

Egg Yolk Phosvitin and Functional Phosphopeptides—Review

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Abstract: Phosphopeptides are among the most interesting biomolecules with characteristic molecular structure and functions. They usually contain clusters of phosphoserines, which can effectively bind calcium and iron, and inhibit formation of insoluble calcium phosphates or iron complexes. Therefore, phosphopeptides can increase calcium or iron bioavailability and prevent lipid oxidation in foods. Milk protein casein has been currently used by industry to produce phosphopeptides. Egg yolk phosvitin is considered as the most phosphorylated protein found in the nature. Phosvitin from egg yolk can be much better source for producing phosphopeptides with varying sizes and functions than casein because it contains much greater number of phosphates in the molecule than casein. However, still phosvitin has not been subjected to considerable attention with regard to bioactive peptides production.

Keywords: antioxidant, lipid oxidation, mineral binding, phosphopeptides, phosvitin

Introduction

The demands for the naturally derived bioactive molecules and their potential uses to promote human health and food preservation have been increased dramatically over the past few decades (McCann and others 2005). Among the functional bioactive molecules, bioactive peptides have shown the most promising potentials as therapeutic or health promoting agents (Wyvratt 1988; Wimart and Komajda 2000). 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 and have been generated *in vivo* in various living organisms or found in various proteins (Smacchi and Gobetti 2000). 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 (Meisel 1997a; Korhonen and Pihlanto 2006). These peptides can be ranged from simple dipeptides to complex linear and cyclic structures. They can also be further modified through glycosylation, phosphorylation, and/or acylation and can exert more than one biological or physical effect (Gill and others 1996; 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, antidiabetics, and so on (Shahidi and Zhong 2008). Bioactive peptides with antioxidant and mineral-binding capabilities have high potentials to be used in food industry because the use and application of synthetic antioxidants become challenging due to their potential health hazards (Brannen 1975; Becker 1993; Mendis and others 2005). Recently, there has been

a growing interest for natural and safe antioxidants in food products (Chow 1988; Finkel and Holbrook 2000) and a significant increase in the demand and utilization of natural antioxidants (Shahidi and others 2006).

Among the bioactive peptides, phosphorylated peptides or phosphopeptides are the most interesting biomolecules because they have characteristic molecular structure and functions. Phosphopeptides usually contain clusters of phosphoserines, which can effectively bind calcium and inhibit the formation of insoluble calcium phosphates, resulting an increased calcium or bioavailability. A great number of phosphopeptides have been derived from casein. However, a subunit of casein has only one to 13 phosphoserine residues to stabilize amorphous calcium phosphate (ACP), whereas a molecule of phosvitin has approximately 120 phosphoserine residues, implying various phosphopeptides with different sizes, and calcium binding and releasing capacities can be produced (Castellani and others 2004). Therefore, the objective of this review article is to discuss the characteristics of phosvitin as a potential candidate for phosphopeptides production and the importance of phosphopeptides as nutraceutical agents.

Phosvitin

Egg yolk is composed of clear, yellow-colored fluid called plasma and suspended particles called granules (Romanoff and Romanoff 1949). After dilution with 0.3 N NaCl and centrifugation for at 10000 g for 30 min, the yolk can be divided into supernatant which is plasma and pellet of granules (McBee and Cotterill 1979). The granular fraction of the egg yolk contains lipovitellin and phosvitin (Burley and Cook 1961; MacKenzie and Martin 1967). Hen egg yolk phosvitin was first isolated by Mecham and Olcott in 1949 by diluting the egg yolk with magnesium sulphate. It has been proven that in nonmammalian 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 total egg yolk phosphoproteins and holds about 90% of the egg yolk phosphorous. The meaning of phosvitin indicates that it contains high amount of phosphorous and its origin of egg yolk (Mecham and Olcott 1949). In

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1967, Taborsky and Mok first determined the molecular weights of minor and major phosvitin as 36000 and 40000 Da. Egg yolk phosvitin is a heterogeneous protein composed of 7 components and in which 2 main components account for 80% and 15%, respectively (Culbert and McIndoe 1971). Wallace and Morgan (1986) reported that the unfractionated phosvitin is composed of 5 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 18500 to 60000 Da. Also, they identified 2 main components of phosvitin in sodium dodecyl sulfate polyacrylamide-gel electropherogram as α and β , which could be the 2 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 40000 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 the carbohydrate components show a triple-branched antenna-like structure (Brockbank and others 1990).

The detailed analysis of amino acid sequence indicated that serine accounts for more than 55% of the total amino acids of phosvitin (Table 1; Figure 1), and β -phosvitin contains more ser-

ine 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 by using circular dichroism (CD), Fourier transform infrared spectroscopy, and Fourier transform infrared photoacoustic and fluorescence spectroscopic methods. They suggested that the presence of β -turn in the proximity of o-phosphoserine residues in the 3 compartments structure model of α -helix, β -sheet, and β -turn. According to Prescott and others 1986, in neutral aqueous solution, phosvitin shows an unusual structure which is lack in both α -helix and β -sheet conformations. Conversely, phosvitin undergoes through a large conformational change in strong acidic condition (pH < 2) by forming large proportion of β -sheet. In addition they confirmed that the phosvitin obtained by lyophilization at pH 7.0 has the β -sheet predominantly. Table 2 shows the secondary structure conformation of phosvitin at different pH as estimated by Chang and others in 1978, using CD spectra. Taborsky (1969) evaluated the freezing effect on the conformational changes of phosvitin and reported that it can undergo from unordered conformation to β -structure upon freezing and thawing. This effect has been seen at acidic conditions, and with the increasing acidity, this effect has been increased.

In 1970, Grizzuti and Perlmann carried out the studies on viscosity, optical rotatory dispersion (ORD), and CD of phosvitin. Accordingly, due to high phosphoric acid bound to serine residues of phosvitin, it 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 0.02 to 0.1 of ionic strength 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. Phosvitin is considered as one of the most phosphorylated proteins found in the nature and phosphorous content ranges from 3% to 10% of phosvitin molecular weight (Burley and Cook 1961; Taborsky and others 1967). Phosvitin is enormously hydrophilic,

Table 1—The amino acid composition of hen phosvitin.

Residue	Nr of residues/mol of phosvitin (Taborsky 1974)	According to the nucleotide sequencing (Byrne and others 1984)	mol per 10 ⁴ g of phosvitin	
			α^*	β^*
Gly	7	8	1.4	0.6
Ala	7+	7	1.9	0.8
Val	3	3	0.5	0.7
Leu	3	3	—	0.3
Ile	2	2	0.4	0.2
Pro	3	3	0.7	0.6
Phe	2	1	0.6	0.2
Tyr	1	1	0.2	0.2
Trp	1	1	0.8	—
Ser	120	123	40.5	42.5
Thr	5	4	1.4	0.3
Cys	0	0	—	—
Met	1	1	—	—
Asp	13	13	2.7	2.4
Glu	13	8	5.3	1.8
His	11	13	0.6	2.3
Lys	17	15	5.6	3.3
Arg	11	4	2.5	1.9
Total	220	216		
Other				
No. of P	112		1.59	2.65
Hexose (%)*	2.23		0.15	1.16
Hexosamine (%)*	1.08		0.20	2.20
Sialic acid (%)*	1.91			

*Adapted from Itoh and others (1983).

Table 2—Secondary structure of phosvitin in water at various pH.

pH	Percentage of α -helix	Percentage of β -sheet	Percentage of β -turns	Percentage aperiodic
7.0	0	4.5	31.0	64.05
2.8	0	32.5	18.5	49.0
2.1	0	64.5	1.0	43.5
1.5	10	71.0	0	19.0

Adapted from Chang and others (1978).

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 (Byrne and others 1984).

and it has a very high number of negative charges (approximately 0180e at pH 7.0) and unusually low percentage of nonpolar hydrophobic side chains (Dickinson and others 1997). Due to large repulsive charges, phosvitin possesses a very low specific volume of 0.545 mL/g (Belitz and others 2009), and its isoelectric point is pH 4.0 (Tenrence 1989). Its frictional ratio reveals the presence of phosvitin as an elongated molecule (Belitz and others 2009).

Due to high phosphoric acid bound to serine residues, phosvitin can behave as a polyelectrolyte (polyanion) in liquid state (Ghizzuti and Perlmann 1970). Consequently, phosvitin shows different functionalities such as metal chelating, antioxidant, emulsifying capacities, and so on.

Metal-chelating activity of phosvitin

Egg yolk phosvitin shows a very strong affinity to bivalent metals such as calcium, magnesium, and iron since it carries a high number of phosphate molecules (Taborsky 1963; Grizzuti and Perlmann 1973). Almost all the Fe present in the yolk is bound to phosvitin (Greengard and others 1964), but it only accounts for 0.3% of the total iron binding capacity of phosvitin.

Binding of iron to phosvitin promotes rapid oxidation of ferrous ion to ferric form (Taborsky 1963). Hegenauer and others (1979) reported that phosvitin can bind ferric ions stronger than that of nitrilotriacetate or citrate. Also, they concluded that the clusters of di-*o*-phosphorylserine residues play the major role in iron-chelating activity of phosvitin. However, free phosphorylserine does not react with Fe³⁺. Phosvitin can bind with ferric iron very strongly whereas it forms weak complexes with ferrous iron (Taborsky 1980). This strong stability of phosvitin-Fe complex could be due to the tetrahedral stoichiometry (once Fe ion is bound with one phosphate molecule, a neighboring phosphate molecule completes the binding process) or octahedral stoichiometry (2 other neighboring phosphate molecules complete the process), which increase the activation energy required for the dissociation of the phosvitin-Fe complex (Castellani and others 2004).

Albright and others (1984) found that heating phosvitin at 110 °C for 20 and 40 min could not release the Fe bound to phosvitin, indicating that phosvitin has a very strong Fe-binding capability. They observed that only ethylenediaminetetraacetic acid (EDTA) was capable of releasing bound Fe from phosvitin. Two phosphate groups can bind with one iron ion, and there are over 120 phosphate groups in a phosvitin molecule. Phosvitin can bind around 60 Fe molecules in the ratio of 0.5 iron/phosphate when Fe is in excess (Taborsky 1980). The Fe-binding sites of phosvitin are not uniform, and the interactions of phosvitin with Fe depend on Fe concentration and the availability of phosphoserine clusters (Taborsky 1991). The best Fe-binding activity of phosvitin has been observed at pH 6.5 and ionic strength 0.15. At saturated level, the Fe-binding capacity of phosvitin was 115 µg iron per mg phosvitin. At combinations of low pH (<3.5) and low ionic strength and high pH (>5.2) and high ionic strength, reduction of iron binding capacity of phosvitin has been observed (Castellani and others 2004).

A heat treatment of phosvitin at 50 °C and 90 °C for 60 min did not influence its iron-binding capacity. High pressure treatment at 300 and 600 MPa (±7 MPa) for 10 min neither affected the Fe-binding activity of phosvitin nor changed the phosvitin structure (Castellani and others 2004). Compared to iron-binding phosphoprotein casein, this value is very high. β-casein and αs1-CN can bind 7 and 14 iron ions, respectively (Baumy and Brule 1988; Gaucheron 2000).

Grizzuti and others (1973) found that phosvitin can bind 103 Mg²⁺ ions and 127 Ca²⁺ ions at pH 6.5 (25 °C). However, the Mg²⁺- and Ca²⁺-binding capacities of phosvitin decrease drastically at pH 4.5 (only 40 Mg²⁺ and 32 Ca²⁺ ions). Thus, the metal-binding activity of phosvitin can be a useful character in the process of production of mineral-binding bioactive peptides.

Antioxidant activity of phosvitin

Lu and Baker (1986) evaluated the antioxidant activity of phosvitin in a phospholipid emulsion system with nonorganic and organic metal ions (Fe²⁺, Cu²⁺, and hemin) at different concentrations. It has been observed that phosvitin can effectively inhibit Fe²⁺- and Cu²⁺-mediated oxidation of phospholipids. Yet, phosvitin was not effective enough to inhibit the hemin-mediated phospholipids oxidation. Phosvitin showed a higher inhibitory effect on phospholipids oxidations in the presence of Fe²⁺ (up to 30:1 Fe²⁺-to-phosvitin molar ratio) than copper ions (only 1:1 molar ratio). Lee and others (2002) observed the maximum antioxidant effect of phosvitin at the concentration of 15 and 40 µm in phosphatidylcholine liposomes system and pork muscle homogenate, respectively. Upon autoclaving, the antioxidant activity of phosvitin has been decreased. However, no change in its antioxidant activity has been observed once it has been subjected to pasteurization (Lu and Baker 1986). It has been shown that the antioxidant activity of phosvitin is maximum at pH 6.1 and phosvitin was not effective enough to inhibit the Cu²⁺-induced oxidation at pH 7.8 in phospholipid emulsion system (Lu and Baker 1987). The antioxidant activity of phosvitin and phosvitin-galactomannan conjugate (PGC) has been evaluated by Nakamura and others (1998) in a powdered oil model system. Conjugation of phosvitin has significantly increased the antioxidant and radical scavenging activities of phosvitin. Unlike native phosvitin, the antioxidant activity of PGC has been not affected by autoclaving it at 121 °C, 1.32 atm for 15 min (Nakamura and others 1998).

Ishikawa and others (2004) reported that phosvitin and its tryptic hydrolysate are more effective than other iron-binding proteins or metal chelators such as ferritin, transferrin, EDTA, diethylene triamine pentaacetic acid and citrate in inhibiting Fe²⁺-catalyzed hydroxyl radicals (•OH) production in Fenton reaction system. Phosvitin accelerates autooxidation of Fe²⁺ to Fe³⁺, and thus, reduce the availability of Fe²⁺ for the Fenton reaction. Phosvitin and its tryptic hydrolysate also showed a protective effect against •OH-mediated damages of DNA *in vivo* (Ishikawa and others 2004). Therefore, the antioxidant activity of phosvitin can be exploited in the production of natural antioxidant agents.

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 (Chung and Ferrier 1991; Khan and others 1998) However, partial removal of phosphates with phosphatase and complete dephosphorylation with alkaline treatment have significantly reduced the emulsifying properties of phosvitin (Kato and 1987). In addition, binding of calcium to phosvitin decreased its emulsifying properties. Thus, it was concluded that the electrostatic repulsive forces of phosphate moieties played the major role with regards to the emulsifying properties of phosvitin (Kato and others 1987). NaCl influences the emulsifying ability and emulsion stability of phosvitin at pH 3 and 10: at low NaCl concentrations (<0.5 M), the emulsion stability of phosvitin decreased drastically (Chung and Ferrier 1992). The emulsifying activity of phosvitin decreased

when it was subjected to a heat treatment of above 70 °C for 1 h, but its emulsion stability was not affected until heating above 67.5 °C for 1 h (Chung and Ferrier 1995).

Dephosphorylation and protease digestion of phosvitin with pepsin, trypsin, and α -chymotrypsin reduced both emulsifying ability and emulsion stability of phosvitin (Khan and others 1998). It has been observed that the conjugation of phosvitin with galactomannan can significantly improve the emulsifying activity and emulsion stability of phosvitin (Nakamura and others 1998). In 1999, Khan and others evaluated the emulsifying properties of Millard type conjugates of phosvitin digested with pepsin, trypsin, and α -chymotrypsin protease (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. Better emulsifying properties have been observed in nonaggregated phosvitin than aggregated phosvitin at pH 6. However, increased stabilization has been shown by aggregated phosvitin against coalescence. In addition, at 0.05 M NaCl, phosvitin has formed finer emulsion than at 1.5 M NaCl. Phosvitin showed poor coalescence destabilization ability and was not effective in reducing oil-in-water interfacial tension. However, the interfacial film characteristics were high at pH 5.0 (Castellani and others 2006).

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% to 32% compared to 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 the facts that chelating of Fe by phosvitin and the 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 indicated that the addition of 1% to 2% phosvitin or egg yolk reduced calcium and magnesium absorption in Wistar rats compared to those fed with casein or soy proteins containing diets. Also, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) band pattern of intestinal contents of phosvitin-fed rats resulted larger peptides of molecular masses of 28, 22, and 15 kDa illustrating lower digestibility of phosvitin in the digestive system (Ishikawa and others 2007). Therefore, it is considered that phosvitin shows negative nutritional attributes. Egg yolk phosvitin is also considered as an allergen (Walsh and others 1988; Walsh and others 2005). A few allergens are susceptible to industrial processing, and the allergenicity of egg proteins can be reduced by means of physical and enzymatic reactions due to modification of structural conformation and demolition of primary structure of proteins (Baumgartner and others 2010). 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 evaluated.

Phosphopeptides

An interesting feature of phosphopeptides is their ability to form soluble organophosphate salts. The phosphorylated serine moiety

of the phosphopeptides played the major role in binding divalent metal ions such as Ca, Mg, Zn, Cu, Fe, and so on (Li and others 1989; Hansen and others 1996, 1997; Kitts 2005) and promoted intestinal absorption of calcium, minerals, and other trace minerals (Konings and others 1999). Mellander (1947) first reported that phosphopeptides derived from casein (casein phosphopeptides [CPP]) enhanced the calcification of bones. Interestingly, phosphopeptides have ability to enhance Ca absorption in the gastrointestinal system even in the absence of Vitamin D. Derman (1977) reported that the absorption of iron in the gastrointestinal tract was low because iron forms heavy molecular weight ferric hydroxide in the guts. However, casein phosphopeptides enhanced iron availability and iron absorption in the gastrointestinal system. Therefore, those peptides can be used as the carriers for metal ions (Sato and others 1986). FitzGerals (1998) reported that the negative charges of phosphate groups and side chains of phosphopeptides made them resistant to the gastrointestinal enzymatic digestion, which made them suitable for the carriers of metal ions. Casein phosphopeptides has been already approved as nutraceuticals in Japan (Jiang and Mine 2000). A product called "Capolac" containing CPP (Arla Foods Ingredients, Amba, Denmark) is also available in Sweden as a mineral absorption facilitator (Korhonen and others 2006). However, the interaction between CPPs and mineral and enhancing mineral absorption at the intestinal level is a controversial issue due to different experimental approaches and methodologies used to assess the above-mentioned aspects (Korhonen and others 2006).

Casein accounts for about 80% of total protein in bovine milk. Casein is composed of α_{s1} -, α_{s2} -, β -, and κ -caseins in the ratio of 3.0:0.8:3.0:1.0 (Swaisgood 1992). 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 -Ser(P)₃-Glu₂ of those CPPs, which is negatively charged at physiological pH, is responsible for the interaction with 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 including calcium absorption, calcium retention, bone calcification, antihypertensive effect, anticarcinogenicity, milk curdling, and stabilization of cream liqueurs (Park and Allen 1998).

Milk casein is the major source of protein used for the production of phosphopeptides, but little work has been reported on the production of bioactive phosphopeptides from egg yolk phosvitin. Although, phosvitin can be considered as a potential source of bioactive phosphopeptides, it is highly resistant to proteolytic digestion *in vitro* due to 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. Pepsin produced 3 peptides of Gly 4-Glu 41 (38 residues), Asn 44-Leu 193 (150 residues), and C-terminal fragment of Leu 193-Glu 214 (21 residues). Trypsin digestion resulted in 2 major peptides Ala 1-Arg 35 (35 residues) and Gln49-Arg 212 (164 residues), and α -chymotrypsin digestion produced 2 major peptides Gly4-Gln 49 (46 residues) and Ala 50-Trp210 (161 residues). The SDS-PAGE of tryptic digest of phosvitin indicated that the molecular weight of the larger fragment was 28 kDa. The dephosphorylation of phosvitin by alkaline treatment (incubation of phosvitin in NaOH at 37 °C) increased the proteolytic susceptibility of phosvitin, and resulted in peptides with 10 to 20

amino acids (1 to 3 kDa) after trypsin digestion (Jiang and others 2000). Therefore, in the process of bioactive peptide production, the limited enzymatic susceptibility of phosvitin can be considered as a major drawback. Thus, it is important to evaluate means and ways that can effectively enhance the enzymatic digestion of phosvitin. Enzymatic dephosphorylation, thermal treatments, and use of very effective proteases might be some solutions to increase the enzymatic susceptibility of phosvitin. However, these strategies are yet to be discovered.

Mineral-binding ability of phosphopeptides

CPPs have been studied extensively as the mineral-binding 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 5 commercial CPPs and β -casein (1 to 25) CPPs transiently increased 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 anticariogenic effect of calcium-binding phosphopeptides is due to their recalcification ability of 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 highly stable at various pH conditions (Emery 1992; Bouhallab and others 2002). The bioavailability of 3 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) can enhance the levels of iron in blood compared with inorganic iron and Fe/ β -casein (Ait-Oukhtar and others 1999).

Donella and others (1976) evaluated the Fe³⁺ binding activity of phosphorylserine blocks (Ser-P)_n isolated from acid hydrolysis of phosvitin and found that phosphorylserine blocks (Ser-P)_n with n \geq 4 binds with Fe³⁺ but with less activity than phosvitin. However, no work has been reported with regard to the use of phosvitin-derived phosphopeptides as Fe-carrying bioactive peptides. The possibility of using peptides derived from partially dephosphorylated phosvitin (PDP) as Ca-binding peptides has been studied by Jiang and others (2001). They found that phosphopeptides of PDP containing 35% of phosphate were more effective in binding Ca and inhibiting formation of insoluble calcium phosphate than phosphopeptides retaining 65% and 17.5% of phosphate.

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 Ca⁺² 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 tryptic hydrolysate for enhancing the intestinal absorption of Ca and accumulation of bones. Inclusion of phosvitin phosphopeptides at low, medium and high levels in rat diets had no effect on 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. These evidences show the possibility of using phosvitin in the production of bioactive mineral binding phosphopeptides in future.

Antioxidant effects of phosphopeptides

CPPs have potentials to be used as antioxidants, because CPPs can bind ionic iron, a strong catalyst of lipid oxidation (Kitts 2005). In the presence of tryptic hydrolysate of β -casein and phosphopeptide of β (1–25), lipid oxidation has been inhibited by 50% after 20 h incubation in a liposome system containing iron and ascorbate (Kansci and others 2004). In cooked ground beef stored at refrigerated temperatures, 0.5% CPP inhibited the development of TBARS by 80% while casein hydrolysates inhibited lipid oxidation by 38% after 4 d of storage (Díaz and Decker 2004). In an oil-in-water emulsion system, addition of 5, 25, 50, and 100 μ M caseinophosphopeptides reduced the formation of lipid hydroperoxides by 12%, 83%, 94%, and 96% and decreased the amount of hexanal by 32%, 84%, 97%, and 98% compared with control after 5 d of storage at 37 °C (Díaz and others 2003). CPPs prevented rancidity in oil-in-emulsion system by changing the chemical reactivity of iron, impairing iron to participate in redox cycling, forming insoluble iron complexes and sterically hindering metal-lipid interactions (McClements and Decker 2000). CPPs sequester ferrous ion and reduce production of hydroxyl radical by the Fenton reaction. CPPs effectively competed with deoxyribose for iron and inhibited the direct interactions of iron catalyst and substrate in the site-specific assay (Kitts 2005). In addition, CPPs exhibit antioxidant activity by scavenging free radicals (Rival and others 2001a, 2001b; Chiu and Kitts 2004; Díaz and Decker 2004; Kim and others 2007).

The possibility of using phosvitin-derived phosphopeptides as antioxidants also has been reported. Three ion-exchange chromatographic fractions of tryptic digest of 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 significantly reduced the production of IL-8 a proinflammatory mediator, which is used as 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 a 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 showed better radical-scavenging activity on 2,2-diphenyl-1-picrylhydrazyl than native phosvitin. They reported that the antioxidant activity of 100 μ g/mL phosvitin is significantly ($p < 0.05$) higher than that of vitamin E. However, at 10 μ g/mL concentration,

phosvitin did not show the same effectiveness as vitamin E. It indicated that the peptide fractions with no phosphorous has a 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 PDP (Xu and others 2007). The antioxidant activity can be determined by metal chelating, free radical scavenging, hydroperoxide-reducing ability, and aldehyde-adduction ability of peptides (Chan and others 1994; Zhou and Decker 1999a, 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

Although it is known that some milk ingredients can be effective in inhibiting the growth of microorganisms, there are limited studies on the antimicrobial effects of CPPs. A recent study showed that phosphopeptides with molecular weight of 3.5 to 4.0 kDa derived from casein inhibited the growth of *Escherichia coli* and *Pseudomonas species* (Arunachalam and Raja 2010). A β -casein f(184–210) peptide isolated from human milk hydrolysate inhibited the growth of both Gram-positive and Gram-negative bacteria including *Enterococcus faecium*, *Bacillus megaterium*, *E. coli*, *Listeria innocua*, *Salmonella spp.*, *Yersinia enterocolitica*, and *Staphylococcus aureus* (Minervini and others 2003). Two peptides derived from α_{S1} called casecidins and isracidin were effective in inhibiting the growth of *Lactobacilli*, *S. aureus*, *Streptococcus pyogenes*, *Diplococcus pneumoniae*, and/or *L. monocytogenes* (Lahov and Regelson 1996). α_{S1} -Casein is also a good source for antimicrobial peptides. These peptides could be derived from chymosin digestion from the C-terminal of bovine α_{S1} -casein including f(181–207), f(175–207), and f(164–207). Pepsin hydrolysis of α_{S1} -casein could lead to the formation of 4 antibacterial peptides, which were f(165–170), f(165–181), f(184–208), and f(203–208) (McCann and others 2005; Haque and Chand 2008).

Due to extraordinary capacity of phosvitin to bind bivalent cations, it can also exhibit antimicrobial effect. However, phosvitin and phosvitin-derived phosphopeptides have not been studied much for their antibacterial activities. Incubation of *E. coli* K12 strain at 50°C for 10 min in the presence of 0.01% or 0.1% of phosvitin significantly reduced the bacterial population. Antibacterial effect exerted by phosvitin was due to the leakage of bacterial DNA from 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). Because phosvitin possess higher Fe-binding activity than lactoferrin, it could be a better candidate than lactoferrin for the production of antimicrobial bioactive peptides.

Conclusion and Future Research

Although phosvitin is an attractive source for phosphopeptides, very high price of phosvitin limits its application for the production of bioactive phosphopeptides. Therefore, development of

improved and cost-effective preparation method for phosvitin 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 antiaging mineral-binding peptides such as copper 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 that 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.

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