Preparation of Phosphopeptides From Phosvitin Using the High-Temperature and Mild Pressure Pretreatment and Enzyme Combinations

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Summary and Implications

Phosvitin is an excellent source for phosphopeptide production, but it is highly resistant to enzymatic hydrolysis. The high-temperature and mild pressure (HTMP) pretreatment hydrolyzed phosvitin at random sites and helped the subsequent enzyme hydrolysis of the peptides produced. With the HTMP pretreatment alone, 154 peptides were produced while the use of trypsin, Protex 6L, and Multifect 14L in combination with pretreatment produced 252, 280, and 164 peptides, respectively. The use of two enzyme combinations (Trypsin + Protex 6L and trypsin + Multifect 14L) helped the hydrolysis further. The number of phosphopeptides produced increased when the modifications within the same amino acid sequences were considered. This study indicated that HTMP pretreatment was a breakthrough method to improve the enzymatic hydrolysis of phosvitin that enabled an easy production of phosvitin phosphopeptides (PPPs) for their subsequent functional characterizations.

Introduction

Although bioactive peptides from various plants and animal proteins have been identified, produced, and characterized, phosphopeptides have shown the most diverse and unique bioactivities, from their ability to deliver bioavailable metal ions (calcium and iron) in intestinal tracts, to their antimicrobial and antioxidant activities, cellular differentiation, anti-inflammatory, and anti-cariogenic effects. Most bioactivities of phosphopeptides are derived from their metal-binding capabilities of the phosphorylated serine moiety that inhibits the formation of insoluble metal phosphates. Phosphopeptides not only effectively bind nutritionally essential metal ions such as calcium, iron, and zinc ions, but also release them in the intestinal tract, resulting in increased calcium or iron bioavailability. Furthermore, phosphopeptides could also be used as biomarkers for diagnosis and therapeutic purposes in several kinds of cancers. Currently, milk casein is used to produce phosphopeptides and a few commercial casein phosphopeptides (CPP) products are sold as a nutraceutical or mineral absorption facilitator in Japan and a few European countries. Phosvitin, one of the major egg yolk proteins (8% of yolk proteins), has 128 amino acid residues that can be phosphorylated and is a much better substrate than casein for producing phosphopeptides. However, phosvitin is not used to produce phosphopeptides because the enzyme hydrolysis of natural phosvitin is extremely difficult. Phosvitin molecule has consecutive phosphoserine residues that can interfere with the formation of the proper enzyme-substrate complex needed for hydrolysis. Also, the negatively charged phosphate groups attract water molecules to surround the phosvitin molecules, which can inhibit the access of enzymes to the peptide bonds of phosvitin molecules. Various strategies, including pretreatment of phosvitin using heat, acid, alkali, and high pressure singly or in combinations, or removing part of the phosphate groups have been tested to enhance the enzyme hydrolysis of phosvitin, but with limited successes. The objectives of this study were to test the new pretreatment called "high-temperature under mild pressure (HTMP)" conditions on the enzymatic hydrolysis of phosvitin and to determine the structural characteristics of the phosphopeptides produced using tandem mass spectrometry. The functional characteristics of the hydrolysates were also studied but are not included here.

Materials and Methods

Phosvitin was prepared from chicken egg yolk using the method that we have developed. Protex 6L (alkaline serine endopeptidase, E.C. 3.4.21.62) from *Bacillus licheniformis*, Multifect® 14L (mainly thermolysin), Multifect® P3000 (serine endopeptidase protease from *Bacillus amyloliquefaciens* EC 3.4.21.62) were from Genencor, and Trypsin Type I from bovine pancreas (E.C. 3.4.21.4), Thermolysin from *Bacillus thermoproteolyticus rokko* (E.C. 3.4.24.27) and acetonitrile (ACN) and formic acid were purchased from Sigma. To prepare phosvitin phosphopeptides (PPP), the lyophilized phosvitin was dissolved in deionized distilled water (50 mg/ml) and pre-treated under high-temperature and mild pressure conditions

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(HTMP, at 121 °C, 1.2 or 1.5 atmospheric pressure) using an autoclave for 30-60 min with or without SDS to unfold and partially break the phosvitin structure. The pretreated phosvitin was subsequently hydrolyzed using trypsin (pH 8.0, 40 ºC), Protex 6L (pH 7.0, 60 ºC), Multifect® 14L (or subtlisin pH 8.0, 70 ºC), trypsin + Protex 6L, and trypsin + Multifect® 14L. The substrate to enzyme ratios were 50:1. Heat-pretreated (at 100 °C for 60 min) phosvitin was also prepared to compare the effect of the HTMP on the enzyme hydrolysis of phosvitin. Digestions were carried out for 6 h in a shaker water bath. For two enzyme combinations, hydrolysis with the second enzyme was done after inactivating the first enzyme after the 6h incubation. The hydrolyzed solutions were lyophilized and stored at -20 ºC freezer until used. The degree of hydrolysis (DH) was monitored using SDS-PAGE and a chemical method (the o-phthaldialdehyde). The peptide profile and amino acid sequence of the phosphopeptides in the hydrolysates were determined using LC-tandem mass spectrometry.

Results and Discussion

The HTMP at 1.5 atm for 30 min was better than that at 1.2 atm in hydrolyzing phosvitin using the subsequent enzyme treatments (Fig. 1A and 1C). With the HTMP at 1.2 atm for 30 min, a significant portion of peptides produced by enzyme hydrolysis was bigger than 10 kDa although the proportion of the large peptides (10 kDa) varied depending upon the enzymes used, and two enzyme combinations helped further hydrolyze the peptides from the single enzyme treatment. The combination of SDS with HTMP for 30 min improved the hydrolysis of phosvitin but the improvement was not significant (Figure 1A and 1B). On the other hand, increasing the HTMP time from 30 min to 60 min at 1.5 atm significantly improved the hydrolysis of phosvitin (Figure 1C and 1D). Figure 1D indicated that > 85% of the peptides produced by the pretreatment (121 ºC at 1.5 atm for 60 min) in combination with the enzyme(s) were < 3 kDa in molecular sizes. Longer hydrolysis time (beyond 6 h) did not help phosvitin hydrolysis. This is a breakthrough method in hydrolyzing phosvitin into small peptides, which enables the use of phosvitin as a substrate to produce functional phosphopeptides. To our best knowledge, no study has shown this level of hydrolysis in phosvitin (Fig. 1D). Although the number and size of phosphopeptides produced could vary significantly depending upon the enzymes and hydrolysis conditions used, pretreatment was the key for the hydrolysis of phosvitin.

From the results of Figure 1, HTMP at 1.5 atm for 60 min and three enzymes (Trypsin, Protex 6L, and Multifect 14L) and their combinations (Trypsin + Protex 6L and Trypsin + Multifect 14L) were selected and used as the final treatments for the structural study of the phosphopeptides in the hydrolysates. In the final treatment, thermolysin was replaced with Multifect 14L because Multifect 14L, a commercial food-grade thermolysin, was available. Figure 2 compares the effects of heat-pretreatment and HTMP on the hydrolysis of phosvitin using the selected proteinases. Figure 2A indicated that heat-pretreatment alone (at 100 ºC for 60 min, lane 3), as well as the heat-pretreatment and enzyme combinations, were not enough to break phosvitin into small peptides (Figure 2A, lanes 4-6). With the heat-pretreatment (at 100 ºC for 60 min) and enzyme combinations, a major part of the peptides produced was bigger than 10 kDa in size even though there was some difference in their sizes depending upon the enzymes used (Figure 2A). On the other hand, the HTMP at 121 °C, 1.5 atmospheric pressure for 60 min (Figure 2B, lane 3) showed effectiveness to break down phosvitin into peptides with a wide range of molecular sizes (Figure 2B, lane 3) and the subsequent enzyme treatments further hydrolyzed the majority of large peptides into smaller ones (< 5 kDa) (Figure 2B, lanes 4-8) as had been shown in Figure 1D. Previous studies showed that the effective hydrolysis of phosvitin to small peptides (< 3 kDa) using proteolytic enzymes alone was extremely difficult.

Partial dephosphorylation of phosvitin, especially 35% dephosphorylation using alkaline conditions, helped the hydrolysis of phosvitin, and the phosphopeptides produced were more effective in binding Ca^{+2} and inhibiting the formation of insoluble calcium phosphate than phosphopeptides retaining 65% and 17.5% of phosphates. Phosvitin with heat pretreatment (at 100 ºC for 60 min) and enzyme combinations showed significantly lower levels of hydrolysis than those with the pretreatment and enzyme combinations, except for Trypsin + Protex 6L (Table 1). Among the three enzymes, trypsin and Protex 6L performed better than Multifect 14L when applied singly, but trypsin showed a higher DH value than Protex 6L. Trypsin produced about 10% hydrolysis rate with the heat pretreatment and over 23% with the pretreatment. Protex 6L (Subtlisin or alcalase) is an enzyme with a broad substrate specificity with high stability at high pH and temperature conditions and is an excellent enzyme for industrial application. However, trypsin performed better than Protex 6L in hydrolyzing the peptides produced by the HTMP pretreatment (Table 1). Multifect 14L (thermolysin) is known to cleave the N-terminus of Leu, Phe, Ile, Val, Met, and Ala; however, its hydrolysis effect was not as good as that of the trypsin and Protex 6L because phosvitin has only a few amino acid residues that can be hydrolyzed by thermolysin. When Protex 6L or Multifect 14L was combined with Trypsin, their DH values increased

significantly. Trypsin + Multifect 14L had higher DH values than Trypsin + Protex 6L. Thus, the use of Trypsin + Multifect 14L combination is better than that of the Trypsin + Protex 6L to improve DH of phosvitin. Currently, it is difficult to explain why Multifect 14L produced the highest degree of hydrolysis when combined with trypsin. When the SDS-PAGE and the DH values were compared (Figure 2 and Table 1), they did not agree well: in heat (at 100 ºC for 60 min), the DH value of the Multifect 14 L-treated phosvitin (5.70%) is about half of the trypsin (9.82%) and Protex 6L (10.26%) treatments. With the HTMP pretreatment (121 ˚C, 1.5 atmospheric pressure for 60 min), all 3 enzyme treatments (trypsin, Protex 6L, and Multifect 14L) showed a similar degree of molecular breakdown in SDS-PAGE (Figure 2B), but the DH value of the Multifect 14L-treated phosvitin (8.43%) showed significantly lower DH value than that of the trypsin and Protex 6L treatments (19.04% and 26.01%). The electrophoresis and the degree of hydrolysis results (Figure 2 and Table 1) indicated that if one enzyme is used to hydrolyze the HTMP pretreated phosvitin, trypsin is the best, but trypsin + Multifect 14L combination is recommended for 2-enzyme combinations (Figure 2B).

The HTMP pretreatment produced phosphopeptides with varying sizes by breaking peptide bonds of phosvitin at random sites (Table 2). With the pretreatment alone, 154 peptides (without considering modifications including phosphorylation sites and numbers) were identified from the MS/MS study, indicating that pretreatment was effective in breaking the peptides bonds in phosvitin randomly, importantly from the core part of the phosvitin, which made the subsequent enzymatic hydrolysis easy. The random breakage of peptide bonds in phosvitin by the HTMP could reduce the repeatability of the phosvitin phosphopeptides (PPP) produced after enzyme hydrolysis, but it may not have negative effects on the production of PPP. Further hydrolysis of the HTMP-pretreated phosvitin using different enzymes or enzyme combinations hydrolyzed the polypeptides at different sites and produced phosphopeptides with different amino acid sequences and sizes.

The number of phosphopeptides identified from the phosvitin with pretreatment plus trypsin or Protex 6L combination was 252 and 280, respectively, which were far greater than that of the HTMP pretreatment alone. The HTMP pretreatment with Multifect 14L and two-enzyme combinations (trypsin + Protex 6L and trypsin + Multifect 14L) produced 164, 158, and 154 phosphopeptides, respectively (Table 2), which are a similar number to that from the HTMP-pretreatment alone. However, these numbers are far greater than those identified in earlier studies. The main reason for the smaller number of peptides detected with the two-enzyme combinations was that the size of peptides became smaller due to the further peptide hydrolysis which made the size of many peptides below the detection limit. If all the possible hydrolysis sites in the polypeptides produced by the pretreatment are broken by trypsin or other enzymes, the number of peptides produced could be greater than those shown in Table 2.

Theoretically, trypsin is an excellent enzyme to hydrolyze phosvitin into a variety of phosphopeptides due to the amino acid composition and distribution in phosvitin, because phosvitin contains 15 lysine and 11 arginine residues and they are spread throughout the phosvitin molecule. If all the lysine, threonine, and tyrosine sites in the natural phosvitin are hydrolyzed by trypsin, it can produce phosphopeptides with 2 to 25 amino acid residues with a varying number of phosphorylation and phosphorylation positions. Most of the peptides listed here had *z* values of 2 or 3, and most of the small phosphopeptides, depending upon the *z* and the number of phosphorylation, could not be detected. It should be pointed out that not all the peptides containing serine and/or tyrosine are phosphorylated and the number of phosphates attached to the possible phosphorylation sites is lower than expected. The degree of phosphorylation in the phosphopeptides produced was expected to be very high because 59% of the amino acid residues in phosvitin can be phosphorylated, and almost all the serine residues are phosphorylated. The number of phosphate groups in the phosphopeptides and the size of peptides are known to have a strong effect on their metal-binding strength as well as other functionalities. A higher degree of phosphorylation in a phosphopeptide is expected to have better metal-binding capability and antimicrobial activity than the ones with a lower degree of phosphorylation.

Conclusion

The HTMP pretreatment is an excellent tool to hydrolyze phosvitin effectively at random sites and the combination of enzymes to the HTMP pretreatment was helping hydrolysis of the peptides further, even in the core part of the phosvitin molecule. Varying sizes and types of phosphopeptides with highly diverse functions can be expected from the phosphopeptides produced from phosvitin. Thus, the PPPs produced are expected to have similar functions to the CPPs and can be used as metal (calcium or iron) supplementing agents to prevent/cure osteoporosis or anemia, anti-cariogenic agents, antioxidants to retard aging processes in the skin, and to improve food quality and safety by inhibiting oxidative changes and preventing microbial growth in foods during storage. However, the hydrolysis data themselves do not show the functionalities and bioactivities of the phosphopeptides produced. Future works on the functionality of the PPP

are very important for the applications of the PPP in nutraceutical, pharmaceutical, and cosmeceutical areas in the future.

Figure 1. SDS-PAGE of (A) the enzyme hydrolysates of phosvitin pretreated under high-temperature and mild pressure (HTMP, 121 ºC at 1.2 atm) for 30 min, (B) HTMP (121 ºC at 1.2 atm) for 30 min plus 2% SDS, (C) HTMP (121 ºC at 1.5 atm) for 30 min, and (D) HTMP (121 ºC at 1.5 atm) for 60 min. Lane 1, molecular weight markers; Lane 2, native phosvitin; Lane 3, HTMP phosvitin; Lane 4, HTMP + trypsin; Lane 5, HTMP + protex 6L; Lane 6, HTMP + thermolysin; Lane 7, HTMP + Multifect P3000; Lane 8, HTMP + trypsin + protex 6L; Lane 9, HTMP + trypsin + thermolysin; Lane 10, HTMP+ trypsin + Multifect P3000. The hydrolysis time for each of the enzyme used was 6 h.

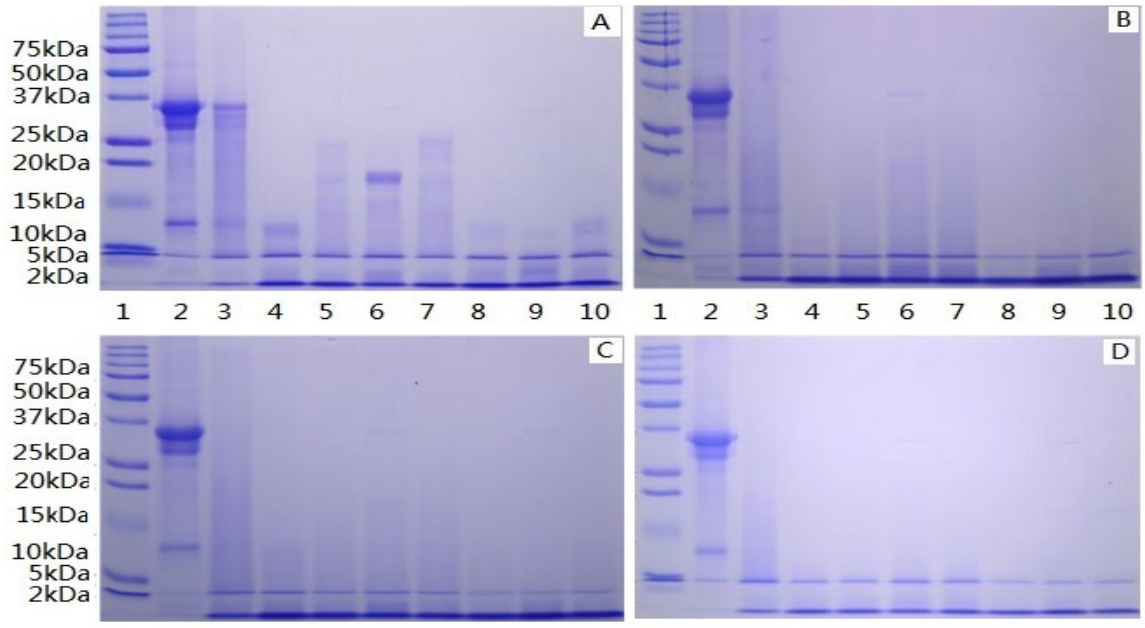

Figure 2. SDS-PAGE of phosvitin hydrolysates with different pretreatments. (A) Heat-pretreated (HP) at 100 ˚C, atmospheric pressure for 60 min, and (B) HTMP pretreated at 121 ˚C, 1.5 atmospheric pressure for 60 min. A: Lane 1, molecular weight markers; Lane 2, native phosvitin; Lane 3, heat-pretreated; Lane 4, HP + trypsin; Lane 5, HP + protex 6L; Lane 6, HP + Multifect 14L; (B): lane 2, native phosvitin; Lane 3, HTMP pretreated; Lane 4, HTMP + trypsin; Lane 5, HTMP + protex 6L; Lane 6, HTMP + Multifect 14L; Lane 7, HTMP + trypsin+protex 6L; lane 8, HTMP + trypsin+Multifect 14L.

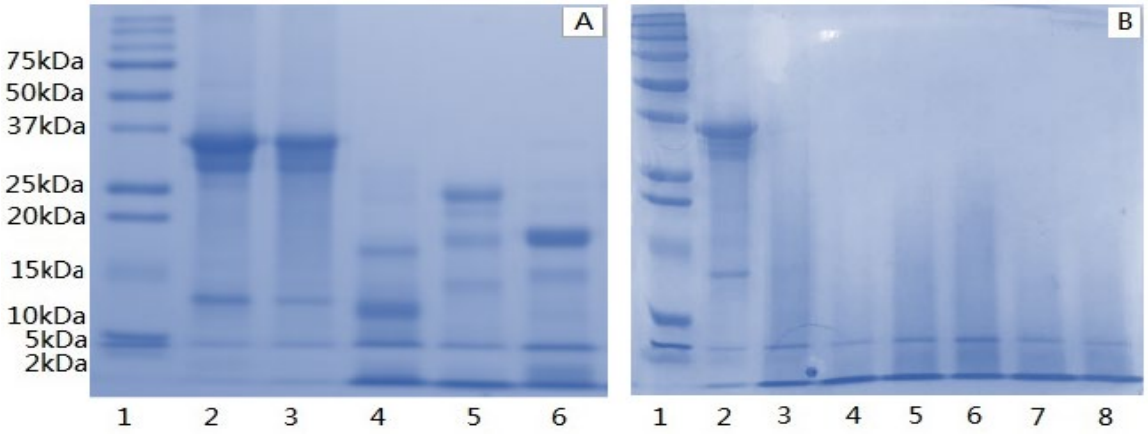

Table 1. Degree of hydrolysis after treating phosvitin with different pretreatment* and enzyme combinations

Treatment	Heat pretreatment	HTMP pretreatment
	---------- Degree of hydrolysis (%) ----------	
No enzyme	3.20±0.15^{a,x}	3.57±0.22^{a,x}
Trypsin	9.82±0.66^{c,x}	23.24±0.58^{d,y}
Protex 6L	10.26±1.49^{c,x}	17.43±0.90^{c,y}
Multifect 14 L	5.70±0.06^{b,x}	8.43±0.21^{b,y}
Trypsin + Protex 6L	19.38±1.87^{d,x}	19.04±0.55^{c,x}
Trypsin + Multifect 14L	21.65±0.59^{d,x}	26.01±1.23^{e,y}

*Heat pretreatment: heating at 100 ˚C, under atmospheric pressure for 60 min; HTMP pretreatment: heating at 121 ˚C, 1.5 atmospheric pressure for 60 min. n = 6.

$^{a-d}$Numbers within a column with different letters differ significantly.

^{x,y}Numbers within a row with different letters differ significantly.

Table 2. Peptide profile of HTMP pretreated phosvitin with different enzyme combinations

Treatment	Number of peptides[1]	Number of peptides[2]
HTMP pretreatment alone	154	310
HTMP pretreatment and trypsin combination	252	599
HTMP pretreatment and Protex 6L combination	280	605
HTMP pretreatment and Multifect 14L combination	164	534
HTMP pretreatment and trypsin + Protex 6L combination	158	396
HTMP pretreatment and trypsin + Multifect 14L combination	154	454

[1]Number of peptides: without considering modifications/phosphorylations.

[2]Number of peptides: Peptides with different modifications/phosphorylations were considered as different peptides. In all treatments, peptides had 0-4 phosphorylations. Modifications observed include deamidation, oxidation, carbamylation, dehydration, methylation, formylation, allylation, and deamidation, but the degree of modifications varied depending upon the pretreatment and enzyme combinations.