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Characterisation of phosvitin phosphopeptides using MALDI-TOF mass spectrometry

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ABSTRACT

Putative phosphopeptides produced from enzyme hydrolysis of phosvitin were identified and characterised using MALDI-TOF/MS. Phosvitin was heat-pretreated and then hydrolysed using pepsin, thermolysin, and trypsin at their optimal pH and temperature conditions with or without partial dephosphorylation. Pepsin and thermolysin were not effective in producing phosphopeptides, but trypsin hydrolysis produced many peptides from phosvitin: 12 peptides, 10 of which were phosphopeptides, were identified from the trypsin hydrolysate. Twelve peptides were also identified from the trypsin hydrolysate of partially dephosphorylated phosvitin, but the phosphate groups remaining with the peptides were much smaller than those from the trypsin hydrolysate of intact phosvitin. This suggested that the phosphopeptides produced from the partially dephosphorylated phosvitin lost most of their phosphate groups during the dephosphorylation step. Therefore, partial dephosphorylation of phosvitin before trypsin hydrolysis may not be always recommendable in producing functional phosphopeptides if the phosphate groups play important roles for their functionalities.

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1. Introduction

Phosvitin is the major glycophosphoprotein in egg yolk, which accounts for 60% of the total phosphoproteins and holds about 80% of the total egg yolk phosphorous (Taborsky & Mok, 1967). Egg yolk phosphoproteins consist of phosvitin and phosvettes (Wallace, 1985). Vitellogenin, the hepatically-derived macromolecular lipophosphoprotein in non-mammalian vertebrates, serves as the precursor for lipovitellin and phosvitin. The molecular weights of the minor and major phosvitin are 36 kDa and 40 kDa, respectively (Taborsky & Mok, 1967). A detailed analysis indicated that phosvitin is composed of 217 amino acids with 123 serine, 15 lysine, 13 histidine, and 11 arginine residues. Serine is the major amino acid, which accounts for more than 55% of the total amino acids in phosvitin, and many of the serines are arranged in clusters of up to 15 consecutive residues (Byrne et al., 1984). Phosphorylation is one of the major post transitional modifications of proteins: in vertebrates, 89.96% of phosphorylation occurs in serine and 9.99% occurs in threonine residue (Mann et al., 2002).

Almost all the serine residues in phosvitin are phosphorylated, and thus phosvitin molecule has extremely strong metal binding capacity and inhibits the bioavailability of metal ions (Byrne et al., 1984). Fragmentation of phosvitin to small peptides using proteolytic enzymes can increase the bioavailability of calcium and iron because the enzymatically hydrolysed peptides inhibit the formation of insoluble calcium phosphates or iron phosphates, which helps the absorption of calcium and iron in guts (Choi, Jung, Choi, Kim, & Ha, 2005). An interesting feature of phosphopeptides is their ability to form soluble organophosphate salts. The phosphorylated serine moiety of the phosphopeptides play the major role in binding divalent metal ions such as Ca, Mg, Zn, Cu, Fe etc. (Hansen, Sandström, Jensen, & Sorensen, 1997; Kitts, 2005; Li, Tomé, & Desjeux, 1989), and promoted intestinal absorption of

calcium, minerals and other trace minerals (Konings, Kuipers, & Huis in't Veld, 1999). Mellander (1947) first reported that phosphopeptides derived from casein (CPP) enhanced the calcification of bones. The absorption of iron in the gastrointestinal tract is low because iron forms heavy molecular weight ferric hydroxide in the guts (Derman et al., 1977). However, in the presence of CPP, enhanced iron availability and iron absorption in the gastrointestinal system has been observed. Therefore, phosvitin is an attractive substrate to produce functional phosphopeptides for various nutraceutical applications as antioxidant or carriers for metal ions, which can be useful in the food and pharmaceutical industries (Kitts, 1994; Kitts & Weiler, 2003). However, the enzymatic hydrolysis of natural phosvitin is extremely difficult because almost all the serine residues in phosvitin are phosphorylated. In the natural phosvitin, the negative charges of the phosphate group surround the phosvitin molecule and prevent enzymes from access to peptide bonds (Gray, 1971; Mecham & Olcott, 1949). Therefore, certain pretreatments that can open phosvitin structure are necessary before enzyme treatment.

With the recent advancement of mass spectrometry (MS), almost all the traditional techniques for amino acid sequencing and molecular characterisation have been replaced by the mass spectrometry. The high sensitivity, resolution and mass accuracy have resulted in mass spectrometry as a major tool in proteomics, especially in phosphopeptide analyses (Griffin, Goodlett, & Aebersold, 2001; Mann et al., 2002). In order to produce functional phosphopeptides with antioxidant and mineral binding activities, hydrolysing phosvitin to smaller peptides (<3 kDa) are desirable. Along with the exploration of functional characteristics of the peptides produced, identification and characterisation of the peptides in the hydrolysates are essential.

The objective of present study was to produce phosphopeptides from phosvitin using heat pretreatment and enzyme hydrolysis, and report the preliminary characterisation of the phosphopeptides produced using Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF/MS).

2. Materials and methods

2.1. Materials

Trypsin Type I from bovine pancreas (E.C. 3.4.21.4; ~15,500 U/mg protein), pepsin from porcine gastric mucosa (E.C. 3.4.23.1; 3,220 U/mg) and thermolysin-Type X from *Bacillus thermoproteolyticus rokko* (E.C. 3.4.24.27; 50–100 U/mg), alkaline phosphatase from bovine liver (E.C. 3.1.3.1; 10 U/mg), acetonitrile (ACN), HPLC grade water, α-cyano-4-hydroxy-cinnamic acid (CHCA), and formic acid were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Phosvitin used was prepared from chicken egg yolk using the method of Ko, Nam, Jo, Lee, and Ahn (2011). Phosvitin (10 mg/mL) was dissolved in distilled water and heated at 100 °C for 60 min to improve hydrolysis before use.

2.2. Partial dephosphorylation of phosvitin

Partially dephosphorylated samples of phosvitin were prepared from phosvitin using alkaline phosphatase. The phosvitin (10 mg/mL) was mixed with the same volume of 200 mM sodium phosphate buffer (pH 10) at an enzyme/substrate ratio of 1:50 (w/w) and incubated at 37 °C for 24 h in a shaker water bath. Alkaline phosphatase was deactivated by placing the sample in a boiling water bath for 10 min. The sample was dialyzed for 24 h at 4 °C and then lyophilized (FreeZone Freeze Dryer, Labconco Corp., Kansas City, MO, USA).

2.3. Enzymatic hydrolysis of phosvitin

The phosvitin and partially dephosphorylated phosvitin were digested using trypsin, pepsin or thermolysin. The pH of phosvitin solutions was adjusted using 1 N HCl or 1 N NaOH prior to addition of the protease. Temperature and pH conditions for each enzyme were as follow: Trypsin at 37 °C and pH 8.0; pepsin at 37 °C and pH 2.0; and thermolysin at 68 °C and pH 6.8. The enzyme/substrate ratio was 1:100 (w/w) in all cases. Digestions were carried out for 24 h in a shaker water bath (C7 – New Brunswick Scientific, Edison, NJ, USA). The enzymatic digestion was arrested by keeping the sample for 10 min in a boiling water bath. The resulting hydrolysates were lyophilized.

2.4. SDS–PAGE electrophoresis

The enzymatic hydrolysate of phosvitin was dissolved in distilled water at 1 mg protein/mL. Sample (10 μl) was mixed with 40 μl of Laemmli sample buffer solution under reducing conditions, heated at 95 °C in a block heater for 5 min, and then 10 μl of sample was loaded on the Mini-PROTEIN Tetra cell (Bio-Rad Laboratory Inc.). Ten percent SDS–PAGE gel and Coomassie Brilliant Blue R-250 (Bio-Rad) containing 0.1 M aluminium nitrate staining solution were used for analysis the proteins and peptides containing phosphorus from the modified Coomassie Blue method (Hegenauer, Ripley, & Nace, 1977). After destaining over-night, the proportion of each peptide bands was calculated by converting the density of each band in the gel picture using the ImageJ software (NIH, Bethesda, MD, USA) as the percent of the total gel density.

2.5. MALDI-TOF/MS analysis

The lyophilized hydrolysate was dissolved in distilled water (10 mg/mL) and centrifuged at 3000g for 10 min. The supernatant was collected, filtered through a 0.45 μm Millipore Millex-FH filter (Billerica, MA, USA), passed through a C_{18} ZipTip Pipette Tip (Millipore, Billerica, MA, USA) to remove some of the salts from the sample, and then deposited on a stainless steel MALDI plate using the dried droplet method (Cohen & Chait, 1996; Hillenkamp, Karas, Beavis, & Chait, 1991). A saturated solution of α-cyano-4-hydroxy-cinnamic acid in 70% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) was prepared for use as matrix. Matrix and sample were mixed in 1:1 (v:v) ratio, dropped on the MALDI plate, and rinsed with 1 μl of 0.1% after drying the droplets. Mass spectra were acquired using a Bruker Microflex Linear TOF Mass Spectrometer (Bruker Optics, Bellerica, MA). Spectra were acquired over the mass range of 600–4000 Da using 50 laser shots in the positive ion mode at a laser power of 25%. Calibration of the mass spectra was achieved using a 1 μm solution of Bradykinin (*m/z* 1060.6) that also contained 1 μm Neurotensin (*m/z* 1672.9).

2.6. Bioinformatics analysis of MALDI data

To generate theoretical peptide digestion patterns, the Protein Prospector MS-Digest software (UC-San Francisco, CA) was used. The software parameters were set to provide digest peptides ranging from 400 to 3000 Da with a maximum number of two missed cleavages, and a variable number of phosphorylation sites. The appropriate protease was selected.

3. Results and discussion

Phosvitin is generally resistant to proteolysis, presumably due to the large number of negative charges present. The SDS–PAGE of
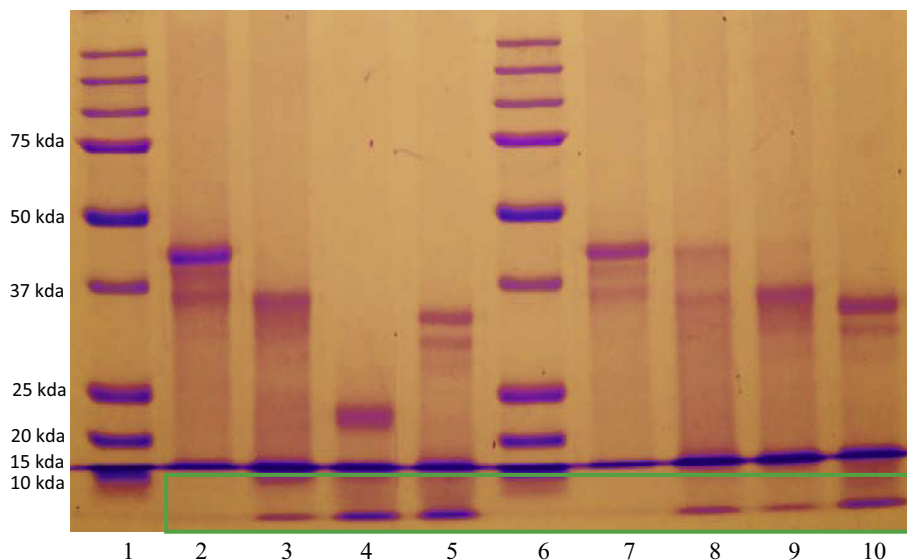
Fig. 1. The SDS–PAGE pattern of pepsin, trypsin and thermolysin digest of phosvitin with and without partial dephosphorylation using alkaline phosphatase. Lane 1, molecular marker; lane 2, heat-treated phosvitin; lane 3, phosvitin hydrolysed with pepsin; lane 4, phosvitin hydrolysed with thermolysin; lane 5, phosvitin hydrolysed with trypsin; lane 6, molecular marker; lane 7, heat-treated phosvitin; lane 8, alkaline phosphatase dephosphorylated phosvitin hydrolysed with pepsin; lane 9, alkaline phosphatase dephosphorylated phosvitin hydrolysed with thermolysin; lane 10, alkaline phosphatase dephosphorylated phosvitin hydrolysed with trypsin.

enzyme hydrolysates of phosvitin resulted in one clear band at the bottom of the gel (<5 kDa), and major bands and smears with molecular sizes >10 kDa implying high resistance of phosvitin to protease activities (Fig. 1). The SDS–PAGE pattern of pepsin, thermolysin and trypsin digestion of the phsovitin indicated that the molecular weight of the larger fragments ranged from 15 to 35 kDa (Fig. 1).

The protease profile of representing peptides tentatively identified from MALDI-TOF analysis of the pepsin, thermolysin, and trypsin hydrolysates of phosvitin are listed in Table 1. Potential peptide identifications were made using the appropriate theoretical digestion of the phosvitin sequence using protein prospector. The MALDI-TOF MS spectra agreed with the theoretical peptide digest patterns predicted by Protein Prospector MS-Digest software.

Pepsin digestion of phosvitin produced 4 peptides, with tentative identifications including phosphopeptides EFGTEPDAKTSSSSS-SASSTA (m/z 2113.4, Pv[2–22]1P) and GTEPDAKTSSSSSSASSTA (m/z 2397.5, Pv[4–22]8P). The non-phosphorylated peptides PDAKTSSSSSSASSTATSSSSS (m/z 1288.3, Pv[7–28]) and TSSSSSSA (m/z 875.7, Pv[23–30]) were also observed. Although it would be tempting to speculate that peptide Pv[4–22] contained eight phosphates, the authors realise that multiply negatively charged ions are detected in positive linear MALDI-TOF MS with extreme low efficiency. It is also likely that there may be counter ions present, potentially even calcium ions since phosvitin is particularly effective at binding calcium. Two calcium ions for example, would add 78.1 Da to the mass of a peptide. At this juncture we cannot

Table 1
Tentatively identified peptides in the pepsin, thermolysin, and trypsin hydrolysates of phosvitin[a] using MALDI-TOF/MS.

Position[b]	Sequence	m/z Observed	m/z Predicted
Pepsin hydrolysis			
2–22	EFGTEPDAKTSSSSSSASSTA (+1 PO$_4$)	2113.4	2113.8–2115.0
4–22	GTEPDAKTSSSSSSASSTA (+8 PO$_4$)	2397.5	2396.2–2397.7
7–28	PDAKTSSSSSSASSTATSSSSS	1288.3	1288.6–1289.4
23–30	TSSSSSSA	875.7	873.3–873.6
Thermolysin hydrolysis			
193–205	EDDSSSSSSSSSV (+2 PO$_4$)	1446.7	1446.5–1447.2
205–214	VLSKIWGRHE (+1 PO$_4$)	1304.7	1304.7–1305.4
209–214	IWGRHE	797.1	797.4–797.9
209–215	IWGRHEI	910.3	910.5–911.1
209–217	IWGRHEIYQ	1201.6	1201.6–1202.4
210–215	WGRHEI	797.1	797.4–797.9
Trypsin hydrolysis			
1–10	AEFGTEPDAK (+1 PO$_4$)	1093.3	1092.5–1093.2
64–80	SSNSSKRSSSKSSNSSK (+8 PO$_4$)	2411.5	2412.6–2413.7
81–94	RSSSSSSSSSSSSR (+8 PO$_4$)	2042.1	2043.4–2044.2
81–94	RSSSSSSSSSSSSR (+9 PO$_4$)	2122.3	2123.3–2124.2
81–94	RSSSSSSSSSSSSR (+10 PO$_4$)	2202.9	2203.3–2204.2
81–94	RSSSSSSSSSSSSR (+11 PO$_4$)	2284.0	2283.3–2284.2
82–94	SSSSSSSSSSSSR (+3 PO$_4$)	1460.6	1459.4–1460.1
82–94	SSSSSSSSSSSSR (+4 PO$_4$)	1540.6	1539.4–1540.1
82–94	SSSSSSSSSSSSR (+5 PO$_4$)	1620.3	1620.4–1621.0
115–121	SSSSSSR	804.6	805.3–805.7
128–154	SSSSSSSSSSSSSKSSSSRSSSSSSK (+11 PO$_4$)	3426.5	3427.7–3429.3
179–208	RSVSHHSHEHHSGHLEDDSSSSSSSSSVLSK	3101.0	3098.4–3100.2

[a] Phosvitin was heat-pretreated for 60 min at 100 °C before the enzyme hydrolysis.
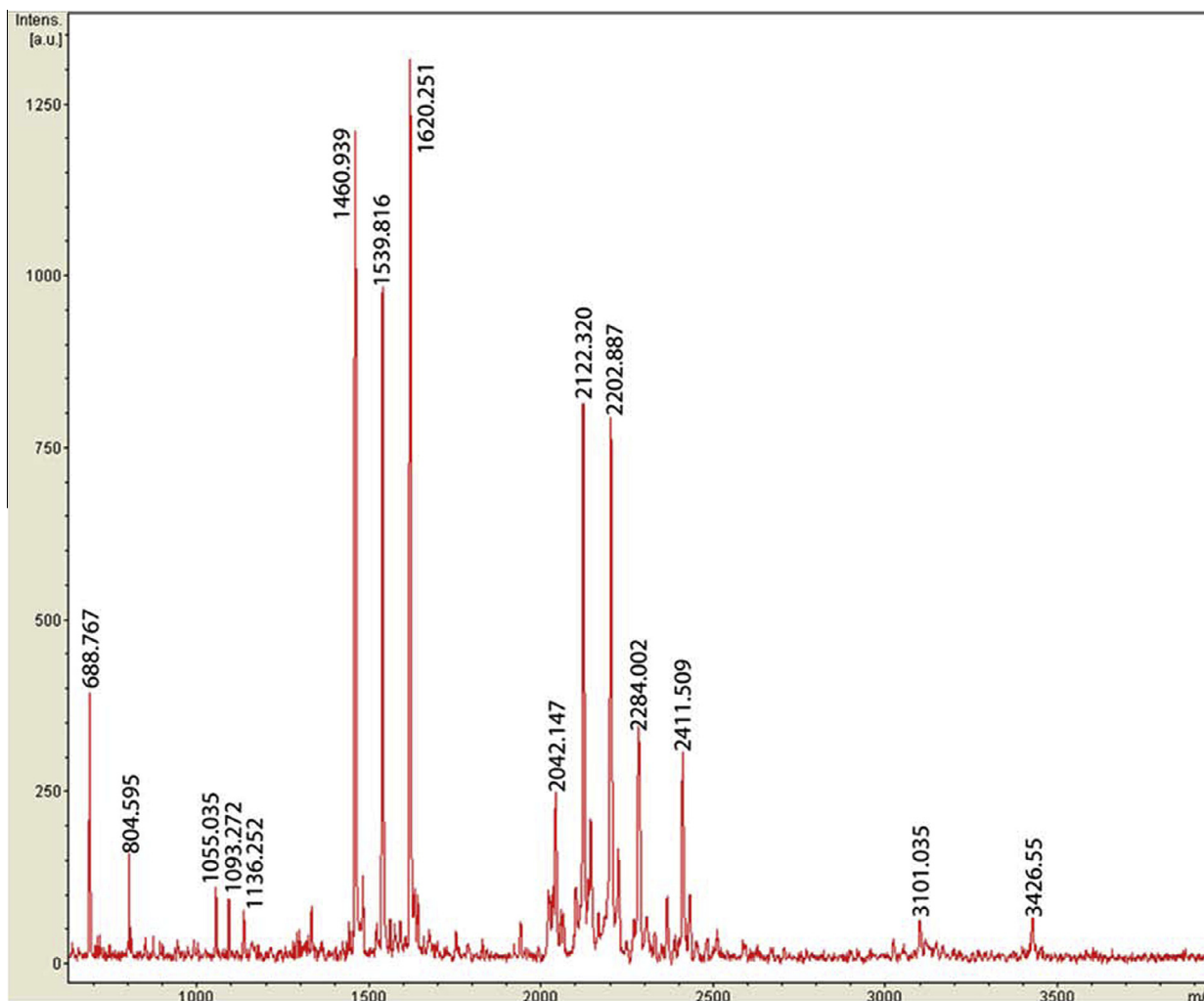[b] Amino acid position in phosvitin.

Fig. 2. MALDI spectra of the peptides from trypsin hydrolysate of phosvitin (phosvitin was heat-pretreated at 100 °C for 60 min and then hydrolysed using trypsin for 24 h at 37 °C).

commit to any particular interpretation of these spectra except to say that we have generated pepsin digest maps and are pursuing confirmation.

Digestion with thermolysin produced 6 peptides, which include EDDSSSSSSSSV (m/z 1446.7, Pv[193–205]2P), VLSKIWGRHE (m/z 1304.7, Pv[205–214]1P), IWGRHE (m/z 797.1, Pv[209–214]), IWGRHEI (m/z 910.3, Pv[209–215]), IWGRHEIYQ (m/z 1201.6, Pv[209–217]), and WGRHEI (m/z 797.1, Pv[210–215]), 2 of which were phosphorylated. It is interesting to note that pepsin hydrolysed only the N-terminal of the phosvitin while thermolysin cleaved peptide bonds in C-terminal of the phosvitin (Table 1).

These results indicated that pepsin and thermolysin were not effective in hydrolysing phosvitin to produce phosphopeptides. Trypsin treatment, on the other hand, was much better than pepsin and thermolysin treatments in hydrolysing phosvitin, and produced 12 putative peptides from phosvitin. Of these trypsin peptides, 10 of them were phosphorylated (Table 1, Fig. 2). The amino acid sequences of the peptides identified include AEFGTEP-DAK (m/z 1093.3, Pv[1–10]1P), SSNSSKRSSSKSSNSSK (m/z 2411.5, Pv[64–80]8P), SSSSSSR (m/z 804.6, Pv[115–121]), SSSSSSSSSS SSSKSSSSRSSSSSSK (m/z 3426.6, Pv[128–154]11P), and RSVSHH SHEHHSGHLEDDSSSSSSSSVLSK (m/z 3101.0, Pv[179–208]). The peptides RSSSSSSSSSSSSSR (m/z 2042.1, 2122.3, 2202.9 & 2284.0, Pv[81–94]8–11P) and SSSSSSSSSSSSSR (m/z 1460.6, 1540.4 & 1620.3, Pv[82–94] 3–5P) were found in multiple phosphorylation states. Trypsin hydrolysis of dephosphorylated phosvitin produced

12 peptides (two peptides with 2 two different numbers of phosphates groups) with the amino acid sequences of KKPMDEEENDQVK (m/z 1589.7, Pv[36–48]), KPMDEEENDQVK-QARNKDASSSSR (m/z 2990.1, Pv[37–60]3P), DASSSSR (m/z 1030.3, Pv[54–60]4P), SSSSSSSSSSSSSR (m/z 1460.6 & 1540.6, Pv[82–94]3P & 4P), SSSSSSKSSSSSSSR (1428.7 & 1986.7, m/z Pv[108–121]1P & 8P), SSSSSSKSSSSSSRSR (m/z 1830.9, Pv[108–123]3P), SSSSSSRSR (m/z 1339.6, Pv[115–123]5P), SSSKSSSSSSSSSSSSSSK (m/z 1754.9, Pv [124–142]), SSSKSSSSSSSSSSSSS-KSSSSRSSSSSSK (m/z 2990.1, Pv[124–154]1P), SSSSSSSSSSSSSSSKSSSSR (m/z 2636.4 Pv[124–154]1P), and SSSHHSHSHHSGHLNGSSSSSSSSR (m/z 2636.4, Pv[155–179]1P), 10 of which were phosphopeptides (Table 2, Fig. 3). This indicated that hydrolysis of dephosphorylated phosvitin was somewhat better than that without dephosphorylation, and all the identified peptides were in the MW range of 1.4–3.0 kDa. However, peptides from a few segments of phosvitin, including 1–35, 61–81, 95–107, and 180–217, could not been identified from the hydrolysate of partially dephosphorylated phosvitin (Table 2). As expected, the phosphopeptides produced from the partially dephosphorylated phosvitin had smaller number of phosphate groups than those from without dephosphorylation, and some of the peptides had only 1 or no phosphate group.

Young, Nau, Pasco, and Mine (2011) evaluated an ion-exchange chromatographic fraction (named PPP3) of hydrolysate obtained by tryptic digestion of partially dephosphorylated phosvitin (produced by incubation in 0.1 N NaOH for 3 h at 37 °C) using

Table 2
Tentatively identified peptides in the trypsin hydrolysates of partially dephosphorylated phosvitin[a] using MALDI-TOF/MS.

Position[b]	Sequence	m/z Observed	m/z Predicted
36–48	KKPMDEEENDQVK	1589.7	1589.7–1590.8
37–60	KPMDEEENDQVKQARNKDASSSSR (+3 PO₄)	2990.1	2989.2–2990.9
54–60	DASSSSR (+4 PO4)	1030.3	1029.2–1029.8
82–94	SSSSSSSSSSSSR (+3 PO₄)	1460.6	1459.4–1460.1
82–94	SSSSSSSSSSSSR (+4 PO₄)	1540.6	1539.4–1540.1
108–121	SSSSSSKSSSSSSR (+1 PO₄)	1428.7	1427.6–1428.3
108–121	SSSSSSKSSSSSSR (+8 PO₄)	1986.7	1987.3–1988.2
108–123	SSSSSSKSSSSSSRSR (+3 PO₄)	1830.9	1830.6–1831.5
115–123	SSSSSSRSR (+5 PO₄)	1339.6	1340.3–1340.8
124–142	SSSKSSSSSSSSSSSSSSK	1754.9	1754.8–1755.7
124–154	SSSKSSSSSSSSSSSSSSKSSSSRSSSSSSK (+1 PO₄)	2990.1	2989.2–2990.8
155–179	SSSHHSHSHHSGHLNGSSSSSSSSR (+1 PO₄)	2636.4	2637.1–2638.5

[a] Phosvitin was heat-pretreated for 60 min at 100 °C, dephosphorylated for 24 h using alkaline phosphatase, and then hydrolysed 24 h using trypsin.
[b] Amino acid position in phosvitin.

the MALDI-TOF and nano-electrospray mass spectrometry (nES–MS) on Q-TOF hybrid quadrupole/TOF instrument and online liquid chromatography tandem mass spectrometry (LC–MS/MS). They observed three main peptides from the C- and N-terminals, and the centre of the phosvitin molecule. Those three peptide sequences were GTEPDAKTSSSSSSASSTATSSSSSSAS-SPNRKKPMDE (Pv[4–41]), NSKSSSSSSKSSSSSSRSRSSSKSSSSSSSSSSSSSSKSSSSR (3 PO₄, Pv[114–147]), and EDDSSSSSSSSSVLSKIWGRHEIYQ (Pv[194–217]). Ten peptides from the N-terminal residues (Pv[194–217]) also have been identified.

Our results are different from those of Goulas, Triplett, and Taborsky (1996) and Young et al. (2011). The exact reason for this observation is not clear, but the heating of phosvitin at 100 °C for 60 min before enzyme hydrolysis may have opened up the compact structure and helped enzyme attack to the peptide bonds of the

inner phosvitin molecule. It is well known that the degree of phosphorylation in phosvitin varies from 3% to 10% (Taborsky & Mok, 1967), and the phosvitin used in the present study contained 7% phosphate (AOAC International, 2000). The current study indicated that the location of phosphorylation in phosvitin and the degree of phosphorylation have significant effects to the access of enzyme to the peptide bonds of the phosvitin (Tables 1 and 2).

One of the noticeable differences between the peptides from the phosvitin and dephosphorylated phosvistin is the number of phosphate groups remaining in the peptides (Table 2). Trypsin hydrolysis of the dephosphorylated phosvitin produced greater number of phosphopeptides, but the number of phosphate groups in the phosphopeptides were lower than the ones from the phosvitin without dephosphorylation. The metal-binding capacity and the binding strength of phosphopeptides are proportional to the
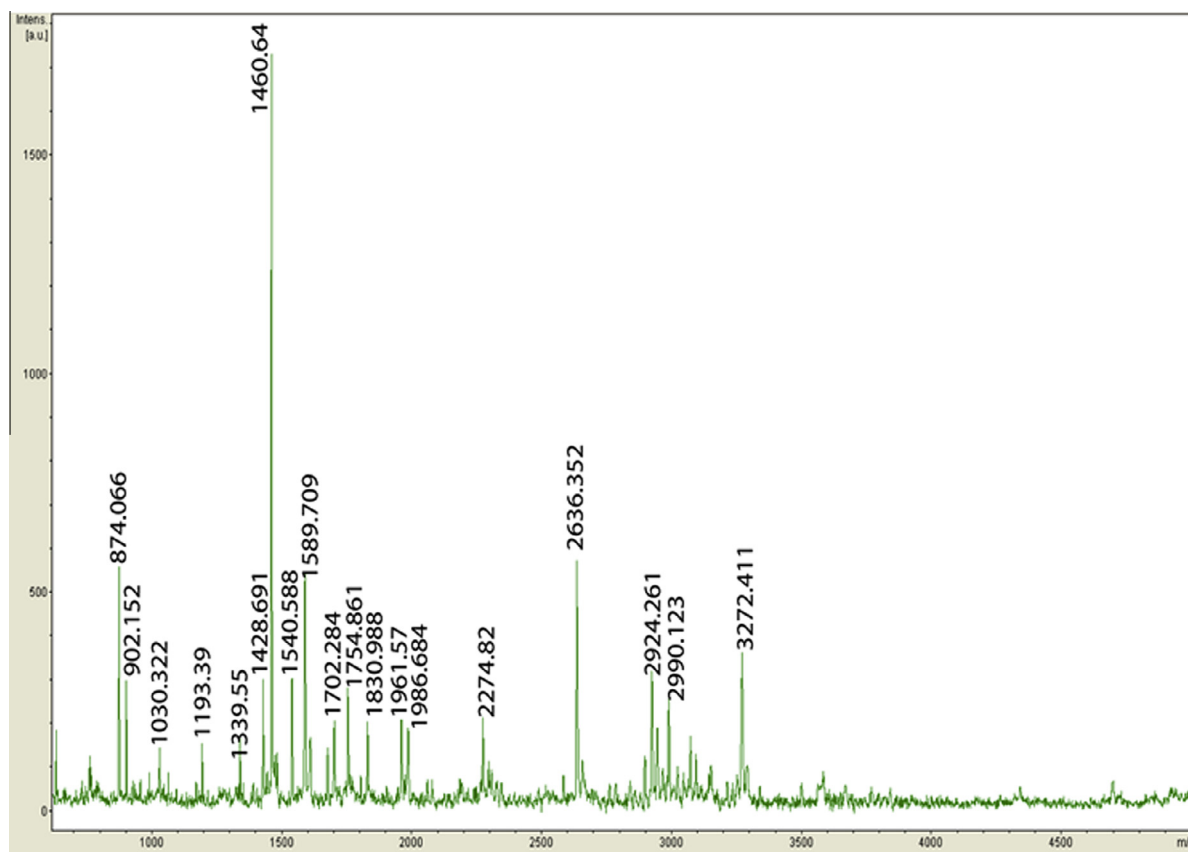

Fig. 3. MALDI spectra of the peptides from trypsin hydrolysate of partially dephosphorylated phosvitin (phosvitin was heat-pretreated at 100 °C for 60 min and then partially dephosphorylated (24 h at 37 °C) using alkaline phosphatase before trypsin hydrolysis for 24 h at 37 °C).

number of phosphophate groups in a peptide, and thus too many or less than 2 phosphate groups in a peptide may not be desirable for them to be used as an antioxidant, or calcium- or iron-supplementing agent. Therefore, removing too many phosphate groups from the phosvitin may not be desirable even though partial dephosphorylation helped enzymatic hydrolysis of phosvitin.

In general, the size of a bioactive peptide can vary from two to twenty amino acid residues (Gill, López-Fandiño, & Vulfson, 1996). Pantzar, Westrm, Luts, and Lundin (1993) studied the small intestinal permeability of different size molecules and found that molecules in the range of 1–3-kDa are highly permeable. Miquel et al. (2005) suggested the potentiality of CPP of 1125–6512 Da, produced by milk-based infant formulas, for intestinal absorption and physiological role in mineral bioavailability. Reynolds (1998) reported that the cluster sequence -Ser(P)$_3$-Glu$_2$ of those CPPs was responsible for the interaction with amorphous calcium phosphate (ACP) and also adjoining Ser(P) residues played an important role for the maximum interaction of ACP. In phosvitin phosphopeptides, the particular cluster sequence of -Ser(P)$_3$-Glu$_2$ could not be found due to its primary structure. However, the identified phosvitin phosphopeptides were composed of less than 30 amino acids and several peptides contained phosphorylated serine residues (Tables 1 and 2), indicating the high possibility of using phosvitin-derived phosphopeptides as bioactive/functional peptides.

One important issue with the trypsin hydrolysis of phosvitin to produce phosphopeptides is its low efficiency in producing small peptides. As shown in Fig. 1, majority of the polypeptides were large (>10 kDa) and only a small portion of phosvitin was degraded to peptides with molecular size of <5 kDa (the bottom band, 23.59% and 21.22% for phosvitin hydrolysed with trypsin and alkaline phosphatase dephosphorylated phosvitin hydrolysed with trypsin, respectively). If the peptide sizes are too large and the number of phosphate groups attached is too many, the metal-binding strength of the peptides would be very high. Therefore, the bivalent cations attached to the phosphopeptides will be very difficult to be released. Currently, we are testing various conditions to improve the enzyme efficiencies to hydrolyse phosvitin and to bring the molecular size of the phosphopeptides down to 1–3 kDa range. Also, the peptides should carry >2 phosphate groups in their sequences to have reasonable metal-binding capacity.

4. Conclusions

Heat pretreatment helped the hydrolysis of phosvitin using trypsin, but partial dephosphorylation of phosvitin was better than heat pretreatment alone, presumably because dephosphorylation either reduced the negative charge on phosvitin or directly exposed trypsin cleavage sites. These initial studies pave the way to a more thorough understanding of effective conditions for the digestion of phosvitin. Improvement of the digestion techniques and the use of chromatographic strategies to enrich phosphopeptides will lead to identification of more phosphopeptides. Future studies using MS/MS methods are continuing in order to confirm the sequence of the phosphopeptides identified in this study.

Acknowledgement

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