MALDI TOF Analysis of the Enrichment of Phosvitin Peptides using Affinity Microchromatography Techniques.

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ABSTRACT

Phosvitin, a protein purified from chicken egg yolk contains 123 serine amino acids, which can become phosphorylated by the addition of a phosphate group (PO42-). This addition yields an ionic charge with the ability to bind 2+ cations such as zinc,

calcium, magnesium, or strontium. Phosvitin's many serines lead to a strong interaction with the ions.

Hydrolysis of Phosvitin may produce phosphopeptides with the same attraction to 2+ cations but with a smaller molar mass and increased ability to stay suspended in solution.

Digested Phosvitin sample will be enriched for phosphopeptides using one of three methods: Strong Anion Exchange (SAX), Titanium Dioxide (TiO2), or Zirconium Dioxide (ZrO2). Chromatography binds negatively charged particles and they are eluted as enriched sample. Salts are removed with C18 ZipTip. Remaining samples peptides will be identified with Matrix-Assisted-Laser-Desorption/Ionization Time-of-Flight Mass Spectrometer (MALDI-TOF).

MATERIALS AND METHODS

Aliquots of phosvitin were digested in parallel with trypsin or thermolysin at an enzyme/substrate ration of 1:50 (w/w), at a pH of 8.0 and 6.8, respectively, for 24 hours at 37^{o} C. 5 µg portions of the digests were subjected to strong anion exchange chromatography (Hypersep SpinTip POROS Strong Anion, Thermo Scientific) , zirconium dioxide affinity chromatography (NT1ZRO.96, Glygen Corp), or titanium dioxide ion affinity chromatography (TT1TIO.96, Glygen Corp) using a variety of commercially available resins. ZipTip C18 (ZTC18S096, Merck Millipore Ltd) were used for every sample to remove ionized salts form the sample in preperation of MALDI-TOF analysis.

Data analysis was performed with a Bruker Microflex LT MALDI-TOF mass spectrometer set at a laser power ranging from 25-30%. 100 shots were collected per sample, and data were acquired over the mass range from m/z 600 to 4000. Peptide assignments based on m/z values were made using the software MS-digest, available through the protein prospector suite (prospector.ucsf.edu).

Chromatography Workflow

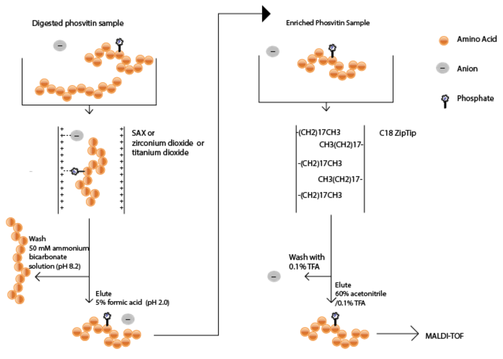

Figure 1: Demonstrates sequential use of two microcolumn

MALDI SPECTRA

Zirconium Dioxide

Table 1

Sample ZrO2	Peaks	Phosphates	Sequence	Shared
NaOH Trypsin	1538.3734-1539.0872	4	(R)SSSSSSSSSSNSK(S)	Yes
	1618.3397-1619.0670	5	(R)SSSSSSSSSSNSK(S)	Yes
	1695.4697-1696.2634	4	(K)RSSSSSSSSSSSR(S)	No
	1910.5967-1911.5173	4	(K)SSSSSSKSSSSSSSR(S)	Yes
	2179.0993-2179.9136	12	(R)SSSSSSSSSSSSR(S)	Yes
	1539.3686-1540.0748	4	(R)SSSSSSSSSSSR(S)	Yes
	1696.6340-1697.4890	3	(R)SSKSSNSSKRSSSK(S)	Yes
	2157.8181-2158.9375	2	(R)SRSSSKSSSSSSSSSSSSSSK(S)	Yes
	2157.6689-2158.7092	5	(K)DASSSSRSSKSSNSSKR(S)	Yes
Heat Trypsin	1696.6340-1697.4890	3	(R)SSKSSNSSKRSSSK(S)	Yes
	2157.8181-2158.9375	2	(R)SRSSSKSSSSSSSSSSSSSSK(S)	Yes
	2157.6689-2158.7092	5	(K)DASSSSRSSKSSNSSKR(S)	Yes
	2179.0993-2179.9136	12	(R)SSSSSSSSSSSSR(S)	Yes
	1539.3686-1540.0748	4	(R)SSSSSSSSSSSR(S)	Yes
	1619.3349-1620.0547	5	(R)SSSSSSSSSSSR(S)	Yes
Heat Trypsin Protex	1936.5330-1937.4285	6	(R)SSKSSNSSKRSSSK(S)	No
Heat Trypsin Multifect	1078.4412-1079.0081	1	(R)SSKSSNSSK(R)	No
Heat DP	1251.3691-1251.9666	3	(K)DASSSSRSSK(S)	No
Heat DP Trypsin	1619.3349-1620.0547	5	(R)SSSSSSSSSSSR(S)	Yes
Heat DP Trypsin Protex	1071.3755-1071.9093	2	(R)SSKSSNSSK(R)	Yes

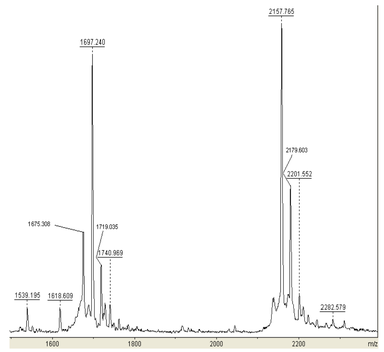

Figure 2: Phosvitin digested with a NaOH Heat Trypsin protocol, enriched using ZrO2 tip method

Titanium Dioxide

Table 2

Sample TiO2	Peaks	Phosphates	Sequence	Shared
NaOH Trypsin	1670.6977-1671.5777	1	(K)SSSSSSKSSSSSSSR(S)	Yes
	1695.4697-1696.2634	4	(K)RSSSSSSSSSSSR(S)	Yes
	1928.6702-1929.6255	4	(K)QARNKDASSSSRSSK(S)	No
	2154.5839-2155.6097	5	(R)SSSKSSSSSSSSSSSSSSK(S)	Yes
	1020.4106-1020.9305	1	(K)SSSSSSSRSR(S)	Yes
	1071.3755-1071.9093	2	(R)SSKSSNSSK(R)	Yes
HCl Trypsin	857.2438-857.6431	2	(K)SSSSSSSR(S)	Yes
	1378.4407-1379.1275	2	(R)SSSSSSSSSSNSK(S)	No
	1539.3686-1540.0748	4	(R)SSSSSSSSSSSR(S)	Yes
	1588.2050-1938.9864	9	(R)SSSSSSSSSSSNSK(S)	No
	2245.1908-2246.0776	11	(K)SSSSSSSSSSSSSSK(S)	No
Heat Trypsin	1020.4106-1020.9305	1	(K)SSSSSSRSR(S)	Yes
	1670.6977-1671.5777	1	(K)SSSSSSKSSSSSSSRSR(S)	Yes
	1695.4697-1696.2634	4	(K)RSSSSSSSSSSSR(S)	Yes
	2154.5839-2155.6097	5	(R)SSKSSNSSKRSSSK(S)	Yes
	2176.4320-2177.3681	9	(R)SSKSSNSSKRSSSK(S)	No
	1539.3686-1540.0748	4	(R)SSSSSSSSSSSR(S)	Yes
	1550.5761-1551.3451	3	(K)RSSSKSSNSSKR(S)	No
	1696.6340-1697.4890	3	(R)SSKSSNSSKRSSSK(S)	Yes
	1910.5967-1911.5173	4	(K)SSSSSSKSSSSSSSR(S)	Yes
	2179.0993-2179.9136	12	(R)SSSSSSSSSSSSR(S)	Yes
	2157.6689-2158.7092	5	(K)DASSSSRSSKSSNSSKR(S)	Yes
	2157.8181-2158.9375	2	(R)SRSSSKSSSSSSSSSSSSSSK(S)	Yes
Heat Trypsin DP Multifect P3000	1605.4602-1606.2388	3	(K)SSSSSSSSSSSSSSK(S)	No
Heat Trypsin DP	1539.3686-1540.0748	4	(R)SSSSSSSSSSSR(S)	Yes

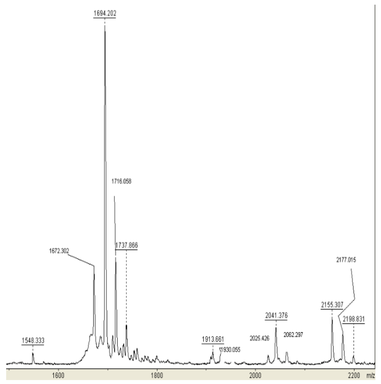

Figure 3: Phosvitin digested with Heat Trypsin protocol, enriched using titanium dioxide

Strong Anion Exchange

Table 3

Sample SAX	Peaks	Phosphates	Sequence	Shared
HCl Trypsin	857.2438-857.6431	2	(K)SSSSSSR(S)	Yes
	1539.3686-1540.0748	4	(R)SSSSSSSSSSSR(S)	Yes
Heat Trypsin	1539.3686-1540.0748	4	(R)SSSSSSSSSSSR(S)	Yes
	1619.3349-1620.0547	5	(R)SSSSSSSSSSSR(S)	Yes
	2088.6028-2089.5852	6	(K)QARNKDASSSSRSSK(S)	No
Heat Trypsin Protex	804.2090-804.5621	3	(K)RSSSK(S)	No
	1939.2003-1939.9741	9	(R)SSSSSSSSSSSR(S)	Yes
	2779.5857-2780.8076	10	(K)SSSSSSKSSSSSSSRSSSK(S)	Yes
	2916.0777-2917.5780	3	(K)SSSSSSRSRSSSKSSSSSSSSSSSSSK(S)	No
Heat Trypsin Thermolysin	2876.9561-2878.4537	4	(K)SSSHHSHSHHSGHLNGSSSSSSSR(S)	No
	2946.9201-2948.3749	5	(R)SSSSSSSSSSNSKSSSSSSKSSSSSR(S)	Yes
Heat Trypsin Multifect P3000	1653.4397-1654.2059	5	(R)NKDASSSSRSSK(S)	No
	1653.3322-1654.0523	6	(K)SSSSRSSSSSSK(S)	No
Heat Trypsin DP	1539.3686-1540.0748	4	(R)SSSSSSSSSSSR(S)	Yes
	1619.3349-1620.0547	5	(R)SSSSSSSSSSSR(S)	Yes
	1761.6688-1762.5811	2	(K)DASSSSRSSKSSKSSNSSK(R)	No
	1859.2339-1859.9942	8	(R)SSSSSSSSSSSR(S)	No
	2779.5857-2780.8076	10	(K)SSSSSSKSSSSSSSRSSSK(S)	Yes
	2946.9201-2948.3749	5	(R)SSSSSSSSSSSNSKSSSSSSKSSSSSSR(S)	Yes
Heat Trypsin DP Protex	1939.2003-1939.9741	9	(R)SSSSSSSSSSSR(S)	Yes
Heat Trypsin DP Thermolysin	1619.3349-1620.0547	5	(R)SSSSSSSSSSSR(S)	Yes
	1904.7747-1905.7709	2	(K)SSNSSKRSSSKSSNSSK(R)	No
Heat Trypsin DP Multifect P3000	1619.3349-1620.0547	5	(R)SSSSSSSSSSSR(S)	Yes
	3589.8849-3591.5414	10	(R)SSSSSSSSSSNSKSSSSSSKSSSSSSRSR(S)	No

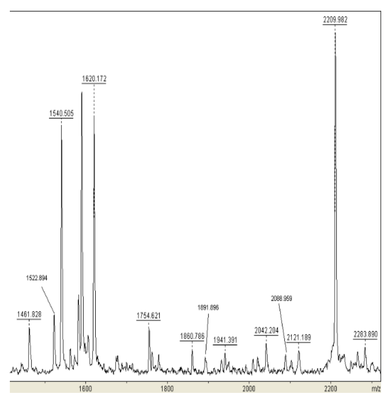

Figure 4: Phosvitin digested with a Heat Trypsin protocol, enriched using SAX tips

Tables 1-3 show the mass per charge of the three chromatography methods: TiO_2, ZrO_2, and SAX respectively. The types of hydrolysis are shown for each sample and the sequence of peaks were determined. Possible number of phosphates per peptide are given. If the peptide is found between different hydrolysis methods it is considered shared.

SDS-PAGE

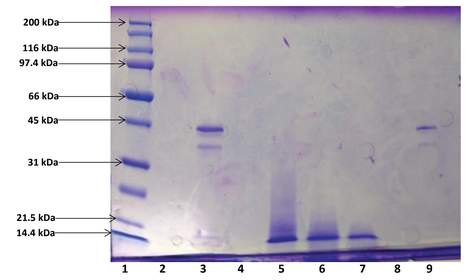

Figure 5. SDS-PAGE analysis of phosvitin and digested phosvitin with different enzymes.

Lane 1) Molecular Marker
Lane 3) 1mg/mL phosvitin (34 or 45 kDa)
Lane 5) Pepsin Hydrolysate of phosvitin
Lane 6) Trypsin Hydrolysate of phosvitin
Lane 7) Thermolysin Hydrolysate of phosvitin
Lane 9) 0.1mg/mL phosvitin.

Our protocol yields thorough phosvitin digestion

DISCUSSION

Trypsin digestion of phosvitin generated the more robust data. Strong anion exchange chromatography on trypsin-digested phosvitin samples identified a potential phosphopeptide at m/z 1541.00 representing the peptide Pv[82-94]. This peak was observed in MALDI-TOF spectra associated with a previous study. A second phosphorylated, Pv[115-121] was also identified at m/z 858.24. Titanium dioxide (TiO2) affinity chromatography, on the other hand, generated potential peptide peaks at m/z 1377.21 representing Pv[95-107] and 1539.41, representing Pv[82-94]. When trypsin digestion was done in the presence of thermolysin, two peptides were tentatively identified; Pv[122-127] at m/z 1013.5 and Pv[1449.78].

We are identifying short peptides that could be used in supplements to increase calcium bioavailability

These data indicate that different columns identify different peptides.

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REFERENCES

Mudiyanselage, C. (2012). Production and Characterization of Phosphopeptides from Egg Yolk Phosvitin. Iowa State University Graduate Thesis

Samaraweera, Himali; Zhang, Wan-gang, Lee, Eun Joo; Ahn, Dong U; (2011). Egg Yolk Phosvitin and Functional Phosphopeptides. Journal of Food Science. Vol. 76, Nr. 7 (R143-R150)

Samaraweera, H., Moon, S.H., Lee, E.J., Grant, J.E., Fouks, J., Choi, I., Suh, J.W., Ahn, D.U. (2014) Characterisation of phosvitin phosphopeptides using MALDI-TOF mass spectrometry. Food Chem. Dec 15;165:98-103. doi: 10.1016/j.foodchem.2014.05.098. Epub 2014 May 27.