ions, conditions of phosphopeptide adsorption and elution and physico-chemical properties and composition of analyzed proteins, peptide sequences and length, and a number and distribution of acidic amino acid residues and phosphorylation sites [42,44,45].

Only one phosphopeptide of α-S1-casein (*m*/*z* 2548) detected in the non-enriched samples was not adsorbed to the used magnetic sorbents, but its shorter forms (*m*/*z* 1952 and 1660) that are formed by more complete cleavage were detected in the eluted fraction. This observation might be explained by different adsorption ability of phosphopeptides.

Our results have shown that IDA-modified magnetic microspheres with immobilized Fe^{3+} or Ga^{3+} ions can be used for an easy enrichment of differently phosphorylated phosphopeptides; no differences were observed in an adsorption of singly and multiply phosphorylated peptides from the proteolytic digest of α-casein. The fact that samples after the enrichment are not necessary to separate using hydrophobic support is one of advantageous properties of the used IMAC microspheres; multiply phosphorylated peptides are not adsorbed to C_{18} trapping columns [46].

Results of our study have shown that IDA-modified magnetic microspheres with immobilized Fe^{3+} or Ga^{3+} ions possess properties that enable to separate phosphopeptides from acidic proteins without necessity to modify multiple acidic amino acid residues [10,42,43]. Single phosphopeptide of porcine pepsin A without an admixture of non-phosphorylated peptides was recovered after IMAC separation from α-chymotryptic digest of porcine pepsin A. The absence of non-phosphorylated peptides in the recovered phosphopeptide fraction was achieved by an increased ionic strength of adsorption buffer containing acetic acid solution and by an addition of acetonitrile.

Contrary to earlier observations [25], Fe^{3+} ions immobilized on IDA-modified P(HEMA–GMA) microspheres did not prefer adsorption of either singly or multiply phosphorylated peptides from α-casein digest under the used conditions. Similarly, we did not observe any significant preference of Ga^{3+}-IDA-magnetic microspheres for the enrichment of singly phosphorylated peptides as compared to immobilized Fe^{3+} ions, as was described by Sykora et al. [25].

Our results have shown that the studied microspheres can be used for the phosphopeptide enrichment not only from the proteolytic digest of model proteins, but also for the phosphoproteomic studies of complex samples.

5. Conclusions

Magnetic non-porous hydrophilic poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) microspheres obtained by the dispersion polymerization and modified with iminodiacetic acid and containing immobilized Fe^{3+} or Ga^{3+} ions represent an advantageous IMAC sorbent for the enrichment of phosphopeptides from proteolytic digests of phosphoproteins differing

in a number of phosphate groups as well as phosphoproteins containing a high number of acidic amino acid residues.

Acknowledgements

The authors would like to thank Jana Vavrova for helping with MS analyses. This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (Grant MSM 0021620806 and project CEH LC 06044). The authors also gratefully acknowledge financial support from AS CR (KAN401220801) and the Czech Science Foundation (Grant Nos. 203/09/0857 and P503/10/0664).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.08.058.

References

[1] P. Cohen, Eur. J. Biochem. 268 (2001) 5001.
[2] Y.P. Lim, Clin. Cancer Res. 11 (2005) 3163.
[3] F. Yang, D.L. Stenoien, E.F. Strittmatter, J. Wang, L. Ding, M.S. Lipton, M.E. Monroe, C.D. Nicora, M.A. Gristenko, K. Tang, R. Fang, J.N. Adkins, D.G. Camp II, D.J. Chen, R.D. Smith, J. Proteome Res. 5 (2006) 1252.
[4] H. Sun, N.K. Tonks, Trends Biochem. Sci. 19 (1994) 480.
[5] J.D. Dunn, G.E. Reid, M.L. Bruening, Mass Spectrom. Rev. 29 (2010) 29.
[6] M. Mann, S. Ong, M. Gronborg, H. Steen, O.N. Jensen, A. Pandey, Trends Biotechnol. 20 (2002) 261.
[7] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598.
[8] J. Reinders, U. Lewandrowski, J. Moebius, Y. Wagner, A. Sickmann, Proteomics 4 (2004) 3686.
[9] D.T. McLachlin, B.T. Chait, Curr. Opin. Chem. Biol. 5 (2001) 591.
[10] R. Raggiaschi, S. Gotta, G.C. Terstappen, Biosci. Rep. 25 (2005) 33.
[11] R. Kange, U. Selditz, M. Granberg, U. Lindberg, G. Ekstrand, B. Ek, M. Gustafsson, J. Biomol. Tech. 16 (2005) 91.
[12] N. Ramadan, J. Porath, J. Chromatogr. 321 (1985) 93.
[13] M. Zachariou, M.T. Hearn, J. Protein Chem. 14 (1995) 419.
[14] V. Gaberc-Porekar, V. Menart, J. Biochem. Biophys. Methods 49 (2001) 335.
[15] C. Baldwin, J. Cumming, A.T. Timperman, Analyst 130 (2005) 318.
[16] T.S. Nuhse, A. Stensballe, O.N. Jensen, S.C. Peck, Mol. Cell. Proteomics 2 (2003) 1234.
[17] M.C. Posewitz, P. Tempst, Anal. Chem. 71 (1999) 2883.
[18] L. Novotna, M. Hruby, M.J. Benes, Z. Kucerova, J. Sep. Sci. 31 (2008) 1662.
[19] M. Franzreb, M. Siemann-Herzberg, T.J. Hobley, O.R. Thomas, Appl. Microbiol. Biotechnol. 70 (2006) 505.
[20] I. Safarik, M. Safarikova, Biomagn. Res. Technol. 2 (2004) 7.
[21] E.F. Petricoin, A.M. Ardekani, B.A. Hitt, P.J. Levine, V.A. Fusaro, S.M. Steinberg, G.B. Mills, C. Simone, D.A. Fishman, E.C. Kohn, L.A. Liotta, Lancet 359 (2002) 572.
[22] J. Villanueva, J. Philip, D. Entenberg, C.A. Chaparro, M.K. Tanwar, E.C. Holland, P. Tempst, Anal. Chem. 76 (2004) 1560.
[23] S. Baumann, U. Ceglarek, G.M. Fiedler, J. Lembcke, A. Leichtle, J. Thiery, Clin. Chem. 51 (2005) 973.
[24] G. Han, M. Ye, H. Zou, Analyst 133 (2008) 1128.
[25] C. Sykora, R. Hoffmann, P. Hoffmann, Protein Pept. Lett. 14 (2007) 489.
[26] F. Tan, Y. Zhang, W. Mi, J. Wang, J. Wei, Y. Cai, X. Qian, J. Proteome Res. 7 (2008) 1078.
[27] S. Akgol, A. Denizli, J. Mol. Catal. B: Enzym. 28 (2004) 7.
[28] P. Prikryl, D. Horak, M. Ticha, Z. Kucerova, J. Sep. Sci. 29 (2006) 2541.
[29] D. Horak, N. Semenyuk, F. Lednicky, J. Polym. Sci. Polym. Chem. Ed. 41 (2003) 1848.
[30] E. Pollert, K. Knizek, M. Marysko, K. Zaveta, A. Lancok, J. Bohacek, D. Horak, B. Babic, J. Magn. Magn. Mater. 306 (2006) 241.
[31] D. Horak, F. Lednicky, E. Petrovsky, A. Kapicka, Macromol. Mater. Eng. 289 (2004) 341.
[32] L. Moravek, V. Kostka, FEBS Lett. 43 (1974) 207.
[33] J. Tang, B. Hartley, Biochem. J. 118 (1970) 611.
[34] B. Sustrova, L. Novotna, Z. Kucerova, M. Ticha, J. Mol. Catal. B: Enzym. 60 (2009) 22.
[35] S.Y. Imanishi, V. Kochin, S.E. Ferraris, A. de Thonel, H. Pallari, G.L. Corthals, J.E. Eriksson, Mol. Cell. Proteomics 6 (2007) 1380.
[36] J.C. Mercier, F. Grosclaude, B. Ribadeau-Dumas, Eur. J. Biochem. 23 (1971) 41.
[37] J.F. Peter, A.M. Otto, Proteomics 10 (2010) 628.
[38] C.L. Gatlin-Bunai, L.H. Cazares, W.E. Cooke, O.J. Semmes, D.I. Malyarenko, J. Proteome Res. 6 (2007) 4517.
[39] X. Liang, G. Fonnum, M. Hajivandi, T. Stene, N.H. Kjus, E. Ragnhildstveit, J.W. Amshey, P. Predki, R.M. Pope, J. Am. Soc. Mass Spectrom. 18 (2007) 1932.
[40] B. Rittich, A. Spanova, P. Salek, P. Nemcova, S. Trachtova, D. Horak, J. Magn. Magn. Mater. 321 (2009) 1667.
[41] D. Horak, I. Hochel, e-Polymers (2005), Available from: http://www.e-polymers.org/061.
[42] A. Stensballe, S. Andersen, O.N. Jensen, Proteomics 1 (2001) 207.
[43] J. Reinders, A. Sickmann, Proteomics 5 (2005) 4052.
[44] U.K. Aryal, D.J.H. Olson, A.R.S. Ross, J. Biomol. Tech. 19 (2008) 296.
[45] P. Cao, J.T. Stults, Rapid Commun. Mass Spectrom. 14 (2000) 1600.
[46] J.C. Smith, D. Fidgeys, Biochem. Cell Biol. 86 (2008) 137.