University of Alberta

Phosvitin extraction and phosphopeptides characterization from chicken egg yolk

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Fall 2012

Edmonton, Alberta

DEDICATION

This thesis is dedicated to my parents: Shumin Yang and Fengqun Ren

ABSTRACT

Phosphopeptides derived from egg yolk phosvitin have been reported to exhibit various physiological functions. However, commercial use of phosvitin phosphopeptides (PPPs) has been hampered due to the lack of a simple and economic method to prepare PPPs. The purposes of this study were to develop a protocol to extract phosvitin from egg yolk, and to prepare and characterize phosphopeptides derived from enzymatic hydrolysis of phosvitin. The phosvitin prepared in this study had comparable or higher purity and recovery than previous methods. The atomic ratio of nitrogen to phosphorus (N/P) describes the density of phosphorus in peptide sequences. A lower value relates to higher density of phosphorus and high phosphorus content is preferred for bioactivities. The phosvitin hydrolysate showed comparable or lower N/P ratio than purified casein phosphopeptides. Novel phosphopeptides with high phosphorylation degree were identified. The protocols developed in this study could be used for laboratory uses and industrial scale-up. The phosphopeptides identified in this study are expected to exert multi-physiological functions and to be developed as functional food ingredients.

ACKNOWLEDGEMENTS

First of all, I want to thank my supervisor Dr. Jianping Wu for his continuous support and encouragement during the past two and half years. The experience that I gained while working with him is precious.

I would like to thank all the people working in Dr. Wu's group. Specifically, Dr. Shengwen Shen, Dr. Sunjong You, Dr. Jianpei Wang, Dr. Aman Ullah, Dr. Chanchan Wang, Dr. Satyanarayana Bejjani, Kaustav Majumder, Sen Li, Wenlin Yu, Ali Akbari, Chamila Nimalaratne, Nandika Bandara, Sahar Navidghasemizad, Alexandra Acero-Lopez, April Milne, Yuchen Gu, Mejo Remanan and Bushra Malik. In the past two and half years, we have spent a great time working together. I also like to thank all the people in Dr. Betti's and Dr. Chen's groups. I am grateful to Bela Reiz and Jing Zheng from Mass Spectrometry Laboratory, Department of Chemistry, for their professional advice in peptide sequencing.

I would like to express my appreciation to Alberta Livestock Meat Agency Inc. and Alberta-Innovates who provided financial support for my thesis.

I am very grateful to Marina Offengenden and Qiyi Li for their help in Mass analysis and protein digestion. I appreciate the friendship with Justina Su Zhang, who gave me great support during my darkest time. Special thanks to Jing Zhao, without her support I could never come this far.

Above all, I am extremely thankful to my dear parents, Shumin Yang and Fengqun Ren, for their endless love and support.

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ABBREVIATIONS

ANOVA - Analysis of Variance

BSA - Bovine Serum Albumin

CPP-ACP - Casein Phosphopeptide Amorphous Calcium Phosphate Nanocomplexes

CPPs - Casein Phosphopeptides

DEAE - Diethylaminoethyl

DH - Degree of Hydrolysis

DPPH - 2, 2-Diphenyl-1-Picrylhydrazyl

EDTA - Ethylenediaminetetraacetic Acid

ESI - Electrospray Ionization Technique

E/S - Enzyme to Substrate

FPLC - Fast Protein Liquid Chromatography

GFC - Gel Filtration Chromatography

Glu - Glutamic Acid

HDL - High-Density Lipoprotein

HPLC - High Performance Liquid Chromatography

IgE - Immunoglobulin E

IgY - Immunoglobulin Y

kDa - Kilo Dalton

LC-MS/MS - Liquid Chromatography Tandem Mass Spectrometry

LDL - Low-Density Lipoprotein

Leu - Leucine

LPS - Lipopolysaccharide

MW - Molecular Weight

N - Nitrogen

NCBI - National Center for Biotechnology Information

N/P - Atomic Ratio of Nitrogen to Phosphorus

P - Phosphorus

pI - Isoelectric Point

PGC - Phosvitin-Galactomannan Conjugate

PPPs - Phosvitin Phosphopeptides

PV - Phosvitin

Q-TOF - Quadrupole Time-of-Flight

ROS - Reactive Oxygen Species

SD - Standard Deviation

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Ser - Serine

UPLC - Ultra Performance Liquid Chromatography

VTG - Vitellogenin

CHAPTER 1 LITERATURE REVIEW

1.1 Overview of phosphoproteins

The 1992 Nobel Prize in Physiology or Medicine was shared by Edmond H. Fischer and Edwin G. Krebs, who first discovered "reversible protein phosphorylation as a biological regulatory mechanism." Nowadays protein phosphorylation is considered to involve in the regulation of many cellular functions, and is extensively studied by researchers worldwide.

Protein phosphorylation is the addition of a phosphate group to a serine, threonine, or tyrosine residue in a protein molecule. It is a widespread cellular event and regulates various activities in both prokaryotic and eukaryotic organisms (Chang and Stewart, 1998). Protein phosphorylation is actually a reversible process that includes phosphorylation and dephosphorylation catalyzed by two types of enzymes: kinases and phosphatases, respectively. During this process, receptors and enzymes are activated or deactivated due to the modifications in their structures. In this way, reversible protein phosphorylation regulates many cellular functions such as cell division, malignant transformation, differentiation, and signal transduction (Swarup, 1992).

1.1.1 Nutritional phosphoproteins from food sources

Protein phosphorylation is a reversible process, and the phosphoproteins that it generates usually have a short lifetime. Eventually the phosphatases will dephosphorylate them. However, there are some other phosphoproteins that will

not be dephosphorylated once synthesized, and they play various functions in organs, cells, bones, and even in the blood of living organisms (Taborsky, 1974). In the food industry, the nutritional phosphoproteins of casein, ovalbumin, lipovitellin, and phosvitin from milk and eggs are quite noteworthy.

1.1.1.1 Casein

Casein, with four major genetic variants of α_{s1}-, α_{s2}-, β- and κ-caseins, represents 76-86% of total bovine milk proteins. Post-translational modifications such as phosphorylation, glycosylation, and limited proteolysis cause several heterogeneities to each casein variant (Swaisgood, 2003). Casein is also known to possess a relatively small molecular weight, ranging from 20 kDa to 43 kDa with a single polypeptide chain (Whitney et al., 1976). The most notable difference among these casein proteins is the variability in the degree of phosphorylation. For example, β-casein contains 4-5 phosphate groups; α_{s1}-casein contains 8-9 phosphate groups, among which one phosphate is located at a threonine residue; α_{s2}-casein contains 10-13 phosphate groups, which is also the most among all the casein proteins; and κ-casein has only one phosphate group located at a serine residue (Ribadeau et al., 1973). However, these are average numbers since each casein protein has several heterogeneities with different degrees of phosphorylation (Eigel et al., 1984). These phosphate groups are esterified to two hydroxyl groups of serine or threonine, and the phosphorylated serine or threonine residues exist in a consecutive run of three or more in casein molecules (Manson and Annan, 1971). The difference in the caseins' phosphorylation degree is due to the phosphorylation and

dephosphorylation extents during the post-translational modification process. Casein is a metal carrier. The phosphoserine residues provide casein proteins with the unique ability to bind metal ions such as calcium and iron. This metal-binding ability is determined by many factors including temperature, pH, ionic strength and especially by the number of phosphoserine residues. When the calcium ions saturate phosphoserine residues, adding calcium will precipitate the casein-calcium complex (Schmidt, 1969; Dalgleish and Parker, 1980).

1.1.1.2 Ovalbumin

Ovalbumin, representing 54% of total egg white proteins, is the major protein component in egg albumen. It has a molecular weight of 42-45 kDa (Li-Chan et al., 1995). Ovalbumin is a phosphoglycoprotein. The single carbohydrate chain is attached to an asparagine residue of the core domain (Nisbet et al., 1981). Depending on the genetic variants, ovalbumin may contain one or two phosphate groups and sometimes no phosphate group at all (Egelandsdal, 1980). All the phosphate groups in ovalbumin are esterified to serine residues (Nisbet et al., 1981). Once ovalbumin is synthesised in the hen's body, it starts to transform towards a more stable form, the S-ovalbumin. About 75% of the native ovalbumin (N-ovalbumin) is transformed to the S-ovalbumin during the first week of incubation (Nguyen and Smith, 1984). The N-ovalbumin gradually loses its activities during the conversion to meet the developmental needs of chicken embryos at different stages (Huntington and Stein, 2001). Ovalbumin is one of the major allergens in egg white. The special amino acid composition and secondary structures are both involved in the formation of binding epitopes for

IgE-mediated allergy reactions (Mine and Rupa, 2003). However, heating and/or irradiating could reduce ovalbumin's allergenicity (Kim et al., 2002).

1.1.1.3 Lipovitellin

Vitellin is the first protein fraction discovered from egg yolks. The name, lipovitellin, was used to describe the lipoprotein purified from vitellin (Chargaff, 1942). Lipovitellin is the most plentiful protein in granules, the basic micro structure in egg yolk. The two variants, α-lipovitellin and β-lipovitellin, together account for 81% of the granules' dry matter (Burley and Cook, 1961). The proportions of α- and β-lipovitellin differ widely among hen breeds (Rao and Mahadevan, 1983), but both contain 20% lipids with a similar composition of phospholipids, lecithin, cholesterol, and triacylglycerols (Bernardi and Cook, 1960a; Bernardi and Cook, 1960b; Martin et al., 1963; Evans et al., 1974). However, the two lipovitellins show significant differences in terms of amino acid composition, carbohydrate content, protein-bound phosphorus content, and apoprotein patterns. As a phosphoprotein, α-lipovitellin and β-lipovitellin contain 0.96% and 0.36% protein-bound phosphorus, respectively. The carbohydrate contents, including mannose, galactose, glucosamine and sialic acid, are 3.60% and 2.85% for α- and β-lipovitellin, respectively (Kurisaki et al., 1981).

1.1.1.4 Phosvitin from chicken egg yolk

In 1906, Levene and Alsberg reported that nitrogen and phosphate accounted for 14% and 10% respectively in "vitellinic acid," a very acidic protein fraction

from chicken egg yolk. Later on they determined that the phosphate existed in the form of phosphoserines by the hydrolysis of vitellinic acid (Levene and Alsberg 1906; Lipmann and Levene, 1932). The first relatively pure phosphoprotein in chicken egg yolk was isolated and characterized by Mecham and Olcott (1949). It was named “phosvitin”, but now this term is more generally used to describe the phosphoproteins discovered from the egg yolks of any species.

1.1.1.4.1 Homogeneity and molecular weight

The homogeneity of phosvitin has inspired extensive attention from researchers worldwide. Although the first preparation of phosvitin appeared homogenous under ultracentrifugation, it was separated into two bands during electrophoresis (Mecham and Olcott, 1949). Later on many researchers reported that phosvitin is not a homogeneous protein. Two or more components were found in different phosvitin preparations by chromatographic fractionation (Connelly and Taborsky, 1961; Wallace and Morgan, 1986a; Wallace and Morgan, 1986b). Connelly and Taborsky (1961) resolved egg yolk phosvitin into two fractions by gradient salt elution from a DEAE-cellulose column. These two fractions were quite similar in terms of nitrogen (N) and phosphorus (P) contents but different in all other aspects including mineral content, amino acid composition, and chemical stability. Taborsky and Mok (1967) obtained a major and minor component from egg yolk phosvitin with molecular weights of 36 and 40 kDa by countercurrent distribution. Clark (1970) resolved phosvitin into two components with MWs of 34 and 28 kDa by size exclusion chromatography. All of the above reports

employed ultracentrifugation techniques to determine the molecular weight (MW) of phosvitin, although their results were slightly different from each other. These phosvitin preparations obtained by different protocols appeared homogenous but might contain more components. In the following report, Abe et al. (1982) employed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the molecular weight of the phosvitin fractions from size exclusion chromatography (SEC). Two fractions were obtained from a Sephadex G-200 column and named as α-phosvitin and β-phosvitin. Their apparent molecular weights, calculated by elution time, were 160 and 190 kDa, respectively. Each fraction showed a single band in native polyacrylamide gel electrophoresis (without SDS), but adding SDS resolved them into several bands, indicating that α- and β-phosvitin are composed of more basic components. Abe et al. (1982) reported that α-phosvitin mainly contains polypeptides with molecular weights of 37.5, 42, and 45 kDa, and β-phosvitin contains a major polypeptide with a molecular weight of 45 kDa. The large molecular weight detected in SEC could be partly due to the electrostatic repulsion between the phosphorylated residues on the phosvitin side chain and the negatively charged groups of Sephadex gels, which will result in a faster elution of phosvitin molecules than neutral molecules (Andrews, 1964). Three less abundant phosphoproteins with molecular weights of 13, 15 and 18 kDa respectively are observed by other workers and named phosvettes (Wallace and Morgan, 1986a). All of these reports come to the conclusion that phosvitin is not a homogeneous protein but is composed of several polypeptides with molecular weights ranging

from 37 to 45 kDa.

1.1.1.4.2 Composition of phosvitin

Phosvitin is one of the most highly phosphorylated proteins in nature and accounts for more than 60% of protein-bound phosphorus in egg yolk (Mecham and Olcott, 1949). Compared with α-phosvitin, which contains 3% phosphorus, β-phosvitin contains a much higher phosphorus content (10%) (Abe et al., 1982). The phosphate groups are mono-esterified to serine residues. Both phosvitin components are abundant in serine residues, which account for 31.5% to 59.2% of total amino acids according to different preparation protocols (Clark 1970; Christmann et al., 1977; Tsutsui and Obara, 1984; Losso and Nakai, 1994). Most of these serine residues are phosphorylated. No sulfur-containing amino acids are found in phosvitin proteins. Glycine, alanine, lysine glutamic acid, and threonine are abundant in α-phosvitin, and histidine is abundant in β-phosvitin (Abe et al., 1982).

Phosvitin is a glycoprotein with 6.5% carbohydrate attached to an aspartic acid through a glycosidic bond. Each molecule in average contains six residues of hexose, five of glucosamine, and two of sialic acid (Shainkin and Perlmann, 1971). However, β-phosvitin contains a higher concentration of carbohydrates than α-phosvitin. The sialic acid moiety is 10 times that of α-phosvitin (Itoh et al., 1983).

1.2 Physiological functions of phosvitin

1.2.1 Antibacterial activity of egg yolk phosvitin

The lipopolysaccharide (LPS) present in the outer membrane of the Gram-negative bacteria has the ability to prevent extraneous agents from entering through an effective permeability barrier (Vaara, 1992). Therefore, LPS becomes a target for antibacterial agents. Amphiphilic proteins are potential antibacterial agents due to their strong chelating ability with LPS. Phosvitin is an amphiphilic protein with hydrophobic amino acids and a carbohydrate chain in the C- and N-terminus and hydrophilic phosphoserine residues in the core domain. Phosvitin is well known for its strong metal chelating ability (Grogan and Taborsky, 1987). Phosvitin also shows a great affinity for lipids, as it has been proved to be an excellent emulsifier (Chung and Ferrier, 1991).

Phosvitin exhibits an impressive antibacterial ability against *Escherichia coli* under thermal stress. Khan et al. (2000) reported that *E. coli* (106/mL) was completely eliminated by 0.1 mg/mL phosvitin when heated at 50 ℃ for 20 minutes, whereas about 10% of the bacteria survived in the control group with heating but without phosvitin. They also noticed the bactericidal activity was reduced by adding Ca^{2+} ions which might chelate with phosvitin molecules and thus block the interaction between phosvitin and LPS. Digestion by α-chymotrypsin could degrade the hydrophobic N- and C-terminus but not affect the chelating ability. However, digested phosvitin lost the bactericidal activity. These results suggest that both hydrophilic phosphoserine residues and hydrophobic N- and C-terminus are necessary for the antibacterial activity of

phosvitin. The hydrophobic groups may anchor to the LPS in the outer membrane of the bacteria while the negatively charged phosphoserine residues will chelate with the positively charged metal ions existing in the outer membrane (Khan et al., 2000). In a more recent report, Wang et al. (2011) observed that the zebrafish embryo phosvitin showed bactericidal activity against pathogenic *Aeromonashydrophila, Escherichia coli, A. hydrophila,* and *Staphylococcus aureus*, and the C-terminal of phosvitin was proved to be essential for the antibacterial activity.

1.2.2 Antioxidant activity of egg yolk phosvitin

The antioxidant activity of phosvitin can be attributed to its strong metal chelating ability to Fe (Ⅱ) ions, the catalyst in the Fenton reaction. The Fenton reaction is an important source for reactive oxygen species (ROS). In the Fenton reaction, Fe (Ⅱ) ions can catalyze hydrogen peroxide to produce a hydroxyl radical, which is more reactive and able to damage many cellular constituents. What is worse, this reaction becomes a redox cycle producing ROS continuously under certain conditions such as the presence of reductants. Usually the Fenton reaction is deactivated because most of the Fe (Ⅱ) ions are bound to transferrin and ferritin. But in some cases when Fe (Ⅱ) ions are released from transferrin and ferritin or the amount of Fe (Ⅱ) ions exceeds the binding sites of these proteins, the Fenton reaction will be activated (Cairo et al., 2002). In these instances, antioxidant reagents could be either free radical scavengers or metal (Fe) chelators. Phosvitin belongs to the latter. Ishikawa et al. (2004) revealed

more details of phosvitin's antioxidant activity by using electron spin resonance (ESR). Phosvitin was actually more competitive than ferritin and transferrin in terms of chelating iron, and therefore it would inhibit the oxidation process. The fact that the concentration of iron ions could affect the inhibitory activity of phosvitin supports the idea that phosvitin's antioxidant activity comes from its strong metal chelating ability. In the same way, phosvitin also prevents DNA damage from oxidation catalyzed by Fe (Ⅱ) (Maheswari et al., 1997; Ishikawa, et al., 2004).

Phosvitin is studied extensively as an antioxidant additive in food processing. Lu and Baker (1986) reported that phosvitin inhibited the metal-catalyzed oxidations in an egg yolk phospholipid emulsion system. Phosvitin's inhibitory capacity on Fe^{2+} catalyzed oxidation was 30 times that of a Cu^{2+} catalyzed oxidation. This inhibitory capacity was not affected by pasteurization (61.1℃, 4 min) but significantly reduced by autoclaving (121.1℃, 10 min). Nakamura et al. (1998) reported that the phosvitin-galactomannan conjugate (PGC) produced by the Maillard reaction was 70% more effective than native phosvitin in preventing iron-catalyzed oxidation in a linoleic acid system. What is more, autoclaving (121℃, 15 min) will not decrease PGC's antioxidant activity. In a phospholipid system, adding salts (NaCl) or proteins (albumen) does not affect phosvitin's antioxidant activity (Lu and Baker, 1987).

1.2.3 Metal-chelating capacity of egg yolk phosvitin

Vitellogenin is the precursor of phosvitin and lipovitellins. It is synthesized in the liver and transferred through the blood system to growing follicles.

Vitellogenin is later hydrolyzed into lipovitellin, phosvitin, and other minor proteins. Although the overall biological function remains unclear, phosvitin acts as a metal carrier and is probably involved in the mineral metabolism during embryonic development (Burley and Vadehra, 1989).

For a long time, chicken eggs have been considered to decrease the intestinal absorption of iron, magnesium, and calcium (Callender et al., 1970; Ishikawa et al., 2007). This phenomenon could be explained by the fact that phosvitin molecules bind nearly all of the iron, and most of the magnesium and calcium in the yolk and form an insoluble complex which is resistant to digestion (Greengard et al., 1964; Sato et al., 1985). Such strong metal-chelating capacity could be attributed to phosvitin's high proportion of negatively charged phosphoserine residues (50% of total amino acids) (Taborsky, 1983; Byrne et al., 1984). The physiological activities of phosvitin, such as antibacterial and antioxidant activity, are usually related to its metal binding ability (Nakamura, et al., 1998; Khan, et al., 2000)

Although there was a dialysis procedure during extraction, the first purified phosvitin still contained a lot of salts, including iron, copper, sodium, and calcium (Mecham and Olcott, 1949). The binding ability with iron, magnesium and calcium has been extensively studied. Phosvitin can bind more than twice the amount of calcium and magnesium ions at pH 6.5 than at pH 4.5. It is not difficult to understand this if we know that at neutral pH 6.5, most of the phosphate groups are dianionic, whereas at pH 4.5, only one hydroxyl is ionized in each phosphate group (Grizzuti and Perlmann, 1973). However, phosvitin can

be precipitated by increasing the concentration of magnesium or calcium (Grizzuti and Perlmann, 1973; Itoh et al. 1983). Phosvitin also binds trace elements such as Mn^{2+} and Co^{2+}. Heating up to 60℃ does not affect the binding capacity with these metal ions (Grizzuti and Perlmann, 1975).

The atomic ratio of iron to phosphorus (Fe/P) was introduced to study the solubility of the phosvitin-iron complex. Phosvitin will be precipitated at a certain Fe/P ratio, but it will recover the solubility when this ratio either increases or decreases (McCollum et al., 1986). Phosvitins from different species have different degrees of phosphorylation; therefore the ratios for maximum precipitation vary in a range from 0.4 to 1.0 (Taborsky, 1991). For chicken egg yolk phosvitin, the ratio for the maximum precipitation is 0.5. The phosvitin-iron complex is so stable that heating at 110℃ for 40 minutes did not release iron at any significant level. Only the metal chelating agent EDTA could release 50% iron from phosvitin without heating (Albright, et al., 1984). Therefore when people prepare the metal-free phosvitin by size exclusion chromatography, the eluting buffer usually contains EDTA (McCollum et al., 1986; Castellani et al., 2003).

1.2.4 Emulsifying properties of egg yolk phosvitin

Egg yolk has been widely used as a natural emulsifier in food production for many years, but the roles of individual yolk proteins in food emulsion are not well elucidated. Phosvitin is one of the major proteins in egg yolk, and is also usually considered to contribute to the overall emulsifying properties.

Many factors, such as protein concentration, oil volume fraction, mixing time,

mixing speed, pH, salt concentration, and temperature, can affect phosvitin's emulsifying properties (Chung and Ferrier, 1991, 1992 and 1995). The emulsifying ability and stability increased with the increase of the protein concentration, oil volume fraction, mixing time, and mixing speed. At the neutral pH, phosvitin exhibits better emulsifying properties than bovine serum albumin (BSA). Phosvitin's emulsion stability and emulsifying ability are significantly affected by pH changes. At the neutral pH, the emulsion stability is much higher than that of other pH but the emulsifying ability is relatively low (Chung and Ferrier, 1991; Chung and Ferrier, 1992). A low pH is favorable to suppress phosvitin's negative charges and to improve the adsorption of its molecules to the oil-water interface. Adding egg yolk lipoproteins improves the emulsion stability at a higher pH of 7.0 and 9.0 (Aluko and Mine, 1997). Heat treatment decreases phosvitin's emulsifying properties by decreasing its denaturation temperature. Heating over 65℃ could decrease the emulsifying ability. Heating over 70℃ for 60 minutes will decrease the emulsion stability (Chung and Ferrier, 1995). The aggregation of phosvitin molecules helps to improve the emulsion stability but not the emulsifying ability. Low ionic strength could help the emulsion formation (Castellani et al., 2005). Phosvitin usually exhibits high emulsifying properties and metal binding ability under the same conditions. What is more, the iron-binding capacity will not be altered once phosvitin is adsorbed to the oil-water interface, where the hydrophobic C-terminus of phosvitin is buried in the oil phase but the hydrophilic core remains in the aqueous solution (Castellani et al., 2006). These findings are quite important

since they eliminate concerns about the additional oxidation risk brought by the phosvitin-released iron in emulsions.

Food systems usually contain many kinds of metal ions. Therefore it is necessary to study the influence of these ions on phosvitin's emulsifying properties. Mg and Fe could flocculate the phosvitin-stabilized emulsions at the neutral pH. This flocculation can be totally avoided by adding EDTA before forming the emulsion, and partially reversed by adding EDTA after forming the flocculation. Sodium dodecyl sulfate (SDS) alone can't de-flocculate the emulsions stabilized by phosvitin, but working with EDTA can completely de-flocculate this system (Castellani et al. 2008). Phosvitin can form aggregates through a phosphocalcic bridge (Grizzuti and Perlmann, 1973; Grizzuti and Perlmann, 1975). Calcium ions will interact with the phosphoserine residues between two phosvitin molecules to form a stable three-dimensional network. As a result, adding calcium ions to phosvitin-stabilized emulsions will increase the amount of phosvitin molecules adsorbed at the oil-water interface, rearrange these molecules towards a more compact structure, and decrease the surface pressure of oil droplets (Belhomme et al., 2008).

Phosvitin can suppress the insolubilization of egg white proteins caused by heating and improve the hardness and transparency of the mixed gels. But adding salt decreases the preventive effect on the heat-induced insolubilization. The mechanism behind this phenomenon remains unknown (Matsudomi et al., 2006).

1.3 Phosvitin extraction and purification

Mecham and Olcott (1949) extracted a phosphoprotein containing 10% phosphorus from chicken egg yolk with a recovery of 60-70%. They diluted egg yolk with magnesium sulphate solution and then centrifuged it to collect the protein fraction. This fraction was dispersed in 0.4 M ammonium sulfate at pH 4.0 and extracted overnight by ethyl ether. The supernatant was filtered and dialyzed against saturated ammonium sulfate. Precipitation was extracted by sodium chloride and dialyzed against water. The phosvitin was extracted in the supernatant and freeze-dried. The purity of this phosvitin preparation, which was described by the atomic ratio of nitrogen to phosphorus (N/P), was 2.72. Mecham and Olcott (1949) were the first to obtain relatively pure phosvitin and also the first to reveal major features of this protein. Many of the following workers are inspired by their research. However, this extraction procedure is tedious and uses many chemicals. It has to be simplified to broaden its potential application.

A series of methods to isolate phosvitin, lipovitellin, lipovitellinin, and livetins from chicken egg yolk were developed, and phosvitin was proved to be an individual protein interacting strongly with but not part of lipovitellin (Turner and Cook, 1958; Martin and Cook, 1958; Joubert and Cook, 1958). The phosvitin extraction protocol developed by Joubert and Cook (1958) is mainly based on the observation of Mecham and Olcott (1949) that phosvitin precipitates in the presence of a low concentration of $MgSO_4$. This phosvitin-enriched precipitation was then extracted with 10% sodium chloride. After

centrifugation, the subnatant was dialyzed against an acetate buffer at pH 4, and phosvitin was obtained in the supernatant. This preparation contained 9.6% phosphorus and 12.6% nitrogen, and showed chemical and physical properties similar to the preparation by Mecham and Olcott (1949).

Sundararajan et al. (1960) reported a more simplified extraction method. It was adopted by Sigma-Aldrich Co. LLC to prepare the commercial phosvitin product. The lipids were first removed by three times water dilution of egg yolk followed by centrifugation. The residue was extracted by 10% NaCl, butanol and ether in series. Phosvitin was first precipitated at pH 1.8 and then re-precipitated in the presence of 0.09 M $MgSO_4$. A repeat process is necessary to obtain higher purity. The yield was close to that of Mecham and Olcott (1949). This report confirmed the observations that phosvitin tends to precipitate at a low pH and low concentration of $MgSO_4$.

Technological developments have helped immensely to increase the understanding of phosvitin. Although these early phosvitin preparations appeared homogeneous following the ultracentrifugation, two or more components were present by electrophoresis (Mecham and Olcott, 1949; Bernardi and Cook, 1960). Three phosvitin precursors were found in chicken plasma supporting the variability of phosvitin (Wang et al., 1983). Wallace and Morgan (1986) developed a method to isolate phosvitin and characterized the constituents of this preparation. Egg yolk was first diluted with two volumes of phenyl methyl sulfonyl fluoride (PMSF) and centrifuged. Precipitation was dissolved in 1 M NaCl and centrifuged again to get rid of low-density

lipoproteins. Phosvitin was obtained in the supernatant after adding saturated $(NH_4)_2SO_4$. The yield was 50-70 mg/egg.

Because most of the existing methods to extract phosvitin involved non-food-grade chemicals and rendered other yolk proteins unsuitable for further use, Losso and Nakai (1994) developed a much simplified method to extract phosvitin from egg yolk using food grade reagents. This method is mainly based on the observations of Tsutsui and Obara (1984), Yamamoto et al. (1990), and Akita and Nakai (1992). The egg yolk was 10 times diluted with water. The supernatant was kept for IgY extraction (Akita and Nakai, 1992). The precipitate was first extracted by hexane to remove the lipids, and then extracted by 10% NaCl. Phosvitin was obtained in the supernatant after dialysis. This phosvitin preparation contained 15.20% nitrogen and 9.34% phosphorus (Losso and Nakai, 1994). This work is of great importance since it is the first attempt to extract phosvitin for applications in the food industry.

Castellani et al., (2003) developed a phosvitin extraction method without organic solvents based on the observation of McBee and Cotterill (1979). The egg yolk was two times diluted with 0.17 M NaCl and centrifuged to obtain granules. The granules were dissolved in 10% NaCl and dialyzed against water. Crude phosvitin was precipitated in the presence of 0.2 M $MgSO_4$. The N/P of this preparation was as high as 3.60, suggesting a low purity. The yield of this method was also inadequate since more phosvitin was not precipitated by $MgSO_4$.

According to previous studies, extracting phosvitin usually involves two steps:

the first is to remove the lipids and obtain the protein fractions containing phosvitin; the second is to break the phosphocalcic bridge between phosvitin and lipovitellin and separate them by differences in solubility. For the second step, workers actually practice two principles. One principle is that phosvitin will precipitate in the presence of Mg at certain concentrations. The other is that lipovitellin will precipitate after being treated with organic solvents followed by salt extraction and dialysis (Chargaff, 1942). In most cases, people adhere a combination of the two principals.

1.4 Phosphopeptides

1.4.1 Development of bioactive peptides

Bioactive peptides can be defined as "*specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health."* (Korhonen and Pihlanto, 2006). Peptides released by proteolytic hydrolysis from inactive food proteins sequences exhibit various physiological activities. These peptides with physiological activities or bioactivities are attracting more and more attention from researchers and the public. Numerous peptides have been identified with positive physiological functions in the cardiovascular, digestive, immune and nervous systems (Hartmann and Meisel, 2007). The physiological functions of these peptides can be attributed to their particular amino acid sequences, which usually cover from two to twenty amino acid residues. However, small peptides with two to three amino acid residues are preferred since these peptides can survive through digestion and be absorbed as

an intact molecule into blood circulatory system to exhibit the activities.

1.4.2 Casein phosphopeptides (CPPs)

The term phosphopeptide was first introduced to describe casein-derived phosphorylated peptides with the ability to enhance vitamin D independent bone calcification in rachitic children (Mellander and Isaksson, 1950; Mellander, 1950). The phosphorylated regions in bovine casein can be released by *in vivo* digestion (Meisel et al., 2003), *in vitro* proteolytic hydrolysis (Kitts et al., 1994), and even during the ripening of cooked curd cheeses (Ferranti et al., 1997). Phosphorylated amino acid residues in CPPs could limit the precipitation of calcium in the intestines by forming soluble organophosphate salts, and thus enhance the absorption of calcium (Meisel et al., 2003).

1.4.2.1 Calcium absorption promoting ability

After Mellander (1950) reported that casein phosphopeptides derived from peptic or tryptic digests could increase calcium absorption of infants, many workers attempted to determine the primary structure of CPPs from both *in vitro* and *in vivo* digestion (Peterson et al., 1958; Bennich et al., 1959). Naito et al. (1972) isolated a phosphopeptide fraction with an N/P ratio of seven to nine from digests of rats fed with a diet containing 20% casein. This fraction represented 30% of the total peptides contents in the digests and exhibited great affinity for calcium. The N/P ratio and molecular size of phosphopeptides varied with the progress of digestion but the presence of phosphopeptides was identified even after nine and half hours. The phosphopeptides generated at

different stages of digestion possessed different characteristics and exhibited a dissimilar ability to stimulate calcium absorption. The N/P ratio and molecular weight are both important for phosphopeptides' physiological functions.

Crude CPPs prepared by *in vitro* tryptic digestion were also proved to promote calcium absorption in normal and rachitic chicks. After chromatography separation, the fractions of CPPs with different molecular weights and phosphorus contents were also proved to enhance the vitamin D independent transportation of calcium in both normal and rachitic chicks (Mykkanen and Wasserman, 1980).

The ability of CPPs to increase calcium absorption can be attributed to the high amount of phosphoserine groups. Both native CPPs and dephosphorylated CPPs were studied in a model of explanted embryonic rat-bone rudiments. Results showed that native CPPs could increase calcium solubility and promote calcification under culture conditions, whereas dephosphorylated CPPs had no significant effect (Gerber and Jost, 1986). This finding was confirmed by many workers and widely accepted (Berrocal et al., 1989).

Although CPPs have been proven to enhance calcium absorption *in vitro* and *in situ* (Sato et al., 1986; Yuan and Kitts, 1991), it took years to confirm the same activity through *in vivo* experiments. Heaney et al. (1994) demonstrated that CPPs promoted calcium absorption in postmenopausal women with low basal absorptive performance. In a model of aged ovariectomized rats, CPPs also showed an inhibitory effect on postmenopausal bone loss (Tsuchita et al., 1995). However, many factors might affect the ability of CPPs to enhance calcium

absorption. Some experiments also produced controversial results. Bennett et al. (2000) found that high CPPs content diet (from 200 to 500 g CPP/kg meal) significantly reduced calcium absorption, but that a high casein content diet could increase calcium absorption. No dose relationship was evidenced. The recent studies mainly focus on the molecular structure of CPPs. It is generally accepted that the metal chelating ability of CPPs could be attributed to a common motif that consists of three phosphoserine residues and two glutamic acids, Ser(P)-Ser(P)-Ser(P)-Glu-Glu (West and Towers, 1976; Schlimme and Meisel, 1995). However, the motif alone does not exhibit the bioactivities. Ferraretto et al. (2003) proved that the synthesized motif structure of Ser(P)-Ser(P)-Ser(P)-Glu-Glu alone was not able to increase calcium absorption *in situ*. Meanwhile they observed that the synthesized CPPs promoted calcium absorption in the same model. But after dephosphorylation or modification to the N-terminus, synthesized CPPs became inactive (Ferraretto et al., 2003). These results suggested that CPPs require a certain molecular structure to exhibit their physiological activities (Gravaghi et al., 2007).

1.4.2.2 Casein phosphopeptide-amorphous calcium phosphate nanocomplexes

Casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) have attracted the most research interest over the past decade. In bovine milk, 68% of calcium and 47% of phosphates exists as nanometer-sized ion clusters in the casein micelles. These calcium phosphate ion clusters are associated with casein proteins through electrostatic and hydrophobic interactions to form the three-dimension structure of casein micelles (Hooydonk

et al., 1986; Kruif, 1999). CPPs, the hydrolysate of casein, carry on the ability of casein micelles to keep calcium and phosphate ions soluble and bioavailable for intestinal absorption and remineralization of subsurface lesions in tooth enamel (Reynolds et al., 1999; Cross et al., 2005). The cluster sequence motif Ser(P)-Ser(P)-Ser(P)-Glu-Glu in CPPs is considered to be critical for calcium and phosphate chelating ability (West and Towers, 1976; Schlimme and Meisel, 1995). The formation of CPPs-calcium phosphate nanocomplex was evidenced and determined by using powder diffraction x-ray crystallography, scanning electron microscopy, and transmission electron microscopy. The current studies suggest that CPPs have great potential to be applied as delivery vehicles for calcium and phosphate ions transportation (Ferraretto et al., 2001; Cross et al., 2005).

Many clinical reports support the inhibitory effect of CPP-ACP on the progression of caries and promoting effect on regression of caries (Andersson et al., 2007; Morgan et al., 2008; Bailey et al., 2009). The promoting effect on remineralization of enamel subsurface lesions is confirmed by both *in vitro* and *in situ* experiments (Reynolds, 1998; Oshiro et al., 2007; Cai et al., 2003; Reynolds et al., 2003; Cai et al., 2007). CPP-ACP can form soluble nanocomplexes with fluoride ions and therefore enhance their fixation at the tooth surface and the remineralization of the enamel (Reynolds et al., 2008). CPP-ACP could be applied as an assistive treatment in addition to fluoride therapy of early stage caries lesions (Reynolds, 2009). Also, when added to beverages or sugar-free chewing gum, CPP-ACP retains the ability to

remineralize enamel subsurface lesions and to increase the acid resistance of tooth (Ramalingam et al., 2005; Manton et al., 2010).

1.4.2.3 Other application of CPPs

CPPs are reported to exhibit immunomodulating activities. Wong et al. (1996) first reported that β-casein significantly increased the mitogenic effect in ovine lymphocytes. In animal experiments with piglets and mice, both the intestinal total and antigen-specific IgA levels were increased (Otani et al., 2000a; Otani et al., 2000b). The phosphoserine residues in CPPs were recognized as an immunomodulating factor (Hata et al., 1998). The commercial CPPs are also proved to reduce allergic symptoms mediated by the IgE antibody, and to stimulate the immune system by increasing cytokine levels in human epithelial intestinal cell lines (Kawahara and Otani, 2004; Otani and Wakatsuki, 2004; Kitts and Nakamura, 2006). The motifs of Ser(P)-Leu-Ser(P) and/or Ser(P)-Ser(P)-Ser(P) are the required structures for the immunoregulating activity of CPPs (Otani et al. 2001).

Compared with the extensive studies on calcium absorption, the studies on the antioxidant activity of CPPs are quite limited. CPPs with small molecular weights were proven to be an effective natural antioxidant to prevent lipid oxidation during meat processing (Sakanaka et al., 2005; Rossini et al., 2009). Although the mechanism is still unknown, it was suggested that CPPs' free peroxyl radicals scavenging capacity was related to the high amounts of antioxidant amino acids such as histidine, lysine, proline, and tyrosine (Kim et al., 2007). However, we could expect phosphoserine residues to play a more

important role in the antioxidant activity of CPPs due to their strong chelating ability with iron, the catalyst of H_2O_2-induced oxidation.

CPPs are also confirmed to enhance the absorption of iron and zinc. More research is needed to elucidate the mechanism behind these observations (Hansen et al., 1996; Yeung et al., 2001; Bouhallab et al., 2002).

1.4.3 Phosvitin phosphopeptides (PPPs)

1.4.3.1 Preparation of PPPs

Due to the high amount of phosphoserine residues, egg yolk phosvitin is expected to be a better protein source than casein for producing bioactive peptides with calcium-absorption promoting ability (Byrne et al., 1984). However, the study of PPPs is hindered by the fact that phosvitin is very resistant to enzymatic hydrolysis (Mecham and Olcott, 1949).

Shainkin and Perlmann (1971) attempted to hydrolyze phosvitin with pronase, a mixture of enzymes isolated from *Streptomyces griseus*, and obtained seven fractions. This process consumed too much time and the hydrolysis of phosvitin is not completed. Goulas et al. (1996) systematically studied the hydrolysis of phosvitin by pepsin, chymotrypsin, and trypsin. All three enzymes could produce only three to four fragments from the N or C-terminus, while the large core of phosvitin molecules remained intact. Also, these three enzymes were more efficient at releasing peptides from the N-terminus than from the C-terminus. Compared with the other two enzymes, pepsin produced a smaller fragment with a single, eight-residue-long oligophosphoserine run from the C-terminus. It was

suggested that the low pH for peptic digestion suppressed the negative charges of phosphoserine residues, making phosvitin more accessible for the proteolytic action. However, such a low pH lead to a conformational change towards a β–type structure, and this conformational change could render the proteolytic action of enzymes (Taborsky, 1968). Besides, the bulk of polysaccharide adjacent to the C-terminus might also render some peptide bonds inaccessible. Therefore the hydrolysis of C-terminal peptide chain is not as complete as that of the N-terminus. Although there are several Lys and Arg residues existing in the core of phosvitin, the negative charges from nearby phosphoserine residues will block the enzymes' access to the peptide bonds close to these residues. Neutralizing the negative charges with metal ions cannot increase the degree of hydrolysis. The addition of metal ions will neutralize the charges of the side chain, but at the same time these ions were bound to phosphoserine residues and blocked the accessible peptide bonds by bulk (Goulas et al., 1996). Khan et al. (1998) confirmed the above observations. They also determined these phosphorylated fragments might be generated from N-terminal 1 to 49 and C-terminal 211 to 217 amino acids, and the remaining large core might cover from 50 to 210 amino acids.

Jiang and Mine (2000) were the first to obtain short-chain PPPs with calcium-binding ability. Their work inspired many of the following researchers. Their protocol to prepare PPPs was widely used in relevant studies. Jiang and Mine (2000) performed a partial dephosphorylation (0.1 N NaOH, 3 h) on phosvitin prior to tryptic digestion. The phosphopeptides released from the

dephosphorylated phosvitin possessed relatively smaller MWs (<3 kDa) compared with the peptides (28 kDa) obtained from native phosvitin. The peptide bonds adjacent to lysine and arginine became more vulnerable to proteolytic action after dephosphorylation. There was no significant difference in the molecular weight of PPPs derived from phosvitin dephosphorylated by different concentrations of NaOH, but the final phosphate retention in PPPs was different and was of great importance for their calcium-binding ability.

1.4.3.2 Calcium-binding ability

The peptides obtained from phosvitin with 17.5% and 35% phosphate retention solubilized more calcium than commercial CPPs. PPPs with 65% phosphate retention exhibited much lower ability to solubilize calcium (Jiang and Mine, 2000). Jiang and Mine (2001) further separated these PPPs into two fractions: PPP1 with a MW of less than 1 kDa and PPP2 with a MW of 1-3 kDa. PPP2 could solubilize more calcium than commercial CPPs whereas PPP1 and O-phospho-1-serine alone did not show significant calcium-binding ability. For PPP2, the amount of bound calcium increased linearly with the increase of free calcium concentration, and the binding constant was close to that of CPPs. In the same way, PPPs can solubilize iron and increase its intake in Caco-2 cells (Feng and Mine, 2006). Since CPPs have been successfully commercialized, PPPs' potential as novel functional food ingredients is very promising.

Choi et al. (2005) hydrolyzed native phosvitin with trypsin to prepare PPPs. This hydrolysate is expected to contain both an intact phosvitin core and small fragments from the C- and N-terminus since they did not perform

dephosphorylation prior to hydrolysis (Goulas et al., 1996; Khan et al., 1998). Interestingly these phosvitin hydrolysates displayed a higher ability to solubilize calcium than commercial CPPs at the ratio of peptides/Ca (w/w) above 1.0. The possible explanation might be that the undigested phosvitin core was also able to solubilize calcium. To explain this result, the binding constant of the hydrolysate should be determined. This binding constant is critical for intestinal calcium absorption and needs to be kept at an appropriate level. For example, the binding constant of CPPs for calcium is between 10^{-2}-10^{-3} M^{-1}. These numbers could be used as a reference when preparing PPPs (Jiang and Mine, 2001). If the binding ability of PPPs is too strong, less calcium will be released and absorbed in digestive systems. On the other hand, if the binding ability is too weak, calcium will precipitate and cannot be absorbed either. In the following animal experiment with SD rats, Choi et al. (2005) reported that diets with 0.125-0.5% phosvitin hydrolysate significantly enhanced the calcium absorption and accumulation in bones. These results are significant for the application of phosvitin phosphopeptides as functional food ingredients. But before that more studies are needed to confirm these results.

1.4.3.3 Antioxidant activity of PPPs

Katayama et al. (2006) prepared PPPs with a MW of 1-3 kDa according to the method of Jiang and Mine (2000). By using anion exchange chromatography, they further separated the PPPs into three fractions: PPP-1, PPP-2, and PPP-3, with phosphorus contents of 0, 7.2% and 18.9%, respectively. The inhibitory effects of the whole PPPs and fractions against hydrogen peroxide-induced

oxidative stress in Caco-2 cells were investigated. IL-8 secretion and malondialdehyde formation, which are considered to be biomarkers of oxidative stress, were significantly reduced by pretreatments of PPPs but not by intact phosvitin. Phosphorus content played an important role in the antioxidant activity of PPPs. Higher phosphorus content produced higher antioxidant activity. However, the free phosphoserines alone were found inactive. These results suggested that both phosphorus content and molecular structure are critical for the antioxidant activity of PPPs (Katayama et al., 2006). PPPs could also up-regulate γ–glutamylcysteine synthetase and other antioxidant enzymes in Caco-2 cells against oxidative stress (Katayama et al., 2007). Similarly, PPPs with higher phosphorus content displayed a better ability to enhance the intracellular antioxidant defense systems.

Xu et al. (2007) reported that PPPs showed 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging ability, iron-chelating ability, and antioxidant activity in a linoleic acid system. They proposed that the amino acid composition of PPPs, rather than phosphorus content, might play a more important role in the antioxidant activity. Histidine, tyrosine, and methionine, which have been demonstrated to be antioxidant amino acids, were found to cover a larger proportion in PPPs than that in native phosvitin. In a recent study by Chay Pak Ting et al. (2011), PPPs fractions with a lower MW (<5 k Da) exhibited much higher antioxidant activity. Their PPPs were also featured with the high phosphorus content and high amounts of His, Met, Leu, and Phe.

1.5 Hypothesis

1.5.1 Phosvitin extraction

If phosvitin interacts with lipovitellin through a phosphocalcic bridge, then phosvitin can be separated from lipovitellin by using high ionic strength (e.g. 10% NaCl) to disrupt this bridge. Phosvitin and lipovitellin have different electric points (PI); therefore it is possible to further separate these two proteins by pH adjustments.

In this study granules were first obtained after two times water dilution of egg yolk and then mixed with 10% NaCl to disrupt lipovitellin-phosvitin complex. After dialysis, lipovitellin was partially precipitated from the granules solution, whereas phosvitin was presented in the supernatant. Lipovitellin, also recognized as a high-density lipoprotein (HDL) in egg yolk, is consisted of α-lipovitellin and β-lipovitellin (Burley and Cook, 1961). Both lipovitellins are soluble at alkaline pH; however, α-lipovitellin and β-lipovitellin would precipitate at pH 7.5-7.8 and pH 6.5-7.0, respectively (Sugano, 1957). Castellani et al. (2003) observed the solubility of phosvitin was not significantly affected when the pH was decreased from 7.0 to 5.0, whereas lipovitellin was completely precipitated at pH below 5.5. Considering the isoelectric point (pI) of phosvitin was around pH 4 (Ternes, 1989), we hypothesized that phosvitin might be separated from lipovitellin by solubility difference as the pH was adjusted from 7.0 to around 5.5. Therefore, effects of the pH of phosvitin extracts (the supernatant of dialysate) on their purity and recovery were studied.

1.5.2 Preparation of PPPs

If the phosphate groups on phosvitin's side chain are alkali liable as proved in literatures (Khan et al, 1998; Jiang and Mine, 1999), then appropriate treatment with NaOH could partially remove the phosphate groups from phosvitin molecule and thus makes the molecule vulnerable for enzymatic hydrolysis. We hypothesized that phosvitin phosphopeptides could be prepared through enzymatic hydrolysis.

1.6 Objectives

PPPs, exhibiting impressive antioxidant activity and calcium-absorption promoting ability, can be used as novel functional food ingredients or nutraceuticals. However, the application of PPPs is hindered by the lack of food industry compatible protocols to extract phosvitin, and efficient and economic protocols to prepare PPPs from phosvitin.

Therefore the objectives of this research were:

- To develop a food-industry-compatible protocol to extract phosvitin from chicken egg yolk;
- To prepare and characterize phosvitin phosphopeptides.

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CHAPTER 2 PHOSVITIN EXTRACTION FROM EGG YOLK

2.1 Introduction

Phosvitin (PV) is the principal phosphoprotein in chicken egg yolk, and the most highly phosphorylated protein in nature (Mecham and Olcott, 1949). It represents about 11% of yolk proteins or 4% of yolk solids (Burley and Cook, 1961). Phosvitin contains 10% phosphorus which is mono-esterified to the 123 serine residues out of its total 217 amino acids (Mecham and Olcott, 1949; Byrne et al., 1984; Clark, 1985). Phosvitin shows great potential for food or functional food uses, as it has excellent emulsifying, antioxidant, antibacterial and metal chelating properties (Khan, et al., 2000; Nakamura, et al., 1998; Albright, et al., 1984; Chung and Ferrier, 1992).

Phosvitin was first purified by Mecham and Olcott (1949) using a lengthy protocol including precipitation by 0.08 M $MgSO_4$, and extraction subsequently with 0.4 M $(NH_4)_2SO_4$, ethyl ether and 0.25 M NaCl. The recovery of phosvitin was 60-70%. Sundararajan et al. (1960) simplified the protocol by preparing egg yolk granules, followed by extraction with 10% NaCl, butanol and ether and precipitation at pH 1.8 in the presence of 0.4 M $MgSO_4$. Their yield and purity were comparable with that of Mecham and Olcott (1949). Most of the subsequent extraction methods were developed based on these two protocols, first involving organic solvent to remove lipids and then salt extraction step to obtain phosvitin (Joubert and Cook, 1958; Tsutsui and Obara, 1984; Losso and Nakai, 1994; Ko et al., 2011).

Ion exchange chromatography is a separation process based on the charge of the analytes. Phosvitin is negatively charged due to its high amount of phosphate groups on side chain; therefore it is feasible to purify phosvitin by anion exchange chromatography. Anion exchange chromatography was used to study the heterogeneity of phosvitin. Connelly and Taborsky (1961) resolved phosvitin into two fractions by using a DEAE-cellulose column. These two fractions were similar in terms of nitrogen and phosphorus contents but different in metal content, amino acid composition, and chemical stability. McBee and Cotterill (1979) developed a method to separate lipovitellin, livetin and phosvitin from egg yolk granules, and further purified phosvitin using a DEAE cellulose column (Sundararajan 1960; Joubert and Cook 1958). Wallace and Morgan (1986b) purified phosvitin by using a Pharmacia Mono Q column and identified another phosphoprotein, the phosvettes. Based on these studies, Castellani et al. (2003) applied anion exchange chromatography to purify phosvitin from crude phosvitin, prepared in the presence of Mg^{2+}. The corresponding yield of 1.7 g/100 g yolk solids is low and the use of Mg^{2+} is not food compatible. Lei and Wu (2011) developed a much simpler method without extraction of crude phosvitin prior to chromatography, but their recovery is only 35.4%.

The objective of the study was to improve phosvitin extraction from egg yolk; crude phosvitin was initially prepared by NaCl extraction and then further purified by ion exchange chromatography.

2.2 Materials and methods

2.2.1 Chemicals and materials

Sodium chloride, sodium hydroxide, and hydrochloric acid were purchased from Fisher Scientific (Nepean, ON, Canada). Glycine, Precision Plus Protein Standard, sodium dodecyl sulfate (SDS), and precast gels (10-20% Tris-HCl) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Phosvitin standard (P1253, from chicken egg yolk) was purchased from Sigma-Aldrich, Ltd. (Oakville, ON, Canada). The deionized distilled water (DD water) used for in the study was produced by a Barnstead water purification system (Thermo Scientific, Asheville, NC, USA).

2.2.2 Egg yolk

Eggs were purchased from a local supermarket. Egg shell was manually broken, and yolk was separated from white and then carefully rolled on a Whatman filter paper (Whatman Inc., Florham Park, NJ, USA) to remove albumen and chalazas residue on the vitelline membrane. The membrane was then punctured by a needle and egg yolk was collected in a beaker cooled with ice.

2.2.3 Granules preparation

Granules were prepared according to the protocol of McBee and Cotterill (1979) with slight modifications. Yolk was two times diluted with deionized water, mixed by magnetic stirring for one hour at 4^{o}C, and centrifuged at 10,000 g for 45 minutes at 4^{o}C to obtain the precipitate, which was called granules.

2.2.4 Phosvitin extraction

Granules prepared above were suspended in 10 times weight of 10% NaCl solution, keeping the pH to 7.25 by adding 3 M NaOH with magnetic stirring at 4^{o}C overnight (Castellani et al., 2003). The solution was dialyzed against DD water for 24 hours with 4-5 water changes, and centrifuged at 10 000g, 4^{o}C for 25 min. The supernatant obtained after dialysis was divided into aliquots. The pH of each aliquot was adjusted to 8 ("as is"), 7, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0 and 3.5, respectively; after stirring for 1 hour, the aliquots were centrifuged at 10 000 g, 4^{o}C for 25 min. The supernatants were collected for further analysis.

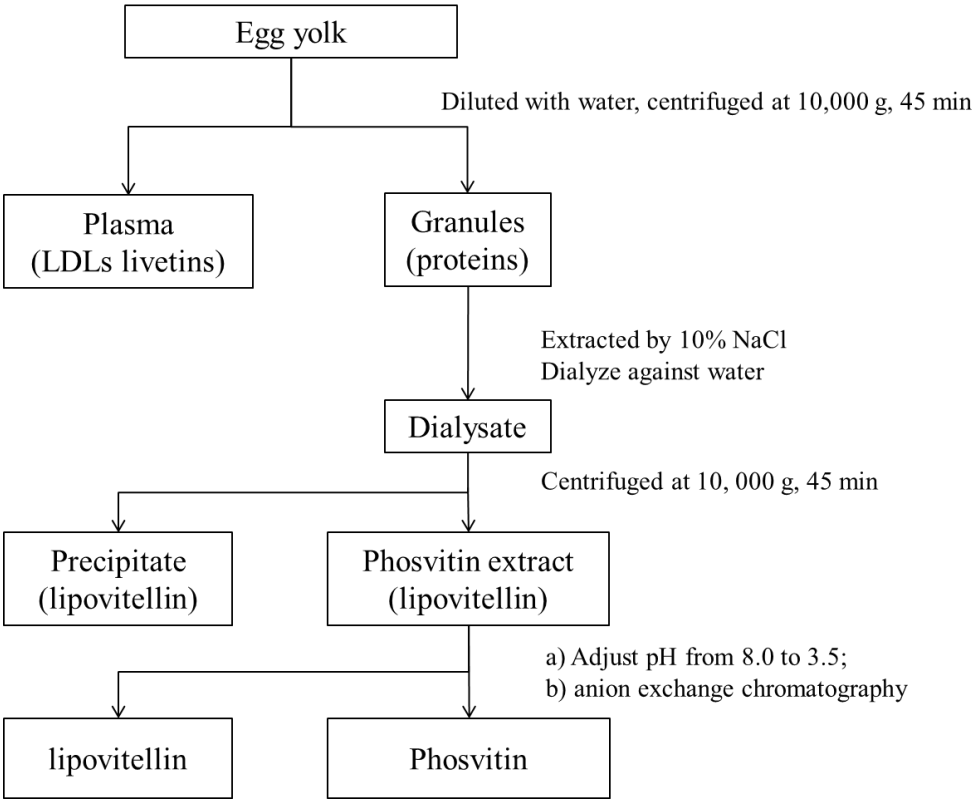

Figure 2-1 Flowchart of phosvitin extraction protocol developed in this study

2.2.5 Gel filtration chromatography

Gel filtration chromatography was carried out using a Waters HPLC system (Waters, Milford, MA, USA) in a TSK-Gel G3000SWxL stainless column (0.78 x 30 cm, Tosoh Bioscience, Inc., South San Francisco, CA, USA). The phosvitin extracts were 1:1 (v/v) diluted with 2× volume of running buffer and filtered with 0.45 μm PVDF filter (13 mm x 0.20 μm, MANDEL, Guelf, ON, Canada). The injection volume was 30 μL. The column was eluted by 0.1 M sodium phosphate buffer (containing 0.2 M NaCl, pH 7.0) at a flow rate of 0.5 mL/min. The elution was monitored at 215 nm. The experiment was controlled by Empower II Software (Waters, Milford, MA, USA). The content of phosvitin in the extracts was calculated from a standard curve prepared by the standard phosvitin at concentrations ranging from 0.1 to 5.0 mg/mL.

2.2.6 Anion exchange chromatography

Crude phosvitin extract was prepared at 15 mg/mL with buffer A (0.05 M tris-HCl at pH 8.0) and filtered through 0.45 μm membrane (Millipore, Billerica, MA, USA) before loading to a HiPrep 16/10 Q FF anion exchange column (GE Healthcare, Piscataway, NJ, USA). The elution was monitored at 215 nm and operated by an AKTA explorer 10S system (GE Healthcare, Piscataway, NJ, USA) from 100% buffer A to 100% buffer B (1.0 M NaCl in buffer A). Fractions were collected and lyophilized for analysis.

2.2.7 Calculation of phosvitin purity, recovery and yield

Phosvitin purity was calculated based on peak area integration in the HPLC chromatograms. Phosvitin recovery was calculated as a percentage based on the weight ratio of amount of phosvitin in the extracts to the total phosvitin in the egg yolk, which was assumed as 4% of egg yolk solids according to Joubert and Cook (1958). Phosvitin yield was calculated as the amount of phosvitin in extracts from 100 g yolk solids. The phosvitin content in extracts was calculated by gel filtration chromatography using Sigma's phosvitin standard.

2.2.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was carried out according to Shapiro et al. (1967) using continuous system (10-20%) gels obtained from Mini-Protean Tetra Cell (Bio-Rad, Hercules, CA, USA). The running buffer was Tris-HCl at pH 8.8 with 0.1% SDS. All samples were prepared in Leammli buffer with 5% (v/v) 2-mercaptoethanol and heated at 95^{o}C for 5 min. After centrifugation at 15,000 g for 5 min, 20 µL of supernatant was loaded to the gel. Electrophoresis was conducted at a constant voltage of 200 V for about 40 min. Precision Plus Protein Standards (Bio-Rad, Hercules, CA, USA) with molecular weights of 250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa were also loaded as the molecular weight standards. After electrophoresis, the gels were stained with 0.05% Coomassie brilliant blue R-250 in a solution of 0.1 M aluminum nitrate/25% isopropanol/10% acetic acid/1.0% Triton X-100, prepared according to the method of Hegenauer et al. (1977), and destained in 7% acetic acid solution. Images of gels were scanned in an Alphachem SP machine, and molecular

weight (MW) was analyzed by AlphaEase FC software version 6.0.0 (Alpha Innotech Corporation, Santa Clara, CA, USA) based on molecular weight markers.

2.2.9 Nitrogen and protein determination

Nitrogen was determined in duplicate by using the Leco-N nitrogen determinator (Model FP-428, Leco Corporations, St. Joseph, MI, USA) and the protein content was calculated using a factor of 6.25.

2.2.10 Phosphorus determination

Phosphorus content was determined according to the protocol of the manufacture using malachite green phosphate assay kit provided by Bioassay Systems (Hayward, CA, USA).

2.2.11 Statistic analysis

Values are presented as mean ± S.D. Analysis of variance (ANOVA) was used to determine differences between means, and Tukey range post-hoc comparisons were used to determine the source of significant differences where appropriate. A $p < 0.05$ was considered statistically significant. All data were analyzed using Statistical Analysis System Software, SAS version 9.0 (SAS Institute, Cary, NC).

2.3 Results and discussion

2.3.1 Effect of pH on phosvitin purity and recovery

The phosvitin extracts showed light orange color at pH around 7.9-8.1. No change was observed until the pH of the extracts was lowered to 6.5, where

significant precipitate was evident and the extracts became transparent and colorless. Phosvitin extracts prepared at different pHs were analyzed by gel filtration chromatography (GFC) in comparison with Sigma's phosvitin standard (Fig. 2-1). Phosvitin was known to be heterogeneous analyzed by both gel filtration chromatography and electrophoresis (McBee and Cotterill, 1979; Abe et al., 1982; Wallace and Morgan, 1986a; Wallace and Morgan, 1986b). Three major peaks were obvious in the phosvitin standard: the first fraction (B) with the largest molecular weight (MW) was usually considered as lipovitellin, while the following two fractions were previously identified as β-phosvitin and α-phosvitin, with MW of 190 and 160 kDa, respectively (Abe et al., 1982). The purity of the standard phosvitin (fractions of β- and α-phosvitin) was estimated to be 90.5% (at 1mg/mL concentration) according to peak area integration. The phosvitin extract prepared at pH 8.0 (the as is pH of the extract) showed five peaks (in addition to two phosvitin peaks), which were designated as A, B, C, D. and E in the order of their elution time from the column (Fig 2-2). Peaks A and B could be considered as low-density lipoproteins (LDLs) and lipovitellin according to their molecular weight (Abe et al., 1982; Jolivet et al., 2006), while peaks C, D and E could be phosvettes or apoproteins derived from lipovitellin according to their relatively small molecular weights (Kurisaki et al., 1981; Wallace and Morgan, 1986b). There was a trend that the area of peaks A-E decreased at decreasing pHs, and peaks A and B were almost not detected at pHs below 5.5. This observation indicated the complete precipitation of lipovitellin at

pHs below 5.5, which was in good agreement with previous results (Castellani et al., 2003).

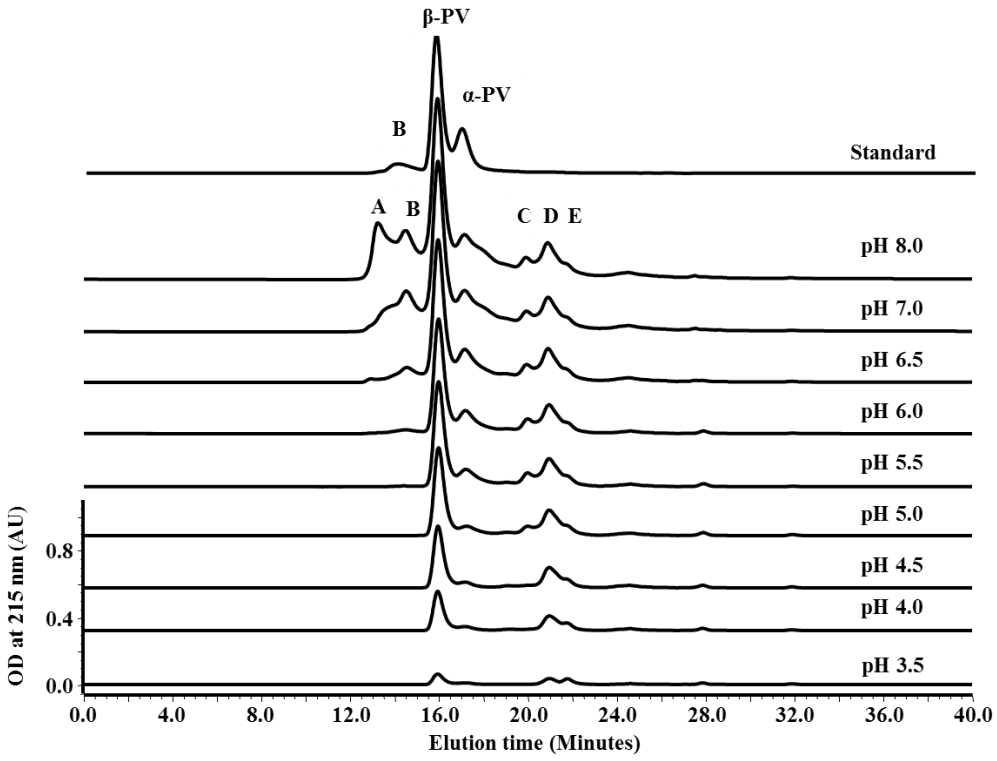

Figure 2-2 Gel filtration chromatograms of phosvitin extracts at different pHs (8.0 (the "as is" pH of the extract, ranging from 7.9 to 8.1), 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5 as indicated on the right of the chromatograms). Phosvitin extracts were prepared from egg yolk granule by dissolving in 10% NaCl, followed by various pH adjustments as described in Materials and Methods. Phosvitin standard was purchased from Sigma.

The purity of phosvitin extracts was increased from 54.5% at pH 8.0 to 63.7% at pH 5.5, and then started to decrease at pH 5.0 (Table 1-1). The purity of phosvitin was significantly decreased to 39.6% at pH 3.5. The highest purity of

63.7% was obtained at pH 5.5 with a recovery of 40.1%. The highest recovery of 109.6% was obtained at pH 8.0. Both the yield and the recovery were significantly decreased at decreasing pHs, especially at pHs close to its pI (pH 4.0) (Ternes, 1989). This study showed that the purity of phosvitin was not increased, but the recovery and yield was significantly decreased at decreasing pHs from 7.0 to 5.0, indicating the occurrence of co-precipitation of phosvitin with lipovitellin at decreased pHs. This was in conflict with the observation of Castellani et al. (2003) who reported that phosvitin was almost not precipitated at pHs from pH 7.0 to pH 5.5. This controversy might be due to the differences in the methods of phosvitin preparation (their phosvitin was prepared with NaCl, $MgSO_4$ and at extremely low pH of 1.8), so the structure of lipovitellin and phosvitin might be modified). In this experiment, we would have expected that 10% NaCl could disrupt the phosphocalcic bridge between phosvitin and lipovitellin, and then to precipitate lipovitellin from supernatant after dialysis at pHs around 5.5 (Castellani et al., 2003). A recovery of 109.6% suggested completely disruption of phosvitin-lipovitellin complex by 10% NaCl; however, the corresponding low purity of 54.5% indicated the existence of a large quantity of impurities, mainly lipovitellin, probably due to co-precipitation of phosvitin with lipovitellin at decreasing pH.

Table 2-1 Phosvitin recovery and purity at different pH

pH	Purity (%)	PV yield (g/100 g yolk solids)	PV recovery (%)

8.0	54.5±1.2 a	4.4±0.1 a	109.6±3.1 a
7.0	57.8±3.2 a	3.8±0.1 b	94.4±2.1 b
6.5	61.2±1.3 a	2.4±0.2 c	60.2±3.8 c
6.0	63.5±0.2 a	1.9±0.0 d	46.5±1.0 d
5.5	63.7±0.5 a	1.6±0.0 d	40.1±1.0d
5.0	60.0±0.3 a	1.2±0.0 e	30.8±0.9 e
4.5	56.6±2.4 a	0.8±0.0 f	20.9±1.0 f
4.0	51.9±4.6 a	0.5±0.1 g	11.9±1.4 g
3.5	39.6±6.4 b	0.2±0.0 h	4.0±0.5 h

*Data with different letters within each column are significantly different at $p < 0.05$. PV: phosvitin.

Phosvitin standard showed a major band with molecular weight (MW) ranging from 32 to 48 kDa, in addition to two minor contaminants at 135 and 54 kDa (Fig 2-2). Granules are composed of 70% lipovitellin, 16% phosvitin and 12% low-density lipoproteins (Joubert and Cook, 1958). In SDS-PAGE, granules were resolved into eight bands with MW of 149, (92-121), 85, 48, 31, 13, 11and 9 kDa, and another wide band with MW of 31-43 kDa referring to phosvitin. Our results were in good agreement with the reports of Tsutsui and Obara (1982) and Wallace and Morgan (1986). Tsutsui and Obara (1982) reported delipidated granules consisted of at least seven polypeptide chains with MW of 130, 92, 84, 71, 39, 22 and 10 kDa. Wallace and Morgan (1986b) identified phosvitin with MW ranging from 28 to 43 kDa, as well as other proteins with MW of 207, 118,

97, 79, 74, 65 and 30 kDa in the granules. The MWs of lipovitellin's subunits deduced from the parent protein vitellogenin were 125, 80, 40 and 30 kDa (Groche et al., 2000); therefore, the bands at MW of 92-121, 85, 48 and 31 kDa could be considered as the four subunits of lipovitellin. Low density lipoprotein was composed of at least six apoproteins with MW ranging from 10 to 180 kDa (Raju and Mahadevan, 1976; Yamauchi et al., 1976; Burley and Sleigh, 1980; Nakamura et al., 1977), but the most abundant components were apoprotein V and VI with MW of 90 and 170 kDa, respectively, corresponding to the wide band of 92-121 kDa and 149 kDa in our results. The three small bands (at 13, 11, and 9 Kda) detected in our study were similar to three phosvette bands (18, 15 and 13 KDa) reported by Wallace and Morgan (1986b). Phosvitin extracts at pH 8.0 had a significant band ranging from 31 to 43 kDa, as well as other eight bands with MWs of around 149 kDa, 116 kDa, 88 kDa, 72 kDa, 50 kDa, 13 kDa, 11 kDa and 9 kDa. After extraction, the bands with MW of 92-121, 48 and 31 were greatly reduced, indicating partial precipitation of LDLs and HDLs; starting from pH 6.0 and below, the band at 92-121 KDa was absent, and the intensity of contaminated bands was significantly reduced. However, the intensity of phosvitin band was also reduced, indicating the co-precipitation of phosvitin with other components at reduced pHs (Fig 2-2).

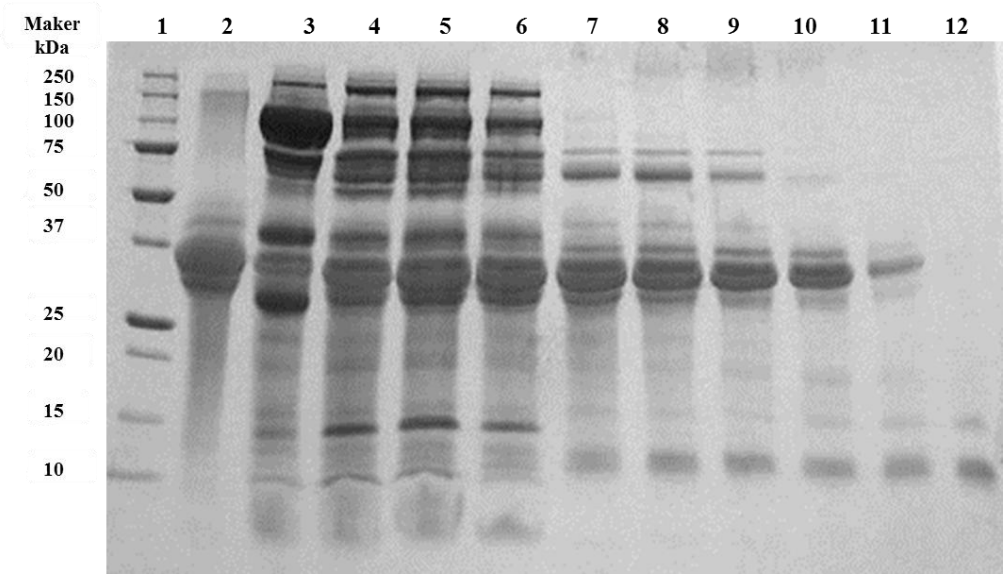

Figure 2-3 SDS-PAGE profile of phosvitin extracts prepared at different pH. 1, molecular weight markers; 2, phosvitin standard from Sigma; 3, egg yolk granules; 4, phosvitin extract at pH 8.0; 5, phosvitin extract at pH 7.0; 6, phosvitin extract at pH 6.5; 7, phosvitin extract at pH 6.0; 8, phosvitin extract at pH 5.5; 9, phosvitin extract at pH 5.0; 10, phosvitin extract at pH 4.5; 11, phosvitin extract at pH 4.0; 12, phosvitin extract at pH 3.5. Phosvitin extracts were prepared from egg yolk granule by dissolving in 10% NaCl, followed by various pH adjustments as in Materials and Methods. Phosvitin standard was purchased from Sigma.

2.3.2 Purification of phosvitin by anion exchange chromatography

The highest recovery of phosvitin was obtained at pH 8.0 (the "as is" pH of phosvitin extracts), but the purity was low probably due to the insufficient separation of the contaminants. Phosvitin is negatively charged because of the high amount of phosphate groups; therefore, anion exchange chromatography

has been successfully used for the purification of phosvitin in previous studies (McBee and Cotterill, 1979; Castellani et al., 2003; Lei and Wu, 2012).

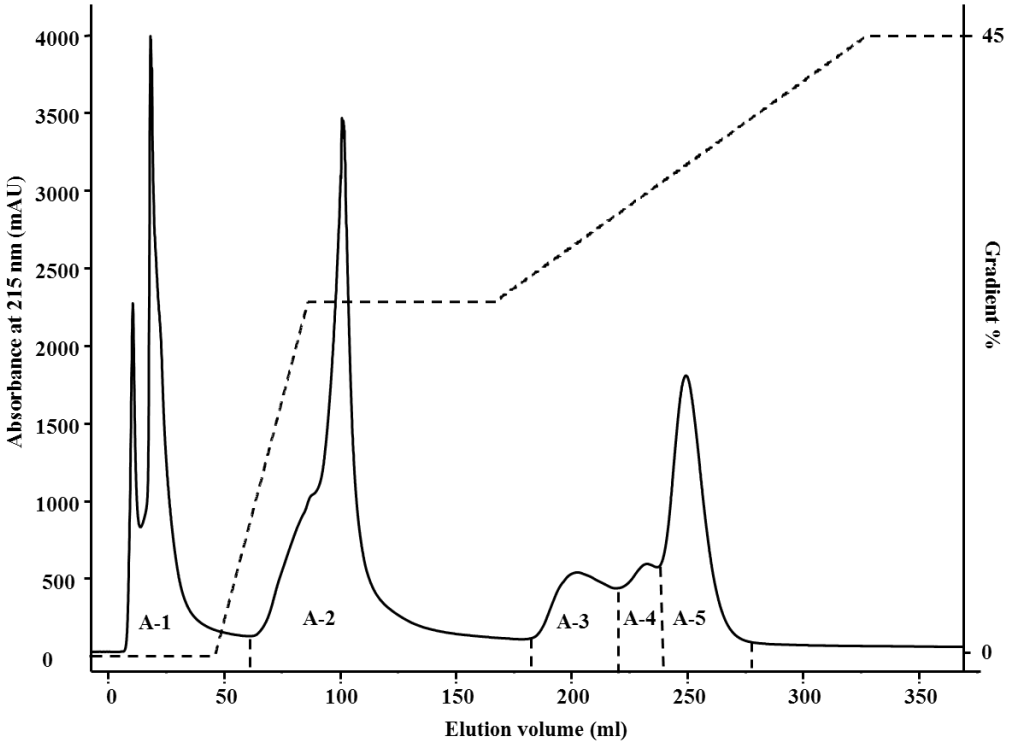

Figure 2-4 Anion exchange chromatograms of extracted phosvitin (pH 8.0, the "as is" pH of the extract, ranging from 7.9 to 8.1). Phosvitin extract was prepared from egg yolk granule by dissolving in 10% NaCl, followed by pH adjustments as described in Materials and Methods.

As shown in Fig 2-3, crude phosvitin extract (at pH 8.0) was resolved into 5 fractions named as A-1 to A-5. All of the fractions were analyzed by gel filtration chromatography against phosvitin standard as shown in Fig 2-4. The fractions A-5 and A-4 had peaks overlapped with the peak position of phosvitin standard, but fractions A-1, A-2 and A-3 had no peaks at the position of phosvitin standard. Two major peaks were detected in fraction A-5 at the

positions of β- and α-phosvitin and these two peaks together represented a purity of 97.1±0.2%, which is higher than the purity of Sigma phosvitin standard (90.5%). Our study showed that phosvitin purified from anion exchange chromatography eliminated HDL. Fraction A-4 also presented peaks at position corresponding to β- and α-phosvitin, along with another two low molecular weights peaks. Usually β-phosvitin is the most abundant, but in fraction A-4, α-phosvitin was more abundant than β-phosvitin. It was reported that α-phosvitin contains less phosphorus than β-phosvitin (Abe et al., 1982); therefore, the binding of α-phosvitin to anion exchange column will be less tight than that of β-phosvitin and resulted in a faster elution time. The purity of purified phosvitin was confirmed by SDS-PAGE as shown in Fig 2-5. Both purified phosvitin and phosvitin standard showed a major band with molecular weight (MW) ranging from 32 to 48 kDa.

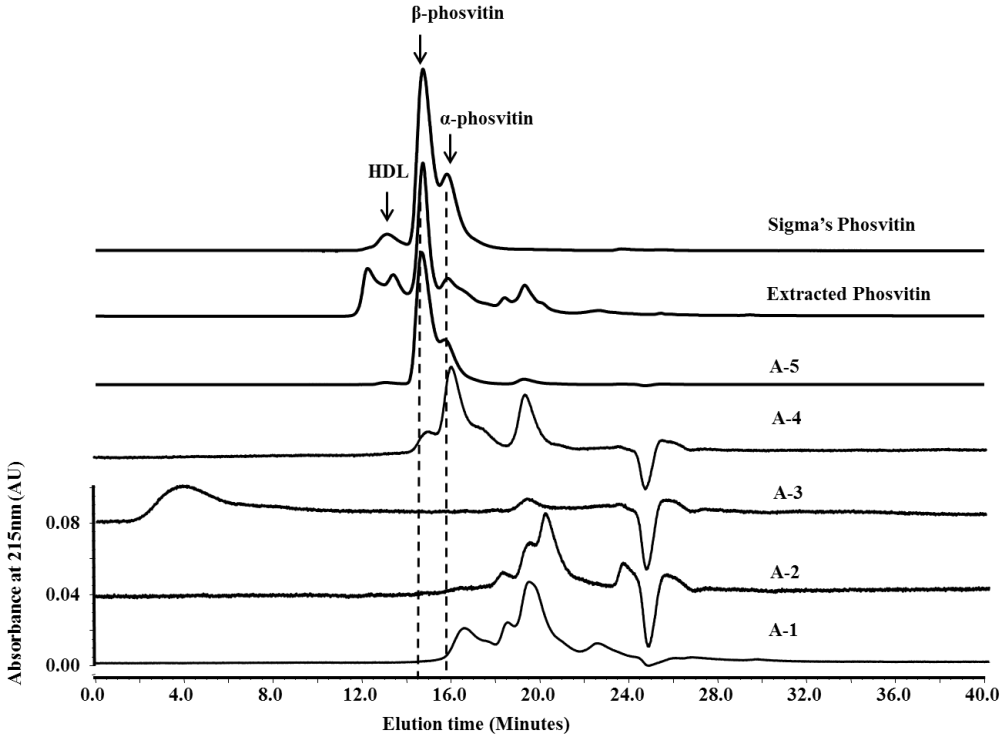

Figure 2-5 Gel filtration chromatograms of extracted phosvitin and FPLC fractions A-1 to A-5. Phosvitin extract was prepared from egg yolk granule by dissolving in 10% NaCl, followed by pH adjustments (at pH 8.0, the "as is" pH of the extract, ranging from 7.9 to 8.1) as described in Materials and Methods. Fractions of A-1 to A-5 were obtained from anion exchange chromatography. Phosvitin standard was purchased from Sigma.

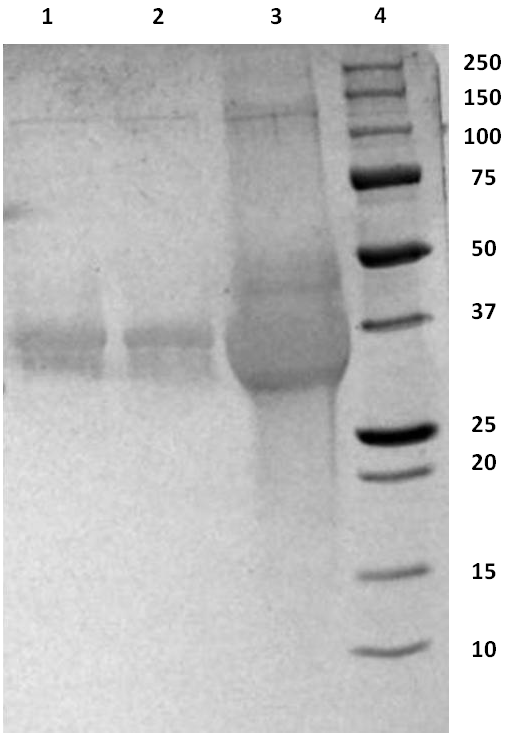

Figure 2-6 SDS-PAGE profile of fraction A-5 from anion exchange chromatography. 1 and 2, fraction A-5; 3, phosvitin standard from Sigma; 4, molecular weight marker. Phosvitin standard was purchased from Sigma.

Although the purity of extracted phosvitin (at pH 8.0) is low, the yield of 4.4±0.1 g/100 g yolk solids (or 109.6±3.1% overall recovery) was the highest among all the results reported. Such high recovery suggested that probably all the phosvitin was extracted by 10% NaCl at pH 8.0. After purification by anion exchange chromatography, the purity was increased from 54.5±1.2 to 97.1±0.2% whereas the yield was reduced from 4.4±0.1 to 2.2±0.1 g/100 g yolk solids (or 55.6±1.3% overall recovery). The yield of the study compared favourably to previously reports, such as 100 mg/egg (or ~1.0 g/100 g yolk solids) by Losso and Nakai

(1994), ~1.0 g/100 g yolk solids (Wallace and Morgan 1986a), 1.7 g/100 g yolk solids by Castellani et al. (2003), and 1.9 g/100 g yolk solids (Lei and Wu, 2012).

2.3.3 Conclusion

Phosvitin is the most phosphorylated protein in nature with great potential to be developed as a valuable functional food ingredient. Previous methods of phosvitin extraction involved either organic solvents or non-food compatible chemicals, and the procedure of extraction are lengthy and tedious, resulting in low purity and yield. Our study showed that 10% NaCl extraction could disrupt the lipovitellin-phosvitin complex leading to a recovery of 109.6% phosvitin from granules but with a low purity of 54.5% at "as is" pH of ~8.0; decreasing pH during the extraction, however, did not improve either the purity or the recovery. The purity of the phosvitin extract could be significantly improved to 97.1% after anion exchange chromatography whereas the recovery was reduced to 55.6% (or yield of 2.2 g / 100 g yolk solids). The developed protocol in the study is simple and efficient, and could be scaled up for industrial production.

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CHAPTER 3 CHARACTERIZATION OF PHOSPHOPEPTIDES FROM PHOSVITIN HYDROLYSATE

3.1 Introduction

Chicken egg yolk phosvitin (PV) is the major phosphoprotein in egg yolk with molecular weight of 37-45 kDa (Abe et al., 1982). It is also the most highly phosphorylated protein in nature since 124 (123 serine and 1 threonine residues) out of total 217 amino acid residues are monoesterified with phosphate (Byrne et al., 1984; Clark, 1985; Grogan et al., 1990). Phosvitin is derived from the multidomain precursors of vitellogenin, which are synthesized in the liver of vertebrates under the stimulation of estrogen and later cleaved into phosvitin, lipovitellin and other minor proteins (Finn, 2007). Due to the large amount of negatively charged phosphoserine residues, phosvitin exhibits strong metal chelating ability, and it is believed to carry and provide metal ions during the embryo development (Taborsky, 1983).

Phosvitin is usually considered nutritionally negative due to its strong affinity to metal ions and resistance to proteolytic digestion (Goulas et al., 1996). It is reported that 95% of the iron in egg yolk is bound to this protein but only 30% is available for intestinal absorption (Greengard et al., 1964; Morris and Greene, 1972). Animal experiment proved that both phosvitin and egg yolk proteins could inhibit calcium, magnesium and iron absorption (Ishikawa et al., 2007). In contrast, the phosphorylated fragments derived from bovine milk casein digests, known as casein phosphopeptides (CPPs), have been demonstrated to enhance

vitamin D independent bone calcification in rachitic children (Mellander and Isaksson, 1950; Mellander, 1950). Phosphoserine residues in CPPs play a key role by forming soluble organophosphate salts with calcium to limit its precipitation in the distal ileum (Meisel et al., 2003). The common motif, consisting of three phosphoserine residues and two glutamic acids, Ser(P)-Ser(P)-Ser(P)-Glu-Glu, is widely recognized to contribute to the metal chelating ability of CPPs (West and Towers, 1976; Schlimme and Meisel, 1995). Unlike CPPs, the understanding of phosvitin derived phosphopeptides (PPPs) is very limited due to the resistance of phosvitin to hydrolysis. The strong negatively charged side chain of phosvitin hinders the enzymatic access to cleavage sites, leaving the phosvitin core an intact structure after hydrolysis (Goulas et al., 1996; Khan et al., 1999). To obtain oligophosphopeptides, dephosphorylation prior to hydrolysis is necessary (Jiang and Mine, 2000). Compared with casein, which contains less than 15 phosphoserine residues in total and 3 phosphoserine residues in a consecutive run (Swaisgood, 1992), phosvitin contains 123 phosphoserine residues and 113 of them are consecutive (Grogan et al., 1990). Phosvitin phosphopeptides (PPPs) were reported to exhibit antioxidant, anti-inflammatory and calcium-absorption promoting ability *in vitro* and *in situ* (Jiang and Mine, 2000; Feng and Mine, 2006; Katayama et al., 2006; Katayama et al., 2007; Xu et al., 2007; Young et al., 2011). Therefore, PPPs have great potentials as novel functional food ingredients or nutraceuticals. Recently, Young et al. (2011) identified 13 peptide sequences from tryptic hydrolysate of phosvitin, but only two of them were phosphorylated. However, the

understanding of PPPs' characteristics is quite limited. In this study we aimed to prepare PPPs by enzymatic hydrolysis and characterize the peptide sequences and phosphorylation by LC-MS/MS.

3.2 Materials and methods

3.2.1 Reagents

Glycine, Precision Plus Protein Dual Xtra Standards, sodium dodecyl sulphate (SDS), and precast gels (12% Tris-HCl) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Phosvitin standard (from chicken egg yolk) was purchased from Sigma-Aldrich, Ltd. (Oakville, ON, Canada). Deionized distilled water (DD water) was prepared from a Barnstead water purification system (Thermo Scientific, Asheville, NC, USA). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada) or Fisher Scientific (Nepean, ON, Canada).

3.2.2 Enzymatic hydrolysis

Phosvitin was prepared according to Chapter 2 and suspended into 5% (w/w) slurry in 0.2 M NaOH solution at room temperature (25^{o}C) for 0.5 h. After adjusting the pH of slurry to 8.0 with 3 M HCl, enzyme-2 was added at a ratio of 1/50 (enzyme/phosvitin, w/w). Incubation was carried out for 3 h as described in the manufacturer's specification. Hydrolysates were centrifuged at 10 000 g for 30 min at 4^{o}C and then lyophilized for the following assay.

3.2.3 Calculation of recovery and yield

The yield was defined as the amount of protein in hydrolysate produced from 100 g yolk solids. The recovery was calculated as a percentage of amounts of protein in the hydrolysate to the total protein in the egg yolk (w/w).

3.2.4 Anion exchange chromatography of phosvitin hydrolysates

Hydrolysate was prepared at 10 mg/mL with buffer A and 4 mL was loaded to a HiPrep 16/10 Q FF anion exchange column (GE Healthcare, Piscataway, NJ, USA) coupled to an AKTA explorer 10S system (GE Healthcare, Piscataway, NJ, USA). The column was eluted by a gradient from 100% buffer A (0.05 M Tris-HCl, pH 8.0) to 100% buffer B (1.0 M NaCl in buffer A). Fractions were collected and lyophilized. Before LC-MS/MS, fraction P-4 was dissolved in Milli-Q water and eluted on a Superdex peptide 10/300 GL column (GE Healthcare, Piscataway, NJ, USA) using Milli-Q water as running buffer. Elution was operated by the same FPLC system and monitored at 215 nm. The protein/peptide fractions were collected and lyophilized for LC-MS/MS analysis.

3.2.5 Nitrogen and protein determination

Nitrogen was determined in duplicate by using the Leco-N nitrogen determinator (Model FP-428, Leco Corporations, St. Joseph, MI, USA) and the crude protein was calculated by multiplying a factor of 6.25.

3.2.6 Phosphorus determination

Phosphorus content was determined by using a malachite green phosphate assay kit provided by Bioassay Systems (Hayward, CA, USA).

3.2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Shapiro et al. (1967) as described in Chapter 2. Samples were prepared at 4 mg/mL. Gels were stained by 0.05% Coomassie brilliant blue R-250 in a solution of 0.1 M aluminum nitrate/25% isopropanol/10% acetic acid/1.0% Triton X-100 and destained by 7% acetic acid solution (Hegenauer et al., 1977). Images were obtained and analyzed by using AlphaEase FC software (version 6.0.0, Alpha Innotech Corporation, Santa Clara, CA, USA).

3.2.8 Gel filtration HPLC

Samples were prepared at 2 mg/mL with 0.1 M sodium phosphate buffer (containing 0.2 M NaCl, pH 7.0) and filtered through a 0.45 μm PVDF filter (13 mm x 0.20 μm, MANDEL, Guelf, ON, Canada) before loading to a TSK-Gel G3000SWxL stainless column (0.78 x 30 cm, Tosoh Bioscience, Inc., South San Francisco, CA, USA). Samples (30 μL) were eluted by 0.1 M sodium phosphate buffer (containing 0.2 M NaCl, pH 7.0) at a flow rate of 0.5 mL/min on a Waters HPLC system (Waters, Milford, MA, USA), and the elution was detected at 215 nm. System control and data analysis were carried out by using Empower II Software (Waters, Milford, MA, USA).

3.2.9 Dephosphorylation of fraction P-4 prior to LC-MS/MS

Fraction P-4 was dephosphorylated by phosphatase (E/S=1/50, w/w) at 37^{o}C for 3 h according to manufacturer's specification. The incubation was stopped by raising temperature to 80^{o}C and held for 10 min. After desalting by ZipTip

pipette tips (reverse phase, 10 µL, Millipore, Billerica, MA, USA), the sample was concentrated by Savant SpeedVac Concentrator (Fisher Scientific ltd., Nepean, On, Canada) prior to LC-MS/MS.

3.2.10 Liquid chromatography-mass spectrometry/mass Spectrometry (LC-MS/MS)

Identification of peptides with/out dephosphorylation of P-4 was carried out on a Waters ACQUITY UPLC system connected to a Waters Micro mass Q-TOF Premier Instrument (Milford, MA, USA). Samples were first separated by a Waters Atlantis dC18 UPLC column (150 mm×75 µm, 3 µm; Milford, MA, USA) and then ionized through a nanoLockspray ionization source in a positive ion mode. The mass of peptides was determined by a Q-TOF analyzer. MassLynx 4.1 software (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K.) was used to control the experiment and collect data. Peaks Viewer 5.2 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) and Mascot search engine (Matrix Science Inc., Boston, MA, USA) were used for peptide sequencing from National Center for Biotechnology Information (NCBI) database.

3.2.11 Statistic analysis

Values are presented as means ± SD. Statistical analyses were carried out using Statistical Analysis System Software, SAS version 9.0 (SAS Institute, Cary, NC). One way Analysis of variance (ANOVA) was used and significant

difference was determined by the Tukey range post-hoc comparisons and accepted at $p < 0.05$.

3.3 Results and discussion

3.3.1 Hydrolysis of dephosphorylated phosvitin and egg yolk proteins

Egg yolk proteins and purified phosvitin were digested under identical conditions. As shown in Fig 3-1, no intact phosvitin was detected in phosvitin or egg yolk proteins hydrolysate. It was reported that without dephosphorylation, hydrolysis of phosvitin by pepsin, trypsin and α–chymotrypsin would produce only 2-4 small fractions from C- and N-terminus, leaving one major fraction of 28-29 kDa as phosvitin core (Goulas et al., 1996; Khan et al., 1998; Jiang and Mine, 2000). Jiang and Mine (2000) demonstrated that dephosphorylation of phosvitin with 0.1 N NaOH for 3 h prior to trypsin hydrolysis could obtain peptides with MW less than 3 kDa. Our SDS-PAGE showed three large protein fragments in phosvitin hydrolysate with MWs of 19.6, 13.3 and 9.3 kDa, and two small fragments with MWs less than 5 kDa (Fig 3-1, lanes 3 and 4). The small fragments had similar MWs with what Jiang and Mine (2000) reported (< 3 kDa). The three large fragments may be derived from the phosvitin core which is resistant to enzymatic hydrolysis due to the high phosphorylation degree (Taborsky, 1983). All three large fragments and two small fragments were also detected in the egg yolk proteins hydrolysate but these bands were faint. Compared with phosvitin hydrolysate, egg yolk proteins hydrolysate may contain higher proportion of small peptides derived from other egg proteins (e.g.

high density lipoproteins and low density lipoproteins) which were difficult to be stained and detected by regular SDS-PAGE.

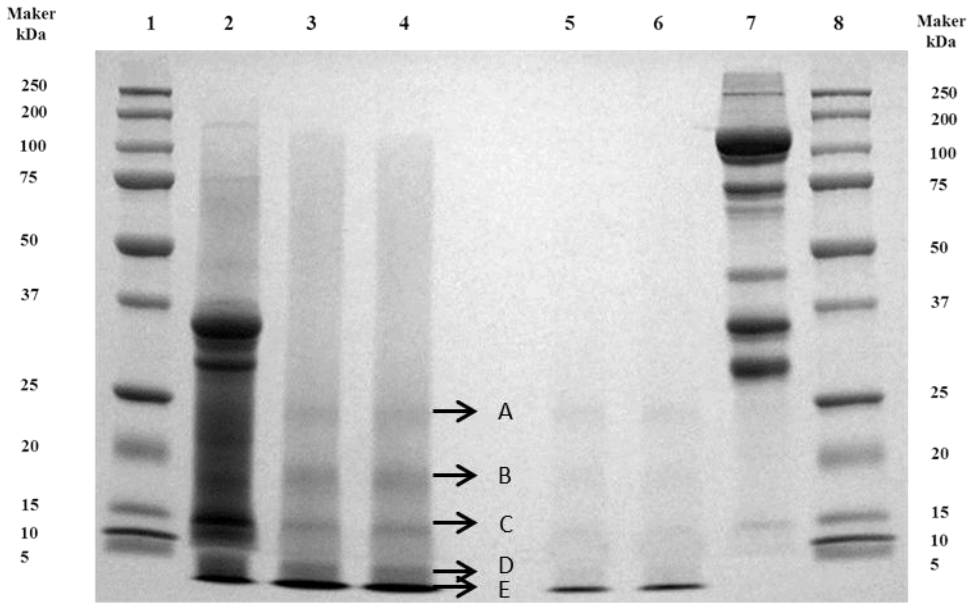

Figure 3-1 SDS-PAGE profiles of hydrolysates of egg yolk proteins and phosvitin. 1 and 8, molecular weight markers; 2, phosvitin prepared by our protocol; 3 and 4, hydrolysates of phosvitin; 5 and 6, hydrolysates of egg yolk proteins; 7, egg yolk proteins. Bands A, B and C were the large fragments with MWs of 19.6, 13.3 and 9.3 kDa; bands D and E were small fragments with MWs lower than 5 kDa. Molecular weights were calculated by using AlphaEase FC software (version 6.0.0, Alpha Innotech Corporation, Santa Clara, CA, USA). Hydrolysis was prepared as described in Materials and Methods.

Gel filtration chromatography further supported that phosvitin can be degraded after dephosphorylation (Fig. 3-2). Before hydrolysis, egg yolk proteins were resolved into four contaminated peaks, A, B, C and D, and two phosvitin peaks,

α- and β-phosvitin. Fractions A and B could be low density lipoprotein (LDL) and high density lipoprotein (HDL) according to their large MW. After hydrolysis, the MW of the hydrolysate was less than 12.4 kDa (Fig 3-2). Similarly, the purified phosvitin could also be hydrolyzed, but the molecular weight of the hydrolysate was larger than that of egg yolk proteins hydrolysate. This is because the major constituent of egg yolk was HDL and HDL which were not as resistant to enzymatic hydrolysis as phosvitin. So we could expect to see more peptides from HDL and LDL other than phosvitin in egg yolk proteins hydrolysate. Both the MWs of β and α-phosvitin were over 200 kDa, which were much larger than their MWs in SDS-PAGE (Fig 3-1). This is mainly due to the linear structure of phosvitin making it elute much faster than granular protein molecules with same MW. TSK G3000SWxL is a silica based gel with negatively charged silanol groups. Phosvitin and phosphopeptides are highly negatively charged and therefore will be eluted faster than neutral molecules due to the ionic repulsion between phosphoserine residues and silanol groups. As a result, phosvitin hydrolysate showed larger MW (at 150 kDa and below) than egg yolk proteins hydrolysate. However, the last four fractions G, H, I and J in phosvitin hydrolysate were also found in egg yolk proteins hydrolysate. It may be feasible to produce the phosvitin phosphopeptides from egg yolk proteins. The fractions larger than 12.4 kDa in phosvitin hydrolysate was not found in egg yolk proteins hydrolysate; this could be due to their relatively low abundance.

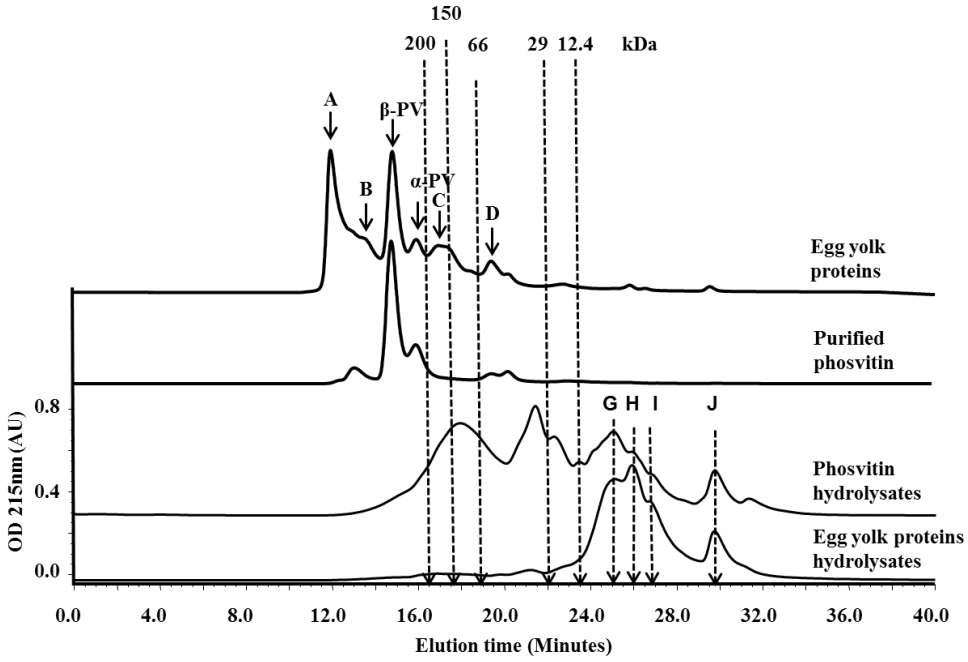

Figure 3-2 Gel filtration HPLC patterns of egg yolk proteins, purified phosvitin and their hydrolysates

The degree of hydrolysis (DH), recovery and yield were summarized in table 3-1. The DH of phosvitin hydrolysis in the study was 12.9%, significantly higher than a DH of 5.2% that was previously reported (Chay Pak Ting et al., 2011). The DH of egg yolk proteins hydrolysate was 24.2%, almost two-fold of the DH of phosvitin hydrolysate; this could be due to the presence of other proteins in the egg yolk proteins that contributed to the higher DH value (Burley and Cook, 1961). A low N/P relates to a high degree of phosphorus; thus it is preferable to have a low N/P ratio for phosphopeptides. In this study the N/P ratio of phosvitin hydrolysate was 3.9, lower than both purified CPPs with the N/P ratio ranging from 6 to 8 (Peterson et al., 1958; Bennich et al., 1959) and the phosvitin hydrolysate with the N/P ratio of 11.8 (Chay Pak Ting et al. 2011). The N/P

value for the egg yolk proteins hydrolysates is 19.1, close to the value of 6-18 for CPP purified by Ca^{2+}/ethanol precipitation method (Zhao et al., 2007). Egg yolk proteins hydrolysate showed significantly higher protein recovery than that of phosvitin hydrolysate.

Table 3-1 DH, protein yield, protein recovery and N/P value of hydrolysates of egg yolk proteins and phosvitin

Hydrolysate	DH %	Yield mg/g yolk solids	Recovery from yolk %	N/P atomic ratio
Egg yolk proteins	24.2±0.2a	138.4±0.3a	41.8±0.1a	19.1±0.4a
phosvitin	12.9±3.1b	20.1±0.7b	6.1±0.2b	3.9±0.1b

*Data with different letters within each column are significantly different at $p<0.05$. DH: degree of hydrolysis

3.3.2 Fractionation of phosvitin hydrolysate by anion exchange chromatography

Phosvitin hydrolysate was fractionated by an anion exchange column as shown in Fig 3-3, resulting in four fractions representing 0.3%, 24.2%, 2.4% and 73.0% of the total phosphorus content. The last fraction P-4 containing the highest phosphorus content, which was in good agreement with the report of Katayama et al. (2006), was subjected to LC-MS/MS analysis.

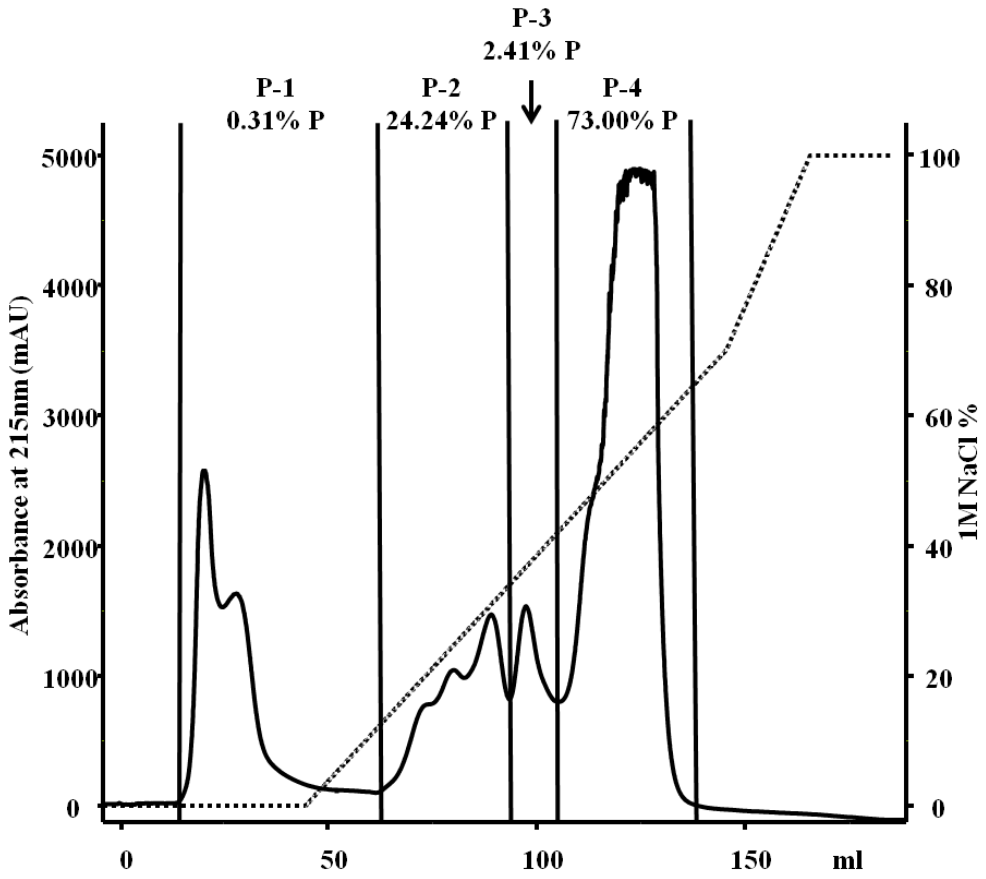

Figure 3-3 Anion exchange chromatography of hydrolysates of phosvitin

3.3.3 Identification of phosphopeptides in fraction P-4 by LC-MS/MS

The fraction P-4 prepared above was used for structure characterization. Due to the negatively-charged phosphoserine residues, peptides derived from phosvitin hydrolysate might not be retained by the dC-18 column before MS/MS analysis, or ionized by regular ionization methods. Therefore we dephosphorylated P-4 with phosphatase before LC-MS/MS to identify the amino acid sequences of these peptides. At the same time, we also analyzed the sample without dephosphorylation by LC-MS/MS, focusing on identification of the phosphorylated amino residues. A representative example was shown in Fig 3-4.

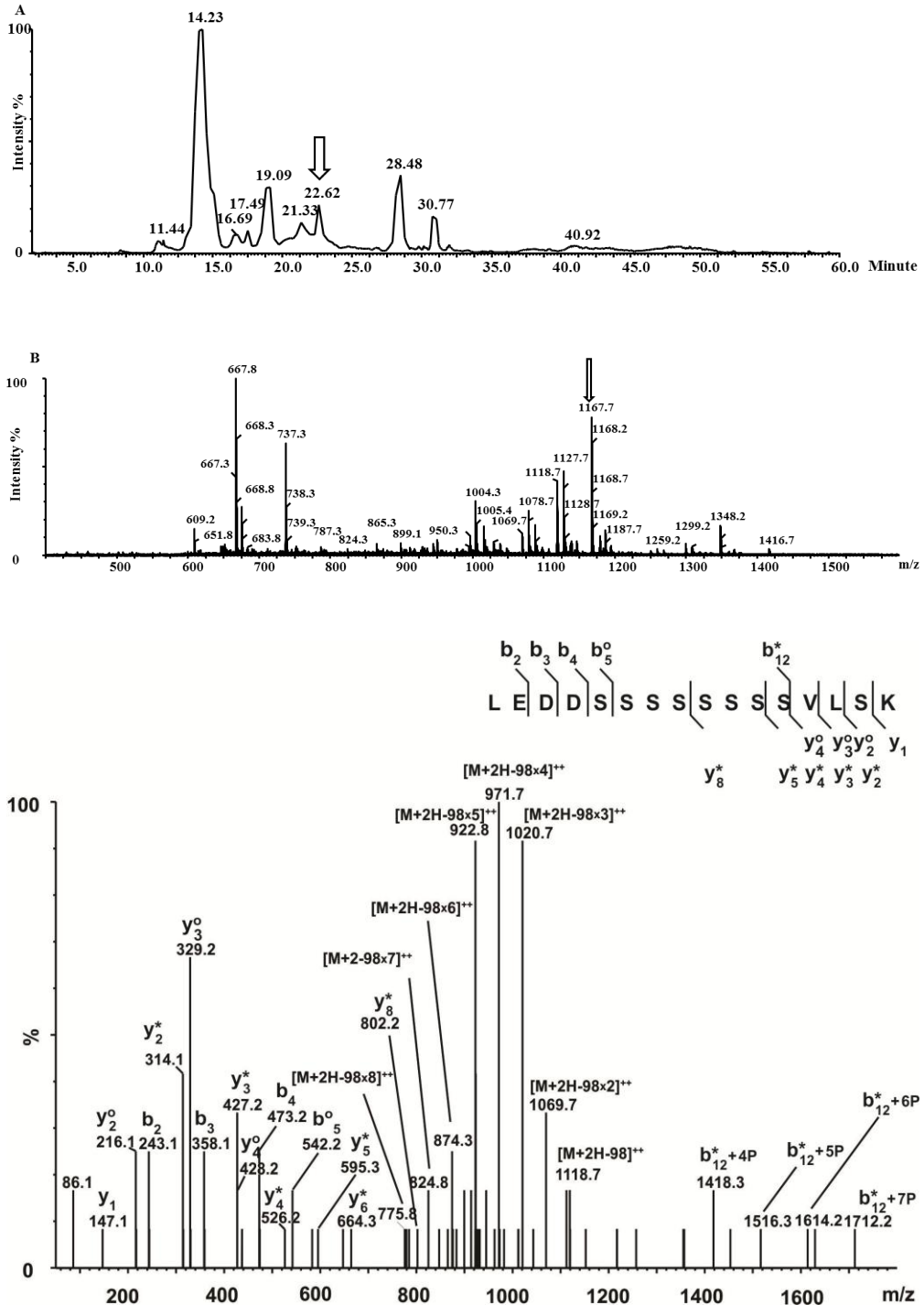

Figure 3-4 A representative peptide LEDDSSSSSSSSVLSK with 9 phosphate groups from fraction P-4 was de novo sequenced by using MS/MS spectra: (A) Total ion chromatogram of P-4 fraction. (B) Mass spectrum of a selected time at

22.62 min. (C) MS/MS spectrum of the ion m/z 1167.7. Losses of H_3PO_4 (98 Da) were detected as can be seen in the MS/MS spectrum. Ions with a (*) represent phosphorylated form of the ions. Ions with a (^{o}) represent dephosphorylated form of the ions.

A total of thirty two peptides were identified from the fraction P-4 derived from three phosvitin domains: AEFGTEPDAKTSSSSSSASSTATSSSSSSASSPN (PV 1-34), KPMDEEENDQV (PV 37-47) and SGHLEDDSSSSSSSSVLSKIWG (PV 190-211); and six peptides were identified from one lipovitellin domain: IITEVNPESEEEDESSPYEDI (VTG 1056-1076) (Table 3-2). Peptides with 6-10 phosphate groups were identified from domain SGHLEDDSSSSSSSSVLSKIWG (PV 190-211). Although no phosphorylation was identified in the peptides derived from domain AEFGTEPDAKTSSSSSSASSTATSSSSSSASSPN (PV 1-34), it was possible that there were phosphopeptides generated but they could not be retained by the dC-18 column prior to MS/MS analysis. Young et al. (2011) reported phosvitin peptides from three domains: PV 4-40, PV 155-197 and PV 244-257. Two of them, PV 4-40 and PV 244-257, were partially overlapped with the domains detected in this study; the last domain PV 244-257 (EDDSSSSSSSSVLSKIWGRHEIYQ) reported (Young et al. 2011) was actually overlapped with the domain PV 190-211 detected in this study. It should be noted that phosvitin contains 217 amino acids in total (Byrne et at., 1984; Goulas et al., 1996). Peptides from the domain PV 155-197 were not detected in this study. This could be due to that these two studies used different enzymes. The

peptides derived from PV 48-154 were not reported by Young et al. (2011) and our study. The three large fragments detected in SDS-PAGE (Fig 3-1) with MW of 19.6, 13.3 and 9.3 kDa might be derived from this phosvitin domain. Young et al. (2011) reported two phosphorylated peptides with one and three phosphate groups out of total thirteen peptides although the same group previously reported that these peptides contained 18.9% phosphorus (Katayama et al., 2006), whereas in this study five phosphorylated peptides were identified containing 6-10 phosphate groups. It is interesting to note peptides with the same amino acid sequences might contain different number of phosphate groups (Table 3-2).

In egg yolk proteins hydrolysate, we identified fifteen peptides from three domains in phosvitin sequence: AKTSSSSSSASSTATSSSSSSASSPN (PV 9-34), DEEENDQV (PV 40-47) and SGHLEDDSSSSSSSSVLSKIWG (PV 190-211); fifty four peptides from five domains in lipovitellin sequence: IGNIYAPE (VTG 116-123), TVDLNNCQEK (VTG 182-191), MTPPLTGDF (VTG 821-829), TSILPEDAPLDVTEEPF (VTG 929-945) and IITEVNPESEEEDESSPYEDIQA (VTG 1056-1078). All the three phosvitin domains, PV 9-34, PV 40-47 and PV 190-211 were also found in phosvitin hydrolysate, and fourteen out of total fifteen phosvitin peptides in egg yolk proteins hydrolysate were also characterized in phosvitin hydrolysate. We successfully identified highly phosphorylated peptides which were not identified as phosphorylated peptides in egg yolk proteins hydrolysate (eg. SGHLEDDSSSSSSSSVLSK (10P), SGHLEDDSSSSSSSSVLS (8P), LEDDSSSSSSSSVLSK (7, 8, 9P), LEDDSSSSSSSSVLSKI (7P)). This

observation suggested that it is feasible to produce phosvitin phosphopeptides from egg yolk proteins hydrolysate. In fact, most of the peptides derived from phosvitin might be phosphorylated, but it is difficult to identify the phosphorylation due to the limitation of current Mass analysis technique.

Table 3-2 Peptides identified from anion exchange chromatography fraction P-4

	Position	**Sequence**	**M/Z**	**Z**	**Mr (Expt)**	**Mr (Calc)**
Domain-I	1112-1145	AEFGTEPDAKTSSSSSSASSTATSSSSSSASSPN (PV 1-34)				
1	1115-1133	GTEPDAKTSSSSSSASSTA	879.4	2	1756.8	1756.8
2	1112-1120	**AEFGT(P)EPDA**	936.4	1	935.4	935.4
3	1112-1131	AEFGTEPDAKTSSSSSSASS	966.9	2	1931.9	1931.8
4	1112-1119	*AEFGTEPD*	865.4	1	864.4	864.4
5	1120-1145	*AKTSSSSSSASSTATSSSSSSASSPN*	1169.5	2	2337.1	2337.0
6	1115-1145	GTEPDAKTSSSSSSASSTATSSSSSSASSPN	1419.1	2	2836.2	2836.2
7	1122-1145	*TSSSSSSASSTATSSSSSSASSPN*	1070.0	2	2137.9	2137.9
Domain-II	1148-1158	KPMDEEENDQV (PV 37-47)				
8	1148-1158	**KPMDEEENDQV**	667.3	2	1332.6	1332.6
9	1151-1158	***DEEENDQV***	489.2	2	976.4	976.4
10	1148 - 1157	KPMDEEENDQ	667.3	2	1332.6	1332.6

11	1150 - 1158	MDEEENDQV	554.7	2	1107.4	1107.4
Domain-III	1301-1322	SGHLEDDSSSSSSSSVLSKIWG (PV 190-211); 6-10 phosphorylation**				
12	1308-1320	SSSSSSSSVLSKI	628.3	2	1254.6	1254.6
13	1306-1318	DDSSSSSSSSVLS	622.8	2	1243.5	1243.5
14	1301-1319	***SGHLEDDSSSSSSSSVLSK*** (10P)	948.4	2	1894.9	1894.8
15	1307-1319	*DSSSSSSSSVLSK*	629.3	2	1256.6	1256.6
16	1304-1317	LEDDSSSSSSSSVL	700.3	2	1398.6	1398.6
17	1306-1319	*DDSSSSSSSSVLSK*	1372.6	1	1371.6	1371.6
18	1307-1320	*DSSSSSSSSVLSKI*	685.8	2	1369.7	1369.7
19	1312-1320	SSSSVLSKI	454.3	2	906.5	906.5
20	1310-1320	SSSSSSVLSKI	541.3	2	1080.6	1080.6
21	1301-1318	***SGHLEDDSSSSSSSSVLS*** (8P)	884.4	2	1766.8	1766.7
22	1305-1322	EDDSSSSSSSSVLSKIWG	929.4	2	1856.8	1856.8
23	1304-1322	LEDDSSSSSSSSVLSKIWG	986.0	2	1969.9	1969.9
24	1301-1320	**SGHLEDDSSSSSSSSVLSKI** (6-7P)	1005.0	2	2007.9	2007.9
25	1306-1320	*DDSSSSSSSSVLSKI*	1485.7	1	1484.7	1484.7
26	1304-1318	*LEDDSSSSSSSSVLS*	743.8	2	1485.6	1485.6

27	1304-1319	***LEDDSSSSSSSSVLSK*** (7, 8, 9P)	807.9	2	1613.7	1613.7
28	1307-1322	DSSSSSSSSVLSKIWG	807.4	2	1612.8	1612.8
29	1301-1317	SGHLEDDSSSSSSSSVL	840.9	2	1679.7	1679.7
30	1304-1320	***LEDDSSSSSSSSVLSKI*** (7P)	864.4	2	1726.8	1726.8
31	1306-1317	DDSSSSSSSSVL	1157.5	1	1156.5	1156.5
32	1306-1322	*DDSSSSSSSSVLSKIWG*	864.9	2	1727.8	1727.8
Domain-IV	1056-1076	IITEVNPESEEEDESSPYEDI (1056-1076) (lipovitellin)				
33	1056-1072	***IITEVNPESEEEDESSP***	952.4	2	1902.8	1902.8
34	1060-1076	*VNPESEEEDESSPYEDI*	984.4	2	1966.8	1966.8
35	1059-1076	***EVNPESEEEDESSPYEDI***	1048.9	2	2095.8	2095.8
36	1068-1076	DESSPYEDI	1054.4	1	1053.4	1053.4
37	1059-1072	***EVNPESEEEDESSP***	788.8	2	1575.6	1575.6
38	1060-1073	*VNPESEEEDESSPY*	805.8	2	1609.6	1609.6

*Peptide sequences identified from native and dephosphorylated peptides were combined together in this table. Sequences with bolded letters were found in both native and dephosphorylated peptides. Sequences with Italic letters were found in both phosvitin hydrolysate and egg yolk proteins hydrolysate. M/Z values were obtained by mass spectrometry. Mr (expt) is the uncharged molecular mass of the peptides, and Mr (calc) is the theoretical mass of the closest matching peptides. Phosphorylation was identified from native peptides

without dephosphorylation. **In some case, one peptide contains different number of phosphate groups.

3.4 Conclusion

Phosphopeptides derived from phosvitin hydrolysate (PPPs) are considered as a novel functional food ingredient with antioxidant, anti-inflammatory and calcium-absorption promoting abilities (Choi et al., 2005; Katayama et al., 2006; Katayama et al., 2007; Xu et al., 2007; Young et al., 2011). In this study, the hydrolysate represented a DH of 12.9% with a recovery of 6.1% from egg phosvitin. The N/P value of 3.9 was lower than purified casein phosphopeptides (CPPs) with N/P ratio ranging from 6 to 18; further study is needed to determine if a lower N/P is favourable for the bioactivities. A total of thirty two peptides were characterized by LC-MS/MS derived from three phosvitin domains: AEFGTEPDAKTSSSSSSASSTATSSSSSSASSPN (PV 1-34), KPMDEEENDQV (PV 37-47) and SGHLEDDSSSSSSSSVLSKIWG (PV 190-211); five peptides were highly phosphorylated with 6-10 phosphate groups. All three phosvitin domains identified in phosvitin hydrolysate were also found in egg yolk proteins hydrolysate, indicating that it is possible to prepare PPPs from egg yolk egg yolk proteins hydrolysate.

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CHAPTER 4 FINAL REMARKS

4.1 Phosphopeptides derived from casein and phosvitin

Casein phosphopeptides (CPPs) identified from *in vivo* digestion have been proven to enhance vitamin D independent bone calcification in rachitic children (Mellander and Isaksson, 1950; Mellander, 1950). Due to the negatively charged phosphoserine residues, CPPs are thought to form soluble organophosphate salts with calcium in the intestines to increase calcium absorption (Meisel et al., 2003). The common motif, which consists of three phosphoserine residues and two glutamic acids (Ser(P)-Ser(P)-Ser(P)-Glu-Glu) was reported to be important for the physiological activities of CPPs (West and Towers, 1976; Schlimme and Meisel, 1995). The most recent research about CPPs focuses on their ability to keep calcium and phosphate ions soluble and bioavailable for remineralisation of subsurface lesions in tooth enamel (Reynolds et al., 1999; Ferraretto et al., 2001; Cross et al., 2005). The nanocomplexes formed by casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) are reported to inhibit caries progression and promote caries regression (Andersson et al., 2007; Morgan et al., 2008; Bailey et al., 2009). Addition of CPP-ACP during fluoride treatment can promote the localization of fluoride ions at the tooth surface and remineralization of enamel with flourapatite (Reynolds et al., 2008; Reynolds, 2009). Besides, CPPs are also proved to be an immunomodulating factor which can increase the antigen-specific IgA levels in animal intestines, reduce IgE-mediated allergic symptoms, and stimulate cytokine release in human epithelial intestinal cell (Hata et al., 1998; Otani et al., 2000a; Otani et al., 2000b;

Kawahara and Otani, 2004; Otani and Wakatsuki, 2004; Kitts and Nakamura, 2006). In the food industry, CPPs serve as an effective natural antioxidant to prevent lipid oxidation during meat processing (Sakanaka et al., 2005; Rossini et al., 2009). The antioxidant activities are due to the presence of antioxidant amino acids such as histidine, lysine, proline and tyrosine as well as phosphoserine residues which have metal chelating ability (Kim et al., 2007). Currently CPPs are widely used as functional food ingredients in dairy products. Besides, the major merchants of dental health care products, such as Colgate-Palmolive Company and GC Corporation, have already launched tooth paste products featured with CPP-ACP.

Study on phosphopeptides derived from egg yolk phosvitin (PPPs) is still fairly new. Phosvitin is the principal phosphoprotein in egg yolk (Mecham and Olcott, 1949). It is considered as an ideal protein source in comparison with casein to produce phosphopeptides, as it contains 123 phosphorylated serine residues and 113 of them are in consecutive runs (Grogan et al., 1990). However, phosvitin is resistant to enzymatic hydrolysis due to its extremely high phosphorylation degree, and also difficult to be purified since it exists in form of complex with lipovitellin. These factors might hinder the study on PPPs, although PPPs have been demonstrated to exert antioxidant, anti-inflammation and mineral-absorption promoting activities (Feng and Mine, 2006; Katayama et al., 2006; Katayama et al., 2007; Xu et al., 2007; Young et al., 2011). Bonepep$^{@}$ is a commercial egg yolk peptide product developed by Pharma Foods International

Co., Ltd. It is featured with activities to increase bone density and prevent osteoporosis. The functional ingredient in Bonepep$^{@}$ is the PPPs.

4.2 A summary of present research

Previous studies indicated that phosvitin and its PPPs have great potentials for functional food uses. However, current phosvitin extraction protocols are lengthy and tedious, involving organic solvents or non-food compatible chemicals, and both the purity and yield are low. An efficient method of PPPs preparation is not available in literature and the structure of PPPs has not been extensively characterized. In this study we first developed a simple and efficient protocol to extract phosvitin, consisting of two major procedures: 10% NaCl extraction and anion exchange chromatography. The purity (97.1%) and yield (55.6%) were comparable favourably with previous results (usually purity less than 92% and recovery less than 50%). Furthermore, phosphopeptides were prepared and novel peptides were characterized. The hydrolysate of phosvitin represented an N/P of 3.9, which is lower (better) than purified CPPs (6-18). The peptides were identified from three domains within phosvitin: AEFGTEPDAKTSSSSSSASSTATSSSSSSASSPN (PV1-34), KPMDEEENDQV (PV37-47) and SGHLEDDSSSSSSSSVLSKIWG (PV190-211).

4.3 Implications of the present study

Phosvitin derived phosphopeptides exhibit great potentials in food or functional food uses with the increasing public awareness of the health benefits of peptide

products and the success of CPPs' commercialization. However, the study on PPPs is hindered by the lack of efficient protocols to purify phosvitin and to prepare oligophosphopeptides through enzymatic hydrolysis. The phosvitin extraction protocol developed in this study might pave the way for further industrial exploration. The protocol is simple and feasible for scale-up preparation with high purity and recovery. It is noteworthy that the phosphopeptides derived from phosvitin hydrolysate were identified with six to ten phosphate groups. This is the first time to obtain such highly phosphorylated peptides, compared to maximum three phosphate groups reported previously in CPPs and PPPs. Considering the fact that the phosphorylated residues play a key role in physiological activities of CPPs, a higher content of phosphorylated residues indicates their promising properties. Although further study is needed, most of the findings in this study are unique and significant and will benefit the relevant research as well as industrial application of egg yolk phosphopeptides.

4.4 Recommendations for future research

- The current study focused on the preparation and characterization of phosphopeptides. In the future, we will investigate a wide range of physiological activities of these peptides *in vitro* and *in vivo*. We hope these work will provide a better understanding about the relationship between phosphopeptides' structure and activities.
- Pilot scale for phosvitin extraction and phosphopeptides preparation is necessary.

- The dialysis of salts during phosvitin extraction may be inconvenient for industrial production. However, it could be solved by ultra-filtration or electro-dialysis.

4.5 Literature cited

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