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Production and characterization of phosphopeptides from egg yolk phosvitin

by

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in partial fulfillment of the requirements for the degree of

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Egg yolk phosvitin, as a highly phosphorylated protein shows high metal chelating, antioxidant and emulsifying properties. The phosphorylated serine moieties of phosvitin play major roles with regard to its extraordinary functionalities. Phosvitin is considered as a nutritionally negative protein since it forms insoluble complexes with the bivalent cations, and it shows potential allergenicity and resistance to enzymatic digestion. However, if it is fragmented, the peptides containing phosphates (phosphopeptides) can greatly enhance the mineral bio-availability, thus its negative attributes can be avoided effectively. The aims of this study were 1) to investigate effective pre-treatment(s) for improved enzymatic hydrolysis of phosvitin, 2) to evaluate the chemical digestion of phosvitin, 3) to find out the functional properties of phosvitin and its hydrolysates, and 4) to characterize the peptides of phosvitin hydrolysates using MS-MS based approach.

Pre-treated phosvitin with SDS (sodium dodecyl sulfate), SDS + heat, acid + heat, alkali+heat, and heat alone were subjected to circular dichroism to evaluate the secondary structural changes of the protein. Enzymatic digestion of phosvitin was done with trypsin, pepsin, α-chymotrypsin, Multifect ® P-3000, alkalase (Bacterial alkaline protease: Protex 6L), and thermolysin (from Bacillus thermoproteolyticus Rokko). Consecutive digestion of phosvitin with trypsin and pepsin, Multifect ® P-3000 & α-chymotrypsin, and alkalase & α-chymotrypsin were also performed. All the enzymatic digests of phosvitin were subjected to SDS-PAGE analysis. The chemical hydrolysis (acid and alkaline) of phosvitin was also performed for high level of hydrolysis of phosvitin as a novel approach to produce functional peptides. Phosvitin and the enzymatic hydrolysates of thermolysin, Multifect ® P-3000 and alkalase of phosvitin and pre-treated phosvitin (100 °C, 1h) were evaluated for their antioxidant and mineral-binding activities. The acid and alkali hydrolysates of phosvitin were also evaluated for their functionalities. The identification and characterization of phosphopeptides present in different hydrolysates were carried out using LCMS/MS (Liquid chromatography-Tandem Mass Spectrometry) and MALDI (Matrix-Assisted Laser Desorption Ionization) approaches.
Significant changes in β-sheet and coil structures of phosvitin were caused by SDS with and without heat treatments. Phosvitin underwent alkaline hydrolysis at 70 to 85 °C at pH 13, which implied the decomposition of protein. Heat treatment at 100 °C for 20 to 60 minute significantly affected the secondary structures of phosvitin. Phosvitin subjected to heat treatment at 100 °C for 1 h showed visible differences in SDS-PAGE compared to the control sample.

Among the six enzymes tested, Multifect ® P-3000 and alkalase showed higher proteolytic digestion than the other four enzymes tested. Thermolysin was better than the other three enzymes tested. Consecutive use of enzymes did not show any further digestion of the hydrolysates obtained from the first enzyme. Even though SDS was capable of denaturing phosvitin, it did not improve the enzymatic digestion of phosvitin. Combination of alkali (pH 12) and heating at 85 °C for 30 min resulted in higher degree of enzymatic digestion possibly due to dephosphorylation of phosvitin. Pre-treatment of phosvitin at 100 °C for 1h clearly showed improved enzymatic digestion of phosvitin. Based on these results, the effective enzymatic digestion of phosvitin for bioactive peptide production can be achieved by pre-treating phosvitin at 100 °C for 1 h followed by hydrolysis using Multifect ® P-3000 or alkalase. Phosvitin showed higher level of hydrolysis in alkaline than acidic conditions. Incubation of phosvitin in 0.075 N NaOH solution at 37 °C for 1 h, and 2 N HCl at 60 °C for 6 h partially hydrolyzed phosvitin. But 0.1 N NaOH at 37 °C for 3 h or 3 N HCl at 60 °C for 6 h incubation was needed for the complete hydrolysis of phosvitin.

Phosvitin and its hydrolysates showed a powerful antioxidant effect in iron-induced oxidation in oil emulsion system. Both Fe$^{3+}$-binding and Cu$^{2+}$-binding efficiencies of phosvitin decreased with increasing levels of phosvitin in the assays. Hydrolysates of phosvitin showed different levels of Fe$^{3+}$-binding and Cu$^{2+}$-binding activities. Phosvitin significantly reduced the solubility of calcium in solution, but phosvitin hydrolysates increased calcium solubility. Preparation of hydrolysates using pre-treated phosvitin did not help in improving the antioxidant or mineral-binding capacities of hydrolysates compared with those without pre-treatments.

Increasing degree of hydrolysis in phosvitin either by HCl or NaOH negatively affected their antioxidant and Fe$^{3+}$-chelating capacities. However, hydrolyzing phosvitin with
0.1 N NaOH, 2 N HCl, or trypsin after 0.05 N NaOH treatment significantly increased Cu^{2+}-chelating activity. The acid hydrolysates of 3 N and 6 N HCl significantly increased the solubility of Ca^{2+} in sodium phosphate buffer (pH 6.8) compared to the negative control implying the possibility of using acid hydrolysates for functional phosphopeptides as mineral supplementing agents.

The amino acid sequences and structural information observed in this study shows the possibility of using phosvitin derived phosphopeptides in the area of bioactive/functional peptides production. However, more studies have to be implemented in order to discover the physiological mechanisms and biological effect *in vivo*. 
CHAPTER 1. INTRODUCTION

The demand for the therapeutic and functional products has been increased dramatically over the past few decades. Especially, naturally derived bioactive molecules and their potential uses in food and health care industry have become more attractive (McCann and others 2005). The term bioactivity has been defined based on two major aspects: 1) In order to be considered as a bioactive substance, a compound must be able to exert an assessable biological effect at a physiologically rational level, and 2) it must be measurable and able to render health benefits by eliminating the potential harmful effects (Schrezenmeir and others 2000). Among the bioactive compounds, bioactive peptides are the most interesting substances since they can be the starting points for the development of drugs and drug-related compounds (Wyvratt 1988; Wimart and Komajda 2000). The contribution of bioactive peptides has become significant in the area of functional foods because of their beneficial roles once they are released from the native food proteins through in vitro processing or in vivo digestion (Ariyoshi 1993).

Peptides are short polymers of amino acids linked by peptide bonds (Shahidi and Zhong 2008). Peptides which can exert different biological functions or physiological effects are known as bioactive peptides. They have been generated in vivo in various living organisms or can be found in various proteins (Smacchi and Gobbetti 2000). However, the bioactive peptides embedded in proteins are usually inactive within the native protein, and are supposed to be released during proteolytic enzyme digestion or food processing (Meisel 1997a; Korhonen and Pihlanto 2006). Generally the size of a bioactive peptide can vary from two to twenty amino acid residues (Gill and others 1996), and most of the bioactive peptides exert more than one biological or physical effects (Meisel and FitzGerald 2003).

There are many kinds of bioactive peptides with various functions including antihypertensive, antioxidant, anticancer, antimicrobial, opioid activities, mineral binding, immunomodulatory, cholesterol-lowering and anti-diabetic etc. (Shahidi and Zhong 2008). Among them, antioxidant and mineral binding peptides have attracted the most interests because of their potential use in food and pharmaceutical industries, especially when the use and application of synthetic antioxidants have become challenging due to their potential
health hazards effects (Branen 1975; Becker 1993; Mendis and others 2005). Utilization of natural antioxidants has recently been significantly increased (Shahidi and others 2006 because of growing interests in the use of natural and safe antioxidants in food systems (Chow 1988; Finkel and Holbrook 2000).

Phosphorylated peptides or phosphopeptides are interesting biomolecules among the bioactive peptides because of their characteristic molecular structure and functions. Phosphopeptides usually contain clusters of phosphoserines, which can effectively bind calcium and iron, inhibit the formation of insoluble calcium phosphates or iron phosphates, and resulting in increased calcium or iron bioavailability. In order to overcome mineral deficiencies, enrichment of foods with minerals in a low concentration has been used in some countries (Vegarud and others 2000). A great number of phosphopeptides have been derived from casein. However, a subunit of casein has only 1 to 13 phosphoserine residues to stabilize amorphous calcium phosphate. But a phosvitin molecule has ~120 phosphoserine residues and it can produce various phosphopeptides with different sizes, which have more affinity for calcium binding and releasing capacities (Castellani and others 2004).

Phosvitin is a principal phosphoprotein present in egg yolk (approximately 2.5% of egg yolk dry matter), and it contains ~10% phosphorus and 6.5% carbohydrates. Its molecular weight is 35,000 - 40,000 Da with 217 amino acid residues. More than 50% of amino acids in phosvitin are serine, and 90% of which are phosphorylated. As a result, it carries a very high number of negative charges with strong metal chelating, antioxidant, emulsifying and antimicrobial activities. Therefore, phosvitin is considered as an excellent candidate for the production of bioactive peptides with antioxidant and metal chelating properties. Phosvitin itself is considered as a nutritionally negative protein because of its ability to form insoluble complexes with metal ions. It has been observed that the absorption of calcium and magnesium in rats has been negatively affected in the presence of egg yolk phosvitin (Ishikawa and others 2007). However if phosvitin molecule is fragmented by enzymatic digestion, all those negative attributes of phosvitin can be eliminated.

Phosvitin is highly resistant to protease activities due to its negative charges (Jiang and Mine 2000). Alkaline dephosphorylation of phosvitin enhances the access of proteases to peptide bonds and thereby increases the protease susceptibility of phosvitin (Jiang and Mine
2001). However, unspecific removal of majority of phosphate molecules from phosvitin will affect the advantage of phosvitin as a substrate for bioactive phosphopeptide production. Thus, finding very specific and effective enzyme digestion is critical in order to exploit phosvitin as a substrate for bioactive phosphopeptides production. We hypothesize that denaturation of phosvitin through different pretreatments, and use of appropriate enzyme/enzymes combination will enhance the enzymatic digestibility of phosvitin and increase its applicability in the field of bioactive peptide production. The objectives of this study are 1) to develop efficient pre-treatments for phosvitin, 2) to produce new enzymatic or chemical digest from phosvitin, 3) to characterize the produced phosphopeptides, and 4) to determine antioxidant and metal binding abilities of phopeptides in model systems.
CHAPTER 2. LITERATURE REVIEW

Antioxidant bioactive peptides

Oxidative reactions lead to unfavorable flavor, aroma, texture and color changes in foods. Lipids and proteins are the major targets for oxidative reactions (Elias and others 2008). In addition, it has been reported that the accumulation of oxidatively-damaged products of lipids, proteins and nucleic acids in the body is well correlated with the age-related diseases (Stadtman 2001). Many literature reports demonstrate oxidation-related occurrences of diseases such as diabetes, atherosclerosis and neurodegenerative disorders etc. (Halliwell 1994). Therefore, prevention and control of oxidation in food systems play a significant role in human health. One of the major claims of functional foods is promoting natural defenses in the body. There is a growing demand for functional foods and overall 10% growth rate per annum for functional foods has been estimated (Weststrate and others 2002).

Many food proteins have been utilized in order to produce bioactive antioxidant peptides. Among them, casein (Chiu 2004; Kansci and others 2004; Díaz and Decker 2004), soy bean protein (Chen and others 1996), whey protein (Pena-Ramos and Xiong 2001, 2004), chick pea (Li and others 2007, 2008), fish skin gelatin (Mendis and others 2005), and muscle proteins (Je and others 2004) have been used considerably. The mode of action of the antioxidative peptides can be through metal binding activity (McClements and Decker 2000; Kitts 2005), radical scavenging (Yen and Wu 1999), active-oxygen quencher, and hydroxyl radical scavenger (Suetsuna and others 2000). However, the real mechanism is not well understood (Elias and others 2008).

Factors affecting antioxidant capacity of peptides

Hernández-Ledesma and others (2007) reported that relatively high radical-scavenging activity of the peptides derived from β-lactoglobulin was attributed to the presence of Trp, Tyr and Met residues. Kawashima and others (1979) evaluated the antioxidant effects of many synthetic amino acids and found that the presence of branched-
chain amino acids (Val, Leu, Ile) was very important for their antioxidant effect. Chang and Linn (1964) showed that linoleic acid salts of basic amino acids (Lys and Arg) had an extraordinary stability towards the oxidation. In addition, His, 4-amino-butyric acid, Lys, and Cys are also known to have antioxidant activities (Karel and others 1966). Thus the presence of antioxidant amino acids in the peptides will affect the antioxidant capacity of bioactive peptides.

Several studies have been done in order to predict the antioxidant activity of bioactive peptides (Peña-Ramos and others 2004). However, the structure-activity relationship or the mode of action of antioxidant peptides has not been well understood (Pihlanto 2006). The antioxidant activity of peptides depends on their structure and the assay system that they have been examined. Saito and others (2003) evaluated 40 peptides which are structurally related sequence of Leu–Leu–Pro–His–His and found that Pro–His–His sequence of those peptides played the main role as antioxidant peptides. In addition some distinctive tri-peptides such as Tyr–His–Tyr, Xaa–Xaa–Trp/Tyr and Xaa–Xaa–Cys(SH) showed a high radical scavenging activity and a high peroxynitrite-scavenging activity, respectively. They have illustrated a strong synergistic activity with phenolic compounds too. Thus, the antioxidant capacity of those peptides is due to the presence of tryptophan and tyrosine in their structure. The phenolic and indolic groups of those two amino acids behave as hydrogen donors and thereby acted as antioxidants. Saito and others (2003) also found that cysteine-containing tripeptides exhibited a strong peroxynitrite-scavenging activity. Thus the primary sequence of bioactive peptides plays an important role with regard to their antioxidant activity. Xu and others (2007) reported that the tryptic digests of partially dephosphorylated phosvitin subjected to fractionation by ion-exchange chromatography resulted in three fractions. The fractions with no phosphorous have shown significantly stronger antioxidant activity than the fraction with highest phosphorous content. Thus it was suggested that amino acid composition plays an important role for the contribution of antioxidant activity of peptides obtained by partial dephosphorylated phosvitin in the linoleic acid system.

Suetsuna and others (2000) evaluated a peptide isolated from casein hydrolysates with strong free radical scavenging activities and found that they have Tyr-Phe-Tyr-Pro-Glu-Leu (YFYPEL) in their amino acid sequence. Also, they characterized the antioxidant effect
of synthetic peptides with the sequences of EL, PEL, YPEL and FYPEL, and found that the antioxidant effect of those peptides varied in the order of EL > FYYPEL > FYPEL > YPEL > PEL. From that they concluded that the order of Glu-Leu sequence is important for their antioxidant activity.

Yamaguchi and others (1975) found that di-peptides with Try and Trp at the amino terminus and His and Met at the carboxyl terminus have strong antioxidant capacities. Chen and others (1996) reported that addition of Leu or Pro to the N-terminus of peptides derived from proteolytic digests of a soybean protein increased its antioxidant capacity of the peptide (Leu-Leu-Pro-His-His). Therefore, the location of some amino acids in the peptide amino acid sequence affects the functionality of antioxidant peptides. In addition, the presence of Tyr at the N-terminal of peptides has been identified as the major determinant for the radical-scavenging activity of ovalbumin-derived peptides (Da´valos and others 2004).

Hernández-Ledesma and others (2007) evaluated the effect of peptide bonds and the conformational arrangement of antioxidant peptides. For that \(\beta\)-lactoglobulin-derived peptides (WYSLAMA, WYSLAM, WYSLA, WYSL, WYS, and WY) and corresponding equimolar amino acid mixture have been evaluated for their antioxidant capacity. All the \(\beta\)-lactoglobulin-derived peptides have shown significantly higher antioxidant capacities than the mixture of amino acids. Therefore, the presence of peptide bonds or the structural arrangement of the peptide or configuration is very important for the antioxidant capacity of the peptides (Pen’à-Ramos and Xiong 2001).

**Metal Chelating peptides**

Metal-chelating activity of peptides can be due to phosphorylated serine moieties (Vegarud and others 2000) or histidine residues present in the peptide (Chen and others 1996). Among the metal-binding bioactive peptides, phosphopeptides play a significant role. Casein has been extensively used for the production of metal-binding phosphopeptides. However, whey proteins, \(\beta\)-lactoglobulin, \(\alpha\)-lactalbumin and lactoferrin also serve as sources for the production of mineral binding peptides (Vegarud 2000). In addition, sunflower seed protein (Megíás and others 2008), chick pea (Megíás and others 2007),
shrimp byproducts (Huang and others 2011) etc. have been studied in order to produce mineral binding bioactive peptides.

**Phosphopeptides**

The interesting feature of phosphopeptides is their ability to form soluble organophosphate salts. The phosphorylated serine moiety of phosphopeptides play the major role in binding divalent metal ions such as Ca, Mg, Zn, Cu, Fe etc. (Li and others 1989; Hansen and others 1996, 1997; Kitts 2005), and promote the intestinal absorption of calcium and other minerals (Koning and others 1999). Mellander (1947) first reported that phosphopeptides derived from casein (CPP) can enhance the calcification of bones. Interestingly phosphopeptides have ability to enhance Ca absorption in the gastrointestinal system even in the absence of Vitamin D. It has been suggested that the absorption of iron in the gastrointestinal tract is comparatively low since it forms poorly soluble heavy molecular weight ferric hydroxide in the gut (Derman 1977). However, CPPs enhanced iron availability and iron absorption in the gastrointestinal track, and thus those peptides can be used as metal ion carriers with enhanced absorbability (Sato and others 1986). In addition, the negative charges of phosphate groups and side chains of phosphopeptides make them resistant to the gastrointestinal enzymatic digestion, which makes them much suitable to be used as carriers for metal ions (FitzGerals 1998). Casein phosphopeptides have been already approved as nutraceuticals in Japan (Jiang and Mine 2000). A product called “Capolac” containing CPP 33(Arla Foods Ingredients) is also available in Sweden as a mineral absorption facilitator (Korhonen and others 2006). However, the interaction between CPPs and mineral and the improved mineral absorption at the intestinal level by CPP are controversial due to large variations in experimental approaches and methodologies used to assess the above mentioned aspects (Korhonen and others 2006).

CPPs are a mixture of phosphorylated peptides with different molecular weights derived from casein degradation by proteolytic enzymes in the digestive tract or obtained from the tryptic digestion of casein *in vitro* (Kitts 1994). It has been found that the cluster sequence of -Ser(P)₃-Glu₂ (which is negatively charged at physiological pH) found in those CPPs is responsible for the interactions of amorphous calcium phosphate (ACP). In addition
the adjoining Ser(P) residues play an important role for the maximum interaction of ACP (Meisel 1997b; Reynolds 1998). CPPs have many biological and technological functions such as calcium absorption, calcium retention, bone calcification, anticarcinogenicity, antihypertensive effect, milk curdling and stabilization of cream liqueurs (Park and Allen 1998).

**Mineral binding ability of phosphopeptides**

As discussed by Meisel (1997b), several amino acid groups are involved in binding of calcium to CPPs including serine-bound phosphate, free glutamine and free carboxyl groups. The hydrophobic tail of CPPs prevents further interactions and formation of insoluble calcium phosphate. Ferraretto and others (2001) found that the mixture of five commercial CPPs and β-casein (1-25) CPPs transiently increased the free intracellular calcium ions in cultured human intestinal HT-29 tumor cells. This increase was independent to the ATP-induced release of calcium from intracellular stores (Ferraretto and others 2001). CPPs acted as calcium carrier peptides and internalized calcium via endocytosis or other processes to provide ionized calcium in cytosol. The calcium-binding ability of CPPs has been applied by clinical dentists. They showed that CPPs stabilized high concentrations of calcium, phosphate ions and fluoride ions on the tooth surface by binding to pellicle and plaque (Reynolds 2009; Walker and others 2010). The anticarogenic effect of calcium-binding phosphopeptides is due to their recalcification ability to dental enamel (Tirelli and others 1997; Clare and Swaisgood 2000).

Compared with other cations, ionic irons have greater strength to bind CPPs, which can be 100 times greater than the binding ability of calcium (Emery 1992; Bouhallab and others 2002). The bioavailability of three different forms of iron (Fe/β(1-25): Fe combined to β(1-25) phosphopeptide; inorganic iron: FeSO₄ or iron gluconate; and Fe/β-casein: iron combined to β-casein) in rats indicated that Fe/β(1-25) enhanced the levels of iron in blood compared with inorganic iron and Fe/β-casein (Aït-Oukhatar and others 1999). Milk casein is the major source of protein in the area of phosphopeptide production, and only a little work has been reported on the production of bioactive phosphopeptides from egg yolk phosvitin.
However, as a highly phosphorylated protein, phosvitin shows a very high metal-binding capacity compared to casein.

**Phosvitin**

Egg yolk is composed of clear yellow-colored fluid called ‘plasma’ and suspended particles called ‘granules’ (Romanoff and Romanoff 1949). After dilution with 0.3N NaCl and centrifugation at 10,000 g for 30 min, the yolk can be divided into supernatant (plasma), and pellet (granules) (McBee and Cotterill 1979). The granular fraction of egg yolk contains lipovitellin and phosvitin (Burley and Cook 1961; MacKenzie and Martin 1967). Hen egg yolk phosvitin was first isolated by Mecham and Olcotte in 1949 by diluting the egg yolk with magnesium sulphate. It was proven that in non-mammalian vertebrates the macromolecular hepatically-derived lipophosphoprotein vitellogenin serves as the precursor for lipovitellin and phosvitin. Egg yolk phosphoproteins are composed of phosvitin and phosvettes (Wallace 1985).

Phosvitin accounts for 60 % of the total egg yolk phosphoproteins and holds about 90% of the egg yolk phosphorus. Taborsky and Mok (1967) first determined the molecular weights of minor and major phosvitin as 36,000 and 40,000 Da. Egg yolk phosvitin is a heterogeneous protein, composed of seven components. Two major components of phosvitin account for 80 and 15 %, respectively (Culbert and McIndoe 1971). Whereas Wallace and Morgan (1986 a,b) reported that the un-fractionated phosvitin is composed of five major components known as B, C, E1, E2 and F and their molecular weights are 40000, 33000, 15000, 18000, and 13000 Da, respectively. Abe and others (1982) reported that molecular weights of phosvitin components ranged from 18,500 - 60,000 Da. Also, they identified two main components of phosvitin in SDS polyacrylamide-gel electropherogram as α and β, which could be the two components, referred by Taborsky and Mok (1967) as major and minor phosvitin. Shainkin and Perlmann (1971) reported that the purified phosvitin with the molecular weight of 40,000 Da contains 6.5 % of carbohydrates consisting of 6 hexose, 5 glucoseamine and 2 sialic acids attached to the N-acetyl derivatives per molecule and carbohydrate components show a triple-branched antenna-like structure (Brockbank and
The detailed analysis of amino acid sequence indicated that serine accounts for more than 55% of the total amino acids in phosvitin (Table 1 & Figure 1), and β-phosvitin contains more serine residues than α-phosvitin. α-Phosvitin is rich in glycine, alanine, lysine, glutamine and threonine, and β-phosvitin is rich in histidine (Itoh and others 1983). Almost all the serine residues in phosvitin molecules are phosphorylated. Byrne and others (1984) reported that phosvitin has a core region of 99 amino acids consisting of 80 serines, which can form groups of residues that are interspersed by arginines, lysines, and asparagines (Figure 1).

Renugopalakrishnan and others (1985) evaluated the secondary structure of phosvitin using Circular Dichroism (CD), Fourier Transform Infrared Spectroscopy, and Fourier Transform Infrared Photoacoustic and Fluorescence Spectroscopic methods. They proposed the three compartments model structure of phosvitin with α-helix, β-sheet and β-turn. And the β-turn is located in between α-helix and β-sheet closer to o-phosphoserine residues. In neutral aqueous solution, phosvitin shows an unusual structure which is lack in both α-helix and β-sheet conformations (Prescott and others 1986). Conversely, phosvitin undergoes a large conformational change in strong acidic condition (pH < 2) by forming large proportion of β-sheet. Table 2 shows the secondary structure of phosvitin at different pH (Chang and others 1978). Taborsky (1970) evaluated the freezing effect on the conformational changes of phosvitin and reported that phosvitin underwent from unordered conformation to β-structure upon freezing and thawing. Also, this effect has been seen at acidic conditions and this effect has been increased with increasing acidity.

Grizzuti and Perlmann (1970) carried out studies on viscosity, optical rotatory dispersion (ORD) and circular dichroism (CD) of phosvitin and reported that due to high phosphoric acid bound to serine residues, phosvitin can behave as a polyelectrolyte (polyanion) in liquid state. Phosvitin molecule accomplishes an extended shape at low ionic strengths due to high electrostatic repulsion between the charged phosphate groups. However, in a narrow range of ionic strength (0.02 to 0.1) it assumes compact shape. Based on the similarity between ORD and CD results it was suggested that phosvitin possesses an unordered conformation at alkaline conditions, and helical regions and β-structures at pH 3.0 to 3.6. The phosphorus content of phosvitin can range from 3 to 10% of its molecular
weight (Taborsky and others 1967; Burley and Cook 1961). Phosvitin is enormously hydrophilic, has a very high number of negative charges (ca. 0.0180e at pH 7.0), and has unusually low percentage of nonpolar hydrophobic side chains (Dickinson and others 1997). It has a very low specific volume of 0.545 ml/g (Belitz and others 2009) and its isoelectric point is pH 4.0 (Ternes 1989). Its frictional ratio reveals the elongated nature of phosvitin molecule (Belitz and others 2009). Due to high phosphoric acid bound to serine residues, phosvitin shows different functionalities such as metal chelating, antioxidant, and emulsifying capacities.

**Biophysical properties of phosvitin**

**Heat stability**

Phosvitin is highly heat stable. Mecham and Olcotte (1949) observed no precipitation of phosvitin or any other changes due to heating of phosvitin preparations at 100 °C for several hours. Phosvitin subjected to 100 °C for 10 min did not show any change in electropherogram. In addition, no coagulation has been observed by heating phosvitin at 140 °C for 10 min (Itho and others 1983). Dixon and Cotterill (1981), however, reported that phosvitin bands disappeared from the electrophoretograms of egg yolk when phosvitin was heated to 69-72 °C in the presence of 10 % of sugar and salt. Chung and Ferrier (1995) evaluated the heat denaturation temperature of phosvitin using Differential Scanning Calorimetry and found that phosvitin became denatured at 79.7 when heated at neutral pH. In the presence of 0.1 and 1.0 M NaCl, its denaturation temperature has been reduced to 77.7 °C and 77.2 °C, respectively.

**Emulsifying activity**

It has been considered that the emulsifying activities of phosvitin are better than that of bovine serum albumin, β-casein and soy protein (Table 3). However, partial removals of phosphates with phosphatase and complete dephosphorylation with alkaline treatment have significantly reduced the emulsifying properties of phosvitin (Kato and others 1987). In addition calcium bound phosvitin showed decreased emulsifying properties. Based on those findings it was concluded that the electrostatic repulsive forces of phosphate moieties play
the major role on the emulsifying property of phosvitin (Kato and others 1987). Chung and Ferrier (1991) reported that phosvitin is a better emulsifying agent than bovine serum albumin at pH 7.0. The emulsifying capacity of phosvitin increased with increasing protein concentrations, oil volume and mixing time, but mixing time showed the greatest effect on the emulsifying ability of phosvitin. Addition of NaCl lowered the emulsifying ability and emulsion stability of phosvitin at pH 3 and 10. At low NaCl concentrations (> 0.5M), the emulsion stability decreased drastically (Chung and Ferrier 1992). The emulsifying activity of phosvitin decreased when it was subjected to a heat above 70 °C for 1 h. The emulsion stability of phosvitin, however, was not affected until heating above 67.5 °C for 1 h (Chung and Ferrier 1995). The dephosphorylation and protease digestion of phosvitin with pepsin, trypsin and α-chymotrypsin has reduced both emulsifying ability and emulsion stability of phosvitin (Khan and others 1998). They also observed that the large fragment (core) of phosvitin resulted from protease activity did not show emulsifying ability, indicating the importance of N-terminal 1 to 49 and C-terminal 211 to 217 parts of the protein with regard to its emulsifying activity. Conjugation of phosvitin with galactomannan (Millard type conjugation) significantly improved the emulsifying activity and emulsion stability (Nakamura and others 1998). Khan and others (1999) evaluated the emulsifying properties of Millard-type conjugates of phosvitin products from pepsin, trypsin and α-chymotrypsin digestion (N-terminal and C-terminal deleted phosvitin) and observed no improvement in emulsifying properties compared to phosvitin. Thereby they concluded that N- and C-terminal moieties of phosvitin are essential for its emulsifying properties for anchoring the oil droplets.

The emulsifying properties of phosvitin have been evaluated at pH 6 in both aggregate and non-aggregate state and two different ionic strength conditions. Better emulsifying properties have been observed in non-aggregated than aggregated phosvitin. However, increased stabilization has been shown by aggregated phosvitin against coalescence. At 0.05 M NaCl, phosvitin has formed a finer emulsion than that at 1.5 M NaCl (Castellani and others 2005). Phosvitin has a poor surfactant activity, but it has shown an excellent emulsion stabilizing activity in conditions (pH and ionic strength) favorable for its iron binding activity. Phosvitin showed poor coalescence destabilization effect and was not
effective in reducing oil-in-water interfacial tension. However, the interfacial film characteristics of phosvitin were high at pH 5.0 (Castellani and others 2006).

Sato and others (1962) reported that phosvitin prolonged chicken blood clotting in vitro. The presence of egg-yolk-like material in plasma reduced the degree of blood clotting by phosvitin. In addition, phosvitin has shown antithrombin action through activation of heparin cofactor II due to its polyanionic nature (Church and others 1988).

**Antioxidant activity of phosvitin**

Antioxidants exert their antioxidant ability either by scavenging of free radicals or by chelating metal ions. The antioxidant activity of phosvitin has been first reported by Lu and Baker (1986). They evaluated the antioxidant activity of phosvitin in the phospholipid emulsion system with nonorganic and organic metals (Fe$^{2+}$, Cu$^{2+}$, and hemin) at different concentrations. Accordingly phosvitin can efficiently inhibit Fe$^{2+}$- and Cu$^{2+}$-mediated phospholipid oxidations by chelating metals. Yet phosvitin was not effective enough to inhibit the hemin-mediated phospholipid oxidation. It showed a higher inhibitory effect on phospholipid oxidations in the presence of Fe$^{2+}$ (up to 30:1 Fe$^{2+}$-to-phosvitin molar ratio) than in the presence of copper (1:1 molar ratio). Autoclaving has decreased the antioxidant activity of phosvitin, whereas pasteurization has not affected it (Lu and Baker 1986). The antioxidant activity of phosvitin was maximum at pH 6.1 but phosvitin was not effective enough to inhibit the Cu$^{2+}$-induced oxidation at pH 7.8 in phospholipid emulsion system. Also, addition of 10% NaCl or 18% egg albumin did not show any detrimental effect on the antioxidant effect of phosvitin (Lu and Baker 1987). Nakamura and others (1998) evaluated the antioxidant ability of phosvitin and phosvitin-galactomannan conjugate (PGC) in a powdered oil model system and found that the conjugation significantly increased the antioxidant ability and radical-scavenging effect of phosvitin (Figure 2). In addition they observed that autoclaving at 121 °C for 15 min did not influence the antioxidant ability of PGC. However the antioxidant activity of native phosvitin was affected by this treatment (Nakamura and others 1998). Lee and others (2002) studied the effectiveness of phosvitin as an antioxidant in phosphatidyl choline liposomes system, pork muscle homogenate and ground pork. The maximum antioxidant effect of phosvitin has been observed when the
phosvitin concentrations are at 15 μm and 40 μm in the phosphatidyl choline liposomes system and pork muscle homogenate, respectively. Maximum antioxidant effect (73 % inhibition than control) was observed at pH 7.0. Heating of phosvitin at 60-100 °C for 10 minutes only decreased its antioxidant activity by 15 % whereas autoclaving (121 °C for 10 minutes) decreased the antioxidant activity by 34 %. Phosvitin was more effective in inhibiting oxidation in cooked ground pork than uncooked ground pork. The inhibition of oxidation was observed at 20 μM phosvitin concentration in both salted and cooked ground pork. However, increasing phosvitin from 20 to 60 μM had no effect on the oxidation of meat (Lee and others 2002).

In addition it has been reported that phosvitin is more effective in inhibiting the generation of hydroxyl radical from H₂O₂ than other iron-binding proteins such as ferritin (Ishikawa and others 2004). The tryptic hydrolysate of phosvitin has shown the similar result. Phosvitin also has shown a protective effect for DNA from Fe²⁺ and hydroxyl radicals in vivo (Ishikawa and others 2004).

**Metal binding activity**

Phosvitin shows a very strong affinity for bivalent metals such as calcium, magnesium and iron since it carries a large number of phosphate molecules (Taborsky 1963; Grizzuti and Perlmann 1973). Almost all the Fe presence in the yolk is bound to phosvitin and its iron binding capacity is about 5.96 mg Fe/g phosvitin (Greengard and others 1964). In addition egg yolk phosvitin contains a significant amount of copper and zinc (Figure 3). According to Taborsky (1963) the percentage of Fe bound in phosvitin is 0.3 %. Phosvitin has an ability to bind ion very strongly and promotes a rapid oxidation of ferrous ion to ferric form (Taborsky 1963). Based on equilibrium dialysis experiments, Hegenauer and others (1979) found that phosvitin can bind ferric ions stronger than that of nitrilotriacetate or citrate and the average association constant for the phosvitin is 10⁻¹⁸. They concluded that the clusters of di-O-phosphorylserine residues of phosvitin play the major role with regards to its iron chelating activity. Phosvitin binds with ferric iron very tightly whereas forms weak complexes with ferrous iron (Taborsky 1980). It has been shown that a free phosphorylserine does not react with Fe³⁺. In 1976 Donella and others, evaluated the Fe³⁺-binding activity of
phosphorylserine blocks \((\text{Ser-P})_n\) isolated from acid hydrolysis of phosvitin and found that phosphorylserine blocks \((\text{Ser-P})_n\) with \(n \geq 4\) can bind with \(\text{Fe}^{3+}\) like phosvitin however, with less activity than phosvitin.

It has been known that some amino acids in phosvitin will be masked due to rearrangement of its structure while releasing acid in the presence of Fe in alkaline pH. In the absence of Fe in phosvitin, however, this reaction was not observed (Taborsky 1963). Albright and others (1984) evacuated the effect of heat on the release of phosvitin-bound Fe. Heating of phosvitin at 110 °C for 20 and 40 minutes was not able to release the bound Fe, illustrating very strong iron binding in phosvitin. Although, heating released some phosphates to the filtrate it did not affect the Fe bound to phosvitin. In addition they evaluated the stability of bound Fe in the presence of NaCl, citric acid and ethylenediaminetetraacetic acid (EDTA) and observed that only EDTA is capable of releasing bound Fe from phosvitin because EDTA forms stronger ligand with iron than phosvitin. Castellani and others (2004) observed a high stability of phosvitin-Fe complex which was prepared under the favorable conditions. They observed that low pH did not affect the stability of phosvitin-Fe complex. However in the presence of \(o\)-phenanthroline which is a strong chelating agent, the bound Fe was released from phosvitin. This strong stability of phosvitin and Fe complex could be due to the tetrahedral stoichiometry (once Fe ion is bound with one phosphate molecule another phosphate molecule completes the binding process) or octahedral stoichiometry (two other phosphate molecules complete the process) which increased the activation energy required for the dissociation of the phosvitin-Fe complexes (Castellani and others 2004). Osaki and others (1975) found that in the presence of phosvitin, \(\text{Fe}^{2+}\) is oxidized by phosvitin and plays a major role in transferring of ion in blood.

Approximately two phosphate groups can bind with one iron ion and there are 135 phosphate groups per phosvitin molecule. In addition, it has been proven that binding Fe to phosvitin does not cause conformational change from ordered structure to \(\beta\)-form. In the presence of iron in excess, phosvitin can bind with Fe in the ratio of 0.5 ion/phosphate (Taborsky 1980).
Grizzuti and Perlmann (1973) evaluated the binding of \( \text{Mn}^{2+} \) and \( \text{Ca}^{2+} \) to phosvitin using dialysis equilibrium and found that at pH 6.5 (25 °C) phosvitin can bind with 103 \( \text{Mg}^{2+} \) ions and 127 \( \text{Ca}^{2+} \) ions. However, they observed drastic reduction of \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \) binding to phosvitin at pH 4.5, which decreased to 40 and 32 \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \) ions, respectively. The optical rotatory and CD studies revealed that upon binding bivalent cations, phosvitin undergoes a conformational change. It has been observed that when phosvitin is titrated with iron salt under acidic conditions precipitation of phosvitin occurred. The maximum precipitation of phosvitin occurred when the Fe/P ratio was 0.5. When the ratio was above 0.5, however, the Fe-phosvitin complex became soluble. When phosvitin was phosphorylated (P/Ser > 7.0), the solubility of phosvitin-Fe complex showed different reliance to the Fe/P ratio (Taborsky 1991). Taborsky concluded that the Fe binding sites of phosvitin is not uniform and the interaction of phosvitin and Fe depend on the Fe concentration and the availability of clusters of phosphoserine (Taborsky 1991).

Lu and Baker (1987) evaluated the Cu binding capacity of phosvitin at two different pH and observed that the Cu binding capacity of phosvitin was lower at pH 7.8 than pH 6.1. \( \text{MnCl}_2 \) or \( \text{CoCl}_2 \) dialysis equilibrium at 25 °C (pH 6.8) revealed that phosvitin could bind 113 \( \text{Mn}^{2+} \) and 120 \( \text{Co}^{2+} \). The binding of cations such as \( \text{Mg}^{2+} \), \( \text{Ca}^{2+} \), \( \text{Mn}^{2+} \), and \( \text{Co}^{2+} \) to phosvitin was not affected by temperature up to 60 °C. The binding of \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) caused a conformational change in phosvitin (Grizzuti and Perlmann 1973).

The Fe-binding capacity of phosvitin is influenced by the interaction of pH and the ionic strength (Figure 4). The best Fe-binding activity has been observed at pH 6.5 and ionic strength 0.15 (Castellani and others 2004). They reported that the best Fe-binding capacity of phosvitin was 115 μg iron/mg phosvitin. Interestingly they found that the nature of buffer does not influence the Fe-binding capacity of phosvitin. A heat treatment of phosvitin at 50 and 90 °C for 60 minutes did not influence its capacity to bind Fe. The CD of heat-treated phosvitin at 50 °C for 60 minutes was not different from the control. Whereas a heat treatment at 90 °C for 60 minutes has shown a slight change in the CD spectrum, indicating changes in its secondary structure. However this change was not capable of affecting the Fe binding capacity of phosvitin (Castellani and others 2004). High pressure at 300 and 600
MPa for 10 min had no effect on the structure of phosvitin and did not affect the Fe binding activity of phosvitin (Castellani and others 2004).

Due to the extraordinary capacity of phosvitin to bind bivalent cations, it also has shown an antimicrobial effect. Khan and others (2000) reported that phosvitin showed an antimicrobial effect under thermal stress conditions. Incubation of E. coli K12 strain at 50 °C for 10 minutes in the presence of 0.01 or 0.1 % of phosvitin showed a significant reduction of the bacterial population. The leakage of bacterial DNA due to cell membrane damages caused by phosvitin under the thermal stress has been identified as the cause of its lethal activity. Binding of Ca to phosvitin and α-chymotrypsin hydrolysis of phosvitin has drastically reduced the antibacterial activity against E. coli under the thermal stress. This indicates the importance of ion chelating activity and hydrophobic domains of phosvitin which has membrane-penetrating ability, for its antibacterial effect (Khan and others 2000).

**Nutritional effect of phosvitin**

Egg yolk is rich with many nutrients such as protein, fat, Fe, Ca, P, Zn and many other vitamins (Watkins 1995). It has been reported that the Fe absorption from egg is about one tenth of that of Fe salt (Callender and others 1970). The relative biological value of egg yolk-Fe has been reported to be 20-32 % compared to that of ferrous ammonium sulfate (Morris and Greene 1972). Fe solubility in the small intestine and the apparent Fe absorption are poor in the presence of egg yolk compared to soybean, egg albumin, casein and pork (Kim and others 1995). This poor bioavailability of egg yolk iron is due to the facts that chelating of Fe by phosvitin and formation of insoluble phosvitin-iron complex (Halkett and others 1958; Taborsky 1963; Greengard and others 1964; Sato and others 1987). The absorption efficiencies of calcium and magnesium from diets containing egg yolk protein and phosvitin have been evaluated. Addition of 1-2 % of phosvitin and egg yolk has reduced calcium and magnesium absorption in Wistar rats compared to rats fed with casein or soy proteins containing diets. Also the SDS-PAGE band pattern of intestinal contents of phosvitin-fed rats has resulted larger peptides molecular masses of 28, 22, and 15 kDa illustrating lower digestibility of phosvitin in the digestive system of rats (Ishikawa and others 2007). Therefore, it is considered that phosvitin shows negative nutritional attributes.
In addition, egg yolk phosvitin is also considered as an allergen (Walsh and others 1988; Walsh and others 2005). According to Baumgartner and others 2010 a few allergens are susceptible to industrial processing, and the allergenicity of egg proteins can be reduced by means of physical and enzymatic reactions through modification of structural conformation and demolition of primary structure of proteins. Therefore, production of phosphopeptides from phosvitin would be an effective solution to overcome all those negative attributes of phosvitin. However, the allergenicity of phosvitin phosphopeptides is yet to be discovered.

Many studies have been carried out to utilize special characteristics of phosvitin in food systems as a natural functional food ingredient or as a pharmaceutical agent. Among them production of functional bioactive peptides from phosvitin has become one of the main interests.

**Production and function of phosvitin phosphopeptides**

The most significant feature of mineral-binding peptides is that they carry a high number of negative charges which can bind with divalent cations and form soluble complexes (Vegarud and others 2000). Phosvitin can be considered as a good source for the production of mineral-binding peptides. However, phosvitin is highly resistant to proteolytic digestion *in vitro* due its extraordinary primary structure, which is composed of long oligophosphoserine blocks uninterrupted by other residues (Byrne and others 1984). Goulas and others (1996) observed a limited hydrolysis of phosvitin with pepsin, trypsin and α-chymotrypsin. They found that pepsin digestion produces three peptides of Asn 44 –Leu 193 (150 residues), Gly 4-Glu 41 (38) and C–terminal fragment of Leu 193- Glu 214 (21 residues). Trypsin digestion resulted in two major peptides Gln49-Arg 212 (164) and Ala 1-Arg 35 (35), and α-chymotrypsin resulted in two major peptides Ala 50-Trp210 (161) and Gly4-Gln 49 (46). The SDS-PAGE pattern of tryptic digest of phosvitin indicated that the molecular weight of the larger fragment was 28 kDa. The dephosphorylation of phosvitin with alkaline treatment (incubation of phosvitin in NaOH at 37 °C) increased the proteolytic susceptibility of phosvitin and tryptic digestion resulted in peptides with 10-20 amino acids (1-3 kDa) (Jiang and Mine 2000).
Mineral binding phosvitin phosphopeptides

The possibility of using peptides derived from partially dephosphorylated phosvitin (PDP) has been studied to produce Ca-binding peptides. Jiang and Mine (2000) found that peptides of phosvitin phosphopeptides (PPP) containing 35 % phosphate was more efficient in binding Ca and inhibiting formation of insoluble calcium phosphate than other phosphopeptides. Also they observed peptides with molecular weight 1-3 kDa were highly efficient in rendering Ca soluble (Jiang and Mine 2001). Choi and others (2005) evaluated the solubility of 0.1 % of CaCl₂ under ileum conditions (pH 7.0, 37 °C) and observed that 1 % phosvitin can increase the solubility of Ca²⁺ by 29 % compared to the control. This observation disagrees with the fact that phosvitin forms insoluble salt complexes in the presence of bivalent cations. In addition, they have studied the effectiveness of phosvitin and phosvitin phosphopeptides (tryptic hydrolysate of phosvitin) in enhancing the intestinal absorption and accumulation of Ca in bones. They found that inclusion of phosvitin phosphopeptides at low, medium and high level did not affect the Ca intake, fecal Ca content or urinary Ca contents in Sprague Dawley rats. However, phosvitin phosphopeptides increased Ca absorption and Ca accumulation in the bones significantly (p<0.05) compared to the control group of rats (Table 4). These evidences show the possibility of using phosvitin in the production of bioactive mineral binding phosphopeptides in future.

Antioxidant phosvitin phosphopeptides

The possibility of using phosvitin-derived phosphopeptides as antioxidants also has been reported. Three ion-exchange chromatographic fractions of tryptic digest of partially dephosphorylated phosvitin (PDP) have been evaluated in the Caco-2 cells to mitigate the H₂O₂-induced oxidative stress in vitro (Katayama and others, 2006). Phosphopeptides of PDP has significantly reduced the production of IL-8 a proinflammatory mediator (a biological indicator of oxidative stress), compared to the control and phosvitin-treated group. In addition, those phosphopeptides have significantly suppressed lipid peroxidation, and increased the production of glutathione (GHS) by Caco-2 cells treated with H₂O₂. Interestingly the peptides fraction composed of more than 50 % serine residues (hence more phosphate molecules) and basic amino acids such as Arg, Lys, and His has been identified as
very effective in mitigating the oxidative stress of Caco-2 cells implying the importance of phosphorylated serine moieties in phosphopeptides with regards to their antioxidant activity. Thus, phosvitin phosphopeptides can be exploited as potential inflammatory response suppressing agents, and inhibitors of lipid peroxidation and oxidative stress, in living cells (Katayama and others 2006).

Xu and others (2007) evaluated tryptic digest of phosvitin and PDP for antioxidant activity in linoleic acid system and found that PDP and its tryptic digest have better antioxidant activity than native phosvitin. Also, tryptic digests of PDP has shown better radical-scavenging activity on 2,2-diphenyl-1-picrylhydrazyl than native phosvitin. They reported that the antioxidant activity of 100 µg/ml phosvitin is significantly (p < 0.05) higher than that of vitamin E. However, at 10 µg/ml concentration, phosvitin did not show the same effectiveness as vitamin E (Figure 5). It indicated that the peptide fractions with no phosphorous have significantly stronger antioxidant activity than the peptide fractions with the highest phosphorous content. Thus it was suggested that amino acid composition plays an important role for the antioxidant activity of peptides obtained from partially dephosphorylated phosvitin (Xu and others 2007). The antioxidant activity can be determined by metal chelating, free radical scavenging ability, hydroperoxide reducing ability and aldehyde adduction ability of peptides (Chan and others 1994; Zhou and Decker 1999 a & b). Also, it could be attributed to amino acid composition and increased exposure of antioxidative amino acids of peptides. However, the real mechanism is not well understood (Elias and others 2008). Further studies are needed in order to exploit phosvitin-derived phosphopeptides as antioxidant agents.

Antimicrobial activity of phosphopeptides

Due to extraordinary capacity of phosvitin to bind bivalent cations, it exhibits antimicrobial effects too. However, phosvitin or its phosphopeptides have not been studied much for their antibacterial activities. Incubation of *E. coli* K12 strain at 50 °C for 10 minutes in the presence of 0.01 or 0.1% of phosvitin has significantly reduced the bacterial population. Antibacterial effect exerted by phosvitin is due to the leakage of bacterial DNA, through the cell membrane damages caused by phosvitin. This indicated that phosvitin alone
or in combination with thermal stress have potentials to be used as an antibacterial agent (Khan and others 2000). Iron binding lactoferrin has been extensively studied for the production of antimicrobial bioactive peptides. The mode of antibacterial effect is supposed to be through its Fe-binding activity (Kontoghiorghes 1986; Rainard 1986; Samuelsen and others 2005). Since Fe-binding capacity of phosvitin is higher than lactoferrin, it could be a better candidate for the production of antimicrobial bioactive phosphopeptides than lactoferrin.

Concluding remarks

Even though phosvitin seems to be an attractive source for the production of phosphopeptides, the high price of phosvitin limits its application for bioactive peptide production. Therefore, development of improved and cost effective preparation methods for phosvitin extraction is vital in order to exploit its applications in food industries. However, utilization of phosvitin-derived phosphopeptides in the nutraceutical industry is not impossible even though it is expensive. Also, there is a huge potential in the cosmetics industry for naturally derived antioxidant and anti-aging mineral-binding peptides such as copper-binding peptides. Thus we propose that the phosvitin-derived phosphopeptides could be very attractive in the cosmetic industry if proper studies on those aspects have been executed. The resistance of phosvitin to protease activities, however, would be a limitation for the production of bioactive peptides. The enzyme digestibility of phosvitin has to be improved in a manner which preserves the specificity and the repeatability of the produced peptides. Furthermore structural, yield and economic aspects and safety issues must be addressed carefully. Thus it is very important to carry out further research in order to utilize egg yolk phosvitin in industrial level.

References


Ala, Glu, Phe, Gly, Thr, Glu, Pro, Asp, Ala, Lys, Thr, Ser (6), Ala, Ser (2), Thr, Ala, Thr, Ser(6), Ala, Ser(2), Pro, Asn, Arg, Lys(2), Pro, Met, Asp, Glu(3), Asn, Asp, Gln, Val, Lys,Gln, Ala, Arg, Asn, Lys, Asp, Ala, Ser (3), Arg, Ser (2), Lys, Ser(2), Asn, Ser(2), Lys, Arg, Ser(3), Lys, Ser(2), Asn, Ser(2), Lys, Arg, Ser(12), Arg, Ser(10), Asn, Ser, Lys, Ser(6), Lys, Ser (6), Arg, Ser, Arg, Ser(3), Lys, Ser(14), Lys, Ser(4), Arg, Ser(6), Lys, Ser(3), His (2), Ser, His, Ser, His (2), Ser, Gly, His, Leu, Asn, Gly, Ser (8), Arg, Ser, Val, Ser, His (2), Ser, His, Glu, His (2), Ser, Gly, His, Leu, Glu, Asp(2), Ser (8), Val, Leu, Ser, Lys, Ile, Trp

Figure 1: Amino acid sequence of phosvitin
(Adapted from Byrne and others 1984)
Table 1: The amino acid composition of hen phosvitin

<table>
<thead>
<tr>
<th>Residue</th>
<th>no. of residues/mol of phosvitin (Taborsky 1974)</th>
<th>According to the nucleotide sequencing (Byrne and others 1984)</th>
<th>mol per $10^4$ g of phosvitin $\alpha^*$</th>
<th>$\beta^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>7</td>
<td>8</td>
<td>1.4</td>
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<td>Ala</td>
<td>7+</td>
<td>7</td>
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<tr>
<td>Val</td>
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<td>0.7</td>
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<td>Leu</td>
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<td>0.3</td>
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<td>Ile</td>
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<td>0.4</td>
<td>0.2</td>
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<tr>
<td>Pro</td>
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<td>0.6</td>
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<td>Phe</td>
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<td>1</td>
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<td>0.2</td>
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<td>-</td>
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<tr>
<td>Other</td>
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<tr>
<td>No. of P</td>
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<td></td>
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<tr>
<td>Hexose (%) *</td>
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<td>Hexosamine (%)*</td>
<td>1.08</td>
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<tr>
<td>Sialic acid (%) *</td>
<td>1.91</td>
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</tbody>
</table>

(*Adapted from Itoh and others 1983)
Table 2: Secondary structure of phosvitin in water at various pH

<table>
<thead>
<tr>
<th>pH</th>
<th>% α-helix</th>
<th>% β-sheet</th>
<th>% β-turns</th>
<th>% aperiodic</th>
</tr>
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<tbody>
<tr>
<td>7.0</td>
<td>0</td>
<td>4.5</td>
<td>31.0</td>
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</tr>
<tr>
<td>2.8</td>
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<tr>
<td>2.1</td>
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<td>64.5</td>
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<tr>
<td>1.5</td>
<td>10</td>
<td>71.0</td>
<td>0</td>
<td>19.0</td>
</tr>
</tbody>
</table>

(Adapted from Chang and others 1978)

Table 3: Emulsifying activities and emulsion stabilities of various food proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Emulsifying activity (OD$_{500}$)</th>
<th>Emulsion stability (min)</th>
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</thead>
<tbody>
<tr>
<td>Phosvitin</td>
<td>0.979 ± 0.054</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.535 ± 0.011</td>
<td>1.3 ± 0.10</td>
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<tr>
<td>β-casein</td>
<td>0.500 ± 0.002</td>
<td>0.6 ± 0.11</td>
</tr>
<tr>
<td>Soy protein</td>
<td>0.352 ± 0.001</td>
<td>0.5 ± 0.10</td>
</tr>
</tbody>
</table>

(Adapted from Khan and others 1998).

Table 4: Effect of phosvitin phosphopeptides on Ca balance experiment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ca intake (mg/day)</th>
<th>Fecal Ca (mg/day)</th>
<th>Urinary Ca (mg/day)</th>
<th>Ca absorption (%)</th>
<th>Ca accumulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>119.1 ± 5.41a</td>
<td>53.4 ± 2.20b</td>
<td>0.16 ± 0.02a</td>
<td>55.1 ± 0.80b</td>
<td>55.0 ± 0.79b</td>
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<tr>
<td>Phosvitin</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>120.9 ± 2.91a</td>
<td>45.4 ± 2.09a</td>
<td>0.13 ± 0.04a</td>
<td>62.4 ± 2.25a</td>
<td>62.3 ± 2.28a</td>
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<tr>
<td>Med</td>
<td>129.1 ± 16.24a</td>
<td>50.2 ± 7.50ab</td>
<td>0.20 ± 0.10a</td>
<td>61.2 ± 2.06a</td>
<td>61.1 ± 2.10a</td>
</tr>
<tr>
<td>High</td>
<td>137.6 ± 14.98a</td>
<td>51.3 ± 3.05ab</td>
<td>0.19 ± 0.09a</td>
<td>62.6 ± 1.92a</td>
<td>62.5 ± 1.97a</td>
</tr>
</tbody>
</table>

Values within a column with different superscripts are significantly different at $p < 0.05$ by Duncan’s multiple-range test (adapted from Choi and others 2005).
Figure 2: Antioxidative effect of PGC in a powder oil model system: (a) Thiobarbituric acid (TBA); (b) Peroxide value (POV); (●) control (no addition); (Δ) 1% phosvitin-galactomannan mixture; (□) 0.1% phosvitin-galactomannan mixture; (◊) 1% PGC; (o) 0.1% PGC. (adapted from Nakamura and others 1998).
Figure 3: Zinc, copper, and iron content of vitellogenin isolated from the plasma of laying turkey hens and phosvitin and lipovitellin isolated from turkey egg yolk (adapted from Richards 1997).

Figure 4: Iron binding activity of at different pH and ionic strength (M) values. The estimated response surface is represented in μg of bound iron per mg of phosvitin. (adapted from Castellani and others 2004).
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CHAPTER 3. PRE-TREATMENTS AND ENZYMATIC DIGESTION OF PHOSVITIN

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Abstract

As a highly phosphorylated protein, egg yolk phosvitin shows high metal chelating, antioxidant and emulsifying properties. The phosphorylated serine moieties play the major role with regard to its extraordinary functionalities. Phosvitin is considered as a nutritionally negative protein since it forms insoluble complexes in the presence of bivalent cations, and shows potential allergenicity and resistance to enzymatic digestion. If fragmented, however, the peptides containing phosphates (phosphopeptides) can greatly enhance the mineral bioavailability, and thus its negative attributes can be effectively avoided. The aim of this study was to develop effective pre-treatment/s for improved enzymatic hydrolysis of phosvitin, and to evaluate the proteolytic activities of various enzymes or enzyme combinations on phosvitin. Phosvitin was pre-treated with SDS (sodium dodecyl sulfate), SDS + heat, acid + heat, alkali+heat and heat alone. Then samples were subjected to circular dichroism to evaluate the changes in the secondary structure of the protein. Phosvitin was digested with trypsin, pepsin, α-chymotrypsin, Multifect ® P-3000, alkalase (Bacterial alkaline protease: Protex 6L) and thermolysin (from Bacillus thermoproteolyticus Rokko). Consecutive digestion of phosvitin with trypsin and pepsin, Multifect ® P-3000 & α-chymotrypsin and alkalase & α-chymotrypsin were also performed. Pre-treated phosvitin was digested with either thermolysin or Multifect ® P-3000 and alkalase separately. All enzymatic hydrolysates were subjected to SDS-PAGE analysis.

SDS with and without heat treatments has caused significant changes in beta-sheet and coil structures of phosvitin. At pH 13, phosvitin may undergo alkaline hydrolysis at 70 to 85 °C, implying decomposition of protein. Heat treatment of 100 °C for 20 to 60 minute affected the secondary structure of phosvitin, significantly. In SDS-PAGE phosvitin subjected to heat treatment at 100 °C for 1 h, showed visible variation compared to the control sample.
Among the six enzymes tested, Multifect ® P-3000 and alkalase showed higher protease activity than other four enzymes tested. Thermolysin was better than the other three enzymes tested. However, consecutive use of enzymes did not show any further digestion of the phosphopeptides from the first enzyme. Even though SDS is capable of denaturing phosvitin it did not improve the enzymatic digestion of phosvitin. Combination of alkali (pH 12) with temperature at 85 °C for 30 min resulted higher degree of enzymatic digestion, possibly due to dephosphorylation of phosvitin.

Heating of phosvitin at 100 °C for 1 h helped enzymatic digestion and clear visible differences were found in the bands of SDS-PAGE. Based on these results, the effective enzymatic digestion of phosvitin for bioactive peptide production can be achieved by pre-treating phosvitin at 100 °C for 1 h and hydrolysis using Multifect ® P-3000 or alkalase.

**Key words:** phosvitin, denature, phosphopeptides, enzymatic hydrolysis, pre-treatments
Introduction

Phosvitin is the principal phosphoprotein present in egg yolk (approximately 1.2% of egg yolk), and contains 3 to 10% phosphorus and 6.5% carbohydrates (1-4). Its molecular weight ranges from 35,000 to 40,000 Da and is composed of 217 amino acids residues. More than 50% of amino acids in phosvitin are serine, 90% of which are phosphorylated (5-6). As a result, phosvitin carries a very high number of negative charges (7-8) and shows a strong metal chelating (9-10), antioxidant (11), emulsifying (12-14) and antimicrobial activity (15). However, phosvitin is considered as a nutritionally negative protein since it forms insoluble complexes in the presence of bivalent cations, and shows potential allergenicity and resistance to enzymatic digestion. If fragmented, however, the peptides containing phosphates (phosphopeptides) can greatly enhance the mineral bio-availability, and thus its negative attributes can be avoided effectively.

The demands for the naturally derived bioactive molecules and their potential uses to promote health and food preservation have been increased dramatically over the past few decades (16). Among the functional bioactive molecules, bioactive peptides have shown the most promising potentials as therapeutic or health promoting agents (17-18). Peptides are short polymers of amino acids linked by peptide bonds (19). Peptides, which can exert different biological functions or physiological effects are known as bioactive peptides and have been generated in vivo in various living organisms or found in various proteins (20). The bioactive peptides embedded in proteins are usually inactive within the native proteins and supposed to be released during proteolytic enzyme digestion or food processing (21-22).

Among the bioactive peptides phosphorylated peptides or phosphopeptides are interesting biomolecules because they have characteristic molecular structure and functions. Phosphopeptides usually contain clusters of phosphoserines, which can effectively bind calcium and iron, and inhibit the formation of insoluble calcium phosphates or iron phosphates, resulting in increased calcium or iron bioavailability. In order to overcome mineral deficiencies, enrichment of foods with minerals in a low concentration has been used in some countries (23). Mellander in 1947 first reported that phosphopeptides derived from casein (CPP) can enhance the calcification of bones (24). Interestingly phosphopeptides have ability to enhance Ca absorption in the gastrointestinal system even in the absence of vitamin
It has been suggested that the absorption of iron in the gastrointestinal tract is comparatively low since it forms poorly soluble heavy molecular weight ferric hydroxide in the gut (25). However, casein phosphopeptides have shown enhanced iron availability and iron absorption in the gastrointestinal tract and those peptides can be used as effective carriers for metal ions for enhanced absorbability (26). Casein phosphopeptides have been already approved as nutraceuticals in Japan (27) and Sweden as a mineral absorption facilitator (22).

A molecule of phosvitin contains ~120 phosphoserine residues while a subunit of casein has only 1 to 13 phosphoserine residues to stabilize amorphous calcium phosphate, implying that phosvitin can be a much better source for phosphopeptides production than casein (28). However, phosvitin is highly resistant to enzymatic hydrolysis due to its extremely high negative charges (1-29). Goulas and others (1996) observed a limited hydrolysis of phosvitin with pepsin, trypsin and α-chymotrypsin (30). Pepsin digestion produced three peptides whereas trypsin and α-chymotrypsin digestions produced two major peptides, indicating that limited enzymatic susceptibility is the major roadblock in the production of bioactive peptide production using phosvitin. Jiang and others found partial dephosphorylation of phosvitin with 0.1 - 0.4 N NaOH at 37 °C for 1-4 h increased the protease susceptibility of phosvitin (27). They observed 31 % and 88 % release of phosphate in 0.1 N and 0.3 N NaOH after 2 h incubation. With alkaline dephosphorylation, however, nonspecific removal of majority of phosphates from phosvitin molecule will compromise the advantage of phosvitin as a substrate for bioactive phosphopeptide production. Therefore, developing conditions or pre-treatments for effective enzymatic digestion of phosvitin is critical in order to exploit phosvitin as a substrate for bioactive phosphopeptides production. Many studies have shown that increased digestion of protein could be achieved with proper pre-treatments (31-33). Therefore, the objectives of the present study are to find effective pre-treatment conditions to improve the enzymatic hydrolysis of phosvitin and to evaluate the proteolytic activities of various enzymes or enzyme combinations to the pre-treated phosvitin.
Materials and Methods

Phosvitin used in this study was extracted and purified using the method described by Ko and others (34). Trypsin-Type I from bovine pancreas, α-chymotrypsin-type II from bovine pancreas, pepsin from porcine stomach mucosa and thermolysin from Bacillus thermoproteolyticus rokko were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Alkalase (bacterial alkaline protease: protex 6L) and Multifect® P-3000 were gifts from Genencor International Inc. (New York, NY, USA).

Pre-treatment of phosvitin

Prior to the enzymatic digestion of phosvitin, it was subjected to pre-treatments with SDS, SDS + heat, pH and heat treatment (acid + heat and alkali + heat) and heat alone in order to denature the structure of phosvitin and thereby increase the subsequent enzymatic digestion.

SDS and SDS + heat treatments

Phosvitin (0.5 %) was dissolved in 0.5 % and 1 % SDS and in double distilled water (DDW). After that one ml of samples in disposable test tubes (13 x 100 mm) were subjected to heating at 100 °C for 15 min in a water bath. Then the samples were cooled down in a cold water bath for 10 minutes. Those samples with or without heat treatments were subjected to centrifugation (Brinkman Instrument Inc., Westbury, NY, USA) at 3,220 x g for 10 min. The supernatants were used for circular dichroism (CD) scanning. Also, the pre-treated samples were vortex mixed and subjected to thermolysin digestion. The resulting hydrolysates were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

pH and temperature treatment

The pH of 0.5 % phosvitin in DDW was adjusted to pH 2, 3, 12 and 13 with 2N HCl or 2 N NaOH and subjected to heat treatments at 70, 75, 80 and 85 °C for 30 min in a water bath (Fisher Scientific, PA, USA). Samples were in disposable test tubes (16 x 100 mm). After that, samples were cooled down in a cold water bath for 10 minutes. Vortex mixed and subjected to SDS-PAGE analysis. The phosvitin samples treated at the highest temperature
(85 °C) at different pH values were subjected to thermolysin digestion and SDS-PAGE analysis.

**Heat treatment of phosvitin**

Phosvitin in distilled water (0.5 %) was subjected to temperature treatments of 100 °C for 10, 20, 30, 40, 50 and 60 min in a conventional water bath. Samples were centrifuged at 3,220 x g for 10 min and the supernatants were subjected to CD scanning. After heat treatments of phosvitin, enzymatic digestion of those treated samples were performed with alkalase, Multifect® P-3000 and thermolysin. The hydrolysates were analyzed by using SDS-PAGE.

**CD scanning**

Before CD scan the samples were centrifuged at 3,220 x g for 10 min. After diluting the samples to final concentration of 0.5 mg/ml, CD measurements were performed on a Jasco J-710 spectrophotometer at room temperature. Data was recorded in a wavelength range of 190-250 nm and CD analysis was done using the JFit program to determine the percentage of alpha helix, beta sheet, and random coil.

**Enzymatic digestion**

Enzymatic digestion of phosvitin/pre-treated phosvitin was carried out by using trypsin, pepsin, α-chymotrypsin, Multifect® P-3000, alkalase, and thermolysin. Phosvitin (1 %) was dissolved in distilled water and the pH was adjusted using HCL/NaOH prior to add the enzymes. The substrate: enzyme ratio was 100:1 (w/w) and was carried out for 6 h. The pH and temperature conditions used were different depending upon enzymes (pepsin, pH 2.0 & 37 °C; trypsin and α-chymotrypsin, pH 8.0 & 37 °C; Multifect® P-3000, pH 7.0 & 50 °C; alkalase, pH 9.0 & 60 °C; thermolysin, pH 6.8 & 68 °C). In addition, two consecutive enzymatic digestions were carried out. The consecutive enzyme combinations were trypsin and pepsin, alkalase and α-chymotrypsin, and Multifect® P-3000 and α-chymotrypsin (pH was again adjusted according to the second enzyme used). The enzymatic digestion was
arrested by keeping samples in a boiling water bath for 10 min. The samples were lyophilized and stored at -20 °C until used. All the samples were subjected to SDS-PAGE analysis.

**SDS-PAGE**

The SDS-PAGE of phosvitin and its enzymatic hydrolysates was conducted 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 low-range SDS-PAGE molecular weight standards (104-19kD)/ Precision plus protein dual color standards (10-250 kD) (Bio-Rad) were used as molecular markers (MM).

**Statistical analysis**

The statistical analysis was done using 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 pre-treatments and CD analyses four replications were used and for the enzymatic digestions three replications were prepared.

**Results and Discussion**

**Effect of SDS and SDS + heat treatments on the structure of phosvitin**

Some proteins in their native state are not digestible by enzymes. However, after denaturing, they become easily digested (35). The denaturants of proteins include heat, acid, alkali, alcohol, urea, salicylate, surface action, ultra violet, high pressure, etc. (36). Phosvitin was subjected to various pre-treatments in order to denature its structure and increase the susceptibility to proteases. SDS is considered as a very effective protein denaturant (41). A defined unique configuration is characterized as a native protein while lack of unique configuration is related to a denatured protein (36). The CD spectra of phosvitin treated with 0.5 and 1 % of SDS indicated that the β-sheet and random coil structure of phosvitin changed significantly (p < 0.05) by SDS (Figure 1). SDS at 0.5 % and 1 % SDS reduced the β-sheet structure in the phosvitin by 6.21 % and 8.45 %, respectively. However, SDS with heat treatment at 100 °C for 15 min increased the sheet structure by 0.9 % and 1.18 % for 0.5 % and 1 % SDS level, respectively compared to SDS without heat treatment. SDS with or
without heat treatment significantly increased the coil percentage of phosvitin compared to control. The effect of SDS and temperature on secondary structure of β-glycosidase proteins has been studied (37). The tertiary structure of the protein has been significantly affected by SDS: 1 % SDS has increased the α-helix structure by 4 % while reducing the unordered structure of the protein. Increase of temperature from 90 to 95 °C has decreased the intensity of α-helix band of β-glycosidase proteins in infrared spectra implying a high degree of protein unfolding. No variation of CD spectrum of phosvitin has been observed after a heat treatment of 50 °C for 1 h (28). However, they observed changes of profiles when phosvitin was heated at 90 °C for 1 h. Therefore, the observed structural changes of secondary structure of phosvitin imply the denaturing of protein and it may influence the subsequent enzymatic digestion of the protein.

**Effects of acid/alkali + heat treatment on phosvitin**

Phosvitin has been subjected to extreme pH conditions in combination with heat treatments in order to increase the denaturation of phosvitin. The structural changes of phosvitin have been observed with pH and ionic strength (7). Phosvitin demonstrates an unordered structure in alkaline pH, but shows predominantly helical and β sheet structures at pH 3.0 to 3.6 (7). When pH was around 1.8 to 1.9 in an aqueous solution, majority of negative charges of phosvitin has been removed and showed more β structure (38). The pre-treatments at pH 2 or pH 3 and heating at 70 °C, 75 °C, 80 °C and 85 °C for 30 min precipitated phosvitin, but SDS-PAGE pattern did not show any clear structural changes other than slight streaking appearance below the phosvitin band. Pre-treating phosvitin at pH 12 or 13 produced a slight yellowish color. Heating of the pre-treated phosvitin at pH 12 at 75 °C, 80 °C and 85 °C also generated slight streaking pattern in the SDS gel, but phosvitin band itself was mostly preserved. After pre-treatment of phosvitin at pH 13 and heating at 70 °C, 75 °C, 80 °C and 85 °C for 30 min, however, the phosvitin was band completely disappeared and a visible band with a streaking appearance at the bottom of the gel showed up (Figure 2). This could be due to the release of phosphate groups from serine and the hydrolysis of phosvitin under strong alkaline conditions (39). Determination of degree of dephosphorylation could be done in order to confirm this. An alkaline treatment of 0.25N
NaOH at 37 °C for 24 h can remove all the phosphate present in phosvitin (40). They observed 67% removal of phosphates from phosvitin in an alkaline (KCl-NaOH buffer in 0.187N NaOH) and heat treatment (at 60 °C for 80 min) combination. Alkaline hydrolysis of protein has been used to determine amino acid composition of proteins, especially tryptophan content (41-42). Therefore, the disappearance of phosvitin band from the SDS-PAGE gel of phosvitin samples, pre-treated at pH 13 and heated could be due to the removal of phosphate moieties and the complete decomposition/alkaline hydrolysis of phosvitin into small peptides/amino acids.

Effects of heat treatments on the structure of phosvitin

Phosvitin shows an unusual structure, which is lack in both α-helix and β-sheet conformations, in neutral aqueous solution (43). The secondary structure of phosvitin has been evaluated using CD, Fourier transform infrared spectroscopy, Fourier transform infrared photoacoustic and fluorescence spectroscopic methods (44). Based on the results, a three compartment structure model of α-helix, β-sheet and β-turn of phosvitin has been suggested. The presence of β-turn in the proximity of o-phosphoserine residues and at the interphase between β-sheet and α-helix was reported. Interestingly it has been confirmed that the phosvitin obtained by lyophilization at pH 7.0 predominantly has β-sheet (44). In the present study lyophilized phosvitin was used at neutral pH and the present findings are in agreement with the above-mentioned facts of phosvitin structure (Figure 3). Phosvitin can undergo from unordered conformation to β-sheet structure upon freezing and thawing (45). The CD analysis showed that heat treatment at 100 °C for different times affected the helix and coil structures of phosvitin. The coil structure was significantly (p<0.05) changed with the heat treatment all the time-interval tested compared to the native phosvitin. The helix structure of the pre-treated phosvitin was significantly (p<0.05) different from the control phosvitin at all-time intervals except for 30 minute heat treatment (Figure 3). No significant change in the sheet structure was observed when phosvitin was treated at 100 °C up to 60 minute alone.

Phosvitin is considered as highly heat stable. After heating phosvitin at 100 °C for several hours, no precipitation or any other changes of phosvitin have been observed (1). In addition no coagulation has been observed by heating of phosvitin at 140 °C for 10 min (46).
However, the heat denaturation temperature of phosvitin as discovered by using differential scanning calorimetry at neutral pH, is $79.7 \pm 1.4 \, ^\circ C$ when heated at the rate of $10 \, ^\circ C/min$ (47). The SDS-PAGE of our samples showed a widening of phosvitin band after heat treatment at $100 \, ^\circ C$ for 1 hr (Figure 4). However, no precipitation or aggregation was observed, indicating that heat treatment at $100 \, ^\circ C$ for 60 min may have resulted in a partial denaturation of phosvitin.

**Effect of enzymes on phosvitin hydrolysis**

Phosvitin shows resistance to proteolytic digestion *in vitro* due to its extraordinary primary structure, which is composed of long oligophosphoserine blocks uninterrupted by other residues (6). However, previous attempts to digest phosvitin enzymatically have been done mostly with trypsin, pepsin and α-chymotrypsin. A limited hydrolysis of phosvitin with pepsin, trypsin and α-chymotrypsin has been reported (30). Pepsin has produced three peptides of Asn 44-Leu 193 (150 residues), Gly 4-Glu 41 (38 residues) and C-terminal fragment of Leu 193-Glu 214 (21 residues). Trypsin digestion has resulted in two major peptides of Gln49-Arg 212 (164 residues), and Ala 1- Arg 35 (35 residues) and α-chymotrypsin resulted in two major peptides of Ala 50-Trp210 (161 residues) and Gly4-Gln 49 (46 residues) (30). Therefore, in this study phosvitin was subjected to alkalase, Multifect® P-3000 and thermolysin too. The SDS-PAGE (15 %) pattern of the phosvitin hydrolysates from the above mentioned three enzymes showed better enzymatic susceptibility of phosvitin than other three enzymes (trypsin, pepsin and α-chymotrypsin). Digestion with trypsin, pepsin and α-chymotrypsin gave a common peptide bands with similar molecular weight in SDS-PAGE (Figure 5). This common peptide produced by the three enzymes is considered as the core part of the protein, which is composed of 99 amino acid residues with clusters of serines and arginines, lysines and asparagines (6). Phosvitin hydrolysates of alkaline proteases- alkalase and Multifect® P-3000 resulted in 5-6 peptides bands in SDS-PAGE and showed the reduction in molecular weight of the first peptide band implying that the core of phosvitin also has been attacked by those two enzymes. This means that phosvitin is more susceptible to alkalase and Multifect® P-3000 than pepsin, trypsin or α-chymotrypsin. In the
production of bioactive peptides from phosvitin improving enzymatic digestion is the key step.

Thermolysin resulted in different peptide patterns in the SDS-PAGE compared with those from the other five enzymes. It showed a continuous streaking pattern implying better protein digestion than pepsin, trypsin and α-chymotrypsin, but the molecular weight of the first band on the SDS-PAGE was not reduced as in alkalase or Multifect® P-3000 treatment.

Thermolysin has a wide substrate specificity with partiality for hydrophobic and bulky amino acid residues (Ile, Leu, Phe), and can cleave proteins at methionine, histidine, tyrosine, alanine, asparagine, serine, threonine, glycine and lysine sites (47-48). Cleavage of Asn 34-Leu 35 at the beginning and slower cut at the site of Thr45-Phe46 by thermolysin in RNase has been observed (49-50). Alkalase has a broad specificity and can cleave carboxyl side of Glu, Met, Leu, Tyr, Lys, and Gln of peptides bonds. It has been used for the production of bioactive peptides through limited hydrolysis of different proteins (52-54). Aromatic (Phe, Trp and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser), and basic (Lys) residues have more susceptibility to alkalase than other amino acid groups (55).

Alkalase cleaves peptides bonds from carboxyl side of Glu, Met, Leu, Tyr, Lys and Gln. Out of 26 cleaving cites of alkalase on casein, it has been found that 17 contains Glu in the P1 position(51). However, the activity of those enzymes on phosvitin is yet to be discovered. Thus, further analyses are currently under way in order to identify and characterize the peptides resulted from the enzymatic digestion of phosvitin by alkalase, Multifect® P-3000 and thermolysin using LCMS/MS.

Consecutive enzyme treatments of phosvitin did not improve phosvitin digestion. The consecutive enzyme combinations with trypsin and pepsin, alkalase and α-chymotrypsin, Multifect® P-3000 and α-chymotrypsin did not result further digestion (Figure 6, consecutive enzyme trypsin and pepsin is not shown) implying that the phosvitin phosphopeptides resulted from one enzyme is not susceptible for further digestion. Phosphopeptides show extraordinary resistance to additional proteolytic action due to o-phosphorylated serine residues in their molecules. It has been observed that the negative
charges of phosphate moiety make surrounding peptide bonds of phosphopeptides insensitive to trypsin (56).

**Enzymatic digestion of phosvitin pre-treated with SDS and SDS + heat**

The phosvitin pre-treated with SDS and SDS + heat was subjected to thermolysin digestion and SDS-PAGE analysis. The results indicated that the susceptibility of protein to protease activity was decreased by SDS treatment. Native phosvitin showed better enzymatic digestion than phosvitin treated with SDS. However, phosvitin subjected to SDS + heat showed better enzymatic susceptibility than phosvitin treated only with SDS (Figure 7). The decreased digestibility of phosvitin with SDS treatment could be caused by the effect of SDS on the enzyme. It has been observed that resistance of proteins to enzymatic digestion in the presence of SDS is related to the binding of SDS on the substrate (57). In addition the presence of SDS might have some negative effect on the enzyme activity. Yu and others in 2003 observed that 0.1 % SDS has affected on the enzymatic action of trypsin, and immediate and complete loss of activity at 0.5 % SDS (58). Even though SDS and SDS + heat treatment significantly affected the sheet and coil structures of phosvitin, implying some degree of denaturation of protein, those treatments did not improve the subsequent thermolysin digestion of phosvitin.

**Enzymatic digestion of acid/alkali + heat treatment**

In order to investigate the effect of acid/alkali pre-treatments plus heating on the enzymatic digestion of phosvitin, the acid/alkali pre-treated samples were heated and subjected to thermolysin digestion. The SDS-PAGE pattern of thermolysin hydrolysates of phosvitin with acid pre-treatments (pH 2 and 3) and heating was not different from that of the hydrolysate obtained from native phosvitin. However, thermolysine hydrolysate of phosvitin pre-treated at pH 12 and heated at 85 °C for 30 min showed one visible band with streaking at the bottom of the gel (Figure 8), indicating complete enzymatic hydrolysis of phosvitin. Similar SDS-PAGE band pattern was observed by Jiang and Mine (2000) for the tryptic hydrolysate of phosvitin after alkaline treatment (0.1 to 0.4 N NaOH at 37 °C for 3 h) (27). They suggested that this increased susceptibility of phosvitin to proteolytic enzymes could be
due to the conformational changes occurred by dephosphorylation of phosvitin, which led the exposure of the core amino acids to enzymatic cleavage. Before and after thermolysin digestion, phosvitin pre-treated at pH 13 and heating at 85 °C for 30 min showed the same pattern in SDS-PAGE. This could be due to complete hydrolysis/decomposition of phosvitin prior to enzymatic digestion as discussed above. This indicated that phosvitin is highly stable at low pH and combination of heat treatment and low pH conditions did not increase the enzymatic susceptibility of phosvitin. The high stability of phosvitin at low pH conditions could be related to the formation of β-sheet structures at low pH ranges (7). High enzyme susceptibility of phosvitin at alkaline condition could be partly because of alkaline dephosphorylation of phosvitin.

**Enzymatic digestion of heat-treated phosvitin**

It has been shown that proteins are more susceptible to enzymatic digestion in their denatured form than in their native form. Thermal denaturation results in unfolding of proteins and hence peptide bonds will become more reachable to enzymatic cleavage (35, 59). The heat-treated phosvitin was subjected to Multifect® P-3000, alkalase and thermolysin to investigate the effect of partial denaturation on the enzymatic susceptibility of phosvitin. The SDS-PAGE band pattern of phosvitin hydrolysates obtained from heat-treated (100 °C for 1 h) and the control phosvitin indicated that, the heat treatment improved the hydrolysis of phosvitin. Especially, the thermolysin digests of heat-treated phosvitin showed clear reductions in molecular weight of the first peptide band appeared on the 15 % SDS-PAGE (Figure 9). The Multifect® P-3000 and alkalase hydrolysates of temperature treated phosvitin did not show any reduction of the molecular weight of the first peptide band in the SDS-PAGE. However, it made clear reduction of the intensities of the first peptide band in the SDS-PAGE (Figure 10). Especially, the first peptide band of alkase hydrolysate has been almost disappeared implying enhanced digestibility of the protein even in the core part of phosvitin molecule. Unusually high amount of negative charges in phosvitin restricted the formation of proper enzyme-substrate complex, which resulted in a poor digestibility of phosvitin (1). Itoh and others (1983) observed that thermal treatment of phosvitin (140 °C for 10 min) can release about 12 % phosphate from phosvitin (46). Therefore, in this heat
treatment partial removal of phosphate moieties could be expected. With the removal of more phosphate moieties, the net negative charge of phosvitin is reduced and more peptide bonds could be exposed to enzymes, allowing more cleavage of the protein (27). This improved digestibility of phosvitin subjected to the thermal treatment at 100 °C for 1 h could be due to both heat-induced denaturation of phosvitin and the removal of phosphate moieties of phosvitin molecules. However, only 0.2 % release of phosphate at neutral pH has been observed when phosvitin was subjected to a heat treatment at 100 °C for 10 minutes (46). Thus still a majority of the phosphate moieties which is important for the functionality of phosvitin might have been preserved even after 1 h heat treatment at 100 °C. Therefore, the enzymatic susceptibility of phosvitin can be improved by a heat treatment of 100 °C for 1 h.

Conclusions
Improving digestion of phosvitin via pre-treatment was challenging. Among the enzymes tested alkalase, Multifect® P-3000 showed better enzymatic activity on phosvitin than other three enzymes tested, this could be due to their broad specificity. Use of consecutive enzyme combinations did not have any effect on further digestion of peptides resulted from the first enzyme used. Combination of alkali and temperature resulted higher degree of enzymatic digestion, possibly due to dephosphorylation of phosvitin. Heat treatment at 100 °C for 1 h enhanced the enzymatic susceptibility of phosvitin slightly. However, present findings suggest that denaturing of phosvitin is difficult and it does not support significantly to improve the enzymatic susceptability of phosvitin.

Abbreviations
SDS - Sodium dodecyl sulfate
PV - Phosvitin

References


Figure 1. Proportions of secondary structures of phosvitin treated with SDS treatment (0.5% and 1%) with/without heat treatment of 100 °C for 15 min, measured by CD spectroscopy. Data represent the mean (n=4) and letters indicate significant difference at p<0.05 (SEM for β-helix =10.185, SEM for random coil = 0.254, SEM for sheet = 0.824).
Figure 3. Proportions of secondary structures of phosvitin treated with heat treatment of 100 °C up to 60 min. Data represent the mean (n=4) and letters indicate significant difference at p<0.05 (SME for β-helix =0.564, SEM for random coil = 0.3547, SEM for sheet = 0.872).
Figure 4. SDS-PAGE patterns of phosvitin and pre-treated phosvitin. lane 1. Phosvitin; lane 2. Phosvitin subjected to heat treatment (HT) of 100 °C, 10 min; lane 3. Phosvitin subjected to HT of 100 °C, 20 min; lane 4. Phosvitin subjected to HT of 100 °C, 30 min; lane 5. Phosvitin subjected to HT of 100 °C, 40 min; lane 6. Phosvitin subjected to HT of 100 °C, 50 min; lane 7. Phosvitin subjected to HT of 100 °C, 60 min; lane 8. Phosvitin.
Figure 5. SDS-PAGE patterns of phosvitin and different enzymatic hydrolysates of phosvitin.
lane 1. Phosvitin; lane 2. 6h trypsin hydrolysate of phosvitin; lane 3. 6h α-chymotrypsin hydrolysate of phosvitin; lane 4. 6h pepsin hydrolysate of phosvitin; lane 5. 6h Multifect® P-3000 hydrolysate of phosvitin; lane 6. 6h alkalase hydrolysate of phosvitin, lane 7. 6h thermolysin hydrolysate of phosvitin; lane 8. MM.
Figure 6. SDS-PAGE of patterns of consecutive enzymatic digestions of phosvitin. Panel (a) lane 1. MM; lanes 2, 5 and 8. Phosvitin (R1, R2, R3); lanes 3, 6 and 9. 6 h enzyme protein Multifect® P-3000 hydrolysate of phosvitin (R1, R2, R3); lanes 4, 7 and 10. 3 h Alphacymotrypsin hydrolysate of Enzyme protein Multifect® P-3000 hydrolysate of phosvitin (R1, R2, R3). Panel (b) lane 1 MM; lanes 2, 5 and 8. Phosvitin (R1, R2, R3); lanes 3, 6 and 9. 6 h alkalase hydrolysate of phosvitin (R1, R2, R3); lanes 4, 7 and 10. 3 h Alphacymotrypsin hydrolysate of alkalase hydrolysate of phosvitin (R1, R2, R3).
Figure 7. SDS-PAGE patterns of phosvitin and phosvitin digests of SDS and SDS+Heat treated phosvitin. lane 1. Phosvitin; lane 2. Thermolysin hydrolysate of phosvitin; lane 3. Thermolysin hydrolysate of phosvitin subjected heat treatment of 100 °C for 15 min; lane 4. Thermolysin hydrolysate of phosvitin treated with SDS (0.5%); lane 5. Thermolysin hydrolysate of 0.5% SDS and heat treatment of 100 °C for 15 min; lane 6. Thermolysin hydrolysate of phosvitin treated with SDS (1%); lane 7. Thermolysin hydrolysate of 1% phosvitin treated with SDS and heat treatment of 100 °C for 15 min; 8. MM.
Figure 8. SDS-PAGE pattern of Thermolysin hydrolysates of phosvitin subjected pH and heat treatments lane 1. Phosvitin; lane 2. Thermolysin hydrolysates of phosvitin; lane 3. Thermolysin hydrolysates of phosvitin treated at pH-2, 85°C for 30 min; lane 4. Thermolysin hydrolysates of phosvitin treated at pH-3, 85 °C for 30 min; lane 5. Thermolysin hydrolysates of phosvitin treated at pH 12, 85 °C for 30 min; lane 6. Thermolysin hydrolysates of phosvitin treated at pH- 13, 85 °C for 30 min; lane 7. MM
Figure 9. SDS-PAGE pattern of thermolysin hydrolysates of phosvitin and pre-treated phosvitin lane 1. Phosvitin; lanes 3,5,7 and 9 thermolysin hydrolysates of phosvitin; lanes 4,6,8 and 10 Thermolysin hydrolysates of phosvitin subjected to heat treatment of 100 °C for 60 min.
Figure 10. SDS-PAGE pattern of phosvitin and hydrolysates (6 h) of phosvitin & heat treated phosvitin.  lane 1. Phosvitin; 2. Phosvitin subjected to temperature treatment of 100 °C for 60 min; 3. Enzyme protein Multifect® P-3000 hydrolysate of phosvitin; 4. Enzyme protein Multifect® P-3000 hydrolysate of heat treated phosvitin; 5. Alkalase hydrolysate of phosvitin; 6. Alkalase hydrolysate of phosvitin subjected to heat treatment; 7. Thermolysin hydrolysate of phosvitin; 8. Thermolysin hydrolysate of phosvitin subjected to heat treatment; 9. MM.
CHAPTER 4. ANTIOXIDANT AND MINERAL-CHELATING ACTIVITIES OF PHOSVITIN AND ITS ENZYMATIC HYDROLYSATES

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Abstract

Phosvitin, and alkalase, thermolysin and Multifect® P-3000 hydrolysates of phosvitin with or without pre-treatment (100 °C, 1 h) were evaluated for their antioxidant and mineral-binding activities. Phosvitin and its hydrolysates showed a powerful antioxidant effect in iron-induced oxidation in oil emulsion system. Presence of phosvitin at more than 0.25 mg/ml in oil emulsion significantly increased lipid oxidation, implying the importance of having the optimum level of phosvitin in order to achieve its fullest potential. Both Fe-binding and Cu-binding activities of phosvitin decreased with increasing concentrations of phosvitin in the assays. Hydrolysates of phosvitin showed different concentrations of Fe-binding and Cu-binding activities. Phosvitin significantly reduced the solubility of calcium in solution, but phosvitin hydrolysates increased it. Preparation of hydrolysates using pre-treated phosvitin did not help in improving the antioxidant or mineral-binding capacities of hydrolysates compared with those without pre-treatment.

Keywords: Phosvitin, phosvitin hydrolysates, antioxidant activity, mineral binding, pre-treatment
1. Introduction

Due to fear of potential hazardous effects of synthetic antioxidants on health and the trends to avoid synthetic food additives (Branen, 1975; Becker, 1993; Frankel, 1993; Chow, 1988), the use of natural antioxidant has been increased dramatically in recent years (Finkiel & Holbrook, 2000, Shahidi, Lyana-Pathirana, & Wall 2006). Peptides derived from proteins showed a great possibility to be used as natural antioxidants in food systems (Xu, Katayama, & Mine 2007). Many enzymatic hydrolysates have been examined and proven to have potent antioxidant activities. Proteins such as casein (Chiu & Kitts, 2004; Kansci, Genot, Meynier, Gauheron, & Chobert, 2004; Díaz & Decker, 2004), soy proteins (Chen, Muramoto, Fumio, & Kiyoshi, 1996), whey proteins (Peña-Ramos & Xiong, 2001, 2004), chick pea proteins (Li, Jiang, Zhang, Mu, & Liu, 2008), fish skin gelatin (Mendis, Rajapakse, & Kim, 2005), muscle proteins (Saiga, Tanabe, Nishimura, & 2003; Je, Park, Kwon, & Kim, 2004) etc. have been evaluated for their antioxidant activity.

In addition, mineral-binding peptides have been considered as useful nutraceuticals since they can enhance the bioavailability and absorption of minerals in the intestine. Phosphopeptides are considered as the major mineral-binding peptides. The interesting feature of phosphopeptides is their ability to form soluble organophosphate salts. The phosphorylated serine moieties of the phosphopeptides play the major role in binding divalent metal ions such as Ca, Fe, Mg, Zn, Cu, etc. (Li, Tomé, & Desjeux, 1989; Hansen, Sandström, & Lönnerda, 1996; Kitts, 2005), and promote the intestinal absorption of calcium and other minerals (Konings, Kuipers, Huis in’st, & Veld, 1999). In addition, the negative charges of phosphate groups and side chains of phosphopeptides make them resistant to the gastrointestinal enzymatic digestion, which makes them much suitable to be used as carriers of metal ions (FitzGerals, 1998). Casein-derived phosphopeptides are considered as the main mineral-binding phosphopeptides and have been already approved as nutraceuticals in Japan (Jiang & Mine, 2000). A product called “Capolac” containing CPP (Arla Foods Ingredients) is also available in Sweden as a mineral absorption facilitator (Korhonen & Pihlanto, 2006). However, the effect of CPPs on mineral absorption has been a controversial issue (Bennett, Desmond, Harrington, McDonagh, FitzGerald, Flynn, and Cashman, 2000).
Phosphopeptides are derived from phosphoproteins. Phosvitin is the major phosphorylated protein found in egg yolk. Phosvitin accounts for 60% of the total egg yolk phosphoproteins and holds about 90% of the egg yolk phosphorous. The molecular weight of phosvitin ranges from 35,000 to 40,000 Da and is composed of 217 amino acids residues. More than 50% of the amino acids in phosvitin are serine and 90% of which are phosphorylated (Taborsky, 1974; Byrne, van Het Schip, van de Klundert, Arnberg, Gruber, & Geert, 1984). As a highly phosphorylated protein, egg yolk phosvitin shows a very strong affinity to bivalent metals such as iron, calcium, magnesium etc. (Taborsky, 1963; Grizzuti & Perlmann, 1973). In addition phosvitin shows antioxidant (Lu & Baker, 1986, 1987; Nakamura, Ogawa, Nakai, Kato, & Kitts, 1998), emulsifying (Khan, Babiker, Azakami, & Kato, 1998; Chung & Ferrier 1991, 1992), and antimicrobial activities (Khan, Nakamura, Ogawa, Akita, Azakami, & Kato 2000). However, phosvitin is considered nutritionally negative since it causes poor bioavailability of iron due to its ability to form insoluble iron complex within the digestive track (Halkett, Peters & Ross, 1958; Taborsky, 1963; Greengard, Sentenac, & Mendelsohn 1964; Sato, Noguchi, & Naito, 1987). Also, egg yolk phosvitin is considered as an allergen (Walsh, Barnett, Burley, Elliott, Hill, & Howden, 1988; Walsh, Hill, Macoun, Cairns, & Howden, 2005). If phosvitin is fragmented to peptides using enzymes, however, all the negative attributes of the protein can be eliminated (Baumgartner & Schubert-Ullrich, 2010). The antioxidant effects of protein hydrolysates depend upon the enzymes utilized for the production of hydrolysates (Amarowicz, 2008). The trypsin hydrolysate of phosvitin and partially dephosphorylated phosvitin showed promising results as an antioxidant (Katayama, Xu, Fan, & Mine, 2006; Xu, Katayama, & Mine, 2007). However, different enzymatic hydrolysates of phosvitin and their antioxidant and mineral-binding activities are yet to be discovered.

Therefore, the objective of the present study was to investigate the antioxidant activity and mineral-binding activity of phosvitin and different enzymatic hydrolysates of phosvitin in model systems.
2. Materials and Methods

Phosvitin was separated according the procedure of Ko, Nam, Jo, Lee, & Ahn (2011). Thermolysin from Bacillus thermoproteolyticus rokko were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Alkalase (bacterial alkaline protease: protex 6L) and Multifect® P-3000 were gifts from Genencor International Inc. (New York, NY, USA). Pre-treatment of phosvitin: Phosvitin was heat-treated at 100 °C for one hour before the enzymatic digestion for improved digestion of phosvitin.

2.1 Preparation of enzymatic hydrolysates of phosvitin

The phosvitin/pre-treated phosvitin solution (1 %) was subjected to digestion with Multifect® P-3000, alkalase, and thermolysin. The substrate: enzyme ratio was 100:1 (w/w) and the hydrolysis was carried out for 6 h. The pH and temperature conditions used were different depending upon enzymes (Multifect® P-3000- pH 7.0 & 50 °C; alkalase- pH 9.0 & 60 °C; thermolysin- pH 6.8 & 68 °C). The enzymatic digestion was arrested by keeping samples in a boiling waterbath for 10 min. The samples were lyophilized and stored at -20 °C until used.

2.2 Preparation of oil emulsion samples

An oil-in-water emulsion was prepared using 1.0 g of corn oil (HyVee Inc., IA, USA) and 100 µl of Tween 20 (Fisher Scientific) in 100 ml of Tris–maleate buffer (pH 6.8) in a 250 ml Erlenmeyer flask. The mixture was homogenized 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. The fatty acid composition of corn oil (Table 1) and the presence of tocopherol was measured using a gas chromatography/mass spectrometry (GC/MS; Hewlett-Packard Co., Wilmington, DE, USA).

2.3 Lipid oxidation assay

For the oxidation assay 8 ml of the oil emulsion was mixed with 0.5 ml of 0.2 % ascorbic acid (Sigma), 0.5 ml of 200 ppm of Fe³⁺(FeCl₃, Sigma), and 1 ml of varying weights
(1-10 mg) of phosvitin/its hydrolysates (2 mg) in a 50-ml test tube. The mixture was then vortex-mixed and incubated at 37 °C for 16 h. At different time intervals, 1 ml of the mixture was taken to determine 2-thiobarbituric acid-reactive substances (TBARS) value.

2.4 Determination of 2-thiobarbituric acid reactive substances (TBARS) value

The oil emulsion mixture (1 ml) was transferred to a disposable test tube (13 x 100 mm), and 2 ml of thiobarbituric acid/trichloroacetic acid solution (20 mM TBA/15 % TCA, w/v) and 50 µl of 10 % butylated hydroxyanisole in 90 % ethanol were added. The mixture was vortex-mixed and then incubated in a 90 °C waterbath for 15 min to develop color. After cooling for 10 min in cold water, the sample was vortex-mixed and centrifuged at 3,000 x g for 15 min at 5 °C. The absorbance of the supernatant was read 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.

2.5 Fe-chelating activity

The Fe-chelating activity of phosvitin and its hydrolysates was determined using the Ferrozine method (Carter, 1971) with some modifications. Varying weights (0-1 mg) of phosvitin or its hydrolysates (0.5 mg) in Tris–maleate buffer (pH 6.8) (100 µl) was vortex-mixed with 1 ml of 10 ppm Fe and 1 ml of 1 % (wt/vol) ascorbic acid in 0.2 N HCl. After incubation for 5 min at room temperature, 0.9 ml of 11.3 % trichloroacetic acid (TCA) was added to the sample and centrifuged at 2,500 x g for 10 min. The supernatant (2 ml) was transferred to a new disposable culture tube, and 0.8 ml of 10 % ammonium acetate (Fisher) and 0.2 ml of Ferroin color reagent (75 mg of ferrzoine, 75 mg of neocuproin and 1 drop of 6 N HCl in 25 ml of distilled water) were added, vortex-mixed, and measured the absorbance at 562 nm after 10 min of incubation at room temperature. The Fe-chelating activity was calculated using a blank (without phosvitin or hydrolysates):

\[
\text{Fe-chelating activity (\%)} = \{1-(\text{sample solution absorbance/blank solution absorbance})\} \times 100
\]
2.6 Cu$^{2+}$-chelating activity of phosvitin and its hydrolysates

Cu-binding activity of phosvitin and its hydrolysates were measured according to the modified method of Kong, & Xiong (2006). One ml of 0.2 mM CuSO$_4$ was mixed with 1 ml of varying amounts of phosvitin (0.5 - 2.5 mg) or its hydrolysates (2 mg) in Tris–maleate buffer (pH 6.8). Then, 1 ml 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, mixed with 1 ml of 10 % pyridine and 20 µl of 0.1 % pyrocatechol violet (Sigma-Aldrich), and incubated at room temperature for 5 min. The 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.

\[
\text{Cu}^{2+}\text{-chelating activity (\%)} = \{1 - (\text{sample solution absorbance/blank solution absorbance})\} \times 100
\]

2.7 Ca$^{2+}$-solubilizing activity

Ca$^{2+}$-solubilizing activity was measured according to the modified method of Jung & Kim (2007). Various concentrations (0.1-0.5 mg/ml) of phosvitin or its hydrolysates (0.5 mg/ml) were added in 5 mM CaCl$_2$ and 20 mM sodium phosphate buffer (pH 6.8). The mixture was stirred at 37 °C for 2 h and centrifuged at 3,200 x g for 10 min. The supernatant was filtered through a 0.45 µm membrane filter and the calcium contents of the filtrate were determined by Inductive Plasma Mass Spectroscopy. *(Iowa State University Soil and plant analysis laboratory, ICP: SPECTRO, Analytical Instruments GmbH).* Ca$^{2+}$-solubilizing activity was calculated as a percentage of available Ca$^{2+}$ in the supernatant compared to the control.

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 The antioxidant activity of phosvitin and its hydrolysates in oil emulsion system

The effect of phosvitin (0.05 - 1 mg/1 ml of oil emulsion assay) on the oxidation of lipids in oil emulsion indicated that the antioxidant effect of phosvitin reached to its maximum when the concentration of phosvitin was between 0.15 to 0.25 mg/ml or at 0.375 - 0.625 x 10^{-8} M in the oil emulsion. Beyond 0.25 mg/ml in emulsion assay, however, the antioxidant effect of phosvitin gradually decreased. Interestingly, this phenomenon is consisitence with all the time intervals evaluated. The present study reveals that phosvitin shows its maximum oxidation inhibitory effect when it is present at the optimum level. At 0.1 mg/ml level, BHA was better antioxidant agent than phosvitin in most of the incubation time. However, during the first two hours of incubation phosvitin performed better than or similar to BHA at 0.1 mg/ml level (Table 2). In addition, the results revealed that phosvitin can be a good antioxidant even at a high Fe concentration (10 ppm).

Lu & Baker (1986, 1987) reported that phosvitin showed a strong antioxidant activity in a phospholipid emulsion system containing non-organic and organic metal ions (Fe^{2+}, Cu^{2+}, hemin) and the antioxidant activity of phosvitin was maximum at pH 6.1. Phosvitin showed a high oxidation inhibitory effect up to 30:1 Fe^{2+}-to-phosvitin molar ratio on phospholipids system in the presence of Fe^{2+} (Lu & Baker, 1986). Lee, Han & Decker (2002) reported that the maximum antioxidant effect of phosvitin was observed when the phosvitin concentrations were at 15 µM and 40 µM in the phosphatidyl choline liposomes system and pork muscle homogenate, respectively.

A number of proteins have been identified as antioxidant proteins. As a metal-binding protein, casein showed antioxidant activity in oxidation-induced system using ferrous iron (50 µM) and ascorbic acid (500 µM) in arachidonic acid phosphatidylcholine liposomes (Cervato, Cazzola & Cestaro, 1999), and the highest antioxidant activity was found when 0.5 mg/ml casein was present. Casein, soy protein and whey protein showed antioxidant activity in oil-emulsion systems (Donnelly, Decker, & McClements (1998); Diaz, Dunn, Mcclements, & Decker (2003); Hu, Mcclements, & Decker (2003). The antioxidant action of protein can be through the chelation of pro-oxidant by proteins (Huang, Satue´-Gracia, Frankel, & German, 1999) or free radical scavenging by amino acid residues such as
cysteine and tyrosine (Suetsuna, Ukeda, & Ochi, 2000; Li, Jiang, Zhang, Mu, Liu, 2008), which diminishes the production of hydroperoxides or inactivates reactive oxygen species (Elias, Kellerby, Decker, 2008).

Diaz and Decker (2004) observed that high amount of CPPs (> 1 mg/ml) in phosphatidylcholine liposome model system exerted prooxidant effect as observed in the present study. Shahidi & Amarowicz (1996) reported that 10 mg of seal protein hydrolysate in 5 ml of β-carotene/linoleate model system showed a significant prooxidant activity even though it showed a significant antioxidant activity at low concentrations. Lactoferrin also has shown prooxidant nature in corn oil emulsion as well as in liposome system when present at high levels (Huang et al, 1999). However, the real mechanism of this prooxidant nature is not understood. Ascorbic acid, a known antioxidant, has shown prooxidant effects in vitro when present higher than certain levels, usually by interacting with transition metal ions (Halliwell, 1996). Gallic acid and its derivatives also showed both antioxidant and prooxidant activities (Aruoma, 1993). Guohua, Emin, and Ronald (1996) reported that tea extract showed a prooxidant activity in the presence of Cu. Thus, if phosvitin is to be used in food systems as an antioxidant, it is important to use an optimal level of phosvitin.

Also, many protein hydrolysates have shown antioxidant activities. The tryptic hydrolysate of phosvitin showed an inhibitory effect on Fe$^{2+}$-catalyzed hydroxyl radicals (•OH) production in the Fenton reaction system. Many protein hydrolysates also showed a protective effect against •OH-mediated damages of DNA in vivo (Ishikawa, Yano, Arihara, Itoh, 2004). In the present study alkalase, Multifect® P-3000 and thermolysin hydrolysates of natural and pre-treated phosvitin were used. The Multifect® P-3000 hydrolysate of phosvitin had significantly (p > 0.05) greater antioxidant effect than BHA in the oil emulsion system except for at 8 h incubation time (0.2 mg/ml of emulsion assay). Among the all hydrolysates tested, the hydrolysate of Multifect® P-3000 showed the best and that of the thermolysine hydrolysate and alkalase hydrolysate of phosvitin showed similar antioxidant effect (Table 3). No significant difference in the antioxidant activity was observed among the three enzyme hydrolysates of both native phosvitin and pre-treated phosvitin. Therefore, the pre-treatment of phosvitin at 100 °C for 1 h did not have any positive effect on the antioxidant activity even though the pre-treatment increased the degree of hydrolysis in
phosvitin. Xu et al., (2007) reported that tryptic hydrolysate of phosvitin and partially dephosphorylated phosvitin (PDP) showed better antioxidant activity than phosvitin in linoleic acid system. In addition, they have observed that tryptic digests of PDP had better radical-scavenging activity than native phosvitin. However, Diaz, Dunn, McClements, & Decker (2003) observed that casein hydrolysates had a higher antioxidant effect than that of the purified CPP in a corn-oil-emulsion system. The use of hydrolysates would be more attractive than purified CPP in practice because it is less expensive (Diaz, & Decker, 2004). Therefore, there is a high possibility that Multifect® P-3000 and alkalase hydrolysates of phosvitin can be used as antioxidants in foods.

3.2 Iron binding activity

At low concentrations of phosvitin (0.01 - 0.06 mg/ml), the Fe$^{3+}$ binding capacity of phosvitin was significantly higher than that of Fe$^{2+}$-binding capacity. With increasing phosvitin concentration (0.07-1 mg/ml), however, Fe$^{2+}$ and Fe$^{3+}$ binding capacities showed similar activity (Figure 1). As a highly phosphorylated protein, phosvitin has a very strong affinity for bivalent metals such as calcium, magnesium and iron (Taborsky, 1963; Grizzuti & Perlmann, 1973). Phosvitin forms very strong complexes with ferric ion whereas form weak complexes with ferrous iron (Taborsky, 1980). Phosvitin may bind with Fe in the ratio of 0.5 ion/phosphate when iron is in excess (Taborsky, 1980). The Fe-binding capacity of phosvitin varied depending on pH and ionic strength of the medium. The best Fe-binding activity of phosvitin was reported to be at pH 6.5 and ionic strength of 0.15, and the iron-binding capacity of phosvitin was 115 μg iron/mg phosvitin (Castellani, Guérin-Dubiard, David-Briand, & Anton, 2004). The above-mentioned observation could be due to the oxidation of Fe$^{2+}$ to Fe$^{3+}$. When the phosvitin concentration in the assay is too low, the oxidation capability of phosvitin might not be sufficient to convert all Fe$^{2+}$ to Fe$^{3+}$. Due to this reason at low concentration of phosvitin, the Fe$^{2+}$ binding activity of phosvitin might have shown lower value. Also, the iron-binding activity of phosvitin was plateaued at 94-95% Fe saturation (Figure 1). The Fe-binding trend indicated that the state of 100% Fe-binding activity may not be possible. However, this finding is controversial to the previous literature on Fe-binding activity of phosvitin and this could be due to the differences in
experimental methods used (Taborsky, 1963; Grizzuti & Perlmann, 1973; Castellani et al., 2004).

The alkalase, Multifect® P-3000 and thermolysin hydrolsates of natural phosvitin and pre-treated phosvitin indicated that thermolysin hydrolysate had similar degree of Fe-chelating capacity to native phosvitin. Alkalase and Multifect® P-3000 hydrolysates of phosvitin showed similar (p>0.05) Fe-chelating activity. However, that is significantly lower than that of the phosvitin. The three enzyme hydrolysates resulted from pre-treated phosvitin showed significantly lower Fe-chelating activity than the hydrolysates from native phosvitin. The thermolysin hydrolysate of pre-treated phosvitin showed significantly higher Fe-chelating activity than that of alkalase and Multifect® P-3000 hydrolysates of pre-treated phosvitin. It has been known that peptides chelated with iron can increase the stability, absorption and bioavailability of iron (Miquel & Farre, 2007). Casein phosphopeptides (CPP) have been evaluated as carriers of different metal ions, and also known to play an important role in their bioavailability (Clare & Swaisgood, 2000).

In phosphopeptides, phosphorylated serine moiety can bind with metal ions such as Ca, Mg, Zn, Cu, Fe etc. (Li et al., 1989; Hansen, Sandström, Jensen, & Sørensen, 1997; Kitts, 2005), and can promote the intestinal absorption of the minerals (Konings et al., 1999). It has been suggested that the absorption of iron in the gastrointestinal tract is comparatively low because iron forms poorly soluble heavy molecular weight ferric hydroxide in the guts (Derman, 1977). However, CPPs are reported to enhance iron availability and iron absorption in the gastrointestinal track, and thus, CPPs can be used as effective carriers for metal ions for enhanced absorbability (Sato, Noguchi, Naito, 1986).

The metal-binding capacity of proteins and their hydrolsates can vary. Wang, Li, Li, Mao, Zhou, & Ren (2011) observed that yak casein hydrolysate had significantly higher Fe-binding activity than native casein. However, significantly lower binding of Zn by five different hydrolysates of yak casein compared to the native protein has been observed (Wang, Zhou, Tong, & Mao, 2011). The SDS-PAGE analysis of enzyme hydrolysates of phosvitin indicated that alkalase and Multifect® P-3000 performed better than thermolysin (Samaraweera, Lee & Ahn, 2011, unpublished data). The Fe-binding activity of alkalase and multifect® P-3000 hydrolysates of phosvitin, however, was lower than that of thermolysin
hydrolysate. Many proteins showed increased metal-binding activity with increasing degree of hydrolysis, and their mode of iron binding could be attributed to the presence of iron binding amino acids such as histidine and arginine (Megías, Pedroche, Yust, Giron-Calle, Alaiz, Millán, Z & Vioque, 2008; Kallay, Varnagy, Malandrinos, Hadjiliadis, Sanna, & Sova, 2009). With phosvitin, however, higher degree of hydrolysis showed the opposite effect (Figure 2). Donella, Pinna, & Moret (1976) reported that phosvitin phosphopeptides with more than 4 phosphorylserine (Ser-P)_n can bind Fe^{3+} as with phosvitin, but with less activity than phosvitin. Therefore, with increased digestibility of phosvitin, less Fe-binding could be possible. In addition, Castellani et al. (2004) explained Fe binding mechanisms as tetrahedral stoichiometry (once Fe ion is bound with one phosphate molecule and another neighboring phosphate molecule completes the binding process) or octahedral stoichiometry (two other neighboring phosphate molecules complete the process). Therefore, with increasing digestibility of phosvitin, it is possible that some phosphate moieties are not involved in the chelating process due to inability to have tetrahedral stoechiometry and octahedral stoichiometry.

All three hydrolysates of pre-treated phosvitin showed significantly lower Fe-binding activity compared to phosvitin and untreated phosvitin hydrolysates. This could be again due to the increased digestibility of phosvitin due to the pre-treatment of phosvitin.

3.3 Cu^{2+}-binding activity of phosvitin and its hydrolysates

The Cu-binding activity of phosvitin was very low. With increasing phosvitin content in the assay solution (from 0.5 to 1.5 mg/ml), the Cu^{2+}-binding activity increased significantly (P < 0.05) and the binding capacity reached a plateau when phosvitin concentration was >1.5 mg/ml (Figure 3). Cu^{2+}-binding capacity of phosvitin was 3.8 Cu molecules per phosvitin molecule. Phosvitin shows different affinities for different metal ions. The metal affinities of phosvitin also vary depending upon the environmental conditions such as pH and ionic strength. Grizzuti and Perlmann (1973) evaluated the binding of Mn^{2+} and Ca^{2+} to phosvitin using dialysis equilibrium and found that at pH 6.5 at 25 °C, phosvitin could bind 103 Mg^{2+} ions and 127 Ca^{2+} ions. However, they observed drastic reduction of Mg^{2+} and Ca^{2+} binding to phosvitin at pH 4.5, which decreased to 40
Mg$^{2+}$ and 32 Ca$^{2+}$ ions. The maximum The Cu$^{2+}$-binding activity of phosvitin is in agreement with the finding of Lu & Baker (1987). They evaluated the Cu$^{2+}$-binding capacity of phosvitin at two different pH and observed that the Cu-binding capacity of phosvitin was lower at pH 7.8 than at pH 6.1. Accordingly one molecule of phosvitin can bind with approximately one molecule of Cu at pH 6.1. Compared to the reported Mn$^{2+}$- and Ca$^{2+}$-binding activities of phosvitin, its Cu$^{2+}$-binding capacity is very low. However, it must be emphasized that the mineral-binding capacity of phosvitin depends on the bivalent metal ions, the environmental conditions, and the method employed for the measuring the binding capacity. In addition, the degree of phosphorylation and the amount of metal already bound to phosvitin molecule may also play important roles to its metal-binding activity. Usually the phosphorous content of phosvitin ranges from 3 to 10% of its molecular weight (Taborsky & Mok, 1967; Burley & Cook 1961). It has been reported that the affinity of β-casein, β-lactoglobulin and β-casein to Cu is much lower than phosvitin (Aulakh & Stine, 1971).

Peters and Blumenstock (1967) reported that albumin and the terminal peptides derived from peptic digestion can bind with one molecule of Cu by the terminal sequence of Asp and Thr. Megías, Pedroche, Yust, Girón-Calle, Alaiz, Millan, & Vioque (2007a &b) purified Cu$^{2+}$-binding peptides from alkalase and flavourzyme hydrolysates of sunflower proteins and from chickpea proteins, and also from pepsin and pancreatin hydrolysates of sunflower proteins (Megías, Pedroche, Yust, Girón-Calle, Alaiz, Millan, Vioque, 2008). The presence of Arg and His in those peptides were identified as the amino acids responsible for their Cu$^{2+}$-binding activity. The Cu$^{2+}$-binding activity of Multifect ®P-3000 hydrolysates of phosvitin showed significantly (p < 0.05) higher Cu$^{2+}$-binding activity than phosvitin. This observation does not agree with the Fe-binding activity of phosvitin and its hydrolysates. All the hydrolysates showed significantly different Cu$^{2+}$-chelating activities (Figure 4). There is a possibility to use the Cu$^{2+}$-binding peptides as mineral fortifying agent in foods for special groups of people who require a higher level of Cu (Megías et al, 2008). This may imply the possibility of using phosvitin hydrolysates as carriers of Cu or an agent that can prevent Cu-mediated oxidation in foods. In the field of cosmetic industry, copper binding peptides is very attractive since it has an anti-aging property (Mazurowska & Mojski, 2008). However, at present synthetic copper peptides are used. Therefore, naturally-derived copper-binding
peptides such as phosvitin-derived phosphopeptides can have a potential to be utilized in the cosmetics industry as antioxidant and anti-aging peptides.

3.4 Ca$^{2+}$- solubilizing activity of phosvitin and its hydrolysates

It has been known that phosvitin has a high Ca$^{2+}$-binding capacity (Perlmann, 1973). The present study was conducted to find out the Ca solubilizing activity of phosvitin and its enzymatic hydrolysates at pH 6.8, 37 °C. Choi, Jung, Choi, Kim, & Ha (2005) reported that the availability of Ca has been increased by 44.1 % at pH 7.0, 37 °C in the presence of 1.0 % phosvitin. However, this finding is controversial to the known behavior of phosvitin and metal ion complexes. It has been known that phosvitin forms insoluble complexes with metal ions. Ishikawa, Tamaki, Arihara, & Itoh (2007) reported that the absorption efficiencies of calcium and magnesium from diets containing egg yolk protein and phosvitin have been decreased. Calcium and magnesium absorption in Wistar rats has been significantly reduced by the addition of 1-2 % of phosvitin compared to the rats fed with casein-containing diets. Present study indicated that phosvitin decreased the Ca$^{2+}$-solubility significantly compared to the control (Figure 5). Phosvitin resulted in significantly lower availability of Ca ion in the supernatant compared to the control. This is due to the formation of insoluble phosvitin-Ca complex. However, with increasing content of phosvitin the reduction of Ca availability in the supernatant decreased, indicating that the binding of phosvitin and Ca reached to a plateau. At phosvitin concentration of 0.4 - 0.5 mg/ml in the assay solution, the Ca binding activity showed no significant variation. Similar behavior has been observed with chelating activities of phosvitin with Fe and Cu. However, it has been known that phosphopeptides containing clusters of phosphoserines can effectively bind calcium and inhibit the formation of insoluble calcium phosphates, which has been known to increase calcium bioavailability (Li et al, 1989; Kitts, 2005). It was first reported that phosphopeptides derived from casein (CPP) can enhance the calcification of bones (Mellander, 1947). CPPs have been well studied and known to have variety of biological and technological functions such as calcium absorption, calcium retention, bone calcification, anticarcinogenicity, antihypertensive effect, milk curdling and stabilization of cream liqueurs (Park & Allen, 1998). However, phosvitin-derived phosphopeptides has been not studied well. Jiang and Mine (2001) evaluated
trypsin-derived peptides from partially dephosphorylated phosvitin for Ca-binding and solublizing activities and found that the peptides retaining 35 % of phosphate were more effective in binding Ca and inhibiting formation of insoluble calcium phosphate than phosphopeptides with 65 % and 17.5 % of retaining phosphate. Choi et al. (2005) reported that the inclusion of phosvitin phosphopeptides at low, medium and high levels had no effect on Ca intake, fecal Ca content or urinary Ca contents in Sprague Dawley rats, but phosvitin phosphopeptides increased Ca absorption and Ca accumulation in the bones significantly (p < 0.05). Therefore, it is important to find out the effect of phosphopeptides derived from different enzymatic digestions of phosvitin for the solubility of Ca. The present findings revealed that at 0.5 mg phosvitin in ml of assay solution reduced the Ca solubility by 20 % compared to the control at pH 7.0, 37 °C (Figure 5). Alkalase, Multifect® P-3000 and Thermolysin hydrolysates of phosvitin showed significantly increased Ca solubility compared to the phosvitin (p<0.05). However, all three hydrolysates and the hydrolysate obtained by pre-treated phosvitin reduced the Ca availability in the supernatant compared to the control at the concentration of 0.5 mg/ml. Thermolysin hydrolysate showed the lowest Ca solubility compared other two hydrolysates. This could be due to less degree of enzymatic digestion resulted from thermolysin compared to other enzymatic hydrolysates tested. The three hydrolysates obtained from pre-treated phosvitin resulted in increased calcium solubility compared to the hydrolysates obtained from phosvitin without pre-treatment. The phosvitin hydrolysates of alkalase and Multifect® P-3000 and thermolysin enzymes showed 55.5 % and 37.5 % and 17.6 % increase in Ca solubility compared to the phosvitin at the same concentration. However, Multifect® P-3000 and thermolysin hydrolysate of pre-treated phosvitin showed an insignificant increase in Ca solubility compared to the hydrolysates of native phosvitin (Figure 6). Therefore, it is clear that hydrolysates of phosvitin can increase the Ca solubility with increasing degree of hydrolysis compared to native protein. Low degree of protein hydrolysis resulted in comparatively larger peptides with higher number of phosphate moieties in it (Goulas, Triplett, Taborsky, 1996), which resulted in higher degree of mineral binding activity and reduced solubility than smaller phosphopeptides. Because the fundamental features of mineral-binding peptides are
to have high number of negative charges that can bind with divalent cations and form soluble complexes (Vegarud, Langsrud, & Svenning, 2000).

Further investigations may be required to increase the degree of digestion of phosvitin to produce much smaller peptides than the ones produced in this study. And also it must be emphasize that it is phenomenally important to find out the amount of phosvitin phosphopeptides to be included in the system in order to get optimum solubility, since the presence of high amount can lead precipitation of Ca-bound peptides.

4. Conclusions

Phosvitin showed a powerful antioxidant activity in oil emulsion system and it can be used as an antioxidant agent for Fe-mediated oxidation in food systems at optimum level. The best antioxidant activity was observed with the hydrolysate of Multifect® P-3000 of phosvitin and the lowest with the thermolysin hydrolysate of phosvitin. The pre-treatment of phosvitin at 100 °C for 1 h did not improve the antioxidant capacity compared to the hydrolysates obtain from phosvitin without pre-treatment. Phosvitin can bind both with Fe$^{2+}$ and Fe$^{3+}$ at similar capacity. However, at low level of iron, more Fe$^{3+}$ molecules bind with phosvitin than Fe$^{2+}$. The Fe-binding and Cu$^{2+}$-binding activities of phosvitin decreased as the level of phosvitin increased. Compared to the Fe-binding activity, the Cu$^{2+}$ -binding capacity of phosvitin was very low. The enzymatic hydrolysis of phosvitin did not increase the Fe-binding capacity of phosvitin. Also, the hydrolysates obtained from pre-treated phosvitin showed significantly lower Fe-binding activity than phosvitin and hydrolysates of native phosvitin. The Ca$^{2+}$-solubility significantly decreased in the presence of phosvitin, but increased significantly in the presence of enzymatic hydrolysate of phosvitin compared to phosvitin. In addition, the hydrolysates obtained from pre-treated phosvitin showed better Ca$^{2+}$-solubilizing activity than those obtained from phosvitin without pre-treatment. According to the results of functional activities of phosvitin hydrolysates it is important to increase the degree of hydrolysis of phosvitin further and to find out the optimum level to be included in a particular system. Therefore, further research should be focus on those aspects in order to exploit phosvitin hydrolysates.
Abbreviations
PV- Phosvitin
BHA- Butylated hydroxyanisole
P-3000 – Phosvitin hydrolysate of multifect® P-3000 enzyme
Alk- Phosvitin hydrolysate of alkalase enzyme
Thermo – Phosvitin hydrolysate of thermolysin enzyme
Pt- P-3000 – Multifect® P-3000 hydrolysate of pre-treated phosvitin
Pt-Alk- Alkalse hydrolysate of pre-treated phosvitin
Pt-Thermo – Thermolysin hydrolysate of pre-treated phosvitin

References


Table 1: Fatty acid composition of corn oil used for oil emulsion system

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>%</th>
</tr>
</thead>
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<tr>
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</tr>
<tr>
<td>Palmitic acid</td>
<td>12.5</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
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</tr>
<tr>
<td>Margaric acid</td>
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</tr>
<tr>
<td>Margaroleic acid</td>
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</tr>
<tr>
<td>Stearic acid</td>
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</tr>
<tr>
<td>Oleic acid</td>
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</tr>
<tr>
<td>Linoleic acid</td>
<td>54.4</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>1.1</td>
</tr>
<tr>
<td>Arachidic acid</td>
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</tr>
<tr>
<td>Arachidonic acid</td>
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</tr>
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</tr>
<tr>
<td>DPA</td>
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</tr>
<tr>
<td>Total</td>
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Table 2: Effect of phosvitin on the TBARS values of oil emulsion during incubation

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<th>mg/ml sample</th>
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</tr>
</thead>
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<tr>
<td></td>
<td>0 h</td>
<td>1 h</td>
</tr>
<tr>
<td>Phosvitin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.16&lt;sup&gt;fwx&lt;/sup&gt;</td>
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</tr>
<tr>
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<td>0.15&lt;sup&gt;cz&lt;/sup&gt;</td>
</tr>
<tr>
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<td></td>
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</table>

<sup>a-f</sup> Means in the same row with different superscript letters differ significantly (p<0.05, n=4).

<sup>u-z</sup> Means in the same column with different superscript letters differ significantly (p<0.05, n=4).

The oil emulsion was added with 10 ppm ionic iron.
Table 3: TBARS values of oil emulsion (mg MDA/L) in the presence of BHA, natural phosvitin, and alkalase, Multifect® P-3000 or thermolysin hydrolysate (6 h-hydrolysis, 2 mg/ml) of phosvitin and pre-treated (100 °C, 60 min) phosvitin in oil emulsion system

<table>
<thead>
<tr>
<th>Sample</th>
<th>0 h</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>16 h</th>
<th>S.M.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>0.179&lt;sup&gt;ew&lt;/sup&gt;</td>
<td>0.257&lt;sup&gt;dw&lt;/sup&gt;</td>
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<td>0.822&lt;sup&gt;by&lt;/sup&gt;</td>
<td>1.452&lt;sup&gt;aw&lt;/sup&gt;</td>
<td>0.015&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.200&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>0.222&lt;sup&gt;by&lt;/sup&gt;</td>
<td>0.239&lt;sup&gt;bz&lt;/sup&gt;</td>
<td>0.341&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>0.012&lt;sup&gt;u&lt;/sup&gt;</td>
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<td>P-3000</td>
<td>0.099&lt;sup&gt;dz&lt;/sup&gt;</td>
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<td>0.137&lt;sup&gt;cz&lt;/sup&gt;</td>
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<td>Thermo</td>
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<td>0.163&lt;sup&gt;bwx&lt;/sup&gt;</td>
<td>0.165&lt;sup&gt;bxy&lt;/sup&gt;</td>
<td>0.188&lt;sup&gt;abyz&lt;/sup&gt;</td>
<td>0.204&lt;sup&gt;az&lt;/sup&gt;</td>
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<sup>a-i</sup>Means in the same row with different superscript letters differ significantly (p<0.05, n=4).

<sup>u-z</sup>Means in the same column with different superscript letters differ significantly (p<0.05, n=4).

The oil emulsion was added with 10 ppm ionic iron.

Abbreviations: Phosvitin- natural phosvitin; P-3000- Multifect® P-3000 hydrolysate of phosvitin; AlK- alkalase hydrolysate of phosvitin; Thermo- thermolysin hydrolysate of phosvitin; Pt-P-3000- Multifect® P-3000 hydrolysate of pre-treated phosvitin, Pt-Alk- alkalase hydrolysate of pre-treated phosvitin ; Pt-Thermo- thermolysin hydrolysate of pre-treated phosvitin; BHA- butylatedhydroxyanisole.
Figure 1: Fe$^{2+}$ and Fe$^{3+}$ chelating activity of phosvitin with 10 ppm Fe at pH 6.8 in Tris-malate buffer.

(This table is repeating with the graph): Fe$^{2+}$- and Fe$^{3+}$-chelating activity of phosvitin at 10 ppm of Fe level and at pH 6.8 (Tris-Malate buffer).

<table>
<thead>
<tr>
<th>Phosvitin (mg/ml)</th>
<th>Fe$^{2+}$</th>
<th>Fe$^{3+}$</th>
<th>SEM</th>
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</table>

SEM 1.30  1.08

<sup>a-b</sup> Means with different superscript letters in rows differ significantly (p<0.05, n=4).

<sup>a-z</sup> Means with different superscript letters columns differ significantly (p<0.05, n=4).
Figure 2: Fe$^{3+}$-chelating activity of phosvitin and its hydrolysates of Multifect® P-3000, alkalase and thermolysin and hydrolysates of pre-treated (100 °C, 60 min) phosvitin at pH 6.8 (Tris-malate buffer).

a-d Treatments with different letters are significantly different (P<0.05, n=4, SEM=0.7915).

Abbreviations: PV-natural phosvitin; P-3000- Multifect® P-3000 hydrolysate of phosvitin; Alk- alkalase hydrolysate of phosvitin; Thermo- thermolysin hydrolysate of phosvitin; Pt-P-3000- Multifect® P-3000 hydrolysate of pre-treated phosvitin, Pt-Alk- alkalase hydrolysate of pre-treated phosvitin; Pt-Thermo- thermolysin hydrolysate of pre-treated phosvitin.
Figure 3: Cu$^{2+}$-chelating activity of phosvitin.

Treatments with different letters are significantly different (p<0.05, n=4, SEM = 0.252).
Figure 4: The Cu^{2+}-chelating activity of phosvitin and its hydrolysates of (P-3000) Multifect® P-3000, alkalase and thermolysin, and hydrolysates of pre-treated (100 °C, 60 min) phosvitin at pH 6.8 (Tris-malate buffer).

\[ a-d \text{Treatments with different letters are significantly different (p}<0.05, n= 4, \text{ SEM}= 0.6485) \]

Abbreviations: PV- natural phosvitin; P-3000- Multifect®P-3000 hydrolysate of phosvitin; Alk-alkalase hydrolysate of phosvitin; Thermo- thermolysin hydrolysate of phosvitin; Pt-P-3000- Multifect® P-3000 hydrolysate of pre-treated phosvitin, Pt-Alk- alkalase hydrolysate of pre-treated phosvitin ; Pt-Thermo- thermolysin hydrolysate of pre-treated phosvitin.
Figure 5: Ca$^{2+}$-solubilizing activity of phosvitin. Reduction of Ca$^{2+}$ in the supernatant of the assay is given compared to the control.

Treatments with different letters are significantly different (p < 0.05, n=4, SEM=0.0563).
Figure 6: Ca\(^{2+}\)-solubilizing activity of phosvitin and its different hydrolysates of (P-3000) Multifect ® P-3000, alkalase and thermolysin, and hydrolysates of pre-treated (100 °C, 60 min) phosvitin at pH 6.8 (Tris-malate buffer) compared to the control.

\(^{a-d}\) Treatments with different letters are significantly different (p<0.05, n= 4, SEM= 0.3114).

Abbreviations: PV, natural phosvitin; P-3000, P-3000 hydrolysate of phosvitin; Alk, alkalase hydrolysate of phosvitin; Thermo, thermolysin hydrolysate of phosvitin; Pt-P-3000, P-3000 hydrolysate of pre-treated phosvitin, Pt-Alk, alkalase hydrolysate of pre-treated phosvitin; Pt-Thermo, thermolysin hydrolysate of pre-treated phosvitin.
CHAPTER 5. CHEMICAL HYDROLYSIS OF PHOSVITIN AND THE FUNCTIONAL PROPERTIES OF THE HYDROLYSATES

Himali Samarakweera, Eun Joo Lee, and Dong U. Ahn

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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. Thus, the chemical hydrolysis of phosvitin was evaluated and the functionalities of chemical hydrolysates were investigated. Phosvitin showed higher stability to acid hydrolysis than alkaline hydrolysis. Incubation of phosvitin in 0.075 N NaOH solution at 37 °C for 1 h and 2 N HCl for 6 h incubation at 60 °C partially hydrolyzed phosvitin, but 0.1 N NaOH at 37 °C for 3 h or 3 N HCl at 60 °C for 6 h incubation was needed for the complete hydrolysis of phosvitin. Increasing degree of hydrolysis in phosvitin either by HCl or NaOH negatively affected their antioxidant and Fe-chelating capacities. Cu^{2+}-chelating activity, however, increased significantly by hydrolyzing phosvitin with 0.1 N NaOH, 2N HCl, or trypsin after 0.05 N NaOH treatment. The acid hydrolysates of 3 N and 6 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), and thus, 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). It has been suggested to use phosvitin as a substrate for producing metal-binding functional peptides (Jiang & Mine, 2000; Katayama, Xu, Fan, & Mine, 2006; Xu, Katayama & Mine, 2007). However, due to its highly phosphorylated nature, it 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, high pressure alone or with heat treatments, have been tested only with slight improvement in enzymatic digestibility. Our previous work indicated that phosvitin is subjected to alkaline hydrolysis at high pH conditions (Samaraweera, Lee, & Ahn, 2011, unpublished data). It is reported that phosphate in phosphoserine is easily dephosphorylated in alkaline conditions (Kellnar, Lottspeich & Meyer, 2008), and Jiang & Mine (2000) have used NaOH in order to achieve dephosphorylation and thereby increase the digestibility of phosvitin.

Protein hydrolysates can be produced by either chemically or by means of enzymes (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 hydrolysates of proteins are extensively used to investigate the amino acid composition of proteins. For that usually 6 N HCl at 110 °C for 6 h is used (Fountoulakis & Lahm, 1998). Under these conditions, proteins are mainly 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 hydrolysis can 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

2.1 Alkaline hydrolysis of phosvitin

Phosvitin was prepared according the procedure of Ko, Nam, Jo, Lee, & Ahn (2011). Phosvitin was dissolved in 0.025 N, 0.05 N, 0.075 N, 0.1 N, 0.2 N and 0.3 N NaOH (25 mg/ml) and incubated in a shaker water bath (C7- New Bruwanswick Scientific, Edison, NJ, USA) at 37 °C for 1 h, 2 h and 3 h. After incubation, the samples were neutralized with 2 N HCl, and the size of peptides was determined using SDS-PAGE.

2.2 Acid hydrolysis of phosvitin

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

2.3 SDS-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 Hydrolysates

In order to measure the functional properties of alkaline hydrolysed phosvitin at 0.05 N & 0.1 N at 37 °C for 3 h and trypsin hydrolysate (pH - 8.0, temperature 37 °C for 6 h, S:E=100:1) of 0.05 N NaOH-treated phosvitin were selected. To determine the functional properties of acid hydrolysates of phosvitin, the samples hydrolyzed in 2 N, 3 N and 6 N HCl solutions at 60 °C for 6 h were selected.

2.5 Antioxidant activity

2.5.1 Preparation of oil emulsion samples

An oil-in-water emulsion containing 1.0 g of 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 homogenizing them 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. The fatty acid composition of corn oil and the presence of tocopherol were measured using a gas chromatography/mass spectrometry (GC/MS; Hewlett-Packard Co., Wilmington, DE, USA).

2.5.2 Lipid oxidation assay

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 (2 mg) in a 50-ml test tube. After vortex-mixing, the mixture was incubated at 37 °C for 16 h. One ml of the mixture was withdrawn to determine 2-thiobarbituric acid-reactive substances (TBARS) value at different time durations of incubation.

2.5.3 Determination of 2-thiobarbituric acid reactive substances (TBARS) value

One ml of oil emulsion assay was mixed with 2 ml of thiobarbituric acid/trichloroacetic acid solution (20 mM TBA/15 % TCA, w/v) in a disposable test tube (13 x 100 mm), and 50 µl of 10 % butylated hydroxyanisole 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 activity

The Fe-chelating activity of alkaline and acid hydrolysates of phosvitin was evaluated by using the Ferrozine method (Carter, 1971) with some modifications. Hydrolysates (0.5 mg) in Tris–maleate buffer (pH 6.8) (100 µl) was vortex-mixed with 1 ml of 10 ppm Fe and 1 ml of 0.1 % (wt/vol) ascorbic acid in 2 N 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) and 0.2 ml of Ferroin color reagent (75 mg of ferrzoine, 75 mg of neocuproin and 1 drop of 6 N HCl 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 hydrolysates):

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\text{Fe-chelating activity (\%) = \{1-(sample solution absorbance/blank solution absorbance)\} x 100}
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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 & Xiong (2006). One ml of 0.2 mM CuSO\(_4\) was mixed with 1 ml of phosvitin/hydrolysates (2 mg) in Tris–maleate buffer (pH 6.8). After that, 1 ml 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), vortex-mixed, incubated at room temperature for 5 min, and then the 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)\} x 100

2.8 Ca$^{2+}$-solubilizing activity

Ca-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 (C7- New Brunswick 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 GmbH). Ca$^{2+}$ solubilizing activity was expressed as a percentage of available Ca$^{2+}$ in the supernatant compared to the control.

2.9 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, Babiker, Azakami, & Kato (1998) evaluated the emulsifying properties of phosvitin treated with 0.25 N NaOH at 35 °C for 24 h and found significant reduction of its emulsifying properties compared to the native phosvitin. Jiang & Mine (2000) dissolved phosvitin in 0.1-0.4 N NaOH solution and incubated at 37 °C up to 4 h 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, implying high degree of hydrolysis. However, they have not evaluated the nature of phosvitin after dephosphorylation with NaOH using SDS-PAGE or any other means. Our previous study indicated that at pH 13, phosvitin underwent
alkaline hydrolysis at temperatures between 70 to 85 °C, implying decomposition of protein (Samaraweera, Lee, and Ahn, 2011a, unpublished data). When phosvitin was treated with low concentrations of NaOH (0.025 N and 0.05 N NaOH) at 37 °C for up to 3 h, only minor changes in phosvitin molecules were observed. However, these treatments produced a few bands not observed in untreated phosvitin sample (Figure 1). With 0.075 N NaOH treatment, most of the phosvitin major band disappeared and resulted in continuous streaking band patterns on SDS-PAGE (Figure 2). This implies that treating phosvitin with 0.075 N NaOH at 37 °C is effective conditions for alkaline hydrolysis. However, 0.1 N NaOH was more effective than 0.075 N NaOH and resulted in almost complete hydrolysis of phosvitin after 3 h at 37 °C. At 0.2 N and 0.3 N NaOH levels, phosvitin bands completely disappeared after 1 h incubation at 37 °C (Figure 3). Therefore, we suggest treating phosvitin in 0.1 N NaOH at 37 °C for 3 h or 0.2 N NaOH at 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 in phosphoserine (> 50 % of amino acids of phosvitin are phosphoserine). Phosphoserine is known to be highly unstable and can be totally destroyed under alkaline conditions (Kellnar, Lottspeich & Meyer, 2008; Pasupuleti, & Braun, 2010).

Also, other amino acids in protein experience racemization in alkaline conditions (Sen, Gonzalez-Flores, Feeney, & John, 1977). Therefore, the alkaline dephosphorylation of phosvitin referred in previous literatures (Sen, Gonzalez-Flores, Feeney, & John (1977); Jiang & Mine, 2000) may not be only the dephosphorylation, but also alkaline hydrolysis of the protein. 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, Gonzalez-Flores, Feeney, & John (1977) treated phosvitin at 0.123 N NaOH at 60 °C for 80 min and observed 87 % removal of phosphate due to β-elimination and 13 % due to hydrolysis. However, our results indicated that treating phosvitin in 0.123 N NaOH at 60 °C for 80 min not only would dephosphorylate but also hydrolyze phosvitin molecule.

Hydrolysis of phosvitin in 0.1 N NaOH at 37 °C for 3 h was selected to examine the antioxidant and mineral binding activities as a mild alkaline hydrolysis of phosvitin even though 0.2 N and 0.3 N at 37 °C resulted better hydrolysis than 0.1 N NaOH at 37 °C for 3 h.
3.2 Acid hydrolysis of phosvitin

According to the SDS-PAGE band pattern, treating phosvitin in 0.05 N, 0.1N and 0.15 N HCl at 37 °C for up to 3 h had no effect on the hydrolysis of phosvitin (Figure 4). Also, treating phosvitin in 2 N, 3 N and 6N at 37 °C for 3 h had no effect on the hydrolysis of phosvitin (data not shown). This high stability of phosvitin in acidic conditions could be related to the formation of high % β-structure in phosvitin (Chang, Wu & Yang, 1978; Grizzuti & Perlmann, 1970). At 2 N, 3 N, and 6 N HCl concentrations, however, phosvitin was hydrolyzed at higher temperature conditions, and 2 N, 3 N and 6 N HCl at 60 °C for 6 h was selected for acid hydrolysis conditions for phosvitin (Figure 5). Crowder, Numan, Haddadian, Matthew, & Neil (1999) hydrolyzed phosvitin in 2 N HCl at 110 -130 °C for various time duration and observed that at 12 h incubation was the optimal time for the hydrolysis and liberation of phosphoserine from phosvitin. The acid hydrolysis of protein at 6 N HCl can destroy tryptophan, serine and threonine due to oxidation, and results in slow hydrolysis of some peptide bonds between bulky and sterically hindered amino acids (Simpson, Neuberger, & Liu,1976; Liu & Chang, 1971). Lee, Veis, & Glonek (1977) observed loss of serine and phosphoserine during acid hydrolysis of phosphoproteins.

The SDS-PAGE band of the phosvitin hydrolysate from 2 N HCl, 60 °C and 6 h treatment showed continuous streaking pattern from phosvitin to the dye line indicating that a significant portion of phosvitin had been hydrolyzed. Treating phosvitin in 3 N or 6 N HCl at 60 °C for 6 h resulted in higher degree of degradation with more streaking patterns shown close to the bottom of the SDS-PAGE gel (Figure 5). This could be due to the hydrolysis of phosvitin into small peptides or in to amino acids. Therefore, as means of obtaining smaller peptides from phosvitin, its hydrolysates of 2 N, 3 N and 6 N HCl, 60 °C and 6 h treatments were selected to determine their antioxidant and mineral binding activities.

3.3 Antioxidant activity of phosvitin hydrolysates

The composition of corn oil was myristic acid 0.1 %, palmitic acid 12.5 %, palmitoleic acid 0.1 %, margaric acid 0.1 %, margaroleic acid 0.1 %, stearic acid 1.7 %, oleic acid 29.0 %, linoleic acid 54.4 %, linolenic acid 1.1 %, arachidic acid 0.4 %, arachidonic acid 0.1 %, eicosapentaenoic acid 0.2 %, and docosapentaenoic acid 0.2 %. The native 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 Fe$^{3+}$ irons binding capacity of phosvitin molecule. The antioxidant activity of phosvitin treated with 0.05 N NaOH at 37 °C for 3 h and its trypsin hydrolysate was not significantly different from that of the native phosvitin (Table 1). The SDS-PAGE band patterns of trypsin hydrolysates of native phosvitin and the trypsin hydrolysate of 0.05 N NaOH, 37 °C and 3 h-treated phosvitin also were not different (Figure 6). This implies that at low normality of NaOH (0.05 N NaOH), phosvitin remained intact and NaOH did not cause significant dephosphorylation from the phosvitin molecule. The phosvitin concentration used for all the treatments was 0.2 mg/ml. At this level, phosvitin treated with 0.05 N NaOH at 37 °C for 3 h and its trypsin hydrolysate showed similar antioxidant activity to BHA in oil emulsion system (Table 1). The phosvitin hydrolysates from 0.1 N NaOH treatment showed similar antioxidant activity to native phosvitin and 0.05 N NaOH treatments until 4 h incubation period. After 4 h incubation, however, its 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 hydrolysed phosvitin exhibited significantly lower antioxidant activity than the native phosvitin implying impaired antioxidant activity of phosvitin after acid hydrolysis. With increasing normality 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 agent in the presence of metal catalysts, due to its capacity to chelate metal ions (Lu & Baker, 1986; Lee, Han & Decker, 2002).

Fadil, Babiker, Fujisawa, Matsudomi & Kat (1996) treated gluten in 0.05 N HCl at 120 °C for 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, Miyazaki, Kawamoto, Kobayashi (1987) evaluated the emulsifying properties of phosvitin after an alkaline treatment (0.25 N NaOH at 37 °C for 24 h to remove phosphate molecules completely) and observed that the emulsifying properties of phosvitin has been greatly decreased. Xu, Katayama & Mine, (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 3 h. 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 phosvitin hydrolysates

Intact phosvitin showed the highest and significantly higher (p<0.05) Fe-chelating activity than the acid & alkaline treated phosvitin and trypsin hydrolysate of 0.05 N NaOH-treated phosvitin (Figure 7). However, the Fe-chelating activities of phosvitin hydrolysates from 0.05 N NaOH-37 °C-3 h treatment and its trypsin hydrolysates, 0.1 N NaOH-37 °C-3 h treatment, and 2 N HCl-60 °C-6 h treatment showed high levels of iron-binding capacity, even though their activities were lower than the natural phosvitin, indicating that they can be used as Fe-carrying functional peptides. In the previous study, hydrolysates from alkalase and Multifect® P-3000 treatments showed lower Fe-binding capacity than that of the native phosvitin (Samaraweera, Lee and Ahn 2011b, unpublished data). Natural phosvitin has a high affinity to Fe ions and tends to form insoluble complexes with Fe (Taborsky, 1963; Grizzuti & Perlmann, 1973; Castellani, Guérin-Dubiard, David-Briand, & Anton, 2004). Thus, lower Fe-binding capacity of phosvitin hydrolysates than 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 problem with phosvitin and may play a significant role in the production of mineral carrying and solubilizing functional peptides. Acid hydrolysates of phosvitin drastically reduced the Fe-chelating activity of the peptides. The phosvitin hydrolysate with 2 N HCl showed comparatively high Fe-chelating activity, implying that hydrolysis of phosvitin with 2 N 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 cause skin hyperpigmentation, arthritis, arthralgias, hypogonadotropic hypogonadism, diabetes mellitus, liver fibrosis, cirrhosis, hepatocellular cancer, and cardiac problem (Papanikolaou &
Pantopoulos, 2005). The acid hydrolysates of phosvitin obtained from 3 N and 6 N HCl treatments 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 3 N and 6 N HCl are not appropriate ways to produce hydrolysates with high levels of Fe-binding activity even though those treatments produced smaller molecular weight peptides (Figure 6). 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 iron carrying agent is still open and further studies on the functional characteristics of the peptides generated are needed.

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). Other studies showed that acid modification of some proteins by deamidation and increasing the functional properties such as solubility, emulsifying and forming activities of the proteins (Chan and Ma, 1999). Nevertheless, acid or alkali hydrolysis can be an approach to produce functional peptides from phosvitin.

3.5 Cu²⁺-chelating activity of phosvitin hydrolysates

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 (Grizzuti & Perlmann, 1973). Lu & Baker (1987) reported that the Cu²⁺-chelating capacity of phosvitin at pH 6.1 is 1:1 ratio, but our previous studies indicated that the Cu²⁺-chelating capacity of phosvitin was 3.8 Cu molecules per phosvitin molecule (Samaraweera, Lee, Ahn, 2011b, unpublished data). In comparison to Mn²⁺- and Ca²⁺-binding activities of phosvitin, the Cu²⁺ chelating activity of phosvitin is considerably low (Grizzuti and Perlmann, 1973). The Cu²⁺-binding activity of phosvitin and its hydrolysates in this study showed different binding capacities. Current study showed that native phosvitin and phosvitin hydrolysates from 0.05 N NaOH treatment have similar Cu²⁺-chelating activity while the phosvitin hydrolysates from 0.1 N NaOH, 2 N HCl, and 0.05 N
NaOH-trypsin treatments showed significantly higher Cu$^{2+}$-binding activity than the native phosvitin (Figure 8). This observation is different from the Fe-binding activity of phosvitin and phosvitin hydrolysates (Figure 7). 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.

**3.6 Ca$^{2+}$-solubilizing activity of phosvitin hydrolysates**

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, Tamaki, Arihara, & Itoh, 2007). However, Choi, Jung, Choi, Kim, & Ha (2005) reported controversial fact showing that the availability of Ca ions has been increased by 44.1 % at pH 7.0, 37 °C in the presence of 1.0 % phosvitin. Our previous study on Ca$^{2+}$-binding capacity of phosvitin indicated that the Ca-binding capacity of phosvitin decreased and come to a plateau as the levels of phosvitin increased. In addition, the solubility of Ca significantly increased as the degree of enzymatic hydrolysis of phosvitin increased (Samaraweera, Lee, & Ahn 2011b, Unpublished data). Trypsin-digested peptides from partially dephosphorylated phosvitin has been evaluated for Ca-binding and solubilizing activities by Jiang and Mine (2001). They found that the peptides retaining 35 % of phosphate were more effective in binding Ca and inhibiting formation of insoluble calcium phosphate than phosphopeptides with 65 % and 17.5 % of retaining phosphate. Choi et al. (2005) reported that the Ca intake, fecal Ca content or urinary Ca 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 they observed that phosvitin phosphopeptides increased Ca absorption and Ca accumulation in the bones significantly.

The Ca$^{2+}$-solubility of phosvitin hydrolysates from 0.05 N NaOH-treatment and 0.05 N NaOH+trypsin hydrolysis was not significantly different from that of the natural phosvitin. This implies that treatment of phosvitin at 0.05 N NaOH at 37 °C for 3 h did not hydrolyze the phosvitin. It is known that trypsin hydrolysis results in a few large peptides and those peptides were unable to increase the solubility of Ca$^{2+}$ (Figure 6 & 9). The phosvitin
hydrolysate obtained from 0.1 N NaOH treatment had higher Ca\(^{2+}\) solubility than those of the native phosvitin or 0.05 N treated phosvitin, but the level of increase was not significant (Figure 9). The increased Ca solubility observed in some of the phosvitin hydrolysates could be due to dephosphorylation of phosvitin and hydrolysis of phosvitin into smaller peptides. Interestingly all three acid hydrolysates of phosvitin showed significantly increased Ca\(^{2+}\)-solubility compared to the native phosvitin and its alkaline hydrolysates (Figure 9). The SDS-PAGE pattern of the 3 N- and 6 N HCl-treated phosvitin showed a streaking pattern at the bottom of the gel, implying the present of very small peptides and 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 increase Ca solubility. In addition, after the acid hydrolysis the hydrolysates were neutralized with NaOH. Hence, the hydrolysates contained high levels of NaCl, and which increases as the normality of HCl to hydrolyze phosvitin increases. It is uncertain that the amount of NaCl present in the solution had any effect on Ca\(^{2+}\) solubility.

However, it is known that the chemical hydrolysis of protein is difficult to be controlled and known to produce various by-products with different functionalities. It also can reduce the nutritional qualities and affect negatively to the functional properties of proteins (Kristinsson & Rasco, 2000).

Further study to determine the size and to characterize the peptides in those phosvitin hydrolysates 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.

4. Conclusions

Phosvitin shows higher stability in acid than in alkaline conditions. Increased degree of phosvitin hydrolysis using either acid or alkali negatively affected on their antioxidant capacity. Iron-binding and Cu\(^{2+}\)-binding activities of acid and alkaline hydrolysates also showed similar behavior, implying that with increased hydrolysis the metal binding capacity of phosvitin is reduced. Acid hydrolysis of phosvitin showed significantly improvement of Ca\(^{2+}\) solubility. This implies the possibility of using acid hydrolysates of phosvitin as
carriers for minerals. However, further studies have to be implemented in order to discover the real effect.

References


Figure 1: SDS-PAGE band pattern of phosvitin and alkaline hydrolysates of phosvitin. Lane 1 - Native phosvitin; lane 2 - 0.025 N NaOH-treated phosvitin at 37 °C for 1 h; lane 3 - 0.025 N NaOH-treated phosvitin at 37 °C for 2 h; lane 4 - 0.025 N NaOH-treated phosvitin at 37 °C for 3 h; lane 5 - 0.05 N NaOH-treated phosvitin at 37 °C for 1 h; lane 6 - 0.05 N NaOH-treated phosvitin at 37 °C for 2 h; lane 7 - 0.05 N NaOH-treated phosvitin at 37 °C for 3 h; lane 8 – Molecular Marker.
Figure 2: SDS-PAGE band pattern of phosvitin and alkaline hydrolysates of phosvitin. Lane 1 - Native phosvitin; lane 2 - 0.075 N NaOH-treated phosvitin at 37 °C for 1 h; lane 3 - 0.075 N NaOH-treated phosvitin at 37 °C for 2 h; lane 4 - 0.075 N NaOH-treated phosvitin at 37 °C for 3 h; lane 5 - 0.1 N NaOH-treated phosvitin at 37 °C for 1 h; lane 6 - 0.1 N NaOH-treated phosvitin at 37 °C for 2 h; lane 7 - 0.1 N NaOH-treated phosvitin at 37 °C for 3 h.
Figure 3: SDS-PAGE band pattern of phosvitin and alkaline hydrolysates of phosvitin. Lane 1-Native phosvitin; lane 2-0.1 N NaOH-treated phosvitin at 37 °C for 1 h; lane 3 - 0.1 N NaOH-treated phosvitin at 37 °C for 2 h; lane 4 – 0.1 N NaOH-treated phosvitin at 37 °C for 3 h; lane 5 - 0.2 N NaOH-treated phosvitin at 37 °C for 1 h; lane 6 - 0.2 N NaOH-treated phosvitin at 37 °C for 2 h; lane 7 - 0.2 N NaOH-treated phosvitin at 37 °C for 3 h; lane 8 - 0.3 N NaOH-treated phosvitin at 37 °C for 1 h; lane 9 - 0.3 N NaOH-treated phosvitin at 37 °C for 2 h; lane 10 -0.3 N NaOH-treated phosvitin at 37 °C for 3 h.
Figure 4: SDS-PAGE band pattern of phosvitin and acid hydrolysates of phosvitin. Lane 1 - Native phosvitin; lane 2-0.05 N HCl-treated phosvitin at 37 °C for 1 h; lane 3 - 0.05 N HCl-treated phosvitin at 37 °C for 2 h; lane 4 – 0.05 N HCl-treated phosvitin at 37 °C for 3 h; lane 5- 0.1 N HCl-treated phosvitin at 37 °C for 1 h; lane 6 - 0.1 N HCl-treated phosvitin at 37 °C for 2 h; lane 7- 0.1 N HCl-treated phosvitin at 37 °C for 3 h; lane 8 - 0.15 N HCl-treated phosvitin at 37 °C for 1 h; lane 9 - 0.15 N HCl-treated phosvitin at 37 °C for 2 h; lane 10 - 0.15 N HCl-treated phosvitin at 37 °C for 3 h.
Figure 5: SDS-PAGE band pattern of phosvitin and acid hydrolysed phosvitin. Lane 1 - Native phosvitin; lane 2 - 2 N HCl-treated phosvitin at 60 °C for 6 h (R1); lane 3 – 2 N HCl-treated phosvitin at 60 °C for 6 h (R2); lane 4 – 3 N HCl-treated phosvitin at 60 °C for 6 h (R1); lane 5 – 3 N HCl-treated phosvitin at 60 °C for 6 h (R1); lane 6 – 6 N HCl-treated phosvitin at 60 °C for 6 h (R1); lane 7 – 6 N HCl-treated phosvitin at 60 °C for 6 h (R2); lane 8 – MM.
Figure 6: SDS-PAGE band pattern of phosvitin and its hydrolysates with different treatments. Lane 1- Native phosvitin; lane 2- Trypsin hydrolysate of phosvitin (6 h); lane 3-0.05 N NaOH-treated phosvitin at 37 °C for 3 h; lane 4- Trypsin hydrolysate of 0.05 N NaOH-treated phosvitin at 37 °C for 3 h, lane 5- 0.1 N NaOH-treated phosvitin at 37 °C for 3 h; lane 6- 2 N HCl-treated phosvitin at 60 °C for 6 h; lane 7- 3 N HCl-treated phosvitin at 60 °C for 6 h; lane 8 – 6 N HCl-treated phosvitin at 60 °C for 6 h; lane 9- MM.
Table 1: TBARS values of oil emulsion (mg MDA/L) in the presence of phosvitin, its hydrolysates, and BHA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>0h</th>
<th>1h</th>
<th>2h</th>
<th>4h</th>
<th>8h</th>
<th>16h</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1059</td>
<td>0.1949</td>
<td>0.2569</td>
<td>0.3920</td>
<td>0.5763</td>
<td>1.5442</td>
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<td>PV</td>
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<td>0.1410</td>
<td>0.1520</td>
<td>0.1984</td>
<td>0.2121</td>
<td>0.2766</td>
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<tr>
<td>0.05 N NaOH-Pv</td>
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<td>0.1771</td>
<td>0.1885</td>
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<td>0.2929</td>
<td>0.0153</td>
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<tr>
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<td>0.1207</td>
<td>0.1419</td>
<td>0.2520</td>
<td>0.4653</td>
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<tr>
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<td>0.1662</td>
<td>0.2891</td>
<td>0.4956</td>
<td>0.7260</td>
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<td>0.0535</td>
</tr>
<tr>
<td>3 N HCl-Pv</td>
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<td>0.2738</td>
<td>0.3364</td>
<td>0.5731</td>
<td>0.8593</td>
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<tr>
<td>6 N HCl-Pv</td>
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<td>0.2772</td>
<td>0.3344</td>
<td>0.5605</td>
<td>0.8515</td>
<td>1.6671</td>
<td>0.0267</td>
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<tr>
<td>Trypsin hy. of 0.05 N NaOH-Pv</td>
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<td>0.2187</td>
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<td>BHA</td>
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<td>0.2387</td>
<td>0.2122</td>
<td>0.2759</td>
<td>0.3152</td>
<td>0.4266</td>
<td>0.0032</td>
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<tr>
<td>SEM</td>
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<td>0.0085</td>
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<td>0.0320</td>
<td>0.0283</td>
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</tr>
</tbody>
</table>

Means in the same raw with different superscript letters (a-f) differ significantly (p<0.05, n=4).
Means in the same column with different superscript letters (v-z) differ significantly (p<0.05, n=4).
The oil emulsion was added with 10 ppm ionic iron.

Abbreviations: 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; Trypsin hy. of 0.05 N NaOH- Pv - Trypsin hydrolysate of phosvitin treated with 0.05 N NaOH at 37 °C for 3 h; butylatedhydroxyanisole; SEM - Standard error of the mean.
Figure 7: Fe$^{3+}$-binding activity of phosvitin, alkaline & acid hydrolysates of phosvitin, and trypsin digest of phosvitin treated with 0.05 N NaOH at 37 °C for 3 h.

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 0.05 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; Trypsin hy. of 0.05 N NaOH-Pv - trypsin hydrolysate of phosvitin treated with 0.05 N NaOH at 37 °C for 3 h; SEM - Standard error of the mean.
Figure 8: Cu$^{2+}$ binding activity of phosvitin, its alkaline & acid hydrolysates, trypsin digested of phosvitin treated with 0.05 N NaOH at 37 °C for 3 h.

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 0.05 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; Trypsin hy. of 0.05 N NaOH-Pv - trypsin hydrolysate of phosvitin treated with 0.05 N NaOH at 37 °C for 3 h; SEM - Standard error of the mean.
Figure 9: Ca^{12+}-solubilizing activity of phosvitin, its alkaline & acid hydrolysates, and trypsin digest of phosvitin treated with 0.05 N NaOH at 37 °C for 3h.

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 0.05 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 6h; Trypsin hy. of 0.05 N NaOH-Pv - Trypsin hydrolysate of phosvitin treated with 0.05 N NaOH at 37 °C for 3 h, SEM - Standard error of the mean.
CHAPTER 6. CHARACTERIZATION OF PHOSVITIN PHOSPHOPEPTIDES BY
MASS SPECTROMETRY
Himali Samaraweera, Derrick Morsat, Eun Joo Lee, Jenifer Grant, R. S. Houk and Dong U. Ahn

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Abstract

The present study identifies and characterizes phosphopeptides obtained from egg yolk phosvitin by mass spectrometry in order to ascertain their applications in the field of bioactive/functional peptide production. Three differently treated enzymatic hydrolysates were used: trypsin, α-chymotrypsin digest of 0.3 N NaOH-treated phosvitin for 3 h at 37 °C and Enzyme Multifect® P-3000 hydrolysate of phosphatase alkaline treated (24h) phosvitin. Peptides were identified either by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) or matrix-assisted laser desorption ionization-mass spectrometry (MALDI-QTOF). Trypsin hydrolysate of phosvitin resulted in peptide sequences of RSSSSSSSSSSSSR (1PO₄) (residues 82-95), SSSSSSSSSSSSR (5PO₄) (residues 83-95), SSSSSKSSSSSSSR (12PO₄) (residues 108-121) along with N- and C-terminal peptides. Enzyme Multifect® P-3000 hydrolysate of phosphatase alkaline-treated phosvitin resulted in AEFGTEPDAKTSSSSSSASST (residues 1-21), SSKSSNSSK (1PO₄) (residues 62-72), SSSSSSSSSSSSR (5PO₄) (residues 83-95), DDSSSSSSSSV (residues 195-206), and DDSSSSSSSSSVLS (residues 195-208). These findings suggest the possibility of using those enzymatic hydrolysates for metal chelating/antioxidant bioactive peptides or functional peptides production.

Key words: Phosvitin, phosphopeptides, mass spectrometry, amino acid sequencing
1. Introduction

Phosvitin is the major glycophosphoprotein in egg yolk, which accounts for 60 % of the total phosphoproteins and holds about 90 % of the total egg yolk phosphorous (Taborsky & Mok, 1967). Egg yolk phosphoproteins consist of phosvitin and phosvettes (Wallace, 1985). The hepatically-derived macromolecular lipophosphoprotein vitellogenin in non-mammalian vertebrates serves as the precursor for lipovitellin and phosvitin. The molecular weights of minor and major phosvitin were reported (Taborsky & Mok, 1967) as 36,000 and 40,000 Da. Phosvitin is a heterogeneous protein and is reported to be composed of seven components. Two major components of phosvitin account for 80 and 15 % respectively (Culbert and McIndoe, 1971). Phosvitin with a molecular weight of 40,000 Da contains 6.5 % carbohydrates, consisting of 6 hexose, 5 glucosamine and 2 sialic acids attached to the N-acetyl derivatives (Shainkin & Perlmann, 1971; Brockbank & Vogel, 1990). A detailed analysis of the amino acid sequence has indicated that phosvitin is composed of 217 amino acids with 123 serine, 15 lysine, 13, histidine, and 11 arginine residues. Serine is the major amino acid, which accounts for more than 55 % of the total amino acids of phosvitin (Byrne, van Het, van de Klundert, Arnberg, Gruber, & Geert, 1984). Almost all serine molecules are supposed to be phosphorylated. Phosphorylation is one of the major post transitional modifications of proteins. In vertebrates, 89.96 % of phosphorylation occurs in serine and 9.99 % occurs in threonine (Mann, Grønborg, Steen, Jensen & Pandey, 2002; Cozzone, 1988; Jensen, 2006).

Amino acid sequencing and characterization of molecular structures play an important role in proteomics. With the advances of mass spectrometry (MS), traditional techniques of amino acid sequencing and characterization of molecules have been replaced. The high sensitivity, resolution and mass accuracy have resulted in tandem mass spectrometry (MS/MS) to be commonly used in proteomics, especially in phosphopeptide analyses (Griffin, Goodlett, & Aebersold, 2001; Mann et al., 2002). Commonly used ionization methods for MS in proteomics are electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). The fragmentation of proteins/peptides is accomplished by various techniques based on either activation through collision-induced
dissociation (CID) or electron capture/transfer dissociation (ECD/ETD) (Tipton, Tran, Catherman, Ahlf, Durbin, & Kelleher, 2011; Aebersold, & Mann, 2003). However, investigation of phosphopeptides by MS/MS is considered one of the most challenging tasks (Mann et al., 2002; Jensen, 2006) due to low profusion of phosphoproteins, low stoichiometry of phosphorylation, ineffective ionization of phosphopeptides, and burden of the phosphate group in CID (Mann et al., 2002; Boersema, Mohammed, Heck, 2009). The key factor of successful proteomics with MS/MS is the ability to generate meaningful detailed ion mass spectra from the peptide fragments (Aebersold, & Mann, 2003). The ionization method implemented and the characteristics of the parent ion subjected to dissociation highly affect the clarity of identification and characterization of phosphopeptides (Smith, Kalcic, Safran, Stemmer, Dantus, & Reid, 2010).

The mass of an amino acid sequence is altered by a covalent modification and hence the change of mass is normally adequate to discover the modification (Yates, Eng, McCormack, & Schietz, 1995). Collision-induced dissociation is frequently used for the fragmentation of molecules in MS/MS by gas-phase β-elimination reactions. Dissociation of phosphorylated peptides results in characteristic loss of phosphoric acid (98 Da) or loss of a phosphate molecule (80 Da) (Mann et al., 2002; Shi, Bajrami, Morton, & Yao, 2008).

In our previous work phosvitin has been evaluated in order to produce functional phosphopeptides with antioxidant and mineral binding activities. Both enzymatically and chemically derived hydrolysates have been evaluated (Samaraweera, Lee, & Ahn, 2011bc, unpublished data). Identification and characterization of peptides is needed to explore their functional characteristics. Thus the present study was conducted with the objective of identification and characterization of phosphopeptides derived from phosvitin hydrolysates by using MS/MS. Many hydrolysates produced from different treatments of phosvitin have been subjected to MS/MS analysis in order to discover the structural information of peptides. Thus, more results are yet to be analyzed. The present study discusses the MS/MS work related to hydrolysates derived from three selected treatments.
2. Materials and Methods

Phosvitin used in this study was extracted according to the method of Ko, Nam, Jo, Lee, & Ahn (2011). Trypsin-Type I and α-chymotrypsin-Type II from bovine pancreas was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Phosphatase Alkaline from bovine intestinal mucosa was from Sigma. Enzyme Multifect® P-3000 was a gift from Genencor International Inc. (New York, NY, USA). Acetonitrile (ACN), HPLC grade water and formic acid were purchased from Sigma.

The following enzymatically treated phosvitin and chemically treated phosvitin samples were selected for MSMS: trypsin hydrolysate of phosvitin, α-chymotrypsin hydrolysate of 0.3 N NaOH treated phosvitin, and Multifect® P-3000 hydrolysate of dephosphorylated phosvitin.

2.1 Trypsin hydrolysate of phosvitin

Phosvitin (10 mg/ml) was dissolved in distilled water and the pH was adjusted to 2 using 1M HCl, prior to addition of the enzymes. The substrate: enzyme ratio was 100:1 (w/w). The enzymatic digestion was carried out at 37 °C for 6 h, 24 h, and 48 h in a shaker water bath (C7- New Brunswick Scientific, Edison, NJ, USA). The enzymatic digestion was stopped by keeping the sample for 10 min in a boiling water bath. The resulting hydrolysates were lyophilized.

2.1.1 Multifect® P-3000 enzymatic digestion of dephosphorylated phosvitin

Phosvitin (10 mg/ml) was dissolved in 100 mM sodium phosphate buffer (pH 10) and incubated at 37 °C for 24 h with the enzyme alkaline phosphatase (E:S- 1:25) in a shaker water bath. The alkaline phosphatase was inactivated by keeping the sample for 10 min in a boiling water bath. After enzymatic dephosphorylation, the samples were dialyzed in double distilled water (4 times water exchange) for 24 h at 4 °C and then lyophilized. The lyophilized sample was dissolved in water (10 mg/ml) and then treated with Multifect® P-3000 (T-50 °C, pH -7.0, E:S-100) for 24 h, and then lyophilized after deactivation of enzyme.
2.1.2 α-Chymotrypsin hydrolysis of 0.3 N NaOH-treated phosvitin

Phosvitin (25 mg/ml) was dissolved in 0.3 N NaOH and incubated for 3 h in a shaker water bath. After that, treated phosvitin was dialyzed in a dialysis tube of 25mm, MWCO-12-14000 Da, Spectra/Por (Fisher) against DDW overnight, water exchange was done for three times. The substrate: enzyme ratio was 100:1 (w/w) and pH was 8.0. The enzymatic digestion was carried out at 37 °C in a shaker water bath (6 h) and after inactivation of the enzyme, lyophilisation was done.

2.1.3 Sample preparation for ESI-MS/MS or MALDI-MS

Each enzymatic hydrolysate (trypsin hydrolysate of phosvitin, α-chymotrypsin hydrolysate of 0.3 N NaOH treated phosvitin and Multifect® P-3000 hydrolysate of dephosphorylated phosvitin) was dissolved in distilled water (10 mg/ml) and centrifuged at 3,000 x g for 10 min. The supernatant was collected, filtered through a 0.45 µm filter and used for HPLC-MS/MS. Samples were prepared according to the method of Hillenkamp & Karas (1991) for MALDI-MS.

2.3 Instrumentation

Digested phosvitin samples were analyzed via high-performance liquid chromatography (HPLC) using a Waters Atlantis dC18 4.6 x 150 mm, 3.0 µm column. Mobile phase A was 100% H₂O with 0.1% formic acid. Mobile phase B was 100% acetonitrile (ACN) with 0.1% formic acid. An isocratic elution from zero to five minutes at 5% mobile phase B was used to remove salts. This was followed by a linear gradient from 5 to 95% mobile phase B over forty-five minutes. The flow rate was 0.5 mL/min throughout. HPLC-MS/MS analysis was performed using a high-resolution Agilent 6540 quadrupole time-of-flight mass spectrometer. Samples were also analyzed via MALDI-MS. MALDI mass spectra were collected on a Bruker Microflex Linear Time of Flight instrument, using alpha-cyano-4-hydroxy-cinnamic acid as matrix. Spectra were acquired over the mass range of 600 Da to 4,000 Da using 50 laser shots and settings favorable to the detection of peptides.
Digested peptides were predicted using the Protein Prospector MS-Digest software (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest), allowing for two missed cleavages and multiple phosphorylations.

3. Results and Discussion

Characterization of peptides is one of the key aspects in bioactive or functional peptide production, which allows understanding and manipulation of the functionalities of those peptides. MS/MS has been effectively used for amino acid sequencing and to investigate the structural information of bioactive peptides derived from different proteins, such as soy protein (Kodera, & Nio, 2006; casein (Hernández-Ledesma, Amigo, Ramos, & Recio, 2004; Schmelzer, Schöps, Reynell, Ulbrich-Hofmann, Neubert, Raith, 2007; Quiro´, Hernández-Ledesma, Ramos, Amigo, & Recio), collagen (Li, Chen, Wang, Ji, & Wu, 2007), ovotransferin (Majumder, & Wu, 2010).

Since phosvitin shows resistance to protease activity it is difficult to use theoretical enzyme cleavages to predict the peptides present in a particular hydrolysate. For example the tryptic digest of phosvitin amino acid sequence with no phosphorylation would result in 53 peptides according to the Peptidemass software: http://web.expasy.org/peptide_mass/ (Table 1) Wilkins, et al., (1997), Gasteiger, Hoogland, Gattiker, Duvaud, Wilkins, Appel, Bairoch (2005).

However, SDS-PAGE of trypsin digestions results in only a few bands, implying resistance of phosvitin to protease activities (Figure 2). A restricted hydrolysis of phosvitin with pepsin, trypsin and α-chymotrypsin has been reported by Goula, Triplett, &Taborsky (1996). They found that pepsin digestion produced three peptides of Asn 44 –Leu 193 (150 residues), Gly 4-Glu 41 (38) and C-terminal fragment of Leu 193- Glu 214 (21 residues). Trypsin digestion resulted in two major peptides Gln 49-Arg 212 (164) & Ala 1- Arg 35 (35), and α-chymotrypsin resulted in two major peptides Ala 50-Trp 210 (161) & Gly4-Gln 49 (46). The SDS-PAGE pattern of tryptic digest of phosvitin indicated that the molecular weight of the larger fragment was 28 kDa.
For a complex mixture of peptide samples, HPLC-MS/MS analysis has shown promising results (Aebersold, & Mann, 2003). Table 2 shows the identified peptides from the three different hydrolysates of phosvitin used in this study. A Mascot database search revealed that the peptide sequences were a match of the MS/MS fragmentation of VIT2_CHICK, Vitellogein-2 OS=Gallus gallus GN=VTG2 PE=1 SV=1 (NCBI BLAST). In addition, the predicted peptide sequences from Protein Prospector MS-Digest software for the MALDI-TOF agrees with the amino acid sequence of phosvitin.

Young, Nau, Pasco, & Mine (2011) evaluated an ion-exchange chromatographic fraction (named PPP3) of hydrolysate obtained by tryptic enzymatic digestion of partially dephosphorylated phosvitin (produced by incubation in 0.1 N NaOH for 3 h at 37°C) using MALDI-TOF and nanoelectrospray mass spectrometry (nES-MS) on Q-TOF hybrid quadrupole/time-of-flight instrument and online liquid chromatography tandem mass spectrometry (LC-MS/MS). Prior to performing HPLC-MS/MS, they dephosphorylated peptides with phosphatase. Accordingly they have observed three main peptides from the C- and N-terminals of phosvitin and from the center of the phosvitin molecules. Those three peptide sequences are GTEPDAKTSSSASSSTATSSSASSPNRKKPMDE (residues 4-41), NSKSSSSSKSSSSRSRSSKSSSSSSSSSSSSSSKSSSSR (3PO₄) (residues 114-147), and EDDSSSSSSSVLSKIWGRHEIYQ (residues 194-217). Ten peptides from the N-terminal residues (mainly from 194-217) also have been identified. However, in the present study we did not find peptide residues representing 114-147 (44 amino acids residues). In the present study peptide sequences of residues from 81-94, 95-107 (with 5 phosphate moieties) and 108-121 (with 12 phosphate moieties) could be identified by MALDI-TOF analysis of trypsin digests (Table 2). This could be due to the fact that they have used 0.3 N NaOH treated phosvitin for trypsin digestion. However, depending on the exact location of phosphorylation of phosvitin and the degree of phosphorylation, the access to enzyme for the peptide bonds of the protein could be affected. It is well known that the degree of phosphorylation of phosvitin can vary from 3 to 10% (Taborsky et al., 1967; Burley & Cook 1961). The phosvitin used in the present study contained 7% phosphate (AOCA, 1994). Further, as a heterogeneous protein, phosvitin is mainly composed of two main components, α (with serine 40.5 mol per 104 g of phosvitin) and β (42.5 mol per 104 g of phosvitin),
which accounts for 80 and 15%, respectively (Culbert & McIndoe 1971; Itoh, Abe, & Adachi, 1983). The amino acid composition, degree of phosphorylation, and the glycosylation of the two major components (α and β) of phosvitin are slightly different. Phosphorylation is a heterogeneous process and could lead to mixture of proteins phosphorylated different sites to different extents (Mann et al., 2002). Therefore, enzymatic specificity on phosvitin is difficult to predict due to the complex nature of phosvitin. It should be emphasized that the ESI-QTOF did not identify the peptides resulted from the center of the phosvitin molecule by trypsin digestion. This could be due to poor ionization of the peptides, poor fragmentation of peptides due to insufficient CID energy or high degree of phosphorylation, which could complicate database searching. For example the neutral loss of a phosphate molecule during CID could result in a complex fragmentation pattern (Schroeder, Shabanowitz, Schwartz, Hunt & Coon, 2004). Further, phosvitin is a glycoprotein with 2.23 % hexose, 1.08% hexosamine and 1.91% of sialic acid (Taborsky, 1974). Both the high extent of phosphorylation and glycosylation in phosvitin complicate the MS/MS based amino acid sequencing. Phosphopeptides from the center of phosvitin molecule from Multifect® P-3000 digestion of dephosphorylated phosvitin could be identified from ESI-QTOF (Table 2). According to the present result MALDI-QTOF was able to identify some phosphopeptides resulted from middle center of phosvitin molecule (without dephosphorylation) due to trypsin digestion (Table 2). Therefore, MALDI for phosvitin phosphopeptide could be more promising compared to ESI. However, the present findings also prove the difficulty of analyzing phosphopeptides by means of MS/MS as discussed in the Introduction.

An interesting feature of phosphopeptides is their ability to form soluble organophosphate salts. The phosphorylated serine moieties of the phosphopeptides play the major role in binding divalent metal ions such as Ca, Mg, Zn, Cu, Fe etc. (Li, Tomé, & Desjeux, 1989; Hansen, Sandström, Jensen, & Sorensen, 1997; Kitts, 2005). Thus promotes intestinal absorption of calcium, minerals and other trace minerals (Konings, Kuipers, & Huis in ‘t Veld, 1999). Mellander (1947) first reported that phosphopeptides derived from casein (CPP) enhanced the calcification of bones. The absorption of iron in the gastrointestinal tract is low because iron forms heavy molecular weight ferric hydroxide in the guts (Derman, Sayers, Lynch, Charlton, Bothwell, & Mayet, 1977). However, in the
presence of casein phosphopeptides, enhanced iron availability and iron absorption in the gastrointestinal system have been observed. Therefore, those peptides can be used as the carriers for metal ions (Sato, Noguchi, & Naito, 1986). Casein phosphopeptides have been approved as nutraceuticals in Japan (Jiang, & Mine 2000). A product called “Capolac” containing CPP (Arla Foods Ingredients) is also available in Sweden as a mineral absorption facilitator (Korhonen, & Pihlanto, 2006). The casein phosphopeptides (CPPs), derived from 3\alpha_{s1}, \alpha_{s2}- and \beta-casein have been studied intensively. These CPPs contain a common motif of three phosphorylated serine residues followed by two glutamic acid residues. This motif is negatively charged at physiological pH and generally considered as highly important for calcium and other cation binding activity of these peptides (Meisel, 1997). The amino acid sequences of four major phosphopeptides derived from trypsin digestion of casein include:

- sequence 1 (\beta-casein(1–25)), Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser(P)-Leu-Ser(P)3-Glu2-Ser-Ile-Thr-Arg;
- sequence 2 (\alpha_{s1}-casin(59–79)), Gln-Met-Glu-Ala-Glu-Ser(P)-Ile-Ser(P)3-Glu2-Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Gln-Lys;
- sequence 3 (\alpha_{s2}-casein(46–70)), Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-Ser(P)3-Glu2-Ser(P)-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys;
- sequence 4 (\alpha_{s2}-casein(1–21)), Lys-Asn-Thr-Met-Glu-His-Val-Ser(P)3-Glu2-Ser-Ile-Ser(P)-Gln-Glu-Thr-Tyr-Lys (Reynolds, 1998).

Milk casein plays the major role in the area of phosphopeptide production. Phosvitin could be a better protein candidate for phosphopeptides production than casein because contains many more phosphoserines. However, very little work has been reported with regard to phosvitin in the area of bioactive peptide production. In general the size of a bioactive peptide can vary from two to twenty amino acid residues (Gill, Lópe-Fandiño, & Vulfson, 1996). Pantzar, Westrm, Luts, & Lundin, (1993) studied the small intestinal permeability of different size molecules and found that intestinal permeable to molecules in the range of 1 to 30 kDa are high. Miquel, Goà mez, Alegrià a, Barberà a, Farreà a, & Recio, (2005) suggested the potentiality of CPP of 1125-6512 Da, produced by milk-based infant formulas for intestinal absorption and physiological role in mineral bioavailability. According to the present study, the identified phosvitin phosphopeptides are composed of less than 20 amino acids and several peptides contain phosphorylated serine residues (Table 2). The cluster sequence -Ser(P)3-Glu2 of those CPPs, is responsible for the interaction with amorphous
calcium phosphate (ACP). Adjoining Ser(P) residues play an important role for the maximum interaction with ACP (Reynolds, 1998). In phosvitin phosphopeptides that particular cluster sequence of -Ser(P)₃-Glu₂ cannot be found according to its primary structure. However, according to the present findings many peptides with phosphorylated serine can be seen, indicating their capacity to react with bivalent cations.

**Conclusions**

Characterization of phosvitin phosphopeptides from mass spectrometry was an extremely challenging task. However, a few peptide sequences with possible phosphorylation could be identified in three different hydrolysates of phosvitin. The peptides originated from N- and C- terminals of phosvitin could be identified mostly. With ESI – QTOP, enzymatic hydrolysates obtained from after dephosphorylation of phosvitin resulted in peptides originated from the central part of phosvitin too.

The amino acid sequences and structural information observed in this study shows the possibility of using phosvitin derived phosphopeptides in the area of bioactive/functional peptides production. However, more studies have to be implemented in order to discover the physiological mechanisms and biological effect *in vivo.*

More information on the amino acid sequences of phosphopeptides can be obtained in the future if the phosphopeptides are enzymatically dephosphorylated, fractionated, and then analyzed using 2D HPLC MS/MS and 2D HPLC MALDI-TOF. The improvement of databases and in-gel digestion can also help identification of all the amino acid sequences of the phosphopeptides from phosvitin hydrolysates. With the information, the characterization of each phosphopeptides can also be possible in the future. One other thing that makes the task difficult is the carbohydrates attached to the phosvitin.
References


Table 1. Peptide could be resulted from theoretical cleavage of trypsin on phosvitin under the assumption of 0% phosphorylation

<table>
<thead>
<tr>
<th>Mass resi</th>
<th>Miss cleavage</th>
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</tr>
</thead>
<tbody>
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<td>5608.4210</td>
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<td>SSSHSHSHSHHSGHLNGSSSS</td>
</tr>
<tr>
<td>3582.6340</td>
<td>180-212</td>
<td>SVSHHSHEHSHEHSGHLEDDSSS SSSSVLSKIWGR</td>
</tr>
<tr>
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<td>3207.3779</td>
<td>148-179</td>
<td>SSSSSKSSSSSHSHSHSHSGH LNGSSSSSSSSSR</td>
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<tr>
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<td>1201.6112</td>
<td>209-217</td>
<td>IWGRHEIYQ</td>
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<tr>
<td>1173.5342</td>
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Table 1 continued

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<td>52-60</td>
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<td>SSSSSSRSS</td>
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<td>SSNSSKR</td>
</tr>
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<td>261.1557</td>
<td>52-53</td>
<td>0</td>
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</tr>
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Figure 1. SDS-PAGE pattern of Trypsin digestion of phosvitin. Lane 1 - Native Phosvitin (Sigma); Lane 2 - Native phosvitin (lab); Lane 3 - Molecular Marker, Lane 4-8 trypsin hydrolystae of phosvitins (different replicates); Lane 9 - Trypsin.
Figure 2. (A) Total ion chromatogram of 24 hr trypsin hydrolysis of phosvitin. (B) Mass spectrum of the peptide KKPMDEEENDQV (position 36-47). Singly charged ion is $m/z = 1461.739$. Doubly charged ion is $m/z = 731.390$. 922.075 is an internal standard. (C) MS/MS spectra of doubly charged ion, 731.390, with labeled b and y fragment ions.
Table 2. Amino acid sequences of peptides identified from the different enzymatic hydrolysates of phosvitin (Mascot data-base/gpm data-base analysis or Protein Prospector MS-Digest software)

<table>
<thead>
<tr>
<th>Residues</th>
<th>Sequence</th>
<th>Observed (m/z)</th>
<th>Mr (exp)</th>
<th>Mr (cal)</th>
<th>Treatment</th>
<th>Instrument</th>
</tr>
</thead>
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<td>1-10</td>
<td>AEFGTEPDAK</td>
<td>532.748</td>
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<tr>
<td>1-21</td>
<td>AEFGTEPDAKTSSSSSSASST</td>
<td>1017.443</td>
<td>2032.871</td>
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<td>ESI-QTOF</td>
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<tr>
<td>4-12</td>
<td>GTEPDAKTS</td>
<td>905.418</td>
<td>904.410</td>
<td>904.403</td>
<td>24 h Phos. Alk. + 24 h Multi.</td>
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</tr>
<tr>
<td>36-48</td>
<td>KKMPDEEENDQVK</td>
<td>795.379</td>
<td>1588.743</td>
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<td>24 h Trypsin</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>0.3 N NaOH trt + 6 h Chymo</td>
<td>ESI-QTOF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24 h Phos. Alk. + 24 h Multi.</td>
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<tr>
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<td></td>
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<td>0.3 N NaOH trt + 6 h Chymo</td>
<td>ESI-QTOF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24 h Phos. Alk. + 24 h Multi.</td>
<td>ESI-QTOF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6 h/48 h Trypsin</td>
<td>MALDI-TOF</td>
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<tr>
<td>37-51</td>
<td>KPMDEEENDQVKQAR</td>
<td>908.829</td>
<td>1815.644</td>
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<td>Trypsin 24 h</td>
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</tr>
<tr>
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<td></td>
<td></td>
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<td></td>
<td>MALDI-TOF</td>
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<tr>
<td>62-72 or</td>
<td>SSKSSNSSK (1 PO₄)</td>
<td>496.205</td>
<td>990.394</td>
<td>990.424</td>
<td>24 h Phos. Alk. + 24 h Multi.</td>
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Table 2. continued

<table>
<thead>
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<td>810.350</td>
<td>1618.686</td>
<td>1618.485</td>
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<td>108-121</td>
<td>SSSSSKSSSSSSSR (12PO₄)</td>
<td>2307.194</td>
<td>2306.183</td>
<td>2306.580</td>
<td>Trypsin 6 h/48 h</td>
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<tr>
<td>195-208</td>
<td>DDSSSSSSSSVLS</td>
<td>622.760</td>
<td>1243.505</td>
<td>1243.494</td>
<td>24 h Phos. Alk + 24 h Multi.</td>
<td>ESI-QTOF</td>
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</table>

Observed (m/z): The molecular mass that was measured by the instrument. For ESI they are 2+ ions, for MALDI they are 1+

ions

Mr (expt) - Mass experimental: The molecular mass of the peptide calculated from the observed mass (without any charge)

Mr (calc) – Mass Calculated: This is the actual uncharged molecular mass of the peptide, calculated using a peptide mass calculator. The difference between the Mr (expt) and the Mr (calc) is due to the fact that the mass spectrometer has some inherent error.
Table 3. Potential neutral loss values following phosphopeptide fragmentation with CID

<table>
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<th>Neutral loss value</th>
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<tr>
<td>H₃PO₄</td>
<td>98</td>
</tr>
<tr>
<td>H₃PO₄ + H₂O</td>
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</tr>
<tr>
<td>2H₃PO₄</td>
<td>196</td>
</tr>
<tr>
<td>2H₃PO₄ + H₂O</td>
<td>214</td>
</tr>
<tr>
<td>2H₃PO₄ + 2H₂O</td>
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<td>312</td>
</tr>
<tr>
<td>3H₃PO₄ + 2H₂O</td>
<td>330</td>
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</table>

As adapted from (Schroeder, Shabanowitz, Schwartz, Hunt & Coon, 2004).
CHAPTER 7. GENERAL CONCLUSIONS AND SUGGESTIONS

Egg yolk phosvitin is the most phosphorylated glycoprotein found in the nature. More than 50% of amino acids of phosvitin are composed of serine and almost all the serine molecules are phosphorylated. As a result, phosvitin carries numerous negative charges and shows extremely high metal ion binding capacity, antioxidant activity, emulsifying activity and antimicrobial activity. However, due to the extremely high negative charges, phosvitin forms insoluble metal complexes, resulting them biologically unavailable, and shows extremely high resistance to protease activities. The objectives of this present study were to investigate the ways for improving enzymatic digestion of phosvitin, to investigate the functional properties of phosvitin hydrolysates, and to characterize the peptides in the hydrolysates.

The secondary structures of phosvitin could be changed by SDS (at 0.5% and 1% SDS with or without heat). However, the enzymatic digestion of phosvitin was not affected by SDS treatments. Phosvitin showed very high heat stability, but treating phosvitin at 100 °C for 1 h resulted in increased enzymatic susceptibility of phosvitin. Among the 6 enzymes tested, alkalase and Multifect® P-3000 showed better enzymatic activity on phosvitin than trypsin, α-chymotrypsin, pepsin and thermolysin. Consecutive treatment of phosvitin with different enzymes did not improve the digestion of phosvitin peptides resulted from the first enzyme used. Phosvitin shows higher stability in acidic conditions than in alkaline conditions. Incubation of phosvitin in strong acid (> 2 N HCl) or weak alkali conditions (> 0.075 N NaOH) induced significant hydrolysis. However, chemical hydrolysis could result in dephosphorylation and destruction of some amino acids. The high susceptibility of phosvitin to alkaline hydrolysis could be due to its high content of phosphoserine, which is considered as highly unstable in alkaline conditions.

Phosvitin showed a powerful antioxidant activity in iron-induced oil emulsion system. Phosvitin exhibited similar Fe³⁺/Fe²⁺-binding capacities, but had higher Fe³⁺-binding capacity than Fe²⁺-binding capacity at low iron concentrations. The Cu-binding capacity of phosvitin was poor compared to its Fe-binding capacity. The enzymatic and
chemical hydrolyses of phosvitin reduced its antioxidant capacity, and calcium and Fe-binding capacities. With increasing degree of hydrolysis, both calcium and Fe-binding capacities of phosvitin hydrolysates decreased while their Cu-binding activity increased. However, the hydrolysates showed comparatively good metal binding capacities. The decrease of calcium and Fe-binding activity after hydrolysis could be a positive attribute because hydrolysates reduce the formation of insoluble ion-complexes. Therefore, increasing degree of phosvitin hydrolysis could be very useful if phosvitin phosphopeptides are to be used as calcium or iron supplementing agents for human.

The identification and characterization of phosvitin phosphopeptides by using MS-MS was extremely challenging task because of extremely high phosphorylated nature of phosvitin, which result highly complicated fragmentation patterns of phosvitin phosphopeptides. However, few amino acid sequences with phosphorylation information were discovered. Information on the amino acid sequences of phosphopeptides can be obtained in the future if the phosphopeptides are enzymatically dephosphorylated, fractionated, and then analyzed using 2D HPLC MS/MS and 2D HPLC MALDI-TOF. With the improvement of data-bases and in-gel digestion can also help identification of all the amino acid sequences of the phosphopeptides from phosvitin hydrolysates. With the information, the characterization of each phosphopeptides can also be possible in the future.
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My deepest gratitude goes to Dr. Dong Ahn, my major professor for his continuous encouragement, guidance, warmth and support throughout my PhD program at Iowa State University. Completing this dissertation would have not been possible without his patience, kindness and help. I was fortunate enough to be one of his graduate students and I consider it as one of the best things happened in my life.

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Thank you very much!!