Production and Characterization of Functional Phosphopeptides from Egg Yolk Phosvitin

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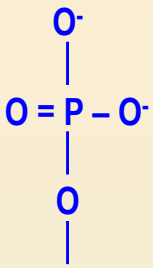
Phosvitin

- A principal phosphoprotein in egg yolk: 1.35-1.50% of yolk (8.0-8.8% of egg yolk proteins)
- Exist in yolk granules in the form of lipovitellin-phosvitin complex through phosphocalcic bridge
- Molecular mass of 35-45 kD and contains ~10% phosphorus
    - An excellent metal (iron and calcium) binding capacity.
    - Can bind 148 mol Ca^{++}/mol phosvitin at pH 7.0.
- Biological functions: Bone formation, antioxidant during embryo development

Amino Acid Sequence of Egg Yolk Phosvitin



1	AEFGTEPDAKTSSSSSSASSTATSSSSSSASSPNRKKPMDEEENDQVKQA	50
51	RNKDASSSSRSSKSSNSSKRSSSKSSNSSKRSSSSSSSSSSSSRSSSSSS	100
101	SSSSNSKSSSSSSKSSSSSSRSRSSSKSSSSSSSSSSSSSSKSSSSRSSS	150
151	SSSKSSSHHSHSHHSGHLNGSSSSSSSSRSVSHHSHEHHSGHLEDDSSSS	200
201	SSSSVLSKIWGRHEIYQ	217

123 phophoserines

↓

Very Strong Metal Chelating Power

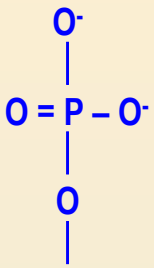
Use of Phosvitin

- Phosvitin is an excellent source for phosphopeptide production
- Phosvitin phosphopeptides
  - Help calcium absorption, calcium retention, bone calcification
  - Can be used as iron supplement
  - Antioxidant and antimicrobial capability
  - Anticancer agent?

Production of Phosphopeptides

- Casein is commercially used to produce phosphopeptides
  - Has only 1 to 13 phosphoserine residues per types (αS1, αS2, β- and κ)
  - Japan: as a nutraceutical
  - Denmark: as a calcium supplement (Capolac)
  - Sweden: as a mineral absorption facilitator
- Phosvitin: has ~120 phosphoserine residues
  - Excellent substrate for phosphopeptides production
  - Various sizes and metal binding capacity
  - Diverse functionality and applications
- Extremely difficult to hydrolyze using enzymes
  - Need pre-treatments to improve enzyme hydrolysis of phosvitin
  - Characterization of peptides produced is needed

Amino Acid Sequence of Egg Yolk Phosvitin

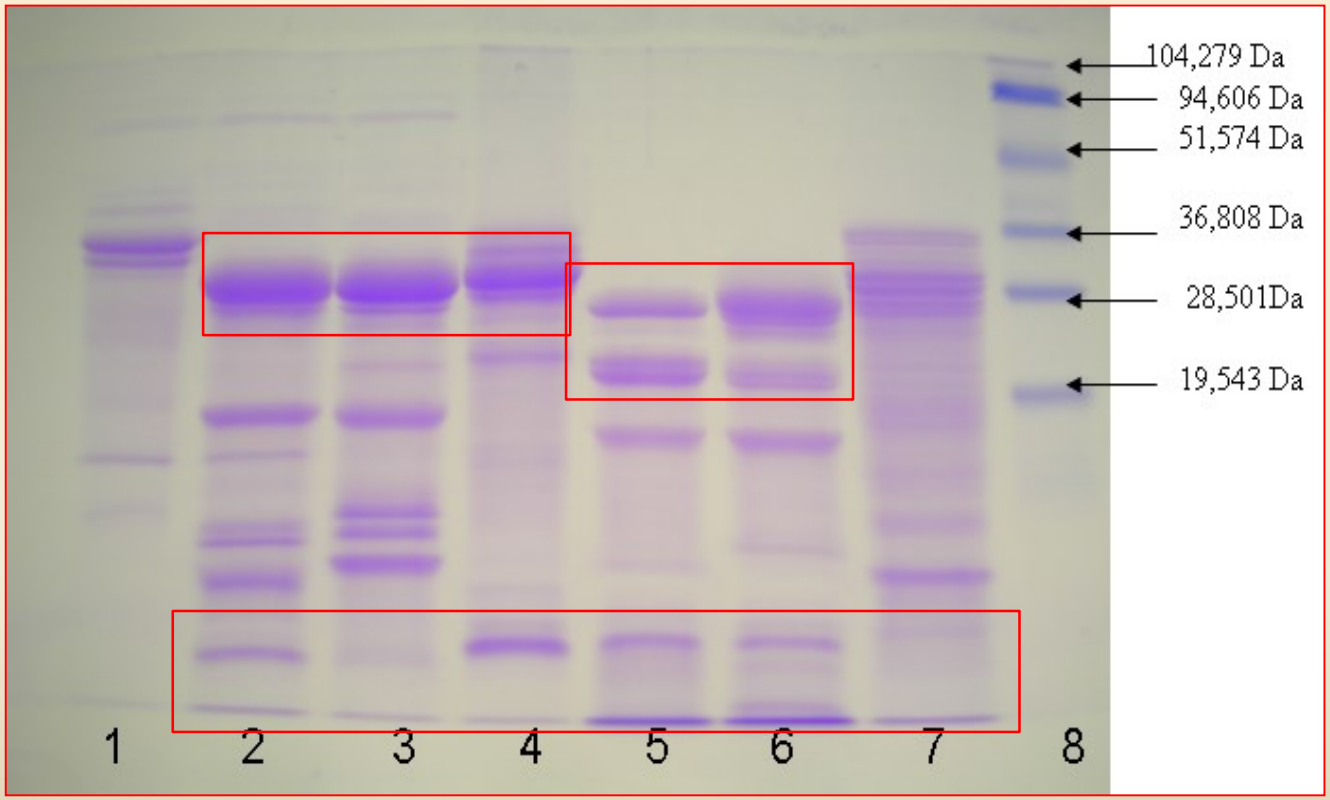


1	AEFGTEPDAKTSSSSSSASSTATSSSSSSASSPNRKKPMDEEENDQVKQA	50
51	RNKDASSSSRSSKSSNSSKRSSSKSSNSSKRSSSSSSSSSSSSRSSSSSS	100
101	SSSSNSKSSSSSSKSSSSSSRSRSSSKSSSSSSSSSSSSSSKSSSSRSSS	150
151	SSSKSSSHHSHSHHSGHLNGSSSSSSSSRSVSHHSHEHHSGHLEDDSSSS	200
201	SSSSVLSKIWGRHEIYQ	217

Pre-enzyme Treatments for Phosvitin

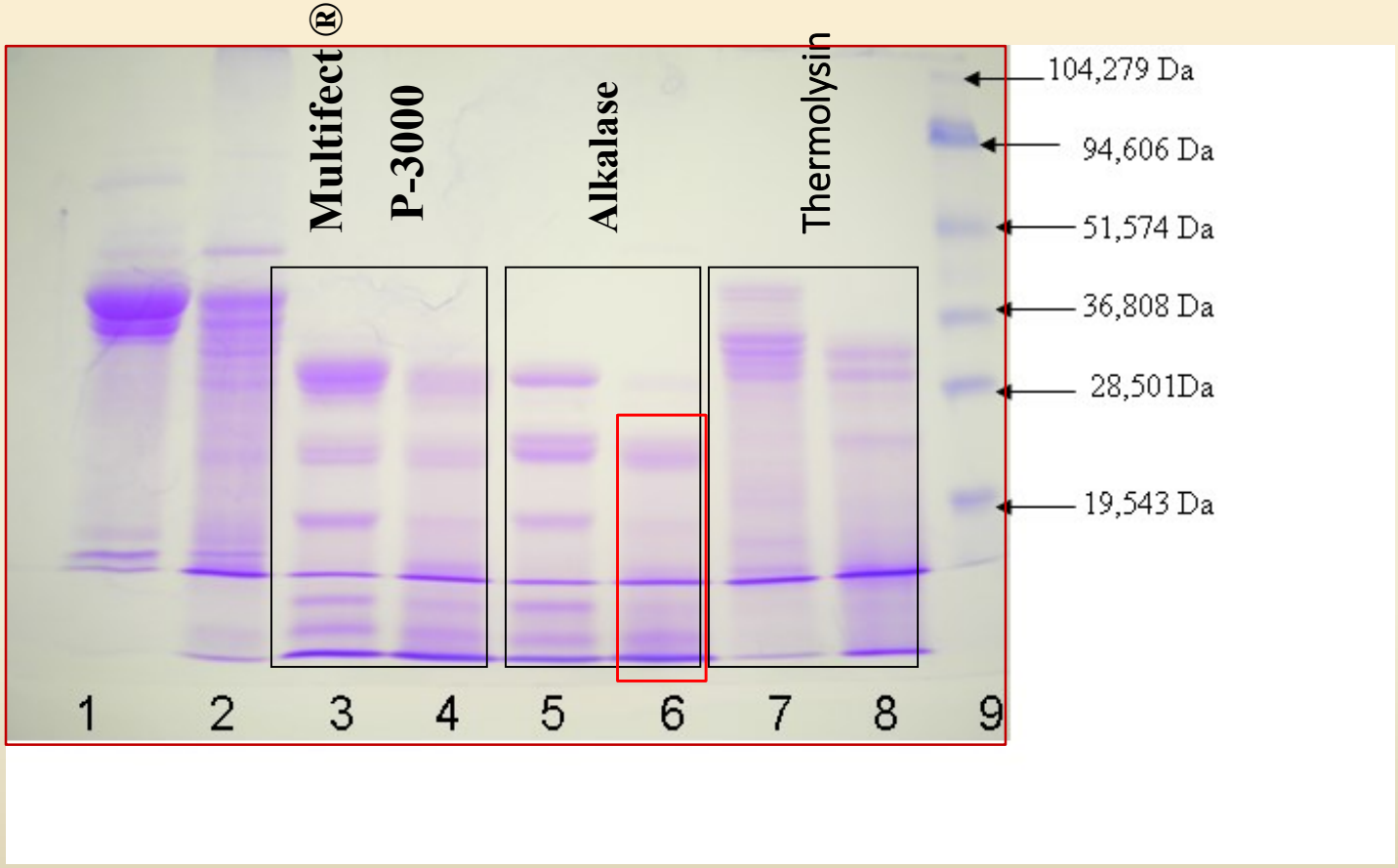
- Acid
- Alkali
- Heat
- Detergents: SDS
- High pressure
- Combinations

Hydrolysis of Phosvitin with Different Enzymes



1. Phosvitin
2. Trypsin
3. α-Chymotrypsin
4. Pepsin
5. Alcalase
6. Multifect ® P3000
7. Thermolysin
8. Marker

Enzyme Hydrolysis of Phosvitin after Heat Treatment



1. Phosvitin (PV)
2. PHT Pv
3. P-3000
4. PHT PV-P-3000
5. Alcalase
6. Alc.-PHT PV
7. Thermo.
8. PHT PV-Thermo.
9. MM

After 1 hr at 100 C pre-heat treatment (PHT) and then hydrolysis

Phosvitin Pre-treated with Heat and then Hydrolyzed with Acid or Alkali

1- Phosvitin

2- pH-2, w/o pre-trt

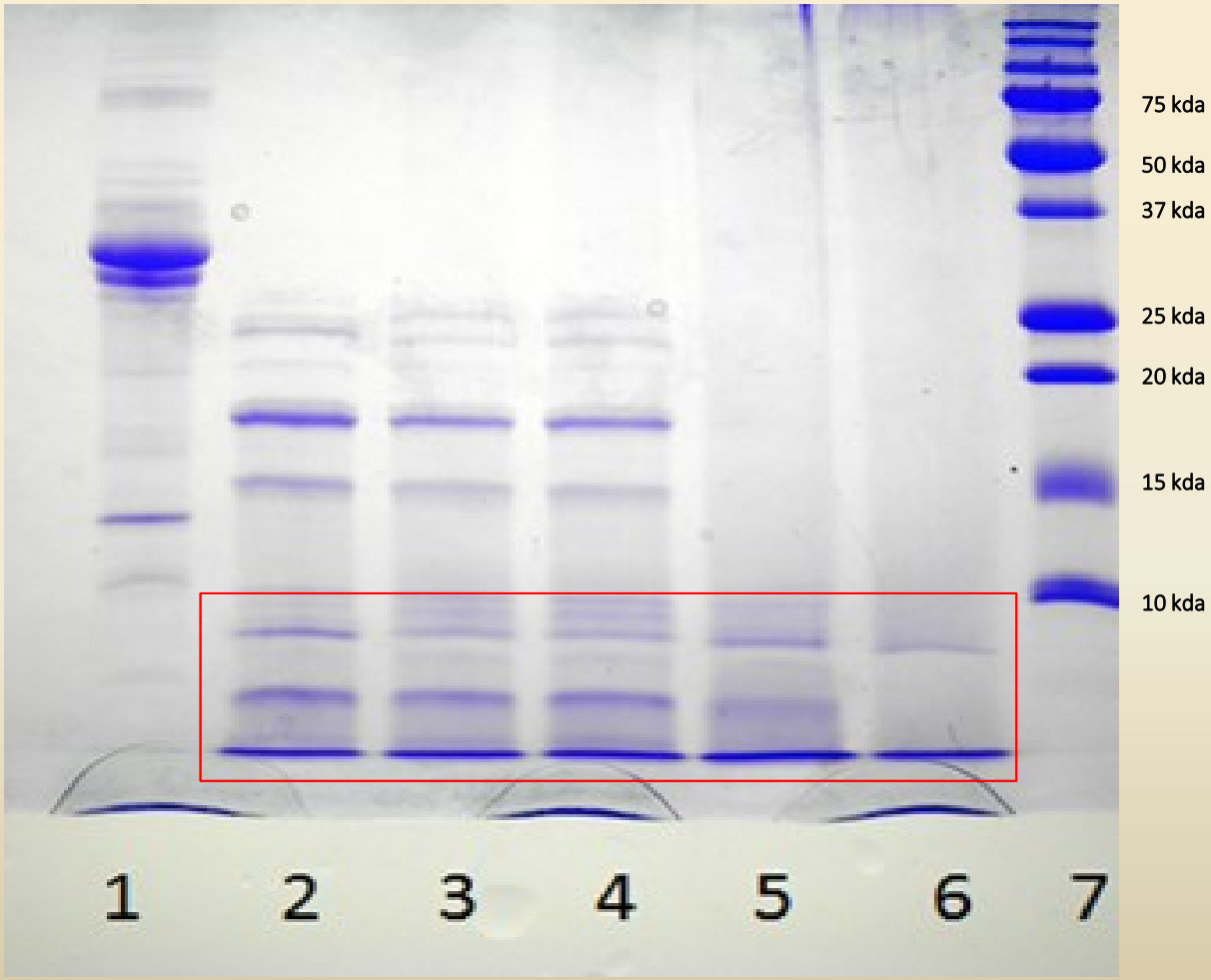
3- pH-2, 85 ^{o}C, 30 min

4- pH-3, 85 ^{o}C, 30 min

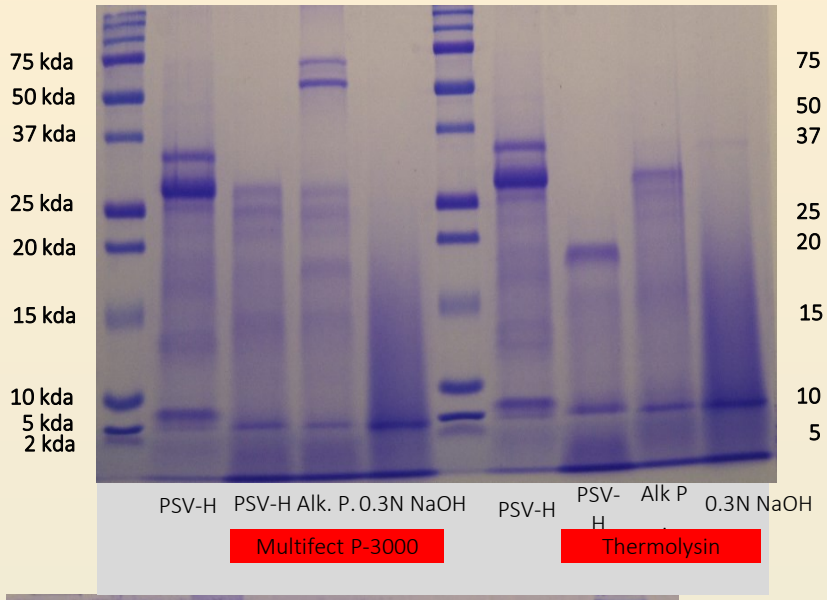
5- pH 12, 85 ^{o}C, 30 min

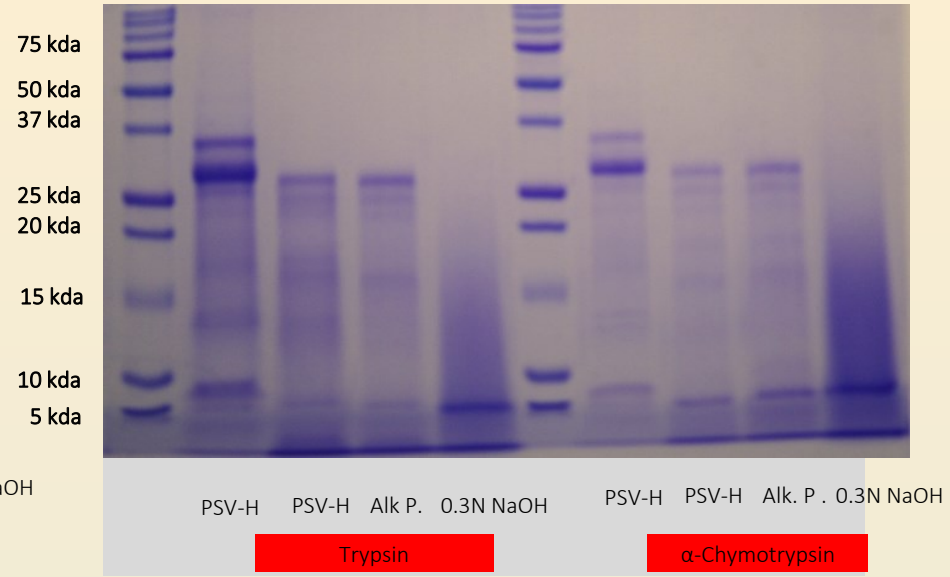
6- pH-13, 85 ^{o}C, 30 min

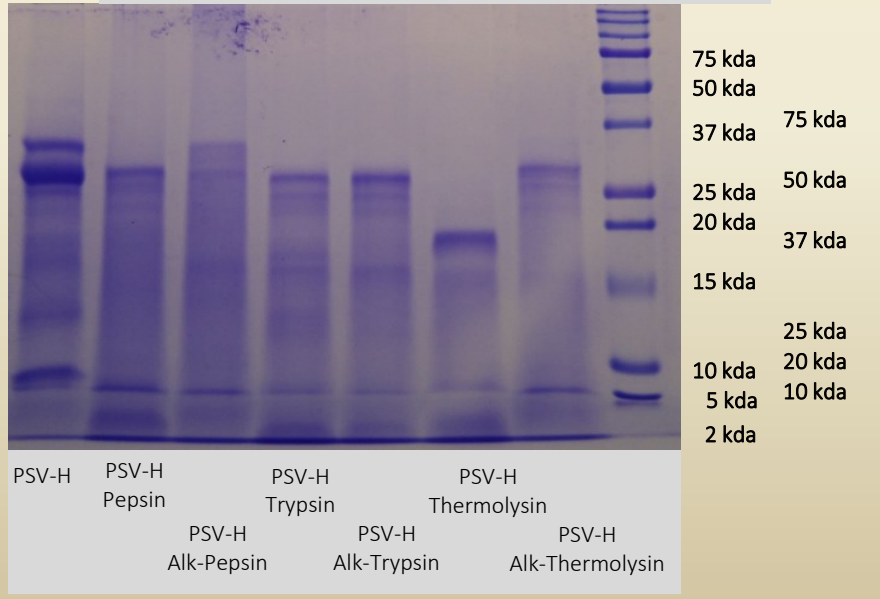
7- Molecular Marker

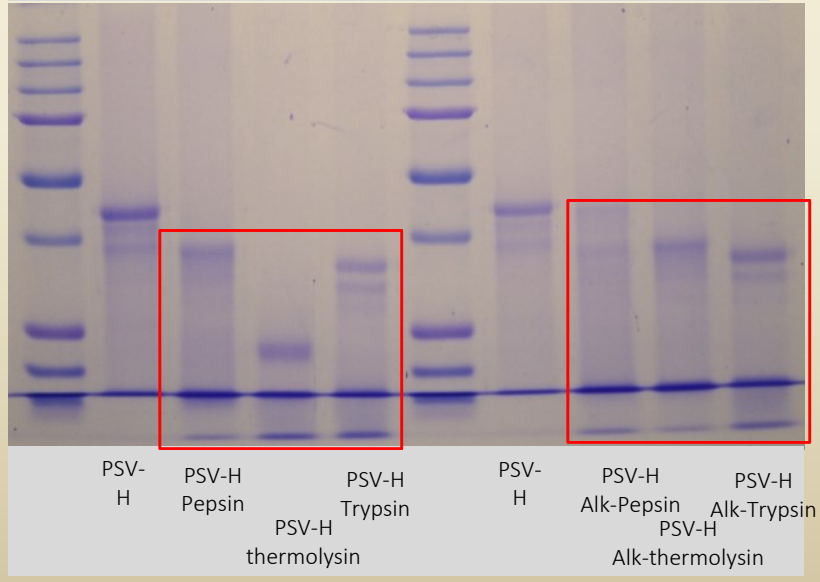


Enzyme Hydrolysis of Phosvitin with Various Pre-Treatment Combinations





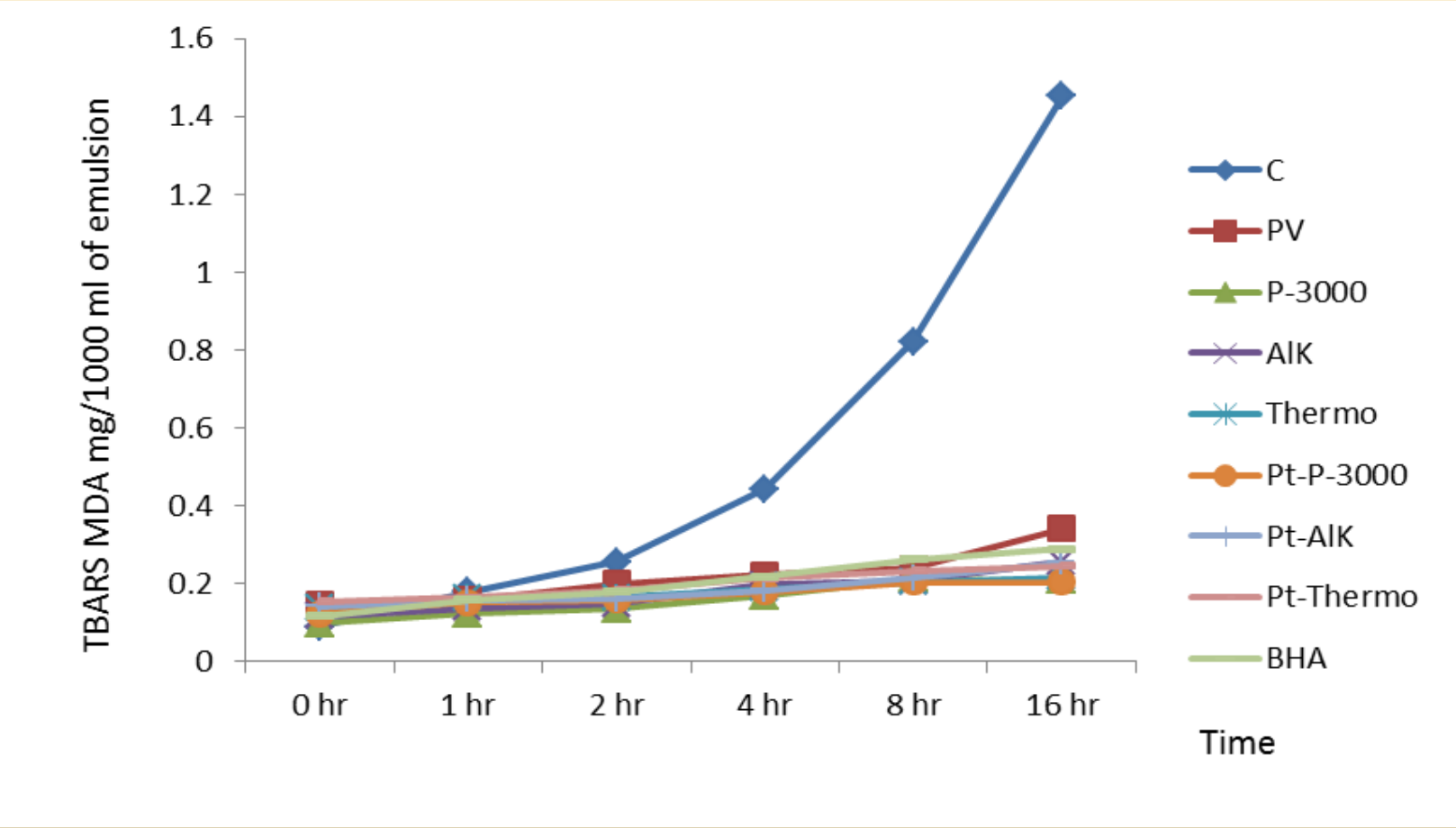




Characterization of Phosphopeptides

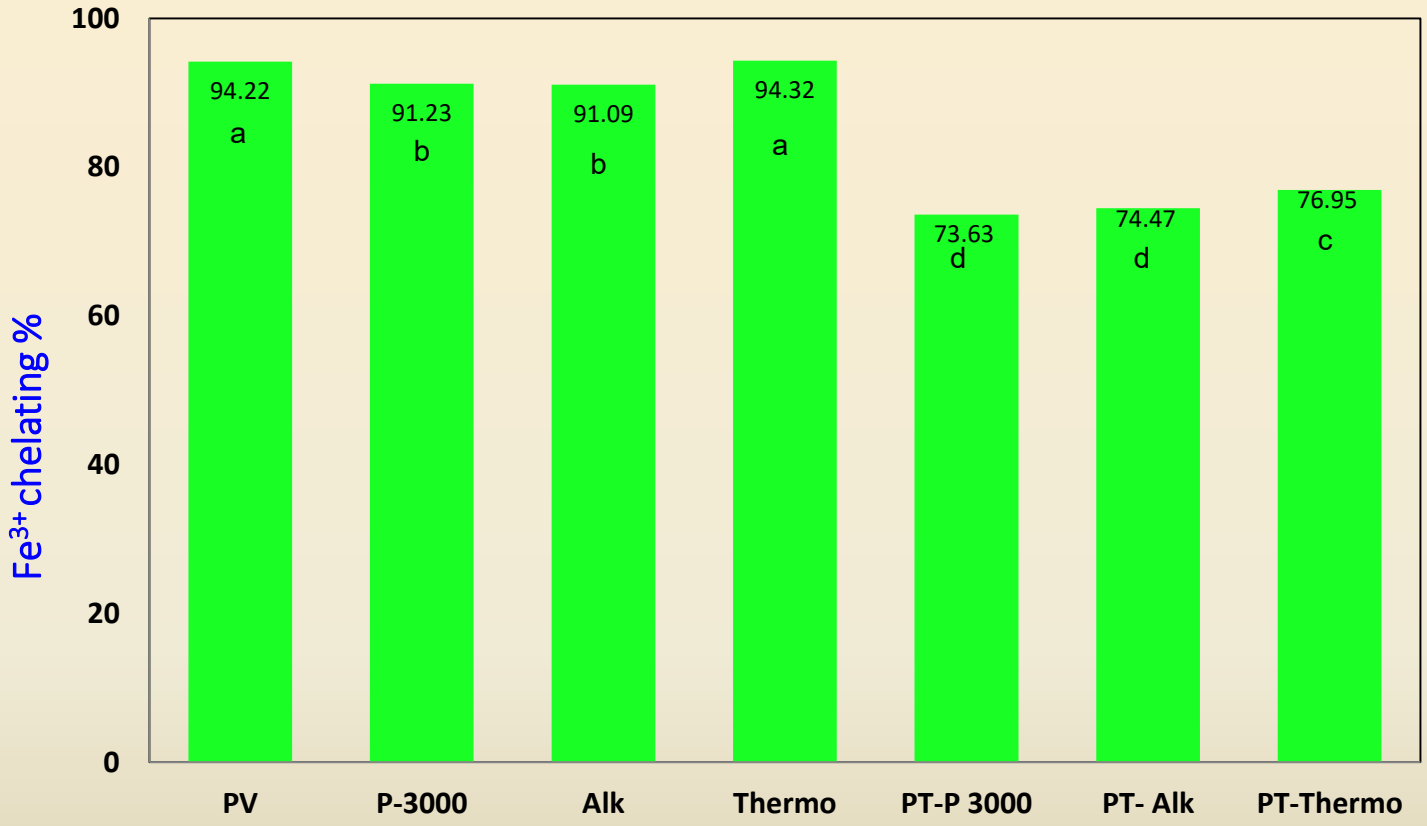
- Chemical
  - Metal binding capacity
  - Free radical chelating
- Functional
  - Antioxidant
  - Anticancer
  - ACE-inhibiting activity
- Structural
  - Mass spectrometry: MS/MS, MALDI-TOF

Antioxidant Activities of Phosvitin and its Enzyme Hydrolysates



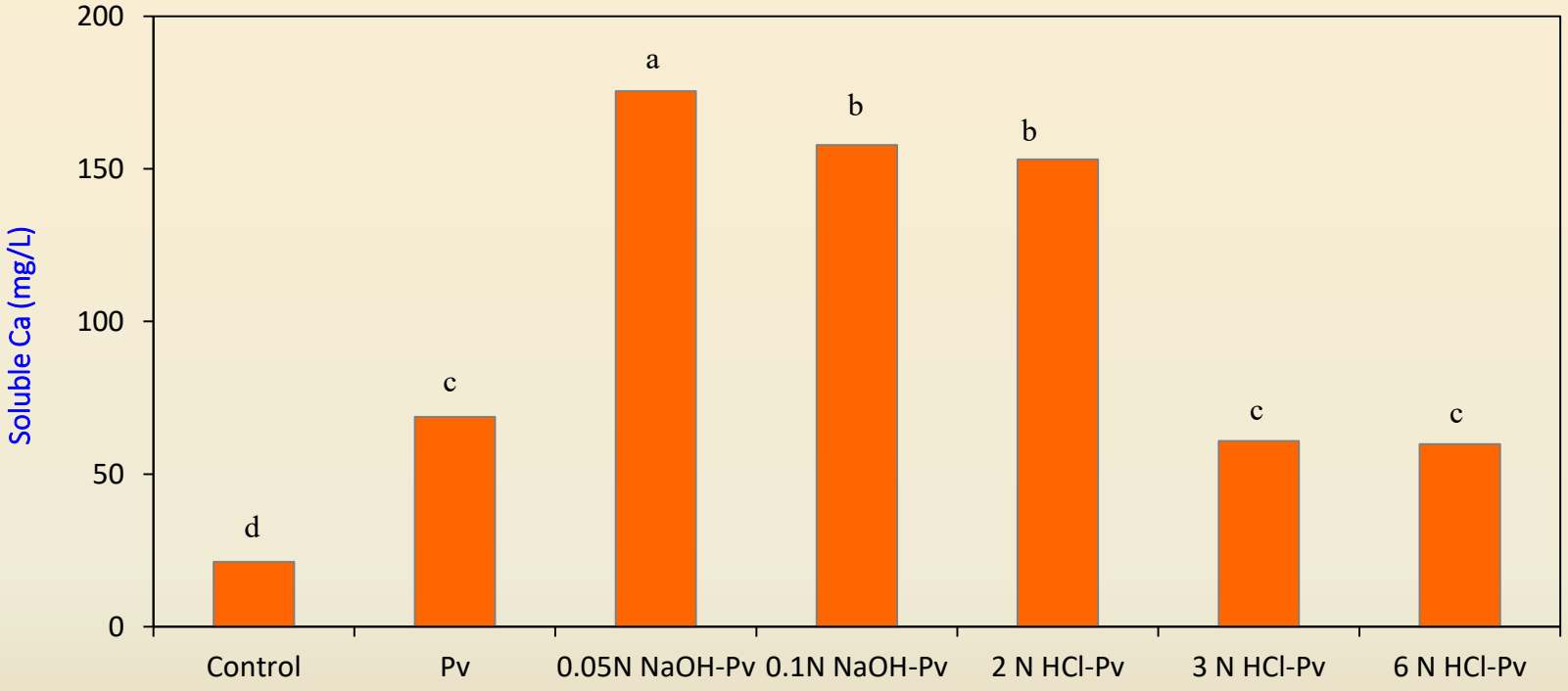
- Oil emulsion was added with 10 ppm Fe^{2+}

Fe^{3+}-Chelating Activity of Heat Pre-Treated Enzyme Hydrolysates of Phosvitin



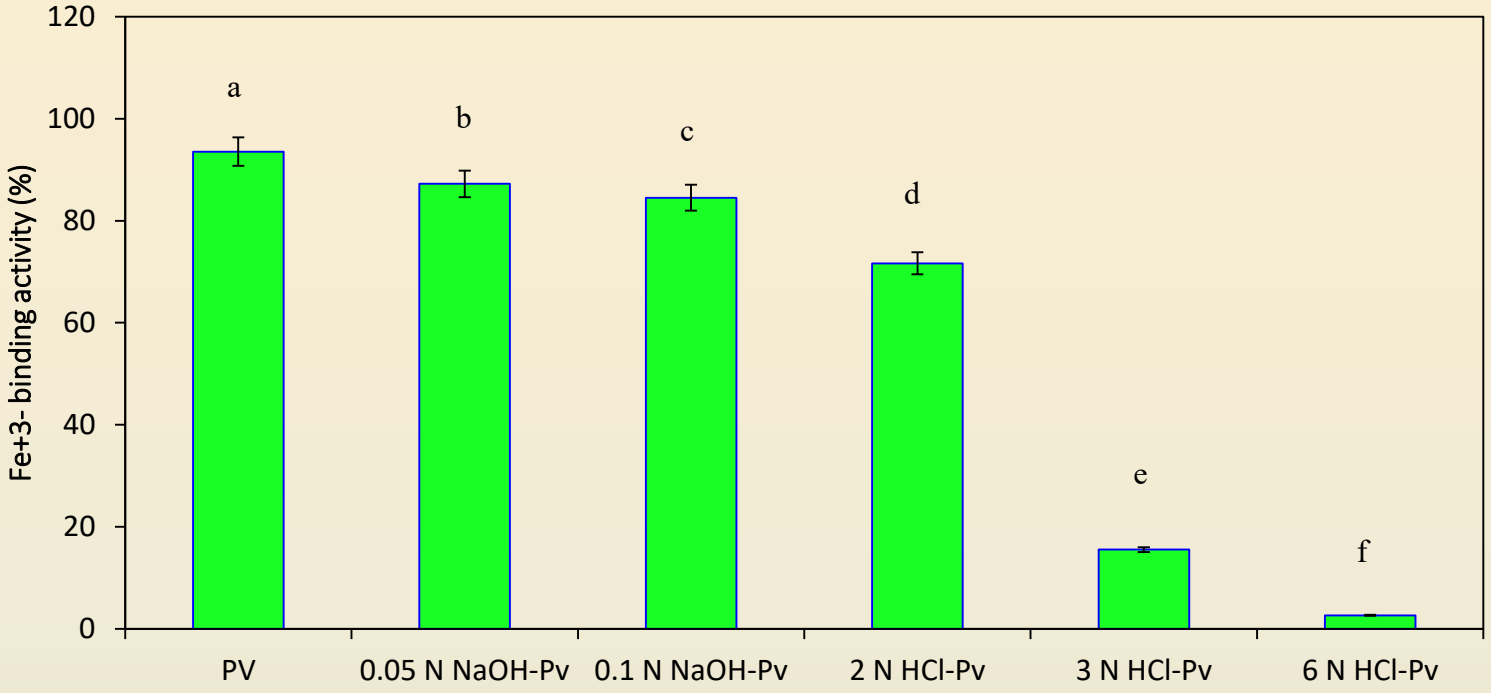
- Tris-malate buffer, pH 6.8 was used
- PT: phosvitin pre-treated at 100 ^{o}C, 60 min.

Ca^{+2}-Solubilizing Activity of phosvitin, and its Alkaline and Acid Hydrolysates



- Pv- Natural phosvitin;
- 0.05 N NaOH-Pv-Phosvitin treated with 0.05 N NaOH at 37 ◦C for 3 h;
- 0.1 N NaOH-Pv- Phosvitin treated with 0.1 N NaOH at 37 ◦C for 3 h;
- 2 N HCl-Pv-Phosvitin treated with 2 N HCl at 60◦C for 6 h;
- 3 N HCl-Pv- Phosvitin treated with 3 N HCl at 60◦C for 6 h;
- 6 N HCl-Pv-Phosvitin treated with 6 N HCl at 60◦C for 6 h,

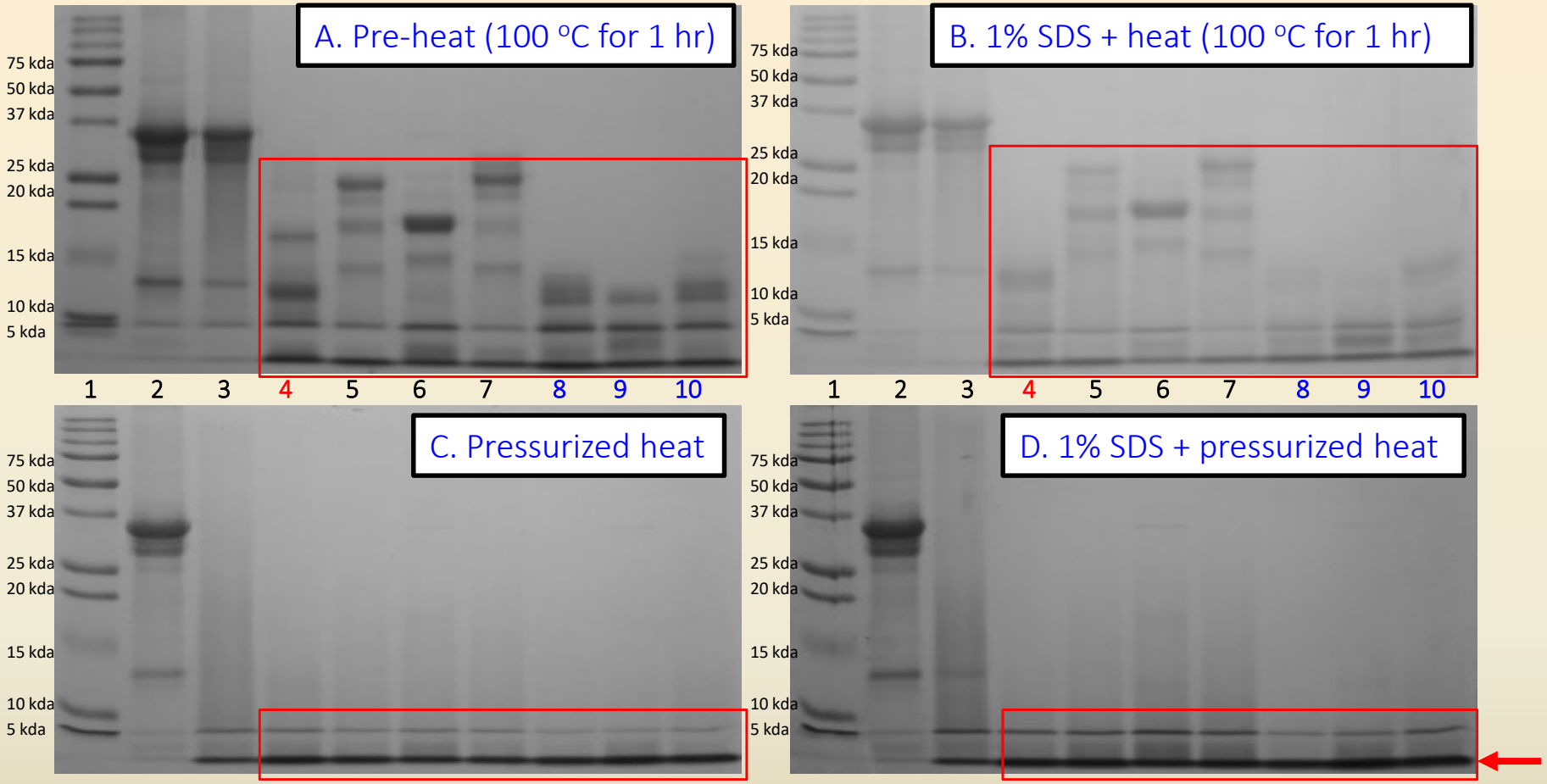
Fe^{+3}-Binding Activity of Acid and Alkali Hydrolysates of Phosvitin



- Pv- Natural phosvitin;
- 0.05 N NaOH-Pv-Phosvitin treated with 0.05 N NaOH, at 37˚C for 3 h;
- 0.1 N NaOH-Pv-Phosvitin treated with 0.1 N NaOH at 37˚C for 3 h;
- 2 N HCl-Pv-Phosvitin treated with 2 N HCl at 60˚C for 6 h;
- 3 N HCl-Pv-Phosvitin treated with 3 N HCl at 60˚C for 6 h;
- 6 N HCl-Pv-Phosvitin treated with 6 N HCl at 60˚C for 6 h;

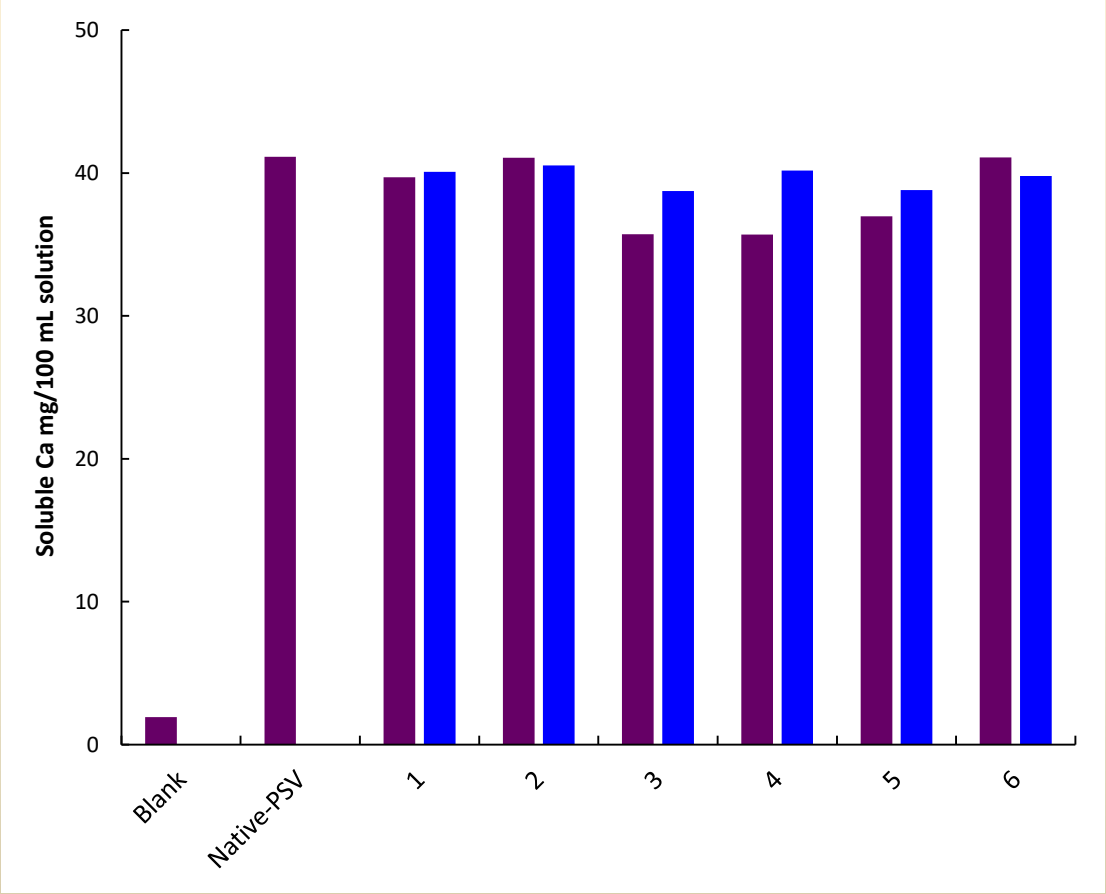
Recent Advancement in Enzymatic Hydrolysis of Phosvitin

Enzyme Hydrolysis of Phosvitin with Various Pre-Treatment Combinations



1. Molecular marker
2. Native phosvitin
3. Heat or **pressurized heat**
4. Trypsin
5. Protex 6L
6. Thermolysin
7. Multifect P3000
8. Protex 6L + trypsin
9. Thermolysin + trypsin
10. Multifect 14L + trypsin

Pressurized-Heat on the Ca^{+2}-Solubilizing Activity of Phosvitin Hydrolysates



Samples 1-6

(■): Normal heat

(■): Pressurized-heat

1: Pre-treatment alone

2: Pre-treatment + Trypsin

3: Pre-treatment + Protex 6L

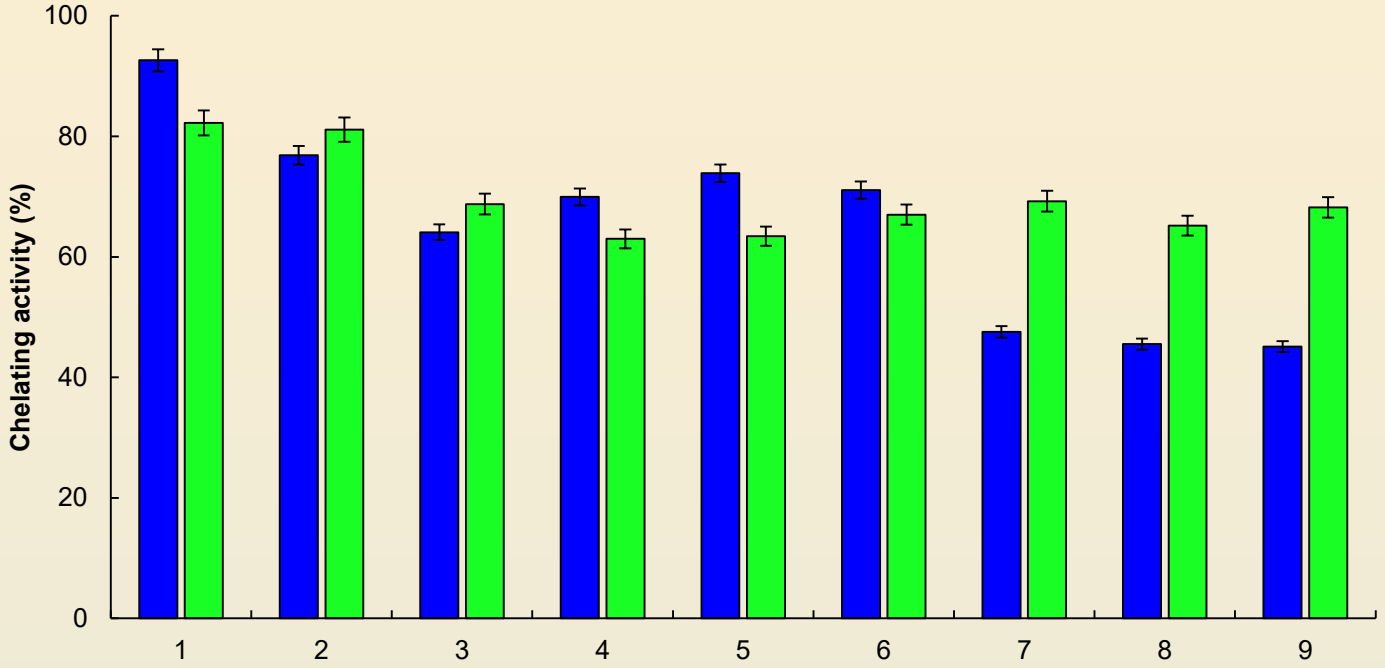
4: Pre-treatment + Multifect 14L

5: Pre-treatment + Protex 6L+Trypsin

6: Pre-treatment + Multifect 14L+Trypsin

- $CaCl_2$ (1 mg/mL) in phosphate buffer (pH 7.6) was used.

Pressurized-Heat Treatment on the Fe^{3+}- and Cu^{2+}-Chelating Activity of Phosvitin Hydrolysates



- (0.5 mg/mL) Fe^{3+} or Cu^{2+} in Tris-maleate buffer was used

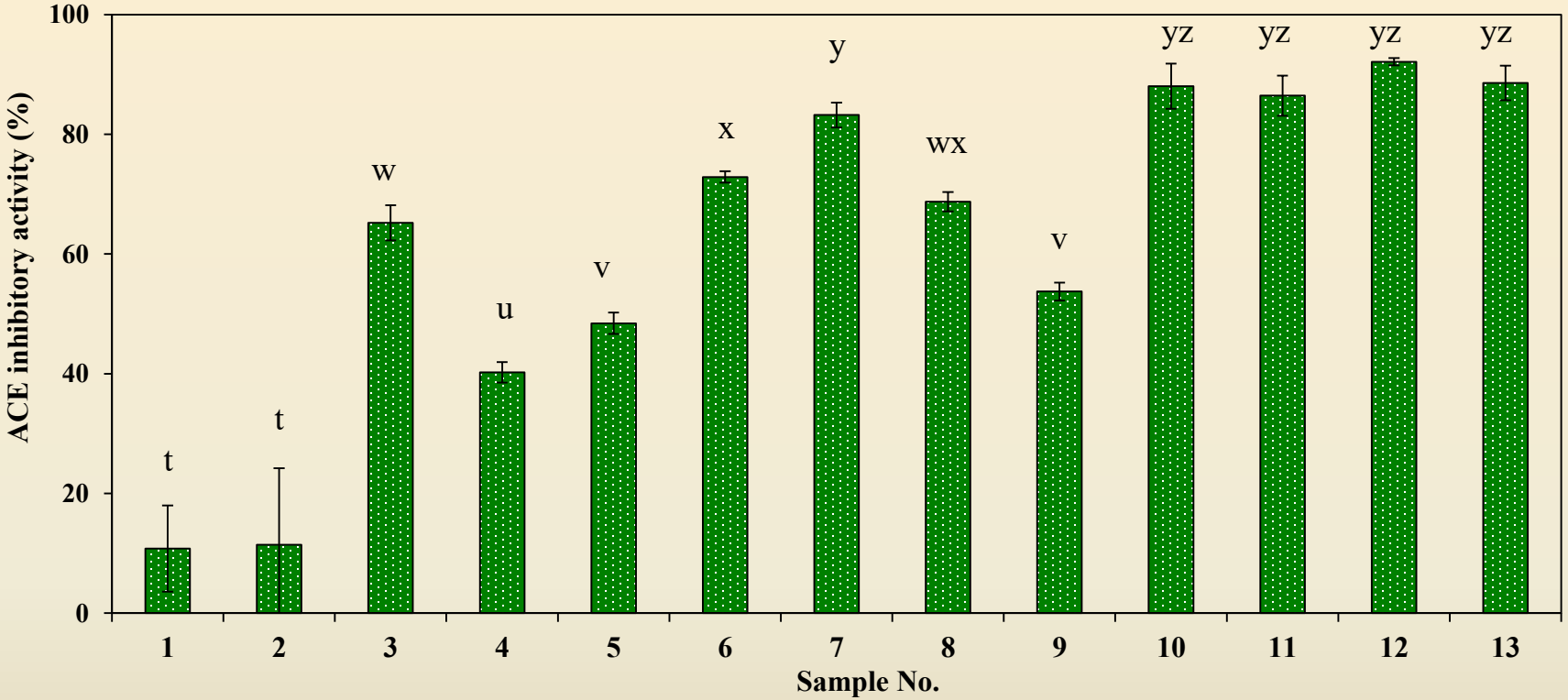
Fe^{3+}-chelating activity (■), Cu^{2+}-chelating activity (□)

1-Native phosvitin; 2-pressurized heat treated phosvitin; 3-Trypsin hydrolysate of pressurized heat PSV; 4-protex 6L hydrolysate of pressurized heat PSV; 5-thermolysin hydrolysate of pressurized heat PSV; 6-multifect P3000 hydolysate of pressurized heat PSV; 7- protex 6L+trypsin hydrolysate of pressurized heat PSV; 8-thermolysin+trypsin hydrolysate of pressurized heat PSV; 9-multifect 14L+trypsin hydrolysate of pressurized heat PSV.

Pressurized-Heat Treatment on the Antioxidant Activity of Phosvitin Hydrolysates

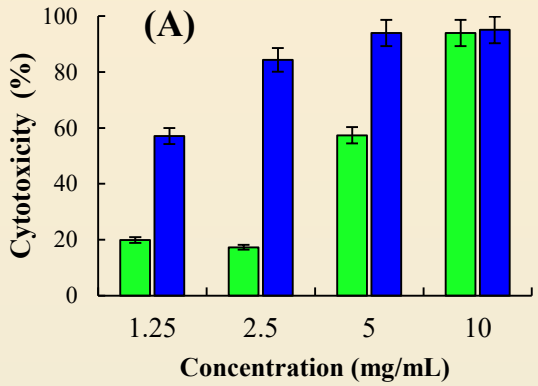
Sample (4 mg/ml sample)	Antioxidant activity (%)
Control	-
Native phosvitin	64.48±0.01
Pressurized heat-treated phosvitin	49.75±0.07
Pressurized heat + Trypsin	52.94±0.03
Pressurized heat + Protex 6L	51.47±0.02
Pressurized heat + Thermolysin	59.47±0.01
Pressurized heat + Protex 6L+ trypsin	45.96±0.08
Pressurized heat + Thermolysin + trypsin	48.76±0.05

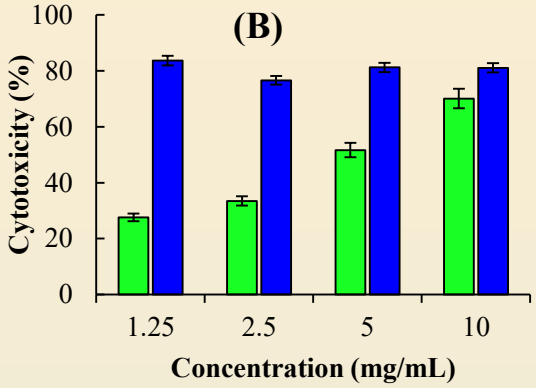
Pressurized-Heat Treatment on the ACE-Inhibitory Activity of Phosvitin Hydrolysates

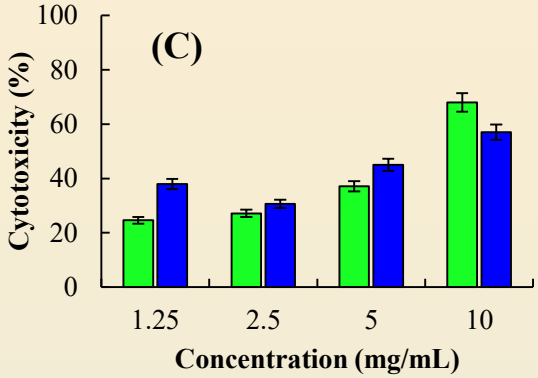


1- Natural phosvitin; 2 – pressurized heat treated phosvitin; 3 - phosvitin hydrolyzed with trypsin; 4 - phosvitin hydrolyzed with elastase; 5 - phosvitin hydrolyzed with pepsin; 6 - phosvitin hydrolyzed with protex 6L; 7 - phosvitin hydrolyzed with thermolysin; 8 -phosvitin hydrolyzed with multifect P3000; 9 - phosvitin hydrolyzed with elastase+trypsin; 10 - phosvitin hydrolyzed with pepsin+trypsin; 11 - phosvitin hydrolyzed with protex 6L+try p; 12 – phosvitin hydrolyzed with thermolysin+trypsin; 13 - phosvitin hydrolyzed with Multifect P3000+ trypsin

Pressurized-Heat Treatment on the Anticancer Effects of Phosvitin Hydrolysates







Protex 6L+trypsin

Thermolysin+trypsin

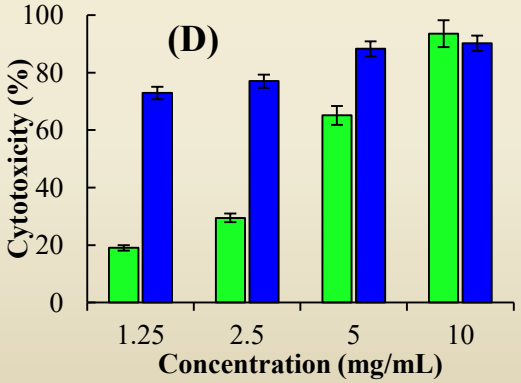
(A) HeLa cell

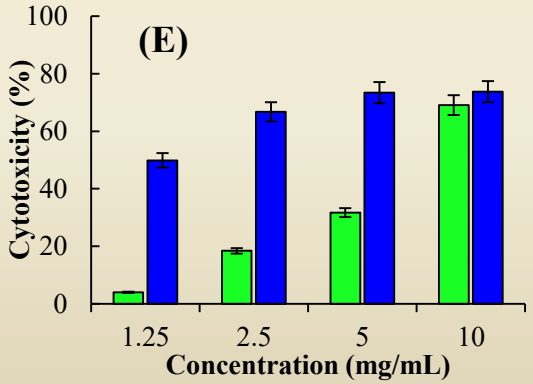
(B) MCF-7 cell

(C) AGS cell

(D) HT-29 cell

(E) LoVo cell





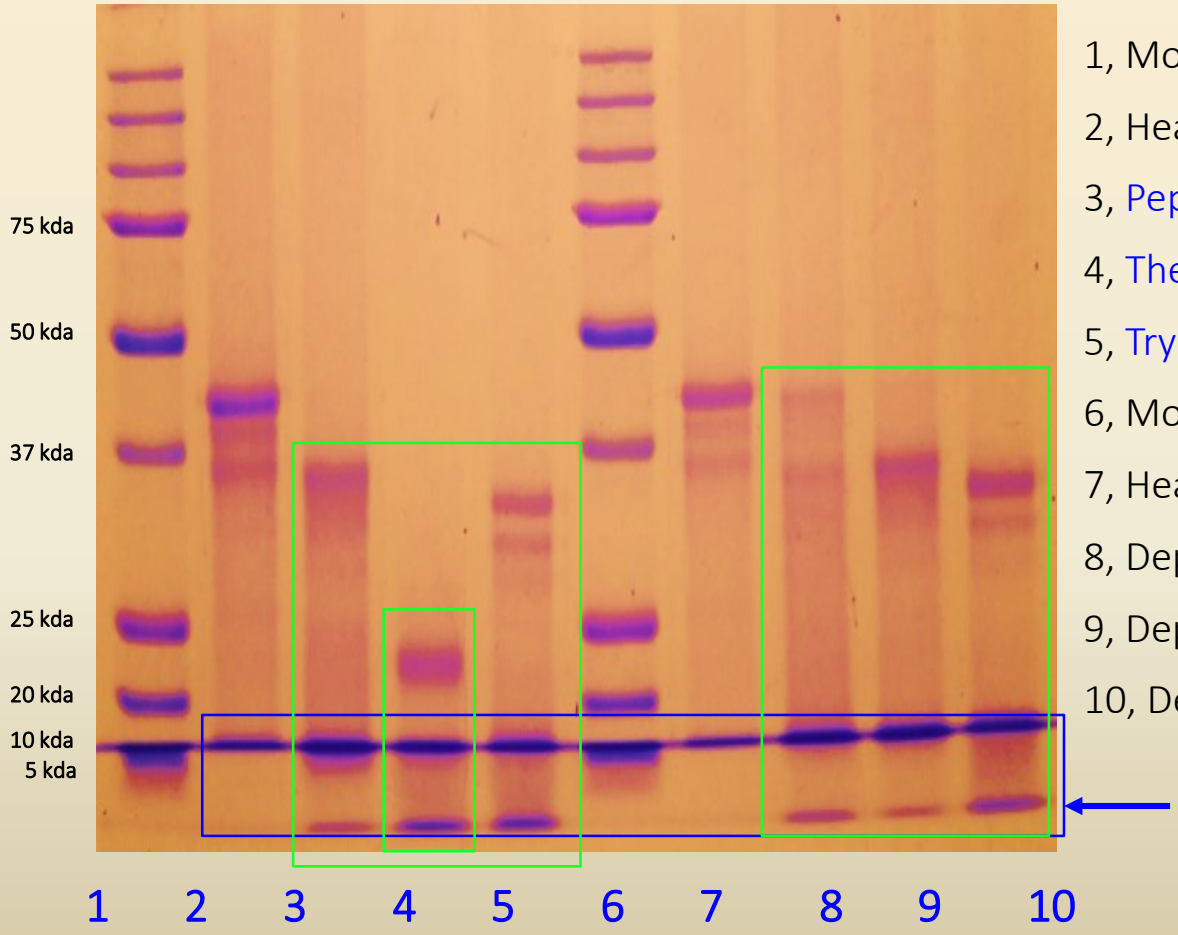
Pressurized-Heat Treatment on the IC_{50} Value of Phosvitin Hydrolysates

Sample \ Cell line	IC_{50} (mg/mL)				
	HeLa	MCF-7	AGS	HT-29	LoVo
pressurized heat alone	> 20	> 20	> 20	> 20	> 20
Pressurized heat + Trypsin	> 20	> 20	> 20	> 20	> 20
Pressurized heat + Protex 6L hydrolysate	> 20	> 20	> 20	> 20	> 20
Pressurized heat + Thermolysin	3.68	0.79	1.33	0.90	1.33
Pressurized heat + Protex 6L + Trypsin	4.98	4.97	5.67	3.79	7.13
Pressurized heat + Thermolysin + Trypsin	1.12	0.67	8.69	0.83	1.27

Structural Characterization of Phosphopeptides

- Structural information is important for the production of highly functional phosphopeptides
  - Types of peptides produced
  - Structure-function relationship
- Improving enzymatic digestion of phosvitin is important
  - Pre-treatments for high degree of digestion
- Use of capillary HPLC coupled with MS, MALDI-TOF

SDS-PAGE of Heat-Pretreated Phosvitin Hydrolyzed with Pepsin, Trypsin and Thermolysin



1, Molecular marker

2, Heat-treated

3, Pepsin

4, Thermolysin

5, Trypsin

6, Molecular marker

7, Heat-treated

8, Dephosphorylated + pepsin

9, Dephosphorylated + thermolysin

10, Dephosphorylated +trypsin

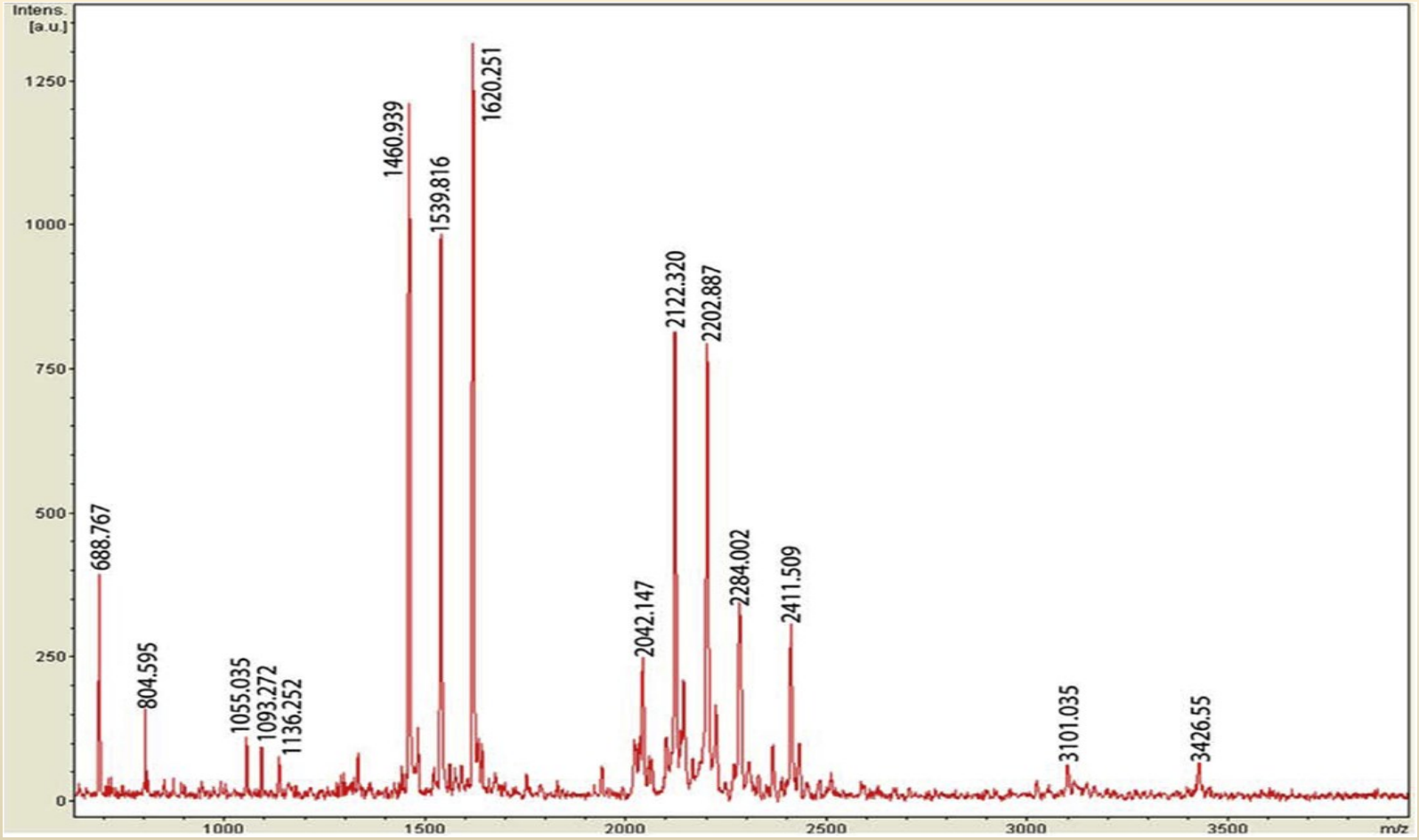
Identified Peptides in the Pepsin and Thermolysin Hydrolysates of Heat Pre-Treated Phosvitin using MALDI-TOF[1]

Position[2]	Sequence	m/z Observed	m/z Predicted
Pepsin hydrolysis			
2-22	EFGTEPDAKTSSSSSSASSTA (+1 PO_4)	*2113.427*	2113.8-2115.0
4-22	GTEPDAKTSSSSSSASSTA (+8 PO_4)	*2397.525*	2396.2-2397.7
7-28	PDAKTSSSSSSASSTATSSSSS	1288.28	1288.6-1289.4
23-30	TSSSSSSA	875.663	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

[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 pepsin or thermolysin.

[2]Amino acid position in phosvitin.

MALDI Spectra of the Peptides from Trypsin Hydrolysate of the Heat-Pretreated Phosvitin



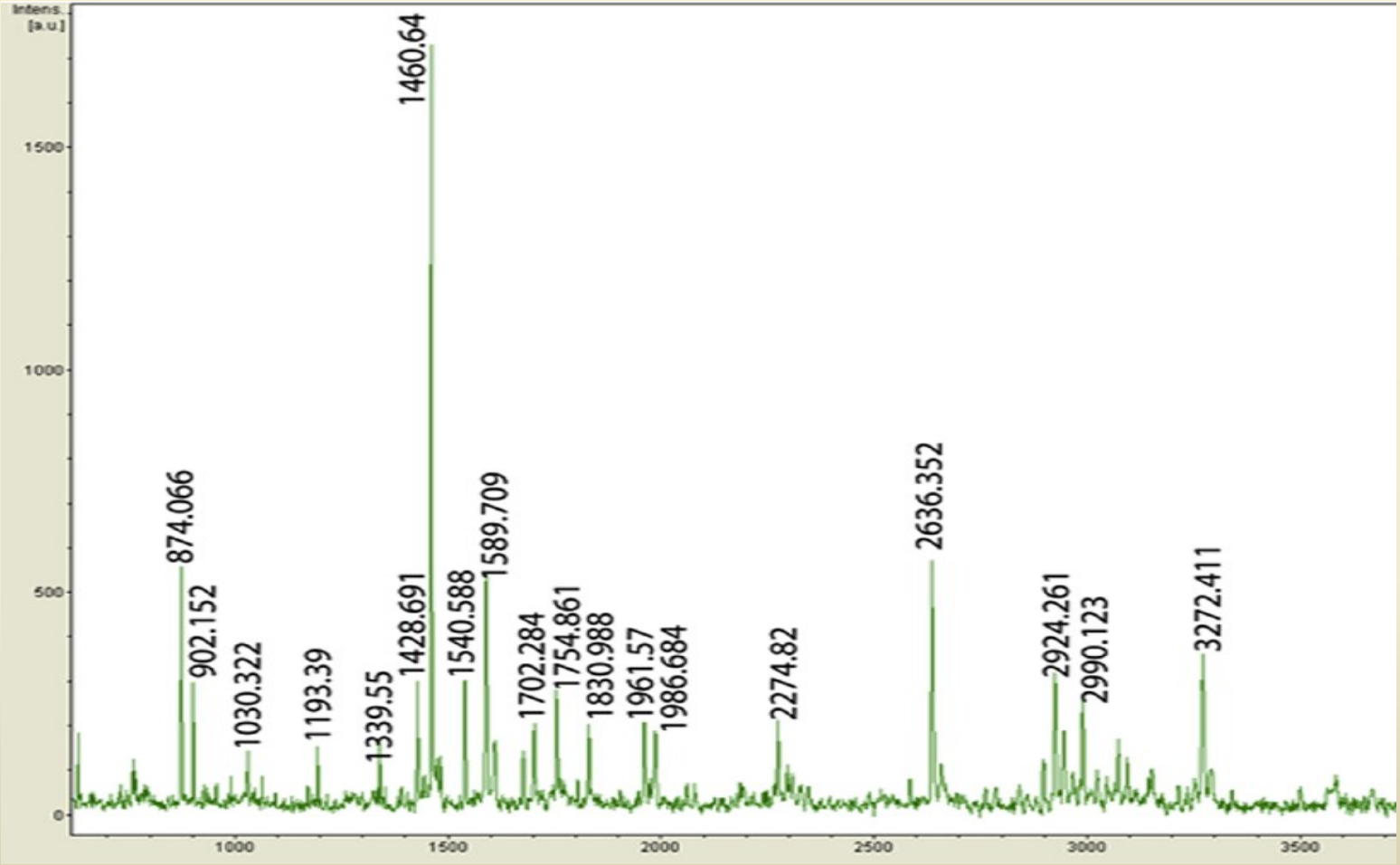
Phosvitin was heat-pretreated at 100 °C for 60 min and then hydrolyzed using trypsin for 24 h at 37 °C.

Identified Peptides in the Trypsin Hydrolysates of the Heat Pre-Treated Phosvitin using MALDI-TOF[1]

Position[2]	Sequence	m/z Observed	m/z Predicted
1-10	AEFGTEPDAK (+1 PO_4)	1093.272	1092.5-1093.2
64-80	SSNSSKRSSSKSSNSSK (+8 PO_4)	2411.509	2412.6-2413.7
81-94	RSSSSSSSSSSSSR (+8 PO_4)	2042.147	2043.4-2044.2
81-94	RSSSSSSSSSSSSR (+9 PO_4)	2122.32	2123.3-2124.2
81-94	RSSSSSSSSSSSSR (+10 PO_4)	2202.887	2203.3-2204.2
81-94	RSSSSSSSSSSSSR (+11 PO_4)	2284.002	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	804.595	805.3-805.7
128-154	SSSSSSSSSSSSSKSSSSRSSSSSSK (+11 PO_4)	3426.55	3427.7-3429.3
179-208	RSVSHHSHEHHSGHLEDDSSSSSSSSVLSK	3101.035	3098.4-3100.2

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

MALDI Spectra of the Peptides from Trypsin Hydrolysate of the Partially Dephosphorylated, Heat-Pretreated 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.

Identified Peptides in the Trypsin Hydrolysates of Partially Dephosphorylated, Heat Pre-Treated Phosvitin using MALDI-TOF

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	SSSKSSSSSSSSSSSSSKSSSSRSSSSSSK (+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.

Summary

- Enzyme hydrolysis of phosvitin was very difficult
- Pre-treatment of phosvitin improved the enzyme hydrolysis, but pressurized heat worked the best
- Phosvitin hydrolysates maintained most of the chemical characteristics of phosvitin
- Hydrolysis of phosvitin improved the anticancer and ACE-inhibitory functions of phosvitin
- Size-function relationship as well as the amino acid sequence of phosphopeptides on their functions remained to be determined

Questions ?