Characterization of Phosvitin Phosphopeptides using MALDI-TOF Mass Spectrometry

Himali Samaraweera[1]; Sun Hee Moon[1]; Eun Joo Lee[2]; Jennifer E. Grant[3]; Jordan Fouks[4]; Inwook Choi[5], Joo Won Suh[6]; Dong Ahn[1,7]

[1]Dept. of Animal Science, Iowa State Univ., Ames, IA; [2]Dept. of Food and Nutrition, Univ. Wisconsin-Stout, Menomonie, WI; [3]Dept. of Biology, Univ. Wisconsin- Stout, Menomonie, WI; [4]Applied Science Program, Univ. Wisconsin-Stout, Menomonie, WI; [5]Functional Materials Research Group, Korea Food Research Institute, Sungnam, Gyeonggi-Do, S. Korea; [6]Center for Nutraceutical/Pharmaceutical Materials, Myongji Univ., Yongin, South Korea; [7]Dept.of Animal Science and Tech., Sunchon National Univ., Sunchon, South Korea

Introduction

Phosvitin accounts for 60% of the total phosphoproteins, or approximately 80% of the total egg yolk phosphorous in egg yolk. (Taborsky & Mok, 1967). Serine, which accounts for more than 55% of its sequence, is often found arranged in clusters of up to 15 consecutive residues (Byrne, et al 1984). Although phosvitin is an attractive source of functional phosphopeptides for various nutraceutical applications (Kitts & Weiler, 2003), it is highly resistant to enzymatic degradation due to both steric and charge effects (Mecham, & Olcott, 1949; Gray, 1971). Production of phosvitin phosphopeptides by enzymatic fragmentation is extremely difficult. Here we report progress in identifying phosvitin petides, and phosphopeptides, that are generated by combining select enzymatic and chemical strategies.

Objective

To produce phosphopeptides from phosvitin using enzyme hydrolysis, and identify and characterize the phosphopeptides produced using Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF/MS).

Material and Methods

Partially dephosphorylation of phosvitin

- Phosvitin (10 mg/mL) was treated using alkaline phosphatase at an enzyme/substrate ratio of 1:50 (w/w).
- Incubated at 37 °C for 24 hr in 200 mM sodium phosphate (pH 10).
- Dialysis for 24 hr at 4 °C & lyophilized.

Enzymatic hydrolysis of phosvitin

- Samples of both untreated and dephosphorylated phosvitin were digested using trypsin (37 °C, pH 8.0), pepsin (37 °C, pH 2.0) and/or thermolysin (68 °C, pH 6.8) at 1:100 (w/w) for 24 hr and then lyophilized.

SDS-PAGE electrophoresis

- Mini-Protein II cell (Bio-Rad), 10% SDS-PAGE gel and Coomassie Brilliant Blue R-250 containing 0.1M aluminum nitrate (Ko et al., 2011).

MALDI-TOF/MS analysis

- Hydrolysate samples (10 mg/mL) were centrifuged at 3,000 *g* for 10 min.
- The supernatant was collected, filtered through a 0.45 µm filter and passed C_{18} ZipTip Pipette Tip to remove some of the salts from the sample
- A saturated solution of a-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 mL of 0.1% after drying the droplets.
- Mass 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% using a Bruker Microflex Linear MALDI Time-of-Flight Mass Spectrometer (Bruker Optics, Bellerica, MA).
- Calibration of the mass spectra was performed using bradykinin (m/z 1060.6) and neurotensin (m/z 1672.9).

Bioinformatics analysis of MALDI data

- The Protein Prospector MS-Digest software suite (UC-San Francisco, CA) was used was used to generate theoretical peptide digests.
- 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

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

Position[2]	Sequence	m/z Observed	m/z Predicted
Pepsin hydrolysis			
2-22	EFGTEPDAKTSSSSSASSTA (+1 PO_4)	2113.4	2113.8-2115.0
4-22	GTEPDAKTSSSSSASSTA (+8 PO_4)	2397.5	2396.2-2397.7
7-28	PDAKTSSSSSASSTATSSSSS	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			
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	RSSSSSSSSSSSR (+8 PO_4)	2042.1	2043.4-2044.2
81-94	RSSSSSSSSSSSR (+9 PO_4)	2122.3	2123.3-2124.2
81-94	RSSSSSSSSSSSR (+10 PO_4)	2202.9	2203.3-2204.2
81-94	RSSSSSSSSSSSR (+11 PO_4)	2284.0	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	SSSSSSSSSSSSKSSSSRSSSSSSK (+11 PO_4)	3426.5	3427.7-3429.3
179-208	RSVSHHSHEHHSGHLEDDSSSSSSSSVLSK	3101.0	3098.4-3100.2

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

Table 2. Tentatively identified peptides in 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	SSSKSSSSSSSSSSSSSK	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 °C, dephosphorylated for 24 h using alkaline phosphatase, and then hydrolyzed 24 h using trypsin. [2]Amino acid position in phosvitin.

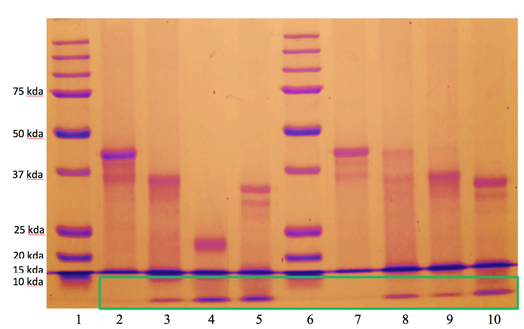

Figure 1. SDS-PAGE digests pattern of phosvitin with and without partial dephosphorylation using alkaline phosphatase. *Lane 1, molecular marker; lane 2, heat-treated phosvitin; lane 3, phosvitin hydrolyzed with pepsin; lane 4, phosvitin hydrolyzed with thermolysin; lane 5, phosvitin hydrolyzed with 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.*

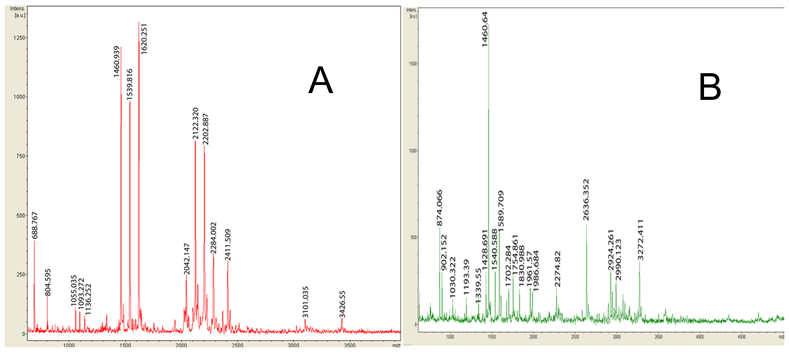

Figure 2. MALDI analysis of the trypsin hydrolysate of phosvitin[1] and partially dephosphorylated phosvitin[2] *[1]Phosvitin (A) and [2]phosvitin that was partially dephosphorylated with alkaline phosphatase (B) were hydrolyzed with trypsin for 24 h at 37 °C.*

Discussion

- Pepsin digestion of phosvitin produced four peptides including two potential phosphopeptides
- Digestion with thermolysin produced one phosphopeptide and two non-phosphorylated peptides.
- Trypsin hydrolysis of phosvitin produced 12 potential peptides and a cluster of ion signals in the m/z range 2000-2500.
- It is not clear whether specific ion signals represent highly phosphorylated peptides, peptides complexed with ions, or other phenomena.
- This analysis is complicated by the potential ion binding nature of these phosphopeptides.
- Clusters of ions, like calcium, complicate the interpretation of what are noted here as potential multiply phosphorylated peptides.
- Trypsin hydrolysis of dephosphorylated phosvitin produced 7 potential multiply phosphorylated and three monophosphorylated peptides.

Conclusions

- These initial studies pave the way to a more thorough understanding of effective conditions for the digestion of phosvitin.
- 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.
- Future studies involve confirming the sequence of the phosphopeptides identified in this study using MS/MS methods.

Acknowledgement

This study was supported jointly by the Korea Food Research Institute (Grant No. E0134115), Republic of Korea, and the Next-Generation BioGreen 21 Program (No. PJ PJ00964305), Rural Development Administration, Republic of Korea.

References

Byrne, B. M., van Het, S. A. D, van de Klundert, J.A.M., Arnberg, A. C., Gruber, M., & Geert, A. B. (1984). Amino acid sequence of phosvitin derived from the nucleotide sequence of part of the chicken vitellogenin gene, *Biochemistry, 23*, 4275-4279.

Gray, H. B. (1971). Structural models for iron and copper proteins based on spectroscopic and magnetic properties, *Advances in Chemistry Series*, *100*, 365-389.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery, *Current Pharmaceutical Design*, *9*, 1309-1323.

Ko, K. Y., Nam, K. C., Jo, C., Lee, E. J., & Ahn, D. U. (2011). A simple and efficient method for separating phosvitin from egg yolk using ethanol and sodium chloride, *Poultry Science, 90*, 1096-1104.

Mecham, D. K. & Olcott, H. S. (1949). Phosvitin, the principal phosphoprotein of egg yolk, *Journal of the American Chemical Society*, *71*, 3670-3679.

Taborsky, G., & Mok, C. (1967). Phosvitin homogeneity and molecular weight, *The Journal of Biological Chemistry, 242*, 1495-1501.