Characterization of Phosvitin Phosphopeptides using MALDI-TOF Mass Spectrometry

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Summary and Implications

Putative phosphopeptides produced from enzyme hydrolysis of phosvitin were identified and characterized using MALDI-TOF/MS. Phosvitin was heat-pretreated and then hydrolyzed 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 number of phosphate groups remaining with the peptides was 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.

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. 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.

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. Fragmentation of phosvitin to small peptides using proteolytic enzymes can increase the bioavailability of calcium and iron because the enzymatically hydrolyzed peptides inhibit the formation of insoluble calcium phosphates or iron phosphates, which helps the absorption of calcium and iron in guts. Therefore, phosvitin is an attractive substrate to produce functional phosphopeptides. 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. 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 characterization 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. In order to produce functional phosphopeptides with antioxidant and mineral binding activities, hydrolyzing phosvitin to smaller peptides (< 3 kDa) are desirable. Along with the exploration of functional characteristics of the peptides produced, identification and characterization 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 characterization of the phosphopeptides produced using Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF/MS).

Materials and Methods

Sample preparation: Phosvitin used was prepared from chicken egg yolk. Phosvitin (10 mg/ml) was dissolved in distilled water and heated at 100 ^{o}C for 60 min to improve hydrolysis before use. Partially dephosphorylated phosvitin were prepared from phosvitin using alkaline phosphatase. The natural phosvitin and partially dephosphorylated phosvitin were digested using trypsin, pepsin or thermolysin. The enzyme/substrate ratio was 1:100 (w/w) in all cases. The degree of enzymatic hydrolysis of phosvitin was determined using SDS-PAGE.

MALDI-TOF/MS Analysis: The lyophilized hydrolysate was dissolved in distilled water, filtered through a 0.45 µm Millipore Millex-FH filter, passed through a C_{18} ZipTip Pipette Tip to remove some of the salts from the sample, and then deposited on a stainless steel MALDI plate using the dried droplet method. Mass spectra were acquired using a Bruker Microflex Linear TOF Mass Spectrometer. Spectra were acquired over the mass range of 600 to 4,000 Da using 50 laser shots in the positive ion mode at a laser power of 25%.

Bioinformatics analysis of MALDI data: To generate theoretical peptide digestion patterns, the Protein Prospector

MS-Digest software 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.

Results and Discussion

Phosvitin is generally resistant to proteolysis, presumably due to the large number of negative charges present. The SDS-PAGE of 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 (Figure 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.

Pepsin digestion of phosvitin produced 4 peptides, with tentative identifications including phosphopeptides EFGTEPDAKTSSSSSASSTA (m/z 2113.4, Pv[2-22]1P) and GTEPDAKTSSSSSSASSTA (m/z 2397.5, Pv[4-22]8P). The non-phosphorylated peptides PDAKTSS-SSSSASSTATSSSSS (m/z 1288.3, Pv[7-28]) and TSSSSSSA (m/z 875.7, Pv[23-30]) were also observed.

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. These results indicated that pepsin and thermolysin were not effective in hydrolyzing 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 AEFGTEPDAK (m/z 1093.3, Pv[1-10]1P), SSNSSKRSS-SKSSNSSK (m/z 2411.5, Pv[64-80]8P), SSSSSSR (m/z 804.6, Pv[115-121]), SSSSSSSSSSS-SSKSSSSRSSSSS-SK (m/z 3426.6, Pv[128-154]11P), and RSVSHHSHEHH-SGHLEDDSSSSSSSSVLSK (m/z 3101.0, Pv[179-208]). The peptides RSSSSSSSSSSSSR (m/z 2042.1, 2122.3, 2202.9 & 2284.0, Pv[81-94]8-11P) and SSSSSSS-SSSSSR (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]), KPMD-EEENDQVKQARNKDASSSSR (m/z 2990.1, Pv[37-60]3P), DASSSSR (m/z 1030.3, Pv[54-60]4P), SSSSSS-SSSSSSR (m/z 1460.6 & 1540.6, Pv[82-94]3P & 4P), SSSSSSKSSSSSSR (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), SSSKSSSSSSSSSSSSSK (m/z 1754.9, Pv [124-142]), SSSKSSSSSSSSSSSSSKSSSSRSSSSSSK (m/z 2990.1, Pv[124-154]1P), SSSSSSSSSSSSSSKSSSSR (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.

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 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. One important issue with the trypsin hydrolysis of phosvitin to produce phosphopeptides is its low efficiency in producing small peptides. As shown in Figure 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 hydrolyzed with trypsin and alkaline phosphatase dephosphorylated phosvitin hydrolyzed with trypsin, respectively). If the peptides 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. Also, the peptides should carry > 2 phosphate groups in their sequences to have reasonable metal-binding capacity.

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. Improvement of digestion techniques and the use of chromatographic strategies to enrich phosphopeptides will lead to identification of more phosphopeptides.

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

Position[2]	Sequence	m/z Observed	m/z Predicted
Pepsin hydrolysis			
22-Feb	EFGTEPDAKTSSSSSSASSTA (+1 PO_4)	*2113.4*	2113.8-2115.0
22-Apr	GTEPDAKTSSSSSSASSTA (+8 PO_4)	*2397.5*	2396.2-2397.7
28-Jul	PDAKTSSSSSSASSTATSSSSS	1288.3	1288.6-1289.4
23-30	TSSSSSSA	875.7	873.3-873.6
Thermolysin hydrolysis			
193-205	EDDSSSSSSSSV (+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			
10-Jan	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	2283.3-2284.7
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	RSVSHHSHEHHSGHLEDDSSSSSSSSVLSK	3101	3098.4-3100.2

[1]Phosvitin was heat-pretreated for 60 min at 100 ^oC before the enzyme hydrolysis.
[2]Amino acid position in phosvitin.

Table 2. Tentatively identified peptides in the trypsin hydrolysates of partially dephosphorylated phosvitin[1] using MALDI-TOF/MS

Position[2]	Sequence	m/z Observed	m/z Predicted
36-48	KKPMDEEENDQVK	1589.7	1589.7-1590.8
37-60	KPMDEEENDQVKQARNKDASSSSR (+3 PO_4)	2990.1	2989.2-2990.9
54-60	DASSSSR (+4 PO4)	1030.3	1029.2-1029.8
82-94	SSSSSSSSSSSSR (+3 PO_4)	1460.6	1459.4-1460.1
82-94	SSSSSSSSSSSSR (+4 PO_4)	1540.6	1539.4-1540.1
108-121	SSSSSSKSSSSSSR (+1 PO_4)	1428.7	1427.6-1428.3
108-121	SSSSSSKSSSSSSR (+8 PO_4)	1986.7	1987.3-1988.2
108-123	SSSSSSKSSSSSSRSR (+3 PO_4)	1830.9	1830.6-1831.5
115-123	SSSSSSRSR (+5 PO_4)	1339.6	1340.3-1340.8
124-142	SSSKSSSSSSSSSSSSSSK	1754.9	1754.8-1755.7
124-154	SSSKSSSSSSSSSSSSSSKSSSSRSSSSSSK (+1 PO_4)	2990.1	2989.2-2990.8
155-179	SSSHHSHSHHSGHLNGSSSSSSSSR (+1 PO_4)	2636.4	2637.1-2638.5

[1]Phosvitin was heat-pretreated for 60 min at 100 ^{o}C, dephosphorylated for 24 h using alkaline phosphatase, and then hydrolyzed 24 h using trypsin.
[2]Amino acid position in phosvitin.

Figure 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 hydrolyzed with pepsin; lane 4, phosvitin hydrolyzed with thermolysin; lane 5, phosvitin hydrolyzed with trypsin; lane 6, molecular marker; lane 7, heat-treated phosvitin; lane 8, alkaline phosphatase dephosphorylated phosvitin hydrolyzed with pepsin; lane 9, alkaline phosphatase dephosphorylated phosvitin hydrolyzed with thermolysin; lane 10, alkaline phosphatase dephosphorylated phosvitin hydrolyzed with trypsin.

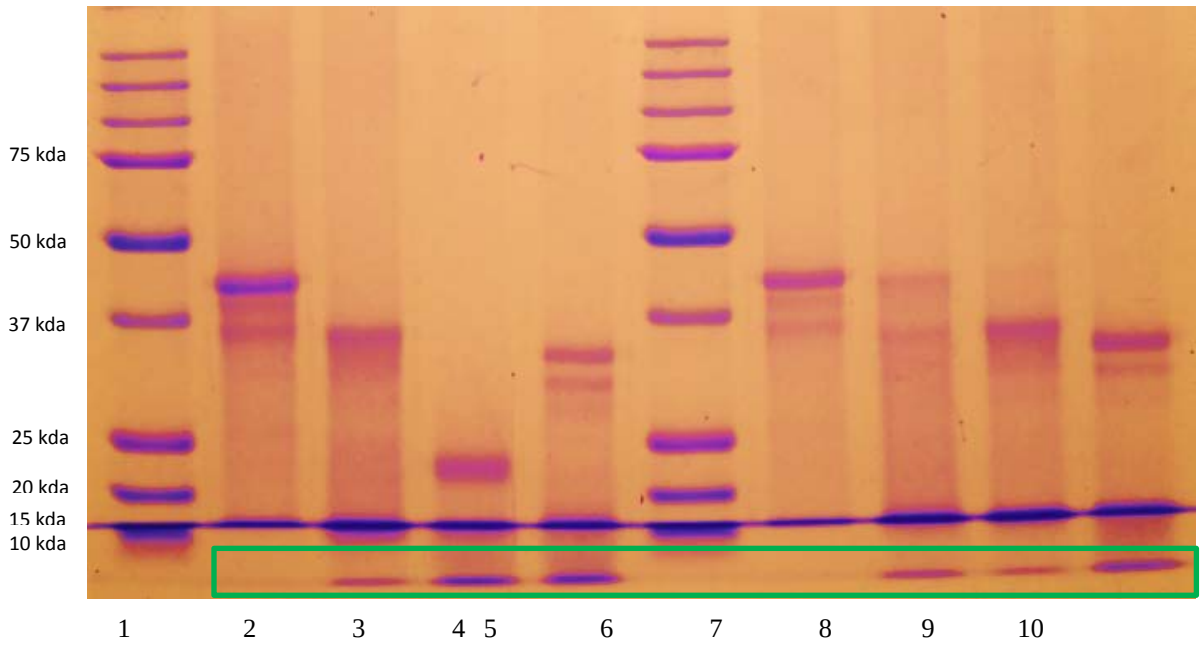
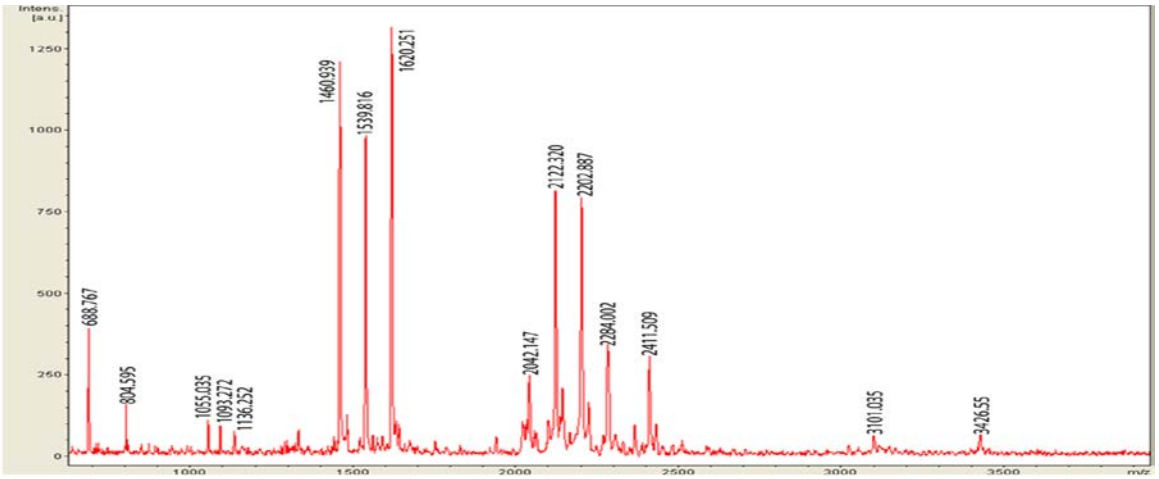
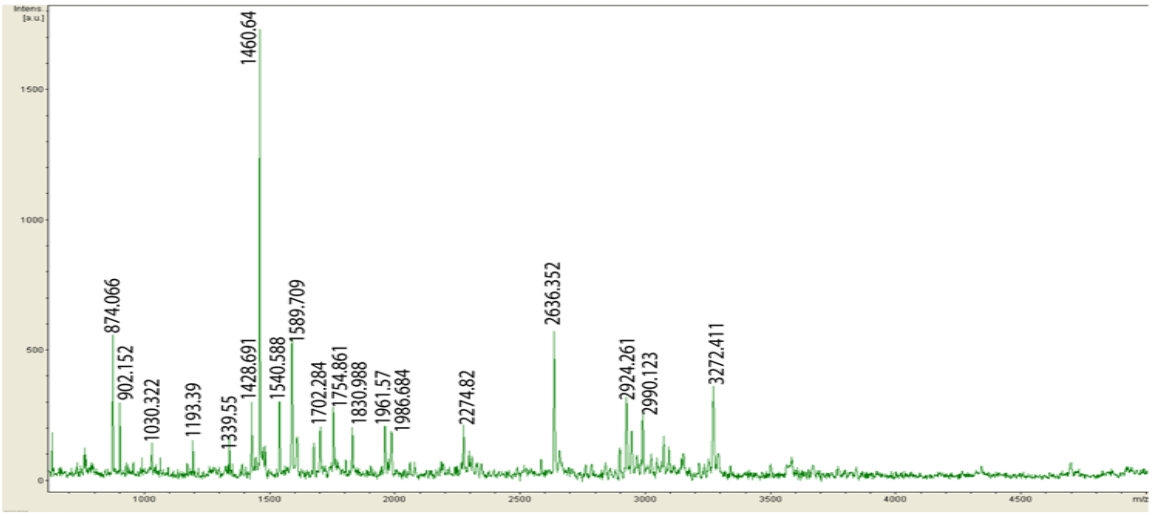


Figure 2. MALDI spectra of the peptides from trypsin hydrolysate of phosvitin[1]



[1]Phosvitin was heat-pretreated at 100 °C for 60 min and then hydrolyzed using trypsin for 24 h at 37 °C.

Figure 3. MALDI spectra of the peptides from trypsin hydrolysate of partially dephosphorylated phosvitin[1]



[1]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.