Chemical Hydrolysis of Phosvitin and the Functional Properties of the Hydrolysates

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Abstract

As a highly phosphorylated protein, phosvitin shows a powerful antioxidant and metal-binding activity. However, its extraordinary resistance to protease activities limits the possibility of using phosvitin as a source for the production of functional peptides. The objectives of this study were to test the possibilities of using alkaline or acid hydrolysis to produce small peptides from phosvitin and to investigate the functional properties of the resulting peptides. Phosvitin was dissolved in various concentrations of NaOH (0.025N-0.3N) or HCl (0.05N–6N) and incubated in a shaker water bath (37°C for NaOH and 60°CforHCl) for 1-6 h. After incubation, the degree of hydrolysis, and the functional properties of the hydrolysateswere determined.Phosvitin showed higherresistanceto acid hydrolysis than alkaline hydrolysis.Incubation of phosvitinin 0.075 N NaOHsolution at 37°Cfor 1h and 2N HCl at 60°Cfor 6 h partially hydrolyzedphosvitin, but 0.1 N NaOH at 37°C for 3 h or 3N HCl at 60°Cfor 6 h incubation was needed forthe complete hydrolysis of phosvitin. Increasing degree of hydrolysis inphosvitin either by HCl or NaOH negatively affected their antioxidant and Fe-chelating capacities.Cu^{+2}-chelating activity, however, increased significantly by hydrolyzingphosvitinwith 0.1N NaOH or2N HCl. The alkalinehydrolysates(0.05 N and 0.1NNaOH)and acid hydrolysates(2 N HCl) significantly increased the solubility of Ca^{+2} in sodium phosphate buffer (pH 6.8) compared to the control.

Key words: *Phosvitin, acid hydrolysis, alkaline hydrolysis, antioxidant activity, metal chelating activity*

1.Introduction

Phosvitin is considered as the nature's most phosphorylated protein (Mecham&Olcotte 1949; Burley & Cook 1961). More than 50% of amino acids in phosvitin are serine and almost all of them are phosphorylated (Taborsky, 1974; Byrne, van Het, van de Klundert, Arnberg, Gruber, & Geert, 1984).Therefore, phosvitin exhibits strong antioxidant, emulsifying and metal chelating activities (Taborsky, 1963; Grizzuti&Perlmann, 1973; Lu & Baker, 1986; Lee, Han & Decker, 2002; Nakamura, Ogawa, Nakai, Kato & Kitts, 1998).Phosvitinhas beensuggested to use as a substrate for producing metal-binding functional peptides (Jiang & Mine, 2000; Katayama, Xu, Fan, & Mine, 2006; Xu, Katayama & Mine,2007). However, due to exyremelyhighphosphorylation, phosvitin shows extraordinary resistance to enzymatic hydrolysis(Mecham&Olcotte, 1949; Anton, 2007). To improve the enzymatic hydrolysis of phosvitin, various pre-treatments, including heat, acid, alkali, non-ionic detergent, and high pressuresingly or in combinations have been tested only with slight improvementin its enzymatic digestibility. It is reported that phosphate in phosphoserine is easily dephosphorylated in alkaline conditions (Kellnar, Lottspeich& Meyer, 2008), andJiang & Mine(2000) have used NaOH in order to achieve dephosphorylation and thereby increased the digestibility of phosvitin.

Protein hydrolysates can be produced by chemically or enzymetically (Fountoulakis& Lahm, 1998). Partial hydrolysis of proteins with acid and alkali has been utilized to enhance the functionality of protein and thereby increase the utilization of proteins in food processing, especially to prepare hydrolysates of soy, zein, casein, yeast and gluten (Kinsella and

Shetty, 1979). Acid hydrolysis of vegetable and animal proteins has been used to produce flavor and taste enhancers in meat, crackers and soups (Kristinsson&Rasco, 2000).

Alkaline and acid hydrolyses of proteinsare extensively used to investigate amino acid composition of proteins. For that usually 6 N HCl at 110˚C for 6h is used (Fountoulakis& Lahm, 1998). Under these conditions, proteinsaremainly decomposed to amino acid monomers with some oligopeptides (Anfinsen, 1965; Kellnar, Lottspeich, & Meyer, 2008). At present, no known studies on the use of alkaline and acid hydrolysis for the production of functional peptides are available. However, if alkaline or acid hydrolsysiscan produce functional peptides from phosvitin, it would be more economical and practical than using enzymes. Yet, the use of chemical hydrolysis has some known disadvantages such as difficulties in controlling degree of hydrolysis, production of D-amino acids from L-amino acids, and production of toxic compounds (Nnanna, 2007). The objectives of present study were to test the possibilities of using alkaline or acid hydrolysis to produce small peptides from phosvitin, and to investigate the functional properties of the resulting peptides.

2. Materials and Methods

2.1Alkaline hydrolysis of phosvitin

Phosvitin wasprepared according the procedure ofKo*et al*. (2011). Phosvitin was dissolved in in 0.025N, 0.05N, 0.075N, 0.1 N, 0.2N and 0.3N NaOH (25mg/mL) and incubated in a shaker water bath (New Bruwnswick Scientific, Edison, NJ, USA) at 37˚C for 1h, 2h or 3h. After incubation, the samples were neutralized with 2N HCl, and the size of peptides was determined using SDS-PAGE.

2.2 Acid hydrolysis of phosvitin

Phosvitin (25mg/mL) was dissolved in different concentrations of HCl and incubated in a shaker water bath at different temperatures and durations. After incubation, the reaction was stopped by neutralizing them with 10 N NaOH andthe degree of hydrolysis was analyzed using SDS-PAGE.

2.3 SDSE-PAGE

The SDS-PAGE of acid and alkaline hydrolysates of phosvitin was done under reducing conditions using Mini-Protein II cell (Bio-Rad). Fifteen percent SDS-PAGE gel and Coomassie brilliant blue R-250 (Bio-Rad) with 0.1M aluminum nitrate staining were used. Pre-stained Precision plus protein dual color standards (10-250 kD) (Bio-Rad) was used as the molecular marker (MM).

2.4 Measurement of functional properties

Thehydrolysates ofphosvitin at 0.05N and 0.1N at 37˚C for 3h and thosehydrolyzed in 2N, 3N and 6N HClsolutions at 60˚C for 6h were selectedto measure the functional properties of alkaline or acid hydrolysates of phosvitin.

2.5 Antioxidant activity

An oil-in-water emulsion containing 1.0 gof corn oil (HyVee Inc., IA, USA) and 100 µL of Tween 20 (Fisher Scientific) in 100 mL of Tris–maleate buffer (pH 6.8) was prepared by homogenizingthem using a Brinkman Polytron (Type PT 10/35; Brinkman Instrument Inc., Westbury, NY, USA) for 4 min in an ice bath at full power. The oil-in-water emulsion was physically stable during incubation period at 37˚C. Samples for lipid oxidation assay was prepared by mixing 8 mL of the oil emulsion, 0.5 mL of 0.2 % ascorbic acid (Sigma) and 0.5 mL of 200 ppm of Fe^{+3}($FeCl_3$, Sigma), and 1 mL of phosvitin and its hydrolysates (2mg) in a 50-mL test tube. After vortex-mixing, the mixture was incubated at 37˚Cfor 16 h.

The mixture was withdrawn to determine 2-thiobarbituric acid-reactive substances (TBARS) value at different incubation time. One mL of oil emulsion was mixed with 2 mL of thiobarbituric acid/trichloroacetic acid solution (20 mM TBA/15% TCA, w/v) in a disposable test tube (13x100 mm), and 50 µL of 10% butylatedhydroxyanisole in 90% ethanol were added. After vortex-mixing, the mixture was incubated in a 90˚C water bath for 15 min to develop color. Then, the samples were cooled for 10 min in cold water, vortex-mixed and centrifuged at 3,000 x *g* for 15 min at 5˚C. One ml of the supernatant was taken to measure the absorbance at 532 nm against a blank prepared with 1 ml DDW and 2 ml TBA/TCA solution.The amounts of TBARS were expressed as mg of malondialdehyde (MDA) per L of emulsion.

2.6 Fe-chelating activityof phosvitin and its hydrolysates

The Fe-chelating activity of alkaline and acid hydrolysates of phosvitinwas evaluated using theFerrozine method (Carter, 1971) with some modifications. Hydrolysates containing 0.5 mg

phosvitin or phosvitinhydrolysate in Tris–maleate buffer (pH 6.8) (100µL) was vortex-mixed with 1 ml of 10 ppm Fe and 1 mL of 0.1% (wt/vol) ascorbic acid in 2N HCl. The mixture was incubated for 5 min at room temperature, added with 0.9 mL of 11.3% trichloroacetic acid (TCA),and then centrifuged at 2,500 x g for 10 min.The supernatant (2 mL) was transferred to a new disposable culture tube, added with 0.8 ml of 10% ammonium acetate (Fisher Scientific) and 0.2 mL of Ferroin color reagent (75 mg of ferrzoine, 75 mg of neocuproin and 1 drop of 6NHCl in 25 mL of distilled water), vortex-mixed, incubated for 10 min, and then the absorbance was measured at 562 nm after 10 min of incubation at room temperature. The Fe-chelating activity was calculated using a blank (without phosvitin or phosvitinhydrolysate)

Fe-chelating activity (%) = {1-(sample solution absorbance/blank solution absorbance)}x100

2.7 Cu^{2+}-chelating activity of phosvitin and its hydrolysates

The Cu^{2+}-binding activity of acid and alkaline hydrolysates was measured according to the modified method of Kong andXiong(2006). One mL of 0.2mM $CuSO_4$ was mixed with 1 mLof phosvitin/hydrolysates (2mg) in Tris-maleate buffer (pH 6.8). After that, 1mL of 11.3% TCA was added to the sample and centrifuged at 2,500 x g for 10 min. The supernatant (2 mL) was transferred to a disposable culture glass tube,added with 1 mL of 10% pyridine and 20 µL of 0.1% pyrocatechol violet (Sigma-Aldrich). The sample was vortex-mixed, incubated at room temperature for 5 min, and thenthe disappearance of blue color due to dissociation of Cu^{2+} (by protein/peptides) was measured at 632 nm. The Cu^{2+}-chelating activity was calculated using the following formula

Cu^{2+}-chelating activity (%) = {1-(sample solution absorbance/blank solution absorbance)}x100

2.8.Ca^{2+}-chelating/solubilizing activity

Ca-chelating/solubilizing activity was measured according to the modified method of Jung & Kim (2007). The phosvitin and its acid and alkaline hydrolysates (4 mg) were added in 8 mL of 5 mM $CaCl_2$ and 20 mM sodium phosphate buffer at pH 6.8. The mixture was incubated at 37°C for 2 h in a shaking water bath (New Bruwnswick Scientific, Edison, NJ, USA), and centrifuged at 3,200 x g for 10 min. The supernatant was filtered through a 0.45 µm membrane filter and the calcium content of the filtrate was determined by Inductive Plasma Mass Spectroscopy (ICP: SPECTRO, Analytical Instruments).

2.8 Statistical analysis

The statistical analysis was done using the SAS-9.2 software.Data were analyzed for one-way analysis of variance (ANOVA). LSD tests were performed for significant differences ($p< 0.05$) among means. For all the assays, four replications were used.

3. Results and Discussion

3.1 Alkaline hydrolysis of phosvitin

NaOH has been used to dephosphorylate phosvitin. Khan*et al.* (1998) evaluated the emulsifying properties of phosvitin treated with 0.25N NaOH at 35°C for 24h and found drastic reduction in its emulsifying properties compared to the native phosvitin. Jiang and Mine (2000) dissolved phosvitin in 0.1-0.4 N NaOH solution and incubated at 37°Cfor up to 4h for dephosphorylation of phosvitin. After that, they have treated the resulting phosvitin with trypsin and found that trypsin digestion resulted in a streaky band pattern at the bottom of the SDS-PAGE gel, implying high degree of digestion. However, they have not evaluated the nature of phosvitin after dephosphorylation with NaOH using SDS-PAGE or any other means. Our preliminary study indicated thatat pH 13, phosvitin underwent alkaline hydrolysis at temperatures between 70°C to 85°C, implying decomposition of protein. When phosvitin was treated withlow concentrations of NaOH (0.025N and 0.05 N NaOH) at 37°C for up to 3h, only minor changes in phosvitin molecules were observed. However, these treatmentsproduced a few bands not observed in untreated phosvitin sample (Figure1A). With 0.075 N NaOHtreatment, most of the phosvitin major band disappeared and resulted in continuous streaking band patterns on SDS-PAGE (Figure 1B). This implies that treating phosvitinwith 0.075 N NaOH at 37°C is effective conditions for alkaline hydrolysis. However, 0.1N NaOHwas more effective than 0.075 N NaOH and resulted in almost complete hydrolysis of phosvitin after 3h at 37°C. At 0.2N and 0.3N NaOHlevels,phosvitin bands completely disappeared after 1 h incubation at 37°C (Figure 1C). Therefore, we suggest treating phosvitin in 0.1 N NaOHat 37°C for 3 h or 0.2N NaOHat 37°C for 1 h for the complete hydrolysis. The high susceptibility of phosvitin to alkaline hydrolysis is probably due in part to its structural characteristics, which is very high inphosphoserine (> 50% of amino acids of phosvitin

are phosphoserine). Phosphoserine is highly unstable in alkaline conditions and phosphoserinecan be totally destroyed under alkaline conditions(Kellnar*et al*, 2008; Pasupuleti and Braun, 2010). Also,other amino acids in protein experience racemization in alkaline conditions(Sen*et al.*, 1977). Therefore, the alkaline dephosphorylation of phosvitin referred in previous literatures (Sen*et al.*,1977; Jiang & Mine, 2000) is in doubt. During the alkaline treatment at 0.1 N NaOH or higher concentrations, probably phosvitin has been broken down into smaller peptides or individual amino acids. Sen et al. (1977) treated phosvitin at 0.123 N NaOH at 60°Cfor 80 min and observed 87% removal of phosphate due to β-elimination and 13 % due to hydrolysis. However,our results indicated that treating phosvitinin 0.123 N NaOH at 60°C for 80 min not only dephosphotylated but also hydrolyzedphosvitin molecules.

Incubation of phosvitin in 0.2N and 0.3 N NaOH at 37°C for 3h resulted in better hydrolysis than that in 0.1 N NaOH, the hydrolysates from 0.1 N NaOHwas selected to examine the antioxidant and mineral binding activities because it wasmilderhydrolysis method forphosvitin than that at higher NaOH concentrations.

3.2 Acid hydrolysis of phosvitin

According to the SDS-PAGE band patterns, treating phosvitinin 0.05, 0.1 and 0.15N HCl at 37°Cfor up to 3h had no effect on the hydrolysis of phosvitin (Figure 2A). Also, treating phosvitin in 2, 3 and 6N at 37°Cfor 3h had no effect on the hydrolysis of phosvitin (data not shown). This high stability of phosvitin in acidic conditionscould be related to the formation of high % β-structure in phosvitin (Chang et al, 1978; GrizzutiandPerlmann, 1970). At 2, 3, and 6N HCl concentrations, however,phosvitinwas hydrolyzed at higher temperature conditions, and 2, 3 and 6N HCl at 60°C for 6h was selected for acid hydrolysis conditions for phosvitin (Figure 2B). Crowder*et al.* (1999) hydrolyzed phosvitin in 2N HCl at 110-130°C for various time duration and observed that 12 h incubation was the optimal time forthe hydrolysis and liberation of phosphoserine from phosvitin. The acid hydrolysis of protein at 6N HCl can destroy tryptophan, serine and threonine due to oxidation, and results in slow hydrolysis of some peptide bonds between bulky andsterically hindered amino acids (Simpson*et al.*1976; Liu and Chang, 1971). Lee*et al.* (1977) observed loss of serine and phosphoserine during acid hydrolysis of phosphoproteins.

The SDS-PAGE bands of phosvitinhydrolysatefrom2N HCl at60°Cfor 6h incubation showed continuous streaking patterns from phosvitin to the dye line indicating that a significant portion of phosvitin had been hydrolyzed. Treating phosvitin in 3N or 6N HClat 60°C for 6h resulted in higher degree of degradation with more streaking patterns shown close to the bottom of the SDS-PAGE gel (Figure 2A). This could be due to the hydrolysis of phosvitin into small peptides or into individual amino acids. Therefore, phosvitinhydrolysatesfrom 2, 3and 6N HCl at60°Cfor6h incubationwere selected to determineantioxidant and mineral binding activities.

3.3 Antioxidant activity of phosvitinhydrolysates

Thenative phosvitin showed the best antioxidant activity in the Fe^{+3}-induced lipid oxidation in oil emulsion system (Table 1). This is due to the extraordinary high iron-binding capacity of phosvitin molecule.

The phosvitin concentration used for all the treatments was 0.2 mg/mL. At this level, native phosvitin and phosvitin treated with 0.05 N NaOH at 37°C for 3h showed similar antioxidant activity to BHA in oil emulsion system (Table 1).The phosvitinhydrolysate from 0.1N NaOH treatmentsfor 4h at 37°Cshowed similar antioxidant activity to the native and 0.05 N-treated phosvitin. After 4h incubation, however, their antioxidant capability was significantly lower than that of the native phosvitin and 0.05 N NaOH treatments. During all the incubation periods, all 3 acid hydrolysedphosvitin exhibited significantly lower antioxidant activity than the native phosvitin implying impaired antioxidant activity of phosvitin after acid hydrolysis. With increasing concentration of HCl, the antioxidant activity decreased drastically. This could be due to high degree of dephosphorylation or destruction of phosvitin molecule. Phosvitin is an excellent antioxidant againstmetal catalysts due to its capacity to chelate metal ions (Lu and Baker, 1986; Lee*et al.*,2002).

Fadil*et al*. (1996) treated gluten in 0.05 N HCl at 120°Cfor 60 min to increase solubility, but acid and alkaline hydrolysis of protein has not been implemented for the production of functional peptides to our knowledge. Therefore, this could be a novel approach for the production of functional peptides in an economical manner compared to enzymatic hydrolysis. Kato*et al.* (1987) evaluated the emulsifying properties of phosvitin after an alkaline treatment (0.25N NaOHat 37°C for 24h to remove phosphate molecules completely) and observed that the emulsifying properties of phosvitin have been greatly decreased. Xu*et al.*,(2007) evaluated the antioxidant activity of three different fractions of peptides derived from trypsin digestion of 0.1 M NaOH-treated phosvitin at 37°C for 3h. The phosphate

contents of the three fractions were reported as 0%, 7.2% and 18.9%, respectively. They found that stronger antioxidant activity of those peptides fractions compared to native phosvitin and suggested that this could be due to the presence of histidine, methionine and tyrosine on those peptides. However, the purification of those three peptides is a tedious procedure and could be expensive.

3.4 Fe-chelating activity of phosvitinhydrolysates

Intact phosvitin showed the highest and significantly higher (p<0.05) Fe-chelating activity than the acid-or alkaline-treated phosvitin (Figure 3). However, the Fe-chelating activities ofphosvitinhydrolysates from 0.05NNaOH at 37°C for 3 h treatment, 0.1N NaOH at 37°C for 3 h treatment, and 2 N HCl at 60°C for 6h treatmentshowedhigh levels of iron-binding capacity, even though their activities were lower than that of the natural phosvitin, indicating that they can be used as Fe-carrying functional peptides. Also, thehydrolysates fromalkalase and Multifect® P-3000 treatments showed lower Fe-binding capacity than that of the native phosvitin(unpublished data). Natural phosvitin has a high affinity to Fe ions and tends to form insoluble complexes with Fe (Taborsky, 1963; GrizzutiandPerlmann, 1973;Castellani et al., 2004). Thus,lower Fe-binding capacity of phosvitinhydrolysatesthan the intact phosvitin may be helpful in reducing precipitation of Fe. Hence the reduction of Fe-chelating activity by hydrolysates can be supportive to overcome the problems with phosvitin and may play a significant role in the production of mineral-carrying and mineral-solubilizing functional peptides. Acid hydrolysates of phosvitin drastically reduced the Fe-chelating activity of the peptides. The phosvitinhydrolysatewith 2N HCl showed comparatively high Fe-chelating activity, implying that hydrolysis of phosvitin with 2N HCl could be another way of producing smaller peptides from phosvitin with good Fe-binding activity. Iron is a powerful catalyst of oxidation in foods and excess amounts of ionic Fe can causeskin hyperpigmentation, arthritis, arthralgias, hypogonadotrophic hypogonadism,diabetes mellitus, liver fibrosis, cirrhosis, hepatocellular cancer, and cardiac problem (Papanikolaou&Pantopoulos, 2005). The acid hydrolysates of phosvitin obtained from 3N and 6N HCltreatments showed drastic reduction in Fe-binding activity. This could be due to the destruction of serine under harsh acidic conditions (Liu and Chang, 1971). Therefore, treating phosvitin at 3N and 6N HCl are not appropriate ways to produce hydrolysates with high levels of Fe-binding activity even though those treatments producedpeptides with smaller molecular weight (Figure 3).However, it does not mean that the small peptides with low iron-binding capacity produced by acid or alkaline hydrolysis of phosvitin cannot be used as iron-carrying agents because the iron binding capacity of the hydrolysates would be still high. This means that the possibility of using acid hydrolysates of phosvitin as an iron carrying agent is still open.Other studies showed that acid modification of some proteins by deamidation increased the functional properties such as solubility, emulsifying and forming activities of the proteins (Chan and Ma, 1999).

It is also known that chemical hydrolysis of proteins can lead to formation of toxic compounds such as lysino-alanine and can reduce the nutritional qualities of some proteins (Lahl&Windstaff, 1989).Nevertheless, acid or alkali hydrolysis can be an approach to produce functional peptides fromphosvitinand further studies on the functionalcharacteristics of the peptides generated by acid and alkali are needed.

3.5 Cu-chelating activity of phosvitinhydrolysates

It is known that phosvitin has different affinities for different metal ions. In addition,environmental conditions such as pH and ionic strength of the medium play a major role with regards to its metal binding activities (GrizzutiandPerlmann, 1973). Lu and Baker (1987) reported that the Cu^{2+}-chelating capacity of phosvitin at pH 6.1 is 1:1 ratio, but our studies indicated that the Cu^{2+}-chelating capacity of phosvitin was 3.8 Cu molecules perphosvitin molecule (data not shown). In comparison to Mn^{2+}- and Ca^{2+}-binding activities of phosvitin, the Cu^{+2}-chelating activity of phosvitin is considerably low (Grizzuti and Perlmann, 1973). The Cu^{2+}-binding activity of phosvitin and its hydrolysatesin this study showed different binding capacities. Current study showed that the native phosvitin and phosvitinhydrolysates from 0.05 N NaOHtreatment have similar Cu^{2+}-chelating activity while the phosvitinhydrolysatesfrom 0.1 N NaOH and 2N HCltreatments showed significantly higher Cu^{2+}-binding activity than the native phosvitin (Figure 4). This observation is different from the Fe-binding activity of phosvitinand phosvitinhydrolysates(Figure 3). There is no clear explanation for the significantly increased Cu^{2+}-chelating activity of those three hydrolysates. However, those three conditions could be useful for developing Cu^{2+}-chelating functional peptides from phosvitin. Further study to determine the size and to characterize the peptides in

thosephosvitinhydrolysates using the tandem Mass Spectrometry (MS/MS) is under way. With the information from the MS/MS study, it is expected that the behavior of the hydrolysates could be explained in detail.

3.5 Ca-chelating/solublizing activity of phosvitinhydrolysates

Phosvitin shows a very high affinity to Ca^{+2} and consequently forms insoluble complexes with Ca^{+2}, which makes the calcium unavailable for absorption in the guts (Perlmann 1973; Ishikawa *et al.*, 2007). Our study indicated that the Ca^{+2}-binding capacity of phosvitin increased as the levels of phosvitin increased. In addition, the solubility of calcium significantly increased as the degree of enzymatic hydrolysis of phosvitin increased (Unpublished data). Jiang & Mine (2001) found that the trypsin-digested peptides retaining 35% of phosphate were more effective in binding $Ca^{+2}$and inhibiting formation of insoluble calcium phosphate than phosphopeptides retaining 65% and 17.5% phosphate. Choi *et al.* (2005) reported that the Ca^{+2}intake, fecal Ca^{+2}content, or urinary Ca^{+2}contents of Sprague Dawley rats fed with diets containing phosvitin/trypsin-derived phosphopeptides at low, medium and high levels were not different from each other. However, phosvitinphosphopeptides increased Ca^{+2}absorption and accumulation in the bones significantly.

When $CaCl_2$ was incubated with phosvitin and its hydrolysates at 37˚Cfor 2 h, the amounts of solubilized Ca^{+2}ions in the supernatant were increased by phosvitin and its hydrolysates addition (Figure 4). Alkaline (0.05 N and 0.1 N NaOH) and acid (2 N HCl)hydrolystes of phosvitin showed significantly increased Ca^{+2}-solubility compared to the native phosvitin and its acid hydrolysates (3 N and 6 N HCl). The increased Calciumsolubility observed in some of the phosvitinhydrolysates could be due to dephosphorylation of phosvitin or hydrolysis of phosvitin into smaller peptides. The Ca^{+2}-solubility of phosvitinhydrolysates from 3 N and 6 N HCl treatments were not significantly different from that of the natural phosvitin (Figure 4). The SDS-PAGE pattern of the 3N- and 6N HCl-treated phosvitin showed smear patterns at the bottom of the gel, implying the presence of numerous small peptides or even amino acid monomers due to extensive acid hydrolysis of the protein. This result suggested that it is important to have very small phosphopeptides in order to decrease Ca solubility. After acid hydrolysis, the hydrolysates were neutralized with NaOH, and thus the hydrolysates contained high levels of NaCl. It is uncertain that the amount of NaCl present in the solution had any effect on Ca^{+2} solubility. It is known that the chemical hydrolysis of protein is difficult to control and known to produce various by-products with different functionalities. It also can reduce the nutritional qualities and affect to the functional properties of proteins negatively (Kristinsson&Rasco 2000). Further study to determine the size and amino acid sequences of the peptides in the phosvitinhydrolysates using the tandem Mass Spectrometry (MS/MS) is under way. With the information from the MS/MS study, it is expected that the behavior of the hydrolysates could be explained better.

4. Conclusions

Phosvitin shows higher stability in acid than in alkaline conditions. Phosvitin moleculescould behydrolyzed by treating it with 0.1N NaOH for more than one hour. Treating phosvitin in 3 N HCl at 60˚Cfor 6h completely degradedthe phosvitin molecule, but 6N HClproduced high degree of acid hydrolysis than that at lower concentrations. Increased degree of phosvitin hydrolysisusing either acid or alkalinegatively affected their antioxidant capacity.Iron-binding and Cu-binding activities of acid and alkaline hydrolysatesalso showed similar behavior, implying that with increased hydrolysis the metal binding capacity of phosvitin is reduced. Hydrolysis of phosvitin in 0.05 and 0.1 N NaOH at 37 for 3 h and 2 N HClhydrolysates improved the Ca^{+2}-solubility significantly, and thus are recommended as acid and alkali hydrolysis conditions, respectively, to produce functional phosphopeptides from phosvitin.

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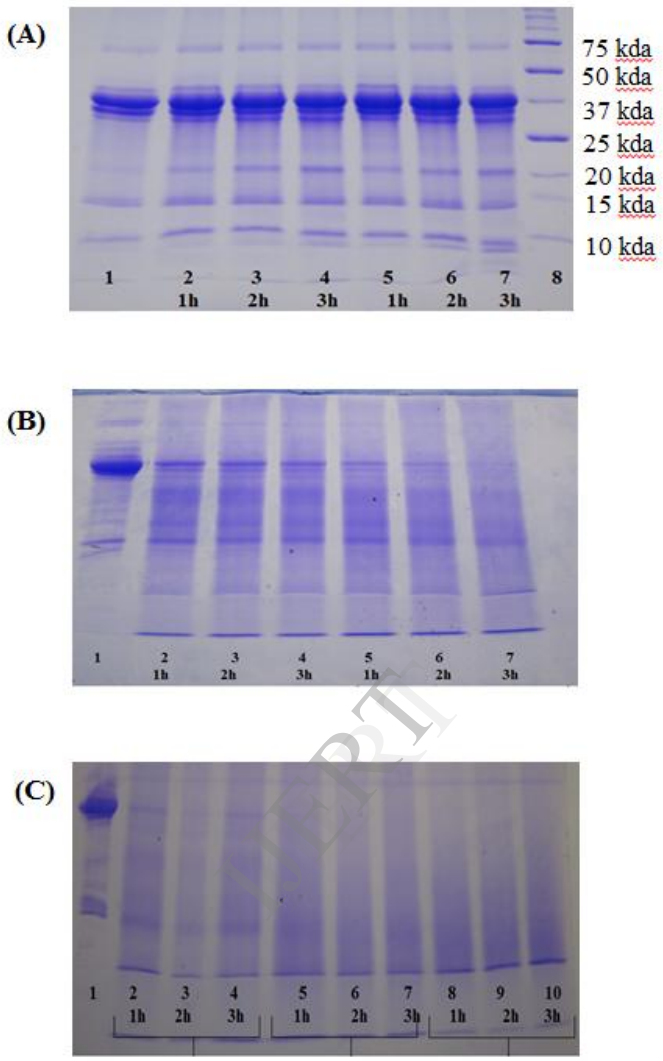

Figure1.SDS-PAGE band pattern of phosvitin and alkaline hydrolysates ofphosvitin.**(A)** Lane 1-Native phosvitin; lane 2-0.025N NaOH-treated phosvitin at 37˚C for 1h; lane 3-0.025N NaOH-treated phosvitin at 37˚C for 2h; lane 4 -0.025N NaOH-treated phosvitin at 37˚C for 3h; lane 5-0.05N NaOH-treated phosvitin at 37˚C for 1h; lane 6-0.05N NaOH-treated phosvitin at 37˚C for 2h; lane 7-0.05N NaOH-treated phosvitin at 37˚C for 3h; lane 8–standard maker. **(B)**Lane 1 -Native phosvitin; lane 2-0.075N NaOH-treated phosvitin at 37˚C for 1h; lane 3-0.075N NaOH-treated phosvitin at 37˚C for 2h; lane 4-0.075N NaOH-treated phosvitin at 37˚C for 3h; lane 5-0.1N NaOH-treated phosvitin at 37˚C for 1h; lane 6-0.1N NaOH-treated phosvitin at 37˚C for 2h; lane 7-0.1N NaOH-treated phosvitin at 37˚C for 3h.**(C)**Lane 1 -Native phosvitin; lane 2-0.1N NaOH-treated phosvitin at 37˚C for 1h; lane 3-0.1N NaOH-treated phosvitin at 37˚C for 2h; lane 4–0.1N NaOH-treated phosvitin at 37˚C for 3h; lane 5-0.2N NaOH-treated phosvitin at 37˚C for 1h; lane 6-0.2N NaOH-treated phosvitin at 37˚C for 2h; lane 7-0.2N NaOH-treated phosvitin at 37˚C for 3h; lane 8-0.3N NaOH-treated phosvitin at 37˚C for 1h; lane 9-0.3N NaOH-treated phosvitin at 37˚C for 2h; lane 10-0.3N NaOH-treated phosvitin at 37˚C for 3h.

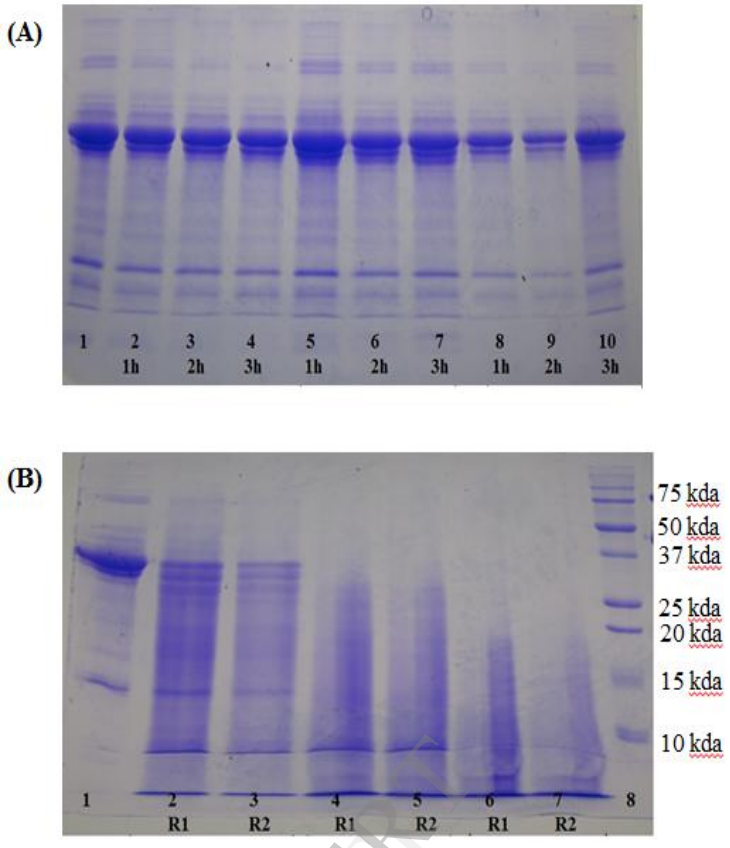

Figure 2. SDS-PAGE band pattern of phosvitin and acid hydrolysates ofphosvitin.(A) Lane 1-Native phosvitin; lane 2-0.05N HCl-treated phosvitin at 37˚C for 1h; lane 3-0.05N HCl-treated phosvitin at 37˚Cfor 2h; lane 4–0.05N HCl-treated phosvitin at 37˚C for 3h; lane 5-0.1N HCl-treated phosvitin at 37˚C for 1h; lane 6-0.1N HCl-treated phosvitin at 37˚C for 2h; lane 7-0.1N HCl-treated phosvitin at 37˚C for 3h; lane 8-0.15N HCl-treated phosvitin at 37˚C for 1h; lane 9-0.15N HCl-treated phosvitin at 37˚C for 2h; lane 10-0.15N HCl-treated phosvitin at 37˚C for 3h.**(B)** Lane 1-Native phosvitin; lane 2- 2N HCl-treated phosvitin at 60˚C for 6h (R1); lane 3-2N HCl-treated phosvitin at 60˚C for 6h (R2); lane 4–3N HCl-treated phosvitin at 60˚C for 6h (R1); lane 5-3N HCl-treated phosvitin at 60˚C for 6h (R1); lane 6-6N HCl-treated phosvitin at 60˚C for 6h (R1); lane 7-6N HCl-treated phosvitin at 60˚C for 6h(R2); lane 8-standard maker.

Table 1: TBARS values of oil emulsion[1](mg MDA/L) in thepresence of phosvitin, phosvitinhydrolysates, or BHA

Sample	Time						
	0h	1h	2h	4h	8h	16h	SEM
Control	0.106^{dxyz}	0.195^{cdy}	0.257^{cdxy}	0.392^{cy}	0.576^{bx}	1.544^{ax}	0.056
Pv	0.111^{cxy}	0.141^{cz}	0.152^{cz}	0.198^{bz}	0.212^{bz}	0.277^{az}	0.012
0.05 N NaOH-Pv	0.140^{bv}	0.177^{by}	0.189^{bz}	0.245^{az}	0.259^{az}	0.293^{az}	0.015
0.1 N NaOH-Pv	0.093^{cyz}	0.121^{cz}	0.142^{cz}	0.252^{cz}	0.465^{by}	0.744^{ay}	0.054
2 N HCl-Pv	0.091^{ez}	0.166^{dey}	0.289^{dwx}	0.496^{cx}	0.726^{bw}	1.400^{ax}	0.054
3N HCl-Pv	0.108^{fxyz}	0.274^{ew}	0.336^{dw}	0.573^{cx}	0.859^{bv}	1.663^{ax}	0.019
6 N HCl-Pv	0.111^{exy}	0.277^{dw}	0.334^{dw}	0.561^{cx}	0.852^{bv}	1.667^{ax}	0.027
BHA	0.122^{fwx}	0.239^{dx}	0.212^{eyz}	0.276^{cz}	0.315^{bz}	0.427^{az}	0.003
SEM	0.005	0.009	0.017	0.032	0.028	0.070	

[1]The oil emulsion was added with 10 ppm ionic iron.
[a-f]Means between incubation time with different superscript differ significantly ($p<0.05$, n=4).
[v-z]Meansbetween treatment with different superscript differ significantly ($p<0.05$, n=4).
Abbreviations: Pv-Natural phosvitin; 0.05 N NaOH-Pv-Phosvitin treated with 0.05N NaOH, at 37°C for 3h; 0.1 N NaOH-Pv-Phosvitin treated with 0.1 N NaOH at 37°Cfor 3h; 2 N HCl-Pv-Phosvitin treated with 2N HCl at 60°Cfor 6h; 3N HCl-Pv - Phosvitin treated with 3N HCl at 60°Cfor 6h; 6 N HCl-Pv-Phosvitin treated with 6N HCl at 60°Cfor 6h; butylatedhydroxyanisole; SEM-Standard error of the mean

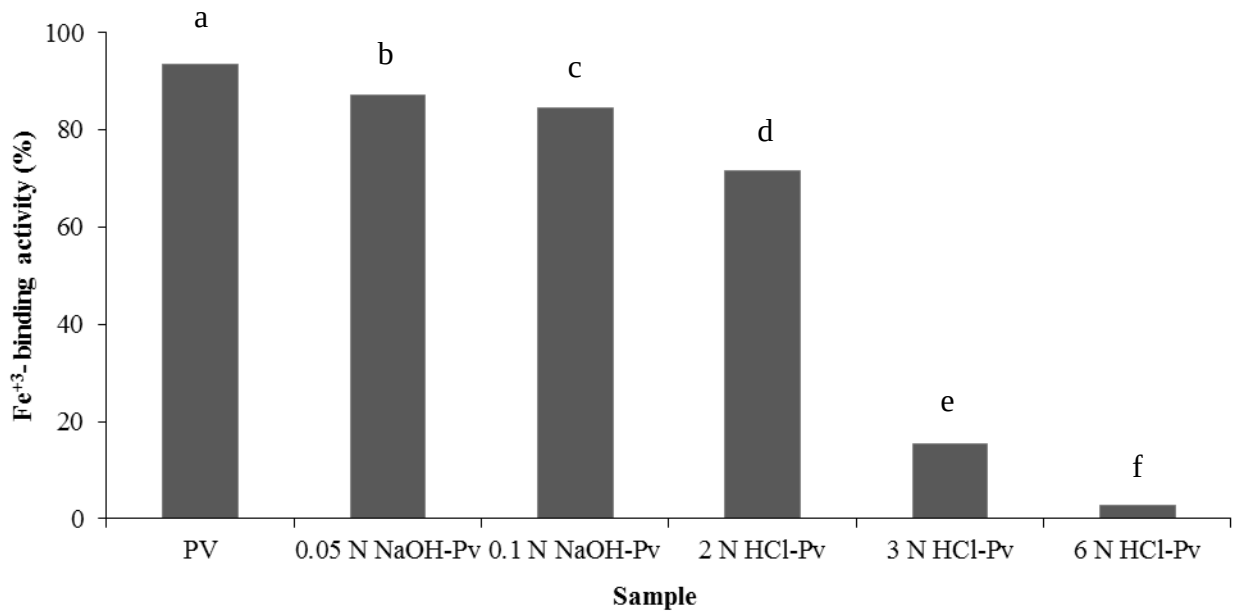

Figure 3.Fe^{+3}-binding activity of phosvitin, alkaline and acid hydrolysates of phosvitin.
[a-f]Treatments with different letters are significantly different ($p<0.05$, n=4, SEM=1.0338).
Abbreviations: Pv- Natural phosvitin; 0.05 N NaOH-Pv- Phosvitin treated with 0.05N NaOH, at 37˚C for 3h; 0.1 N NaOH-Pv-Phosvitin treated with 0.1 N NaOH at 37˚C for 3h; 2 N HCl-Pv-Phosvitin treated with 2N HCl at 60˚C for 6h; 3N HCl-Pv-Phosvitin treated with 3N HCl at 60˚C for 6h; 6 N HCl-Pv-Phosvitin treated with 6N HCl at 60˚C for 6h; SEM - Standard error of the mean.

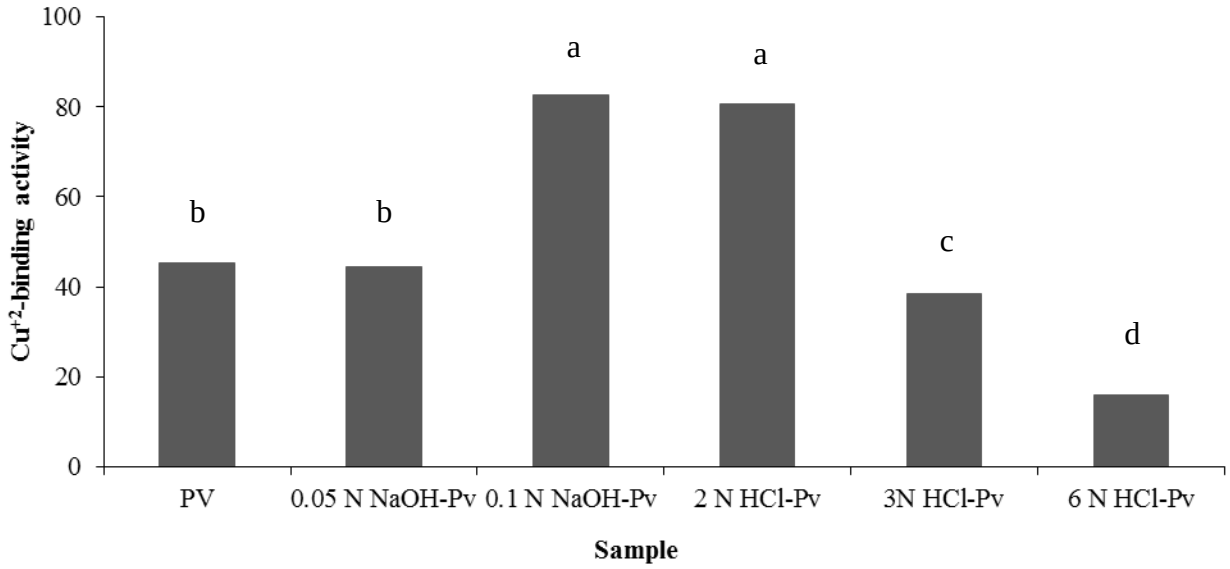

Figure 4.Cu^{+2}-binding activity of phosvitin, its alkaline and acid hydrolysates.

[a-d]Treatments with different letters are significantly different ($p<0.05$, $n=4$, SEM=1.409).

Abbreviations: Pv-Natural phosvitin; 0.05 N NaOH-Pv-Phosvitin treated with 0.05N NaOH at 37˚C for 3h; 0.1 N NaOH-Pv-Phosvitin treated with 0.1 N NaOH at 37˚C for 3h; 2 N HCl-Pv-Phosvitin treated with 2N HCl at 60˚C for 6h; 3N HCl-Pv-Phosvitin treated with 3N HCl at 60˚C for 6h; 6 N HCl-Pv-Phosvitin treated with 6N HCl at 60˚C for 6h; SEM-Standard error of the mean.

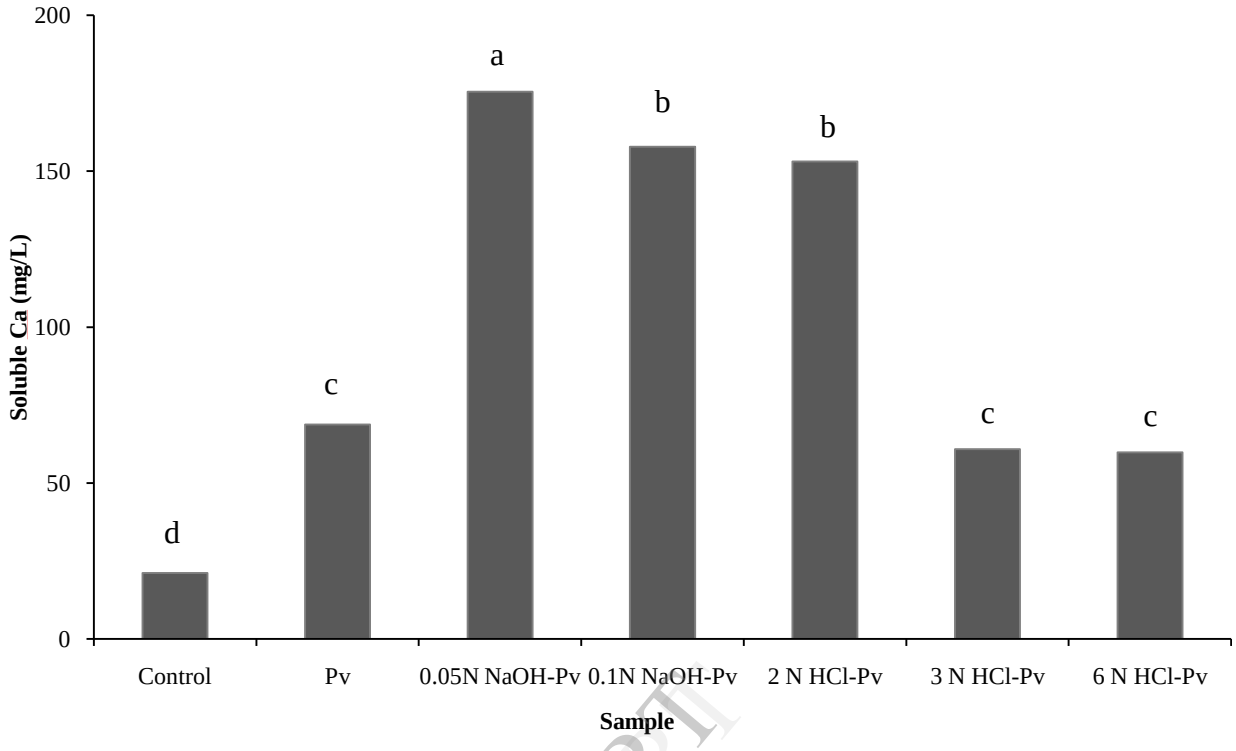

Figure 5.Ca^{+2}-solubilizing activity of phosvitin, its alkaline and acid hydrolysates.

[a-e]Treatments with different letters are significantly different (P<0.05, n=4, SEM=0.8764).

Abbreviations: Pv- Natural phosvitin; 0.05 N NaOH-Pv-Phosvitin treated with 0.05N NaOH at 37˚C for 3h; 0.1 N NaOH-Pv-Phosvitin treated with 0.1 N NaOH at 37˚C for 3h; 2 N HCl-Pv-Phosvitin treated with 2N HCl at 60˚C for 6h; 3N HCl-Pv-Phosvitin treated with 3N HCl at 60˚C for 6h; 6 N HCl-Pv-Phosvitin treated with 6N HCl at 60˚C for 6h,SEM - Standard error of the mean.