**Studies on antimicrobial and antioxidant properties of phosvitin hydrolysates produced by high hydrostatic pressure combined with enzymatic hydrolysis**

by

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## Abstract

Phosvitin (PV) is a metal binding protein in egg yolk with unique amino acid composition (more than 55% serine). Due to a high proportion of phosphorylated serine residues, PV shows metal chelating, antioxidant and antimicrobial activities. The use of PV, however, in nutra- or pharmaceutical application has been restricted mainly due to the formation of insoluble complexes with divalent metal ions in the gastrointestinal tract. The PV hydrolysates (PVH) that are hydrolyzed by proteases may solve the problem and increase their applications without compromising the PV's functional properties. There are many attempts to produce the PVH. However, the yield of the PVH is low due to the PV's negative charges causing the resistance to digestive enzymes. The present technology of the high hydrostatic pressure combined with enzymatic hydrolysis (HHP-EH) is a new method to increase the enzyme efficiency. The present study of the HHP-EH shows a higher degree of hydrolysis containing more peptides with $M_w$< 3 kDa compared to atmospheric pressure (AP). PV Hydrolysis with Alcalase (Alc) under HHP resulted in the highest degree of hydrolysis (31.3%). The PVH treated by Alc and Trypsin (Try) obtained from both HHP and AP treatments showed superior iron chelation capacity (69-73%). Alc-PVH produced by HHP-EH displayed significantly greater reducing power (3.5 μM Trolox equivalent/mg) than AP-PVH (1.3 μM Trolox equivalent/mg). In the second study, a combination of specific IgY (100 μg/mL) and PVH-Alc-HHP (1 mg/mL) as an anti-microbial agent was found to be the most efficient to control

the foodborne Enterotoxigenic *Escherichia coli* (ETEC) K88 and K99 *in vitro*. The synergistic anti-microbial activities of IgY and PVH may support their potential application in feed supplementation to prevent microbial contamination and infectious diseases. In the third study, there is limited information available on the quantification of PV. The double antibody sandwich ELISA (DAS-ELISA) and biotinylated DAS-ELISA developed has a PV detection range of 5.6 – 90 μg/mL and 2.5 – 40 ng/mL, respectively. The biotinylated DAS-ELISA is a superior method for PV quantification regarding accuracy and sensitivity. This highly efficient PV detection method may recuperate the performance of the existing protein assay methods as well as facilitate future research on PV bioactivities and applications.

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## List of Abbreviation

Alc, Alcalase

AP, Atmospheric pressure

Arg, Arginine

Asn, Asparagine

Asp, Aspartic acid

BSA, Bovine serum albumin

CFU, Colony-forming units

DH, Degree of hydrolysis

DHA, Docosahexaenoic acids

DNA, Deoxyribonucleic acid

DPPH, 2,2-diphenyl-1-picrylhydrazyl

EDTA, Ethylenediaminetetraacetic acid

Ela, Elastase

ETEC, Escherichia coli

FRAP, Ferric (Fe III) reducing ability of plasma

GHS, Glutathione

GIT, Gastrointestinal tract

Glu, Glutamine

Gly, Glycine

H, Heavy chains

HDL, High density lipoprotein

HHP-EH, High hydrostatic pressure-enzymatic hydrolysis

HIC, Hydrophobic interaction chromatography

His, Histidine

HP, Horseradish peroxidase

HPLC, High performance liquid chromatography

HRPO, Horseradish peroxidase

IgA, Immunoglobulin A

IEC, Ion exchange chromatography

IgG, Immunoglobulin G

IgM, Immunoglobulin M

IMAP, Immobilized metal affinity electrophoresis

IgY, Immunoglobulin Y

IL-8, Interleukin 8

L, Light chains

LDL, Low density lipoprotein

LOD, The limit of detection

LPC, Lysophosphatidylcholine

LPS, Lipopolysaccharides

LPE, Lysophosphatidylethanolamine

Lue, Leucine

Lys, Lysine

MALDI-TOF, Matrix-assisted laser desorption/ionization

MUFA, Monounsaturated fatty acids

$M_w$, Molecular weights

NHS-$PEO_4$-Biotin, Biotinaimdohexanoic acid-3-sulfo-N-hydroxysuccinimide ester

PBS, Phosphate buffered saline

PE, Phosphatidylethanolamine

PEG, Polyethylene glycol

PC, Phohatidylcholine

PL, Phospholipids

PLA2, Phospholipase A2

PMSF, Phenylmethylsulfonyl fluoride

ROS, Reactive oxygen species

PUFA, Polyunsaturated fatty acids

PV, Phosvitin

PVH, Phosvitin Hydrolysates

SAFA, Saturated fatty acids

SCWL, Single Comb White Leghorn

SDS, Sodium dodecylsulfate

SE-HPLC, Size exclusion high performance chromatography

Ser-P, Phosphoryl serine blocks

Ther, Thermolysin

TMB, Tetramethylbenzidine

TNBSA, 2,4,6-trinitrobenzene sulfonic acid

TPTZ, 2,4,6-tripyridyl-s-triazine

Try, Trypsin

WSF, Water-soluble fractions

# Chapter 1: Literature review

## *1. 1 Introduction of Egg*

## *1.2 Structure of Egg*

The egg is composed of five distinct structures such as egg shell, egg shell membrane, egg white (or albumen), vitelline membrane, and egg yolk. The parts of the egg are shown schematically in Figure 1.1.

The eggshell is formed in a matrix component of calcium and proteins involving in the eggshell calcification to form egg shapes. The main biological function of the eggshell as a first line of defense is to prevent bacterial penetration and to protect the embryo from external aggression during its development. The chicken eggshell is a natural porous matrix formed in the hen's oviduct over a predetermined period (Hamilton 1986; Nys et al., 2004; Tullet 1987). The egg shell has a distinct pattern of pores to allow gas exchange of carbon dioxide. However, the pores may permit bacterial penetration as far as the shell membranes.

The next layers of the eggshell membrane are composed of the inner and outer shell membranes. The relatively thin keratin-like membranes play a role in the second line of defenses against possible bacterial invasion. The inner membrane is thinner than the outer membrane, but together they are only 0.01-0.02 mm thick.

The egg white, or albumen, represents approximately 60% of total egg weight. The main function of egg white plays a role in the third line of the defense during the development of embryo. The egg white consists of 4 layers (see Figure

1.1) in eggs; chalaziferous or inner continuous with the chalaza (layer 1), the inner thick white (layer 2) surrounded by the outer thin white (layer 3), and the outer layer of white is the outer thick layer (layer 4).

The percentage of the total white found in each of the four layers varies widely, depending on strain of the laying hen, age of the hen, and age of the egg.

The vitelline membrane, or yolk membrane, surrounds the yolk and prevents the egg yolk from mixing with the egg white. The membrane is the final barrier to microorganisms invading into the yolk. However, it allows small molecules to cross the membrane during the embryogenesis. The dry weight of the membrane is 5 to 10 mg per egg, depending on the egg size.

The yolk consists of the latebra, germinal disk, and concentric layers of light and dark surrounded by the vitelline membrane. The yolk comprises about 33% of the total egg weight.

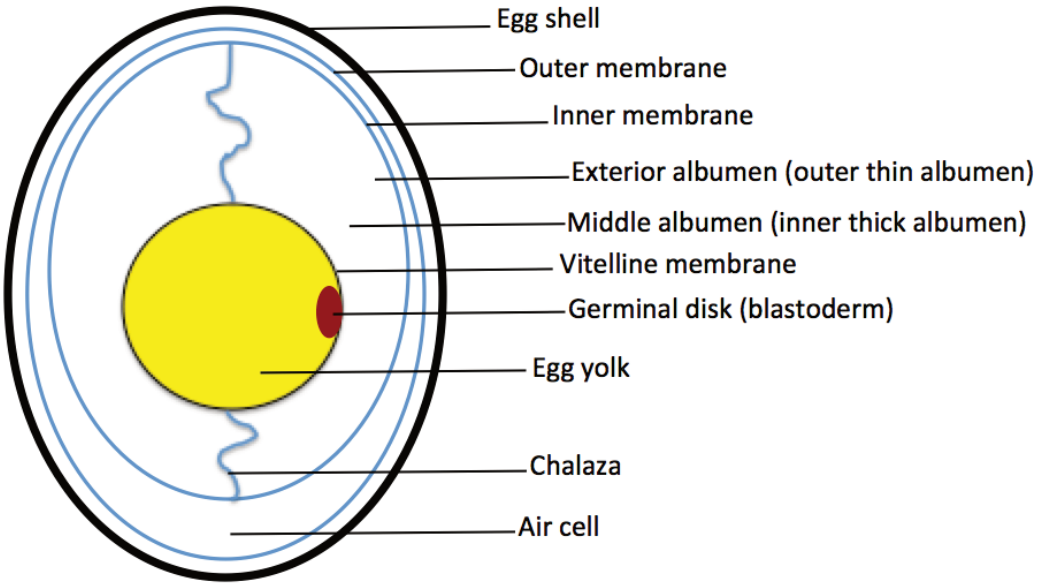



Figure 1.1: Schematic drawing of egg

### *1.3 Composition of Egg*

The nutritional value of whole egg protein is considered to be 100. That is used as standard for measuring nutritional quality of other food proteins. The egg white contains more than 40 different kinds of proteins that make up 11% of its entire composition of egg. Due to their functional and pharmacological properties, egg proteins are desirable ingredients in the pharmaceutical industry.

The major egg white proteins include ovalbumin, ovotransferrin, ovomucoid, ovomucin and lysozyme. They account for > 83% of total egg white proteins. Other minor proteins include ovoinhibitors, ovomacroglobulin, cystain, avidin and ovoglycoprotein at low concentration which accounts for < 17% of total egg white proteins.

The egg proteins distributed in the yolk exists as lipoproteins, of which there are low density and high density. The low density lipoprotein (LDL) is the major protein, accounting for up to 65% of the total yolk proteins. The high density lipoprotein (HDL) includes a phosphoprotein known as phosvitin. About 80% of phosphorus in eggs is contained in phosvitin, which is derived from vitellogenin formed in the liver (Sugino et al., 1997). Other yolk proteins include a water-soluble livetin, non-lipid glycoprotein, and riboflavin-binding protein.

The fat in the egg is exclusively in the yolk, and comprises 5.5 to 6 g in an average 60 g egg. Almost all lipids are present in lipoprotein complexes within the yolk. Trace levels of lipids have been observed in the egg white. The lipids in eggs have attracted attention both at scientific and consumer level due to the link between high dietary fat consumption and coronary heart diseases.

Yolk lipids are classified into triglycerol, phospholipid, and free cholesterol. Triglycerol and phospholipids are the major components of yolk lipids, comprising up to 65% and 32%, respectively. Fatty acids are also found in egg yolk with saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA).

Minerals are contained within the egg yolk. The egg yolk contains 1% mineral that contains with phosphorous as the most abundant mineral component. More than 60% of the total phosphorous in egg yolk is found in phospholipids.

The major inorganic components of egg white are sulphur, potassium, sodium and chloride. Table 1.1 shows phosphorous, calcium and magnesium are major minerals in eggs.

Table 1.1 Mineral content of edible egg portion and their approximate proportion in egg white and yolk

| Nutrient | Whole egg (mg) | White (%) | Yolk (%) |
|---|---|---|---|
| Phosphorous | 89 | 5 | 95 |
| Chlorine | 87.1 | 70 | 30 |
| Sulfur | 82 | 70 | 30 |
| Sodium | 63 | 90 | 10 |
| Potassium | 60 | 80 | 20 |
| Calcium | 25 | 8 | 92 |
| Magnesium | 5 | 80.0 | 20.0 |
| Iron | 0.72 | 5 | 95 |
| Zinc | 0.55 | n/a | 95 |
| Iodine | 0.024 | 5 | 95 |
| Manganese | 0.012 | 10 | 90 |

| | | | |
|---|---|---|---|
| Copper | 0.007 | 35 | 65 |

Based on 59 g shell weight, with 50 g total liquid whole egg, 33.4 g white, and 16.6 g yolk.
Abbreviation: n/a, not applicable
Adapted from: Cherian G (2006) Egg biology. In: Hui YH (ed) Handbook of food science, technology, and engineering. CRC Press-Taylor & Francis Group, USA, Table 153.4, p 153-5.

The egg is considered a good source of most vitamins, except vitamin C. Eggs contain both fat-soluble and water-soluble vitamins. As shown in the Table 1.2, vitamins A, D, and E are exclusively in the yolk. Choline, folic acid, and pantothenic acid are also found in the yolk. Niacin appears to be found mainly in the white.

Table 1.2 Vitamin content of egg edible portion and their approximate proportion in egg white and yolk

| Nutrient | Whole egg (mg or μg) | White (%) | Yolk (%) |
|---|---|---|---|
| Vitamin A (IU) | 317 | n/a | 100 |
| Vitamin D (IU) | 24.5 | n/a | 100 |
| Vitamin E (mg) | 0.70 | n/a | 100 |
| Vitamin B12 (μg) | 0.50 | 15.0 | 85 |
| Biotin (μg) | 9.98 | 25 | 75 |
| Choline (mg) | 215.1 | 0.2 | 99.8 |
| Folic acid (μg) | 23 | 4 | 96 |
| Inositol (mg) | 5.39 | 25 | 75 |
| Niacin (mg) | 0.037 | 80 | 20 |
| Pantothenic acid (mg) | 0.063 | 5 | 95 |
| Pyridoxine (mg) | 0.07 | 5 | 95 |
| Riboflavin (mg) | 0.25 | 60 | 40 |
| Thiamine (mg) | 0.03 | 7 | 93 |

Based on 59 g shell weight, with 50 g total liquid whole egg, 33.4 g white, and 16.6 g yolk.
Abbreviation: n/a, not applicable.
Adapted from: Cherian G (2006) Egg biology. In: Hui YH (ed) Handbook of food science, technology, and engineering. CRC Press - Taylor & Francis Group, USA, Table 153.3, p 153-4.

Most fat-soluble vitamins are concentrated in the yolk. Although several factors, such as age, strain of bird, and age of bird are involved, diet is the most important factor for regulating egg vitamin content. The transfer efficiency of a vitamin depends on vitamin level in the diet, feed intake, rate of egg production, and egg weight. The transfer efficiency may vary between vitamins. For example, vitamin A has a transfer efficiency between 60 and 80%, riboflavin, pantothenic acid, and biotin have transfer efficiencies of 15-25%, and vitamin K, thiamine, and folacin have 5 to 10% transfer efficiencies (Naber and Squires 1993).

### *1.3.1 Egg Shell*

The eggshell is composed of 95% minerals. Among minerals, calcium accounts for more than 98%. Other minor minerals include phosphorous, magnesium, and trace amounts of iron and sulfur comprising less than 0.05%.

Carbohydrates in egg shell are composed of glycosaminoglycans that are anionic polysaccharides consisting of hyaluronic acid (48%) and galactosaminoglycan (52%) (Nakano et al., 2001). These carbohydrates have wide application in the cosmetics, pharmaceutical and food industry. Eggshell powder is considered to be a good source of highly bioactive calcium and could be used as an ingredient for human consumption.

The matrix protein identifies desmosine and isodesmosine, similar of elastin-like proteins (Leach 1982). Immunohistochemistry using antibodies to collagen types I, V, and X showed that the matrix may contain collagens. The proteins also contain hydroxylysine (Wong et al., 1984; Arias et al., 1997; Wang et al., 2002). However, the bulk of the amino acid composition differs from collagen and suggests that collagen is not predominant but that a unique protein containing lysine-derived cross links may be present (Leach 1982).

The outer shell largely consists of calcium carbonate (94%), with other components including magnesium carbonate (1%), calcium phosphate (1%), and organic matter that are mostly protein (4%).

The shell color of colored eggs is due to pigments (ooporphins) deposited on the shell surface. The shell is formed in a distinct pattern with pores for gas exchange. Even though the pores are partially sealed by keratin, they allow carbon dioxide and moisture to escape from the egg. Under some conditions, the pores also permit bacterial penetration as far as the shell membranes.

### *1.3.2 Egg Shell Membrane*

The egg shell membrane is composed of collagen-like proteins (collagen type I and V), in a ratio of 100 of type I to 1 of type V. Coarse fibers (2.5 μm in diameter) contain more type I collagen, while type V collagen predominates in the fine fibres (0.6 μm in diameter), and is largely located in the inner membrane. Other components identified in eggshell membranes are glycosaminoglycans, such as dermatan sulfate and chondroitin sulfate (Baker and Balch 1962), hyaluronic acid (Long et al., 2005), sialic acid (Nakano et al., 2003), desmosine

and isodesmosine (Starcher and King 1980), ovotransferrin (Gautron et al., 2001), lysyl oxidase (Akagawa et al., 1999), lysozyme (Hincke et al., 2000) and β –N acetylglucosaminidase (Ahlborn et al., 2006).

The egg shell membrane contains several bacteriolytic enzymes, such as lysozyme and N-acetyl glucosaminidase and other membrane proteins that have been through to have beneficial effects in treating injuries. The peptides derived from the membrane were shown to stimulate skin fibroblasts *in vitro* (Suguro et al., 2000). The egg shell membrane proteins are currently utilized as a cosmetic ingredient for their emollient properties.

### *1.3.3 Egg White*

The Egg white, albumen, may be regarded as a protein system consisting of ovomucin fibers in an aqueous solution of numerous globular proteins. The albumen proteins and their characteristics are presented in Table 1.3.

Table 1.3 Proteins in egg white

| Protein | % of albumen proteins | Molecular weight (kDa) | Characteristics |
|---|---|---|---|
| **Major proteins** | | | |
| Ovalbumin | 54 | 45 | Heat stable polypeptide containing phosphorous and carbohydrate |
| Ovotransferrin | 12 | 76 | Metal-binding transport protein |
| Ovomucoid | 11 | 28 | Trypsin inhibitor |

| | | | |
|---|---|---|---|
| G2 globulin | 4 | 30 – 45 | - |
| G3 globulin | 4 | - | - |
| Ovomucin | 3.5 | 5,500-8,300 | Maintains structure and viscosity of egg white |
| Lysozyme | 3.4 | 14.3 | Damages cell wall bacteria |
| **Minor Proteins** | | | |
| Ovoinhibitor | 1.5 | 49 | Serine proteases inhibitor |
| Ovoglycoprotein | 1 | 24.4 | Sialoprotein |
| Ovoflavoprotein | 0.8 | 32 | Riboflavin-binding protein |
| Ovomacroglobulin | 0.5 | 769 | Strongly antigenic protein |
| Cystatin | 0.05 | 12.7 | Thiol protease inhibitor |
| Avidin | 0.05 | 68.3 | Biotin-binding protein |

Adapted from: Etches RJ (2008), Production of novel proteins in chicken eggs. In: Mine (ed), Egg Bioscience and Biotechnology. John Wiley & Sons, USA, Table 7.1, p 293.

#### *1.3.3.1 Major protein*

Ovalbumin is the major protein and constitutes up to 54% of total egg white proteins. It typically serves as a major source of amino acids for the developing embryo. Ovalbumin, the predominant protein in albumen is classifed as a phosphoglycoprotein. The carbohydrate and phosphate moieties are attached to the polypeptide. The ovalbumin sequence contains 385 amino acids. The N-

terminal amino acid is glycine, and the C-terminal amino acid is proline. The molecular weight of the polypeptide is 43.6 kDa. Ovalbumin contains two phosphate residues on serines 68 and 344 (Kinoshita and Koike 2012).

Purified ovalbumin is made up of three components. These are A1, A2 and A3, all of which differ in phosphorous content. Ovalbumin A1, A2, and A3 containing two, one and no phosphate groups per molecule, respectively, are present in albumen fraction in relative portions of about 85:12:3. The molecule contains a carbohydrate chain attached at asparagine 292. Ovalbumin is the only albumen protein to contain free sulfhydryl groups. Each ovalbumin molecule contains four sulfhydryl groups, three of which are reactive to p-chloromercuribenzoate in native protein and the fourth in the denatured protein.

Ovotransferrin is a monomeric glycoprotein consisting of a single polypeptide chain of 686 amino acids. The molecular weight of ovotransferrin is about 78 kDa; this constitutes 13% of total proteins in egg white. This protein consists of two lobes, each containing a specific binding site for iron, although ovotransferrin does not contain iron in the egg. Copper, zinc or aluminum may also bind to this site. Inhibition of gram-negative bacteria occurs by depriving the iron source which is essential for their growth and survival (Lock and Board 1992). The antimicrobial activity can result from a direct effect on the membranes: interaction of the cationic ovotransferrin with the anionic outer membrane of gram-negative bacteria (Valenti et al., 1986).

Ovomucin is a macromolecule and heavily glycosylated glycoprotein, consisting of peptide-rich α-subunit and a carbohydrate–rich β-subunit. Ovomucin

is a major egg white glycoprotein (3.5%) with a molecular mass of approximately 254 kDa. It contains *O*-linked carbohydrate moieties that, upon formation of extensive hydrogen bonds with water, can give rise to a characteristic gel-like structure. Ovomucin serves physical functions to maintain the structure and viscosity of egg white albumen, thus serving to prevent the spread of microorganisms (Ibrahim et al., 1994), and to characterize foaming and emulsifying properties.

Ovomucoid is a highly glycosylated protein (20 to 25% carbohydrates, w/w) of 28 kDa, 11% of egg white proteins. Ovomucoid consists of three domains of the amino acid sequences of 1-68, 69-130 and 131-186. Each domain is cross-linked by three disulfide bridges (Kato et al., 1987). The carbohydrate moiety consists of three oligosaccharide units bound to the protein through asparagine residues (Montgomery and Wu 1963). The polypeptide chain is composed of 26% α-helix, 46% β-structure, 10% β-turns and 18% random coil (Watanabe et al., 1981).

Lysozymes can be found in not only egg white but also the shell and the vitelline membrane. It belongs to a class of enzyme that lyses the cell walls of gram-positive bacteria. The lysozyme also known as muramidase or N-acetylmuramic hydrolase is a relatively small secretory enzyme that catalyzes the hydrolysis of beta 1,4 bonds between N-acetylmuramic acid and N-acetylglycosamine in cell walls of bacteria.

Lysozyme is a polypeptide of 129 amino acid residues having a molecular weight of 14.3 kDa. With the isoelectric point (pI) of 10-11, it is a strongly basic

protein in egg white, well known for bacteriostatic, bacteriolytic and bacteriocidal activity, particularly against gram-positive bacteria. The protein represents only 3.4% of total egg proteins. It is a good example of naturally occurring enzymes used in the food industry as a preservative to maintain product quality and reduce the incidence of spoilage.

The molecular weight of penalbumin is 61.6 kDa which is larger than ovalbumin (47.1 kDa). This protein has several features related to ovalbumin. Regarding the composition of penalbumin, it has more carbohydrate and lacks phosphate. The amino acid compositions are significantly different, but the differences could be explained if penalbumin is an extended form of ovalbumin.

The composition of ovoglobulin contains 13.6% of hexose, 13.8% of hexosamine and 3% of sialic acid. Hexose occurs as mannose and galactose in the ratio of 2:1, hexosamine as glucosamine and sialic acid as N-acetylneuraminic acid. It has a minimum molecular weight, calculated from the tryptophan content, of 24.4 kDa. The term ovoglobulin refers to ovoglobulin G2 and G3, each constituting about 4% of egg albumen proteins. Ovoglobulin G2 and G3 are similar in many properties, including their molecular weight (49 kDa).

Ovoglobulins are also of interest for commercial applications of albumen because they denature rapidly and may, therefore, have more effect on the initial foaming of the albumen than the more plentiful albumen proteins.

#### *1.3.3.2 Minor proteins of albumen*

Ovoinhibitor is a glycoprotein in egg white, composed of 447 amino acids with a molecular weight of 48 kDa. Like the ovomucoid, this protein is a

proteinase inhibitor. It inhibits the activities of trypsin, chymotrypsin and some proteinases of microbial origin.

The ovomacroglobulin is the largest globular protein in eggs white. This protein, also known as ovostatin, is composed of four subunits, each having a molecular weight of 175 kDa, with pairs of the subunits joined by disulfide bonds. Ovomacroglobulin inhibits hemagglutination, possesses anti-collagenase activity, and has inhibitory activity against diverse proteolytic enzymes including serine proteases, cysteine proteases, thiol proteases, and metalloproteases (Molla et al., 1987; Li-Chan and Nakai 1989).

As a member of a "superfamily" of cystatins, egg white cystatin belongs to the Type 2 cystatin, which has about 115 amino acids and two disulfide bonds, but no carbohydrates. Secreted cystatin has a theoretical molecular weight of 13.2 kDa. Egg white cystatin has been shown to possess anti-bacterial activity, preventing the growth of group A streptococcus (Bjork et al., 1989), *Salmonella typhimuriu*m (Nakai et al., 2008), and the periodontitis-causing *Porphyromonas gingivitis* (Travis et al., 1997).

Chicken egg white riboflavin-binding proteins are the prototype of a family that includes other riboflavin and folate binding proteins. Ovoflavoprotein binds riboflavin at pH above 4.3 with an association constant of $7.9 \times 10^8$ M. It is composed of 219 amino acids (Hamazume et al., 1984) with a molecular weight of 32 kDa. The carbohydrate content is about 15%, consisting of mannose, galactose, glucosamine and sialic acid. Ovoflavoprotein also referred to as flavoprotein or riboflavin-binding protein, is a phosphor-glycoprotein that is

responsible for binding most of the riboflavin (vitamin $B_2$) in egg white. Flavoprotein is considered to have the highest selenium content (1800 ng/g) among egg white proteins.

The avidin constitutes a maximum of 0.05% of the total protein content of egg white. Avidin is an alkaline (pI 10.5), highly stable, tetrameric glycoprotein that is best known for its biotin-binding properties. Each of the four monomers binds one molecule of biotin and the avidin-biotin interaction, with dissociation constant of $10^{-15}$ M, is the strongest non-covalent interaction reported between protein and ligand. Each avidin chain, composed of 128 amino acid residues, is arranged in an eight-stranded anti-parallel β-barrel, whose inner region defines the D-biotin binding site. A fairly rigid binding site is readily accessible in the apoprotein structure, making it sterically complementary to the shape and polarity of biotin.

Both chicken egg white avidin and its bacterial relative streptavidin are widely used as tools in some affinity-based separations, diagnostic assays, and a variety of other applications. Other applications include the potential of avidin as an insecticide and antimicrobial agent. Due to its high proportion of tryptophan residues, avidin is unstable under oxidizing conditions in strong light.

Thiamin-binding protein can be isolated from egg white by using affinity chromatography. Regarding function, the protein binds thiamine (vitamin $B_1$) in a 1:1 ratio and is similar with avidin in that it is a vitamin scavenger. In terms of structure, the protein has a molecular weight of 38 kDa and does not contain

carbohydrate. It is not usual in that it forms a stoichiometric complex with the albumen riboflavin-binding protein. An identical protein is present in egg yolk.

Vitamin $B_2$-binding protein has a molecular weight 98 kDa. The vitamin-binding ability of this protein was heat-labile in 2 h at 80 °C, but the complex was stable for 6 h at this temperature. Due to this difference, this albumen protein is distinguished from a $B_{12}$-binding protein in egg yolk.

Ovoglycoprotein is a protein of lipocalin family present in egg white. It represents about 1% of egg white protein. It is an acidic glycoprotein (pI 3.9) with a theoretical molecular weight of 20.3 kDa and a sugar content of 30%. Despite the information just previously given, very little is known about this protein.

Albumen contains minor enzymes. These enzymes include phosphatase, catalase, and glycosidase, aminopeptidase (Sugino et al., 1997).

### *1.3.4 Vitelline membrane*

The vitelline membrane is a protein matrix surrounding the yolk (Mann 2008). It is a thick structure of considerable strength separating the yolk form the egg white and forms the last barrier to microbial infection. Subsequently, it was found that it consists of three distinct layers (Kido et al., 1995). A thin continuous layer sandwiched between two fibrous layers, the inner layer in front of the oocyte, and the outer layer facing the albumen. The components of the inner layer are glycoproteins, GP-I, GP-II, GP-III, while the outer layer contains ovomucin, lysozyme, VMO-I, and VMO-II (Kido et al., 1995).

The inner layer, which is the avian parallel to the mammalian zona pellucida, is secreted by the granulosa cells surrounding the oocyte in the follicle

or in the liver from where it is transported to the follicle via blood circulation. (Kerver et al., 2002) The inner layer forms a diffusion barrier between egg white and egg yolk and so affects the properties of the shape and development of the embryo. The main proteins of the inner layer are the glycoproteins GPI (43 kDa), GPII (110 kDa) and GPIII (300 kDa). Some research showed that inner layer contains ZP proteins ZPC/ZP3, ZP1 (ZPB1), and ZPD (Zhang et al., 2011)

The outer layer possibly contains ovomucin, lysozyme C, and the vitelline membrane outer proteins, VMO-I and VMO-II (Kerver et al., 2002). The VMO-I was found to have 163 amino acids with molecular weight of 18 kDa (Kido et al., 1995). VMO-I is a simple protein and some research showed that it inhibits the hemagglutinating activity elicited by wheat germ agglutinin and has a glycan activity similar to the transferase activity of lysozyme (Mann 2008). On the other hand, VMO-II is small protein with 53 amino acids and a molecular mass of 6 kDa (Xiao et al., 2004).

### *1.3.5 Egg Yolk*

The egg yolk is composed of about 17% proteins, 35% lipids, and 1% carbohydrates. The rest of the constituents include cholesterol, vitamins, minerals and water. Egg yolk is constituted of an aqueous phase (called plasma) and an insoluble denser phase (granules).

The granules represent about 19-23% of yolk dry matter, accounting for about 50% of yolk proteins and 7% of yolk lipids (Anton and Gandemer 1997; Dyer-Hurdon and Nnanna 1993). The granules are mainly composed of high-density lipoproteins (HDL) (70%), phosvitin (16%) and 12% Low density

lipoproteins (LDL) (Burley and Cook 1961; Saari et al., 1964). High-density lipoprotein (HDL) consists of α- and β-lipovitellins, which differ in amino acid composition and bound phosphorous and carbohydrates. The proportion of α- and β-lipovitellins in HDL appears to be similar. The LDL is a structural constituent of the granules. HDL-phosvitin complex is the basic unit of granules linked by phosphocalcic bridges between the phosphate groups of their phosphoseryl residues (Causeret et al., 1991).

The VLDL, yolk lipoprotein, consists of apoVLDL II and apolipoprotein-B (Burley et al., 1984). ApoVLDL II is the only apoprotein from blood lipoproteins to be transferred to the yolk without any modification and is called apovitellenin I (Dugaiczyk et al., 1981). The source of yolk LDLs is VLDL. During the transfer from blood to the yolk, apolipoprotein-B is cleaved into several fragments, referred to as apovitellenin I-VI (Burley et al., 1993). Vitellogenin consists of three species designated as vitellogenin I, II, and III (Wang et al., 1983; Wang and Williams 1980). Vitellogenin is cleaved into the yolk granule proteins lipovitellin I and II and the phosphoprotein phosvitin.

Plasma forms the aqueous phase in which yolk particles are in suspension. It contains a large quantity of lipoproteins (LDL). Plasma corresponds to about 78% of yolk dry matter and is composed of 85% LDL and15% livetins (Burley and Cook 1961). It accounts for about 90% of yolk lipids (including nearly all the carotenoids) and 50% of yolk proteins. Yolk proteins consist of lipoproteins (30%) and soluble proteins (8%). The protein contents of lipovitellins are about 80%; the lipid contents are about 20%. The lipids include phospholipids (60% of

the lipid, primarily lecithin), triacylglycerols, and small amounts of cholesterol, sphingomyelin, and other lipids. The lipovitellins include glycoconjugates with mannose, galactose, glucosamine, and sialic acid. However, α-lipovitellin has a much higher sialic acid content than does β-lipovitellin, which explains α-lipovitellin's relatively acidic nature (Seko et al., 1997).

Livetin is a water-soluble protein that accounts for 30% of plasma proteins and is composed of α-livetin (serum albumin), β-livetin (α2-glycoprotein), and γ-livetin [γ-globulin immunoglobulin Y (IgY)] (Sugino et al., 1997). The mean molecular weights of α-, β-, and γ -livetins are reported to be 80 kDa, 45 kDa, and 150 kDa, respectively. The relative proportion of the three livetins is 2:5:3, respectively. Chicken serum albumin (α-livetin) has been implicated as the causative allergen of the bird-egg syndrome. β-livetin has been identified as a 45 kDa α2-glycoprotein. Chemically, its composition includes 14.3% nitrogen and 7% hexose.

#### *1.3.5.1 Lipids of the Egg Yolk*

Lipids are the main components (32 to 36%) of the egg yolk solids. The composition of yolk lipid is generally about 65% triglyceride, 28 to 30% phospholipids, and 4 to 5% cholesterol. However, the composition of yolk lipids can be affected by various factors including hen age, genotype, and changes in the diet of the hens.

##### *1.3.5.1.1 Fatty acids*

The predominant saturated fatty acids in eggs are palmitic (C16:0) and stearic (C18:0). The content of these two fatty acids in chicken eggs may range

from 22 to 26% and 8 to 10%, respectively. In addition to these two fatty acids, there are also other minor amounts of C14 and C20. The total saturated fatty acids may constitute up to 30 to 35% of total fatty acids in egg yolks.

Monounsaturated fatty acids (MUFA) in eggs are C16:1 and C18:1, which constitutes 42-46% of total fatty acids. Oleic acid (C18:1) is the major monounsaturated fatty acid in chicken eggs. The contents of long-chain (20 and 22) omega-6 and omega-3 polyunsaturated fatty acids (PUFA) were 20% and 25%, respectively, in egg yolk.

There are two families of PUFA in egg, namely n-6 and n-3 fatty acids. The predominant n-6 PUFA in egg lipids is C18:2n-6 (linoleic acid). Other n-6 fatty acids in eggs may include C20:4n-6, C22:4n-6, and C22:5n-6. The content of n-3 fatty acids in eggs is made of α-linoleic acids (18:3n-3) and docosahexaenoic acids (DHA, 22:6n-3). Among these, DHA is the major n-3 fatty acid in the egg. The α-linoleic content in regular eggs is under 1% of the total lipids; DHA may constitute between 1 and 3%. The content of n-3 PUFA is a reflection of dietary fat. Addition of flax, fish oil, and marine algae in laying hen diet leads to significant increases in α-linoleic acid and DHA in eggs (Cherian and Sim 1991)

Products enriched with PUFA are prone to oxidation and the enrichment with antioxidants is necessary in order to prevent the risk of oxidative damage. Grune et al. (2001) suggested supplementation of feed with at least 80 IU vitamin E/kg to prevent increase in cytotoxic aldehydic lipid peroxidation during production and storage of omega-3 PUFA-enriched eggs. Dietary vitamin E

resulted in a decrease of PUFA, SFA, and total lipids in fresh yolk lipids, whereas monounsaturated fatty acids (MUFA) did not change. Also, dietary vitamin E supplement slowed down the process of oxidation of egg yolk fatty acid during storage.

***1.3.5.1.2 Phospholipids***

The major components of egg yolk phospholipids (PL) are phohatidylcholine (PC) and phosphatidylethanolamine (PE), which may make up ~81% and 12% of egg yolk lecithin; lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and sphingomyelin are also minor components of yolk PL. The major fatty acids in egg PC are palmitic, oleic, stearic and linoleic acids, represented 32%, 26%, 16% and 13%, respectively; arachidonic and docosahexanoic acids (4.8% and 4%, respectively) are also present in significant amounts.

Kivini et al. (2004) studied the influence of oil-supplemented feeds (containing 15% vegetable-based or fish oils) on the concentration of the phospholipid content and their composition in hen eggs. Also, the total phospholipid contents and proportions of PC, PE and sphingomyelin were similar for all feeding groups. The supplemented feeds had a significant ($p < 0.05$) effect on the fatty acid composition of phosphatidylcholines. Furthermore, supplements decreased the proportion of saturated fatty acids in total fat, but not in the phospholipids.

Many studies have been conducted on methods for extraction and separation of phospholipids or lecithins from egg, as well as preparation of

lysolecithin by the enzymatic action of phospholipase A2 (PLA2), including immobilized PLA2. In addition to providing sources of purified phospholipids for basic research, these methods have been established to meet the demand to produce purified egg lecithin for pharmaceuticals, nutraceutical, and food applications. Examples of beneficial properties of yolk phospholipids are potential industrial applications as nutraceuticals and functional food ingredients (Sugino et al., 1997)

***1.3.5.1.3 Fat-soluble Vitamins and Carotenoids***

Most egg yolk vitamins, especially the fat-soluble vitamins, are contained in the yolk. Hen egg is considered a source of most vitamins necessary for human nutrition, except vitamin C. One egg may supply almost 12% vitamin A, more than 6% of vitamin D, 9% riboflavin, and 8% pantothenic acid of the recommended daily allowance in the United States. Only fish contains more vitamin D than eggs.

The color of the yolk is an important factor for the consumer acceptability of commercial eggs. The natural pigments in egg yolk are carotenoids that are conjugated isoprene derivatives. Among carotenoids, lutein and zeaxanthin are incorporated to a larger extent than β-carotene and astaxanthin. A large proportion of the yolk pigments is transported through the blood from the intestine by lipoproteins, which are normally deposited in the yolk. The functions of pigments are not known clearly, but the health of the chick after hatching may be improved by these carotenoids.

### *1.3.5.2 Egg Yolk Protein*

Yolk lipoprotein precursors such as very low-density lipoproteins (VLDL) and vitellogenin are synthesized in laying hen's liver and are transported in the blood to the oocyte.

Vitellogenin consists of three species designated as vitellogenin I, II and III (Wang and Williams, 1980; Wang et al., 1983). Vitellogenin is cleaved into the yolk granule proteins lipovitellin I, II and the phosphoprotein phosvitin. Amino acid analysis indicated the presence of a highly phosphorylated phosvitin in vitellogenin I and II and small phosvettes derived from vitellogenin III (Wallace and Morgan 1986a). Bessman et al. (1956) separated the granules by subjecting yolk to a centrifugal force of 20,000×g. Granules consist of 11-13% solids in yolk and contained both lipoprotein and phosphoprotein, and most of the iron and calcium of the yolk (Table 1.4)

Table 1.4 Proteins in egg yolk

| Egg yolk Protein (relative % w/v) | Molecular weight (kDa) | Characteristics |
|---|---|---|
| VLDL (37.3%) | 8-190 | |
| • apo VLDL Ⅱ | - | Apoprotein from blood lipoproteins |
| • apolipoprotein-B | - | Precursor of apovitellenin Ⅰ-Ⅵ |
| Vitellogenin (13.4%) | | |
| • lipovitellin Ⅰ-Ⅲ | - | |
| • phosvitin | 32-120 | Precursor of phosvitin and phosvettes |
| | 35 | Highly phosphorylated |

| | | |
|---|---|---|
| Lipovitellin | 400 | |
| Apoproteins (40%) | (α-,β- | Consist of α-,β- lipovitellin |
| • HDL | lipovitellin | Antioxidant effect |
| | complex) | |
| Livetins (9.3%) | | Serum albumin |
| • α | 80 | Fragment of C-termninal in vitellogenin |
| • β | 45 | Immunoglobulin G (IgG) |
| • γ | 150 | |

Adapted from: BCPAK Ting (2012). Development of antioxidant peptide fractions from egg yolk proteins using enzymatic hydrolysis and ultrafiltration membranes

***1.3.5.2.1 Lipovitellin apoproteins***

Yolk proteins consist of lipoproteins and soluble proteins. The high-density lipoprotein consists of α- and β-lipovitellins, which differ in amino acid composition as well as bound phosphorous and carbohydrates. The protein content of lipovetellin is about 80% while lipid content is about 20%, including phospholipids (60% of the lipid, primarily a lecithin), triacylglycerols (40%), and small amounts of cholesterol, sphingomyelin, and other lipids. Both lipovitellins are glycoconjugates with mannose, galactose, glucosamine, and sialic acid, but α-lipovitellin contains much higher sialic acid content than does β-lipovitellin, explaining its relatively acidic nature (Juneja 1997). The apoprotein form of lipovitellins sometimes referred to as vitelline, is present in a dimeric form linked through hydrophobic interactions; delipidation of lipovitellin has been reported to result in loss of solubility (Juneja 1997).

Lipoproteins include VLDL, LDL, and HDL in egg yolk. The LDL of egg yolk is the most abundant non-water phase of egg yolk. It is about 60% of the dry

weight of egg yolk. The LDL can be separated by high-speed centrifuging, gel chromatography and ion-exchange chromatography. Yolk low-density lipoprotein from egg yolks contains about 12% of protein, the rest being neutral and phospholipid.

The VLDL consists of apoVLDL II and apolipoprotein-B (Burley et al., 1984). ApoVLDL II is the only apoprotein from blood lipoproteins to be transferred to yolk without any modification and is called apovitellenin I (Dugaiczyk et al., 1981).

Apovitellenin I is a small protein of low molecular weight that lacks histidine with a small homodimer with disulfide-linked subunits of 9 kDa. Apovitellenin II is also isolated from egg yolk low-density lipoprotein. The protein's molecular weight is 20 kDa with polysaccharide residues of glucosamine, hexose and sialic acid. The functions of both apovitellenins are not clearly understood, but their properties appear to be an essential part of the lipoprotein structure. Apovitellenin III and IV with molecular weight more than 60 kDa are isolated from the total apoprotein mixture of egg yolk low-density lipoprotein by gel and hydrophobic chromatography.

***1.3.5.2.2 α, β-Livetins***

Livetin is a water-soluble protein that accounts for 30% of the plasma proteins and is composed of α-livetin (serum albumin), β-livetin (α2-glycoprotein), and γ-livetins [γ-globulin immunoglobulin Y (IgY) (Sugino et al., 1997). The mean molecular weights of α-, β-, Y-livetins are reported to be 80, 45

and 170 kDa, respectively. The proportion of the three livetins in yolks is 2:5:3, respectively (Sugino et al., 1997).

Egg yolk α-livetin and chicken serum albumin are identical. It has a molecular weight of 70 kDa and isoelectric point of 4.3 and 5.7. Chicken serum albumin (α-livetins) has been implicated as the causative allergen of the bird egg syndrome. Chicken serum albumin is partially heat labile inhalant. IgE reactivity to chicken serum albumin was reduced by nearly 90% by heating for 30 minutes at 90 ºC.

β-livetin has been identified as a 45 kDa α2-glycoprotein. Chemically, its composition includes 14.3% nitrogen and 7% hexose. Unfortunately, not much information is available about this protein and the existing data available is ambiguous.

***1.3.5.2.3 γ-Livetin (IgY)***

The γ -livetins in yolk are transported from the blood serum of hens. Of the three immunoglobulins (IgM, IgA and IgG) found in the serum, the laying hens transfer IgG to yolk at concentration of ~25 mg/ml, whereas IgM and IgA, are transferred to egg white at concentrations of 0.15 and 0.7 mg/ml, respectively. Morrison et al. (2001) identified several regions within the antibody molecule important for its uptake into the egg yolk. Intact Fc and hinge regions, but not the Fc-associated carbohydrate, are required for transport.(Morrison et al., 2001)

The basic structure of all immunoglobulin molecules is a unit consisting of two identical light polypeptide chains and two identical heavy polypeptide chains. These chains are linked together by disulfide bonds. Typically avian plasma

contains IgY plus IgA and IgM, which are evolutionary different from five distinct classes of mammalian immunoglobulins: IgG, IgA, IgM, IgD, and IgE. Although IgY antibody is functionally equivalent to mammalian IgG, they have profound structural differences.

The Y-globulins or γ -livetins in yolk are referred to as immunoglobulin Y (IgY) to distinguish them from mammalian IgG. Although IgY is derived from hen serum IgG, it differs in many chemical and structural features from mammalian IgG (Kovacs-Nolan et al., 2005).

Both IgG and IgY contain Asn-linked oligosaccharides, although their compositions differ. Like IgG, yolk IgY contains two heavy chains (H) and two light chains (L), but the molecular weight of the IgY's H chains is greater than that of the mammalian IgG's H chains, yielding an overall molecular weight of 180 kDa compared to 150-160 kDa for mammalian IgG. Furthermore, IgY H chains lack a hinge region and composed of four constant regions and one variable domain, whereas the IgG H chain contains a hinge region between the first two of three constant domains, which lead to the flexibility of the Fab fragments. The average molecular weights of IgY, heavy-chain, and Fab fragments are 167, 65 and 45 kDa, respectively.

Peptic digestion degrades IgY into Fab fragments, in contrast to disulfide-linked F(ab')2 fragments generated from IgG. IgY is relatively heat-stable even after 30 minutes at 65 ºC. It remains stable over the pH 5–11 range, but the antigen binding activity was rapidly lost at a pH of 2–3 or lower, probably because of conformational changes (Table 1.5).

Table 1.5 Comparison of chicken IgY and mammalian IgG

| Character | IgY | IgG |
|---|---|---|
| Molecular weight | 180 kDa | 150 kDa |
| Isoelectric point | >acidic | <acidic |
| Heat stability | >sensitive | <sensitive |
| pH stability | >sensitive | <sensitive |
| $F_c$ receptor binding activity | Low | High |
| Protein A/ protein G binding | No | Yes |
| Interference with mammalian IgG | No | Yes |
| Interference with rheumatoid factor | No | Yes |
| Complement activation | No | Yes |

The separation of IgY from the egg yolk involves various chemical reactions and a simple water extraction process. The water-soluble fractions (WSF) of egg yolks can be obtained by using the water dilution method based on the aggregation of yolk lipoproteins at low ionic strengths. Centrifugation or filtration is subsequently used to fractionate the WSF from water-insoluble lipid components of egg yolk. Acidic conditions change the integrity of the egg yolk granules and lead to increases in the lipid binding ability. Therefore, lowering pH results in not only increasing the recovery of IgY but also decreasing the amount of LDL in the supernatant. The WSF was almost devoid of lipids under mild acidic conditions and the highest yield of IgY was obtained between pH 5.0-5.2. To obtain a great purity of the IgY fraction collected from the WSF, a variety of methods have been used. These purification steps involved isolation of IgY from other water-soluble proteins by further concentration using specific salts or acids.

Further purification by gel permeation chromatography, ultracentrifugation, and ultrafiltration, resulted in a 95% pure IgY diagnostic agent.

Microbial food-borne diseases are responsible for serious health problems in humans and animals due to pathogens such as *Escherichia coli* O157:H7, Salmonella spp., Listeria spp., Campylobacter spp., enteropathogenic E. coli, viruses and parasites. IgY studies have demonstrated that specific IgY against *E. coli* O157:H7 and Salmonella is able to inhibit the growth of pathogens, eventually resulting in bacterial death. This research offers many advantages over traditional antibiotics and provides the basis of a highly effective means of producing inexpensive antibodies in egg yolks as functional food and nutraceutical ingredients for the prophylactic treatment of humans and animals against enteric diseases. Among these, oral passive immunotherapy may be of value due to the advantages of reduced cost, ease of administration, and potential to treat localized conditions in the gastrointestinal tract (GIT). Chicken egg yolk IgY is ideal for passive immunotherapy, as it may be readily obtained in large quantities from egg yolk, presenting a more cost-effective, convenient, and hygienic alternative to mammalian antibodies. IgY antibody has been proved to neutralize disease causing pathogens i.e., Rotavirus, *E. coli* O157:H7, *Salmonella enteritis*, *Clostridium perfringens* and toxic gluten for Celiac disease (Gujral et al., 2012).

Using chicken as an antibody producer brings a number of advantages over conventional mammalian antibody and recombinant antibody production and serves as an alternative to antibody sources. Combined with the egg industry's

capacity to produce thousands of eggs per day and an existing technology for the efficient fractionation and purification of IgY, it is conceivable that kilogram quantities of antibodies could be produced on a daily basis. Maintenance of a large flock of laying hens is inexpensive and practical, because large-scale feeding of hens and the collection of eggs are less labor intensive and well integrated. Eggs as the source of IgY can be collected from laying hens by the non-invasive method, which is compatible with animal protection regulations, as compared to mammal's sera from which IgG is separated.

Also, immunization of hens (vaccination) has long been applied to prevent hens from infectious diseases, indicating that immunization of hens is much more systematized to be effective than doing it for animals. The immune response of chickens could be maintained for a long period of more than 20 weeks with two injections. On the contrary to the conventional method of sacrificing animals to collect blood, using chicken is simple; eggs laid by super immunized hens. Thus, IgY has been widely used as a passive immunization therapy to treat enteric infections in humans and animals. A laying hen produces an average of 285 eggs in a year with a yolk of approximately 15 g whereas an immunized rabbit provides about 40 ml of sera. One gram of egg yolk contains about 10 mg of IgY whereas 1 ml of rabbit serum yields about 35 mg of IgG. An immunized hen produces about 43 g of antibodies per year. As egg yolk is known as a perfect food package, the isolation of IgY from the yolk is much easier than that of IgG from animal blood sera. For separation of IgY, a large-scale method is now applicable by automatic separation of the egg yolk with a machine.

Another application is the use of IgY as an immunological tool in the field of diagnostics as well as biomedical research.

### *1.4 Phosvitin (PV)*

Phosvitin (PV) is a phosphor-glycoprotein that contains about 10% phosphorus, with α- and β- phosvitin containing about 2% to 9% phosphorus, respectively. It is, therefore, one of the most highly phosphorylated proteins occurring in nature. About 80% of protein-bound phosphorous in egg yolk is located in phosvitin. Serine residues are predominant in the protein, many of which are phosphorylated and occur consecutively in the primary sequence of the molecule.

The relative abundance of phosphoseryl groups in the phosvitin amino acid sequence confers to the protein a large central hydrophilic portion surrounded by two small hydrophobic parts at the N-terminal and C-terminal (Chay Pak Ting et al., 2011). Due to its polyanionic character (pI = 4), phosvitin possesses a strong metal-chelating property (Castellani et al., 2004). The major site of phosvitin binding to carbohydrate is the Aspargine (Asn) residue at position 169 and the carbohydrate moiety is a branched oligosaccharide, consisting of mannose, galactose, N-acetylneuraminic acid and N-acetylglucosamine and linked to protein by the N-glycosidic bond (Shainkin and Perlmann 1971). Fourier transform infrared spectroscopy showed that the secondary structure of phosvitin is composed of 0% α-helix, 50% β-sheet, 7% β-turns and 43% random coil (Losso et al., 1993). However, factors such as pH can affect its secondary structure and shift the percentages of β-sheets to α-helices and

random coils (Prescott et al., 1986; Renugopalakrishnan et al., 1985) The β-turn is located between the α-helix and β-sheet, closer to the o-phosphoserine residues.

Due to its structure, phosvitin is resistant to heat treatments. No precipitate was observed after heating phosvitin solutions at different pHs (4-7) for several hours at 100°C (Mecham and Olcott 1949). Since native phosvitin has a very stable conformation, once iron is bound it is not easily released. After a heat treatment at 90 °C for 60 min, no decrease in the iron binding capacity could be detected (Castellani et al., 2004).

The unique chemical characteristics of phosvitin conferred by its high proportion of ionizable phosphorylated serine residues are accompanied by properties such as high water solubility and resistance to heat denaturation (Anton et al., 2000) and proteolytic attack (Juneja 1997). Because of the phosphate groups, phosvitin is one of the strongest naturally occurring metal binding biomolecule. Under low ionic strength and acidic conditions, phosvitin forms soluble complexes with $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Fe^{2+}$ and $Fe^{3+}$. Heating to 90 ºC and high pressure up to 600 MPa did not lead to a loss of iron binding capacity (Castellani et al., 2004). Nielsen et al (2000) reported that the addition of ascorbic acid and ascorbic acid 6-palmitate gave rise to an increase in amount of free iron ($Fe^{2+}$) in egg yolk dispersions, possibly owing to reaction with phosvitin- Fe (III), which subsequently propagated lipid oxidation.

The iron chelating activity of phosvitin has been associated with protection against oxidative damage**.** Katayama et al (2006) reported that oligophosphopeptides from hen egg yolk phosvitin have novel anti-oxidative

activity against oxidative stress in intestinal epithelial cells. The protective effect of phosphopeptide structure against $H_2O_2$-induced oxidative stress was almost the same as that of glutathione, and egg phosvitin phosphopeptide with a high content of phosphorous exhibited higher protective activity than those without phosphorus. Yet, phosphoserine itself did not show any significant anti-oxidant activity. These studies suggested that both phosphorus and peptide structure have key roles in the antioxidant activity.

Khan et al (2000) reported that phosvitin and its peptides exhibited anti-bacterial and deoxyribonucleic acid (DNA) leakage effects against *E. coli* under thermal stress at 50 °C, and suggested that phosvitin peptides disrupt the bacterial cells by chelating with metals in the outer cell membranes. Anti-bacterial activity was dramatically reduced by treatment with α-chymotrypsin, although the chelating effect remained.

### *1.4.1 Phosvitin (PV) Characteristics*

Phosvitin (PV) is consists of two highly unfolded flexible polypeptides, α- and β- PV (Abe et al., 1982; Itoh et al., 1983; Vogel 1983). Taborsky and Mok (1967) first reported the molecular weights of minor and major PV as 36 and 40 kDa (Taborsky and Mok 1967). Later, Wallace and Morgan (1986 a,b) determined that un-fractionated phosvitin consists of five major components including B, C, E1, E2 and F and that their molecular weights are 40, 33, 15, 18, and 13 kDa, respectively. PV has been shown as a heterogeneous protein with various ranges of molecular weights composed of 7 components and in which α- and β-PV account for 80% and 15%, respectively (Culbert and Mcindoe 1971).

On electrophoresis, PV has been separated into two subunits, namely α– phosvitin and β–phosvitin. Under denaturing conditions in the presence of 0.5% sodium dodecylsulfate (SDS) and Tris-glycine buffer, α-phosvitin dissociates into three subunits with a molecular weight of 37.5, 42.5 and 45 kDa and β-phosvitin into only one subunit of 45 kDa. These two components differ in their amino acid composition, their phosphorus contents, their carbohydrate content and their precipitation in the presence of calcium (Itoh *et al.*, 1983).

The secondary structure of PV is mainly composed of β-sheet, and random coil (Losso et al., 1993). However, PV's secondary structure could be changed due to pH. At mild acidic pH condition, the β conformation is predominant and phosphoryl groups can be changed from amide to ester linkages. But, the structure undergoes a large conformational change in strong acidic conditions (pH < 2) by shifting the proportion of β-sheets to α-helices and random coils (Yasui et al., 1990). These conformational changes can influence solubility (Castellani et al., 2003). In alkaline conditions, free amino groups become masked and release acid from phosvitin (Connelly and Taborsky 1961; Taborsky and Allende 1962).

Taborsky (1970) evaluated the ways in which freezing and thawing affect PV's conformation and determined that the primary change is from an unordered conformation to a β-structure (Taborsky 1970). Increasing acidity has been shown to intensify this effect.

PV can behave as a polyanion in a liquid state and has hydrophilicity with a small percentage of nonpolar hydrophobic side chains (Dickinson et al., 1997; Khan et al., 2000). PV takes on an elongated shape at low ionic strengths due to

high electrostatic repulsion between the charged phosphate groups. However, in a narrow range of ionic strength (0.02 to 0.1), it takes a compact shape.

### *1.4.2 Phosvitin (PV) Composition*

PV accounts for 4% of egg yolk dry matter (Wallace and Morgan 1986b). PV is comprised of 217 amino acid residues that make up a core region of 99 amino acids, grouped in runs of maximally 80 serine residues interspersed by arginine (Arg), lysine (Lys) and aspartic acid (Asp) (Byrne et al., 1984; Nardelli et al., 1987). PV is a phosphoglycoprotein that contains about 10% phosphorus (80% of total yolk phosphorus), 12.3% nitrogen, and 6.5% carbohydrates (Prescott et al., 1986; Xu et al., 2007; Burley and Vadhera 1989).

The α- and β- PV contain about 2% to 9% phosphorus, respectively. It is, therefore, one of the most highly phosphorylated proteins occurring in nature (Hatta et al., 1988). The amino acids sequence analysis of PV determined that β-PV includes more serine and histidine residues than α-PV. α-Phosvitin is rich in glycine, alanine, lysine, glutamine, and threonine. While β-PV is rich in histidine (Itoh et al., 1983).

Due to PV's distinct amino acid sequence, it contains 56% serine residues, 15% basic and 10% acidic amino acid residues (Byrne et al., 1984; Losso et al., 1993; Xu et al., 2007).

Serine residues are predominant in the protein (>55%), many of which are phosphorylated and occur consecutively in the primary sequence of the molecule (Allerton and Perlmann 1965).

Also, the α-PV molecule contains 6% carbohydrate, 2.5% hexose, 1% hexosamine, and 2% sialic acids attached to the N-acetyl derivatives. Carbohydrate components show a triple-branched antenna-like structure (Brockbank and Vogel 1990). However, β-PV includes only 2% carbohydrate. Unlike many of the other yolk proteins, PV does not contain any lipid (Shainkin and Perlmann 1971).

### *1.4.3 Isolation and Purification of Phosvitin*

PV was first purified by Mecham and Olcotte (1949) using dilute magnesium sulfate. Major (α-PV) and minor (β-PV) PV showed two bands in electrophoresis (Mecham and Olcott 1949). Sigma-Aldrich Company adopted a more simplified method that has been used for commercial phosvitin (Sundararajan et al., 1960). In this approach, egg yolks are centrifuged and collected the precipitate. The precipitate is dissolved in 10% NaCl, and extracted with butanol and centrifuged. This precipitate is re-dissolved in $MgSO_4$ to precipitate once more. The process is repeated to obtain a purer protein extract.

Other approaches have simplified Mecham and Olcott's procedure. Clark (1970) modified this process by using the buffer stage at pH 4.5, with an ionic strength of 0.1 and 0.01M ethylenediaminetetraacetic acid (EDTA) (Clark 1970). Later, Wallace and Morgan (1986) isolated egg yolk PV by modification of the procedure of Wallace et al., 1966. Egg yolks were mixed with phenylmethylsulfonyl fluoride (PMSF) and centrifuged, with the precipitate resuspended in PMSF. This step was repeated several times; then the granules were dissolved in NaCl and PMSF and centrifuged. The supernatant was extracted

with ammonium sulfate and centrifuged, and the supernatant of this mixture filtered to obtain purified PV.

Isolation of PV has been studied by using size-exclusion, anion-exchange chromatography, hydrophobic-interaction chromatography, and gel electrophoresis. A study developed a method with food-grade reagents to isolate PV for application in the food industry (Losso and Nakai 1994). In this procedure, egg yolks were diluted in water and spun down, and the precipitate extracted with hexane and then 10% NaCl. PV was then obtained from the supernatant.

Castellani et al.(2003) carried out chromatographic methods to isolate PV without using organic solvents. Egg yolk granules were dissolved in 0.17M NaCl and then centrifuged. The precipitate was collected and mixed with 1.74M NaCl. The solution was dialyzed against distilled water and centrifuged. The supernatant was pooled and diluted with $Mg_2SO_4$ to obtain crude PV in the precipitate. Hydrophobic interaction chromatography (HIC) and ion exchange chromatography (IEC) were applied to fractionate phosvitin. IEC at pH 5.0 allowed for a relatively high yield of purified PV. However, HIC produced a low yield.

Another study showed that immobilized metal affinity electrophoresis (IMAP) is also considered a potential method of isolating phosphoproteins. In this method, different metal ions are immobilized in native polyacrylamide gels and protein mixtures are electrophoresed in the gel. (Lee et al., 2002). Lee et al. (2002) have found that $Al^{3+}$, $Ti^{3+}$, $Fe^{3}+$, $Fe^{2+}$, and $Mn^{2+}$ were all capable of catching phosphoproteins, with $Fe^{3+}$ the most efficient at capturing phosvitin.

Ko et al. (2011) worked to develop a method of isolating PV that could be used for large-scale applications. Egg yolks were homogenized with distilled water, and the pH (4 to 8) was adjusted. After centrifugation, the precipitate was mixed with ethanol to remove any lipids. This step was repeated, and this time after centrifugation, the precipitate was homogenized with either 10% NaCl or 5 to 25% $(NH_4)_2SO_4$. This solution was adjusted to pH 4.0 and centrifuged, and the supernatant was filtered and then concentrated (Ko et al., 2011). Ko et al. (2011) research results show that a higher pH (6, 7, or 8) produces a higher yield of PV and the best ammonium sulfate concentration was found to be 10%. PV isolated using ammonium sulfate had a higher level of purity than PV isolated using NaCl, although the PV isolated with NaCl had a higher recovery rate.

Zhang et al. (2011) attempted to develop a protocol to isolate PV in large scale mass production using polyethylene glycol (PEG). Egg yolk granules were isolated, dissolved in NaCl, and then homogenized with PEG6000 at varying pHs and concentrations. These samples were then used for anion exchange chromatography to obtain pure, metal-free PV. Zhang et al. (2011) found that at pH 4.0 (around the isoelectric point of PV) and 3% PEG6000, they were able to isolate a good yield of PV (47%) containing a high level of purity (99%). This protocol proved to be simple, efficient, and free of organic solvents, allowing it to be a potential candidate for large-scale PV production for food products.

Lately, the anion-exchange chromatography was further studied by Lei and Wu (2012). Egg yolks were diluted with the appropriate amount of water and spun down, and the precipitate re-suspended in a carbonate-bicarbonate buffer.

This solution was filtered, then loaded onto an anion exchange column. PV was recovered at a yield of 35.4% from the yolk or 1.9% from total dry egg matter. This protocol had only three simple steps and demonstrated a recovery comparable to that of previous, more convoluted methods.

### *1.4.4 Functional properties of Phosvitin*

#### *1.4.4.1 Metal Chelating Activity*

PV shows a strong metal binding capacity for bivalent cations such as calcium, iron, and magnesium due to highly negatively charged phosphate molecules in serine residues (Grizzuti and Perlmann 1973; Taborsky 1963). Most of the iron ions (95%) in egg yolk are bound to PV (Greengard et al., 1964).

Each PV molecule contains 136 phosphate groups. Approximately two phosphate groups can bind with one iron ion. PV from fish eggs has a lower iron-binding capacity than that from hen's eggs because it is smaller and has less phosphorus and serine (Guerin-Dubiard et al., 2002).

It has about 60 molecules of iron-binding capacity. Therefore, the iron-binding capacity is about 5.96 mg Fe/g PV and the percentage of Fe-bound in PV is 0.3% (Greengard et al., 1964; Taborsky 1963). Binding Fe to PV does not cause a conformational change in the ordered structure (Mecham and Olcott 1949; Taborsky 1963; Webb et al., 1973; Albright et al., 1984).

PV can bind to both forms of Fe: Fe (II) and Fe (III) (Osaki et al., 1975). Due to the iron binding capacity, it showed the effect of decreasing the generation of hydroxyl radical (•OH) from $H_2O_2$ in the Fenton reaction system (Ishikawa et

al., 2004). PV was also found to protect DNA against oxidative damage induced by Fe (II) and hydroxyl radical (•OH) in vivo (Ishikawa et al., 2004).

Equilibrium dialysis experiment results suggest that clusters of PV's di-O-phosphoryl serine residues play a significant role in PV's iron chelating activity (Hegenauer et al., 1979). Research has also shown that PV binds more tightly with ferric irons than weak complexes bind with ferrous iron (Taborsky 1980).

According to Albright et al. (1984), PV was heated at 110°C for 20 and 40 min. However, it was not able to release the bound Fe from PV, indicating that PV has a high iron binding ability. Also, they studied the stability of Fe-bound in the presence of NaCl, citric acid, and EDTA and only EDTA can release bound Fe from PV because EDTA forms stronger ligand with iron than PV.

Other researchers also determined the stability of PV-Fe complex in the presence of o- phenanthroline as a strong chelating agent. They found that Fe was released from phosvitin in the presence of o-phenanthroline (Castellani et al., 2004). Furthermore, high pressure at 300 and 600 Mpa for 10 min had no effect on PV's structure and did not affect the Fe-binding activity (Castellani et al., 2004). The reason for the strong stability of PV and Fe complex is due to tetrahedral stoichiometry (once the Fe ion is bound with one phosphate molecule, another phosphate molecule completes the binding process) or octahedral stoichiometry (two other phosphate molecules complete the process) (Castellani et al., 2004).

This PV-Fe complex can increase the activation energy required for the dissociation when iron ions are released from phosvitin (Castellani et al., 2004).

PV's Fe-binding capacity is influenced by the interaction of pH and ionic strength. The best Fe-binding activity showed at pH 6.5 and ionic strength 0.15 (Castellani et al., 2004), resulting in 115 μg iron/mg PV.

PV can also bind to $Mn^{2+}$ and $Ca^{2+}$ (Grizzuti and Perlmann 1973). Grizzuti and Perlmann (1973) found that phosvitin binds with 103 $Mg^{2+}$ ions and 127 $Ca^{2+}$ ions using dialysis equilibrium at pH 6.5 (25 °C). However, they observed that bound $Mg^{2+}$ and $Ca^{2+}$ to PV was released at pH 4.5, which then decreased to 40 and 32 $Mg^{2+}$and $Ca^{2+}$ ions, respectively. Choi et al. (2005) observed the solubility of 0.1 % $CaCl_2$ under ileum conditions (pH 7.0, 37ºC) and determined that 1% phosvitin can increase the solubility of $Ca^{2+}$ by 29% compared to the control (without phosvitin). This observation is inconsistent with evidence showing that PV forms insoluble salt complexes in the presence of bivalent cations.

#### *1.4.4.2 Antimicrobial Activity*

PV with a high iron-chelating capacity contributes to its antimicrobial properties (Taborsky 1963). Because iron is an essential element for all living organisms, PV is considered as an antimicrobial agent.

Most pathogenic bacteria are gram-negative with a lipopolysaccharide (LPS) present in the outer membrane that acts as a permeability barrier against external agents (Vaara 1992). LPS is a good target for antibacterial agents such as surfactants or chelators. PV with a strong metal-binding ability affects the outer membrane of bacteria and act as a chelator to inhibit bacterial growth. Although this ability has not been extensively studied, PV is one of the promising proteins that can be used as an antimicrobial agent.

PV exhibited a strong chelating ability with LPS and eliminated *Escherichia coli* in the presence of 0.1% of phosvitin under 50 °C for 20 minutes (Khan et al., 2000). The antibacterial effect of PV is due to the leakage of bacterial DNA by PV damages the cell membrane (Khan et al., 2000). Under thermal stress, PV has the potential to be used as an antibacterial agent (Khan et al., 2000). Wang et al. (2011) evaluated the antimicrobial effects of zebrafish (*Danio rerio*) PV. They found that both the embryos and protein extracts from the embryos showed antibacterial activity and that this activity could be highly reduced by adding anti-fish PV antibodies. They also studied that recombinant PV could directly inhibit the growth of *Escherichia coli, Aeromonas hydrophilia*, and *Staphylococcus aureus*.

The antimicrobial activity of chitosan/PV-layer by layer (LBL) coated mats was measured (Zhou et al., 2014). The chitosan/PV-LBL-coated mats had a greater inhibition effect against *Escherichia coli* and *Staphylococcus aureus* under 37°C and 50°C than cellulose mats (control). Also, antimicrobial activity was improved when bilayer numbers increased proportionally to the amount of PV (Zhou et al., 2014). This study indicates that chitosan/PV composite mats have potential applications in the areas of the antibacterial coating, tissue engineering, and wound dressing.

#### *1.4.4.3 Antioxidant Activity*

Assessments of antioxidant properties of natural compounds are important because of their uses in medicine, food and cosmetics (Halliwell 1997; Liu 2003; Sánchez-Moreno 2002). In living systems various metabolic processes and

environmental stresses generate various reactive oxygen species (ROS). The ROS can damage effect on biological functions. It leads to cellular dysfunction and cell death. The increase of ROS can trigger oxidative stress in systemic level, and it is manifested in the form of a variety of health problems such as cancer, age-related disease and cardiovascular diseases (Grune et al., 2001; Noguchi and Niki, 2000). Cellular ROS are regulated by the interplay of complex antioxidant machineries in living systems. Nature has bestowed living systems with numerous antioxidant molecules. These natural antioxidants are known to minimise the adverse effects of free radicals in living system. PV is a strong metal chelating agent that can capture the free iron ions Fe (II) and Fe (III) to inhibit the production of reactive oxygen species (ROS). Although iron is an essential nutrient for living organisms, Fe (II) ions can react with $H_2O_2$ and produce toxic hydroxyl radicals (·OH) via the Fenton reaction. This reaction also produces Fe (III) ions, which in turn react with $H_2O_2$, causing a continuous build-up of ROS (Ishikawa et al., 2004).

PV's antioxidant activity was first observed by Lu and Baker (1986). They evaluated the activity in the phospholipid emulsion system with different concentrations of nonorganic and organic metals such as $Fe^{2+}$, $Cu^{2+}$ and, hemin. PV can efficiently inhibit $Fe^{2+}$- and $Cu^{2+}$-mediated phospholipid oxidations by chelating metals. PV was not effective at inhibiting hemin-mediated phospholipid oxidation. It showed a higher inhibitory effect on phospholipid oxidation in the presence of $Fe^{2+}$ (up to 30:1 $Fe^{2+}$-to-PV molar ratio) than in the presence of copper (1:1 molar ratio).

PV's antioxidant activity was high at pH 6.1, but was not effective at inhibiting the oxidation induced by $Cu^{2+}$ at pH 7.8 (Lu and Baker 1986). Antioxidant ability did not change when PV was pasteurized (61.1°C); however, autoclaving (121.1°C, 10 min) decreased its inhibitory capacity on iron catalysis (Lu and Baker 1986). Also, PV inhibited lipid peroxidation induced by UV irradiation in a dose-dependent manner in the presence of excess iron (Ishikawa et al., 2004).

These results suggest that PV might be useful to prevent other iron-mediated oxidative stress-related diseases such as colorectal cancer (Ishikawa et al., 2009; Katayama et al., 2006).

#### *1.4.4.4 Emulsifying Activity*

The emulsifying activities of phosvitin are better than that of other proteins such as bovine serum albumin, β-casein, and soy protein at pH 7 (Chung and Ferrier 1991; Khan et al., 1998). Chung and Ferrier (1991) observed that the PV's emulsifying activity is affected by several factors, such as increasing protein concentration, oil volume, and mixing speed. The mixing speed range of 10,000-22,000 rpm showed the greatest influence on emulsifying activity (Chung and Ferrier 1991).

Dephosphorylated PV using phosphatase with alkaline treatment drastically reduced the emulsifying activity (Kato et al., 1987). This indicates that electrostatic and repulsive forces of phosphate moieties play a significant role in the emulsifying property (Kato et al., 1987). Dephosphorylation and enzymatic hydrolysis of PV with pepsin, trypsin, and α-chymotrypsin reduced phosvitin's

emulsifying ability and stability. The hydrophobic N- and C- terminal portions are essential for emulsifying activity (Khan et al., 1998). PV forms a better emulsion at a low ionic strength; for example, at 0.05 M NaCl, PV has formed a finer emulsion than that at 1.5 M NaCl (Castellani et al., 2005).

The optimal pH range for the emulsifying activity of PV was pH 3 to 9. Emulsifying activity was significantly reduced upon pH increasing to 10. Castellani et al., (2005) concluded that charge and ionic strength could affect PV's emulsifying properties. Examining the way in which heat affects PV emulsions, Chung and Ferrier (1995) found that emulsifying activity decreased when PV was subjected to heat above 70$^{o}$C for 1 h. The emulsion stability, however, was not affected by heating above 67.5$^{o}$C for 1 h (Chung and Ferrier 1995).

### *1.5 Phosvitin Hydrolysates (PVH)*

PV is known as a potential source of bioactive phosphopeptides because it contains highly phosphorylated serines. PV is resistant to proteolytic digestion *in vitro* due to its extraordinary primary structure, which is composed of long oligo-phosphoserine blocks containing negatively charged phosphate groups (Byrne et al., 1984). Developing innovative ways to more efficiently enhance the yield of phosvitin hydrolysates (PVH) could be challenging. Solutions to increase PV's enzymatic susceptibility include enzymatic hydrolysis after treatment in acidic or alkaline conditions, thermal treatment, and high-pressure treatment. The use of very effective proteases also plays a role in optimal conditions. However, these strategies have yet to be studied.

Novel hen egg phosvitin phosphopeptide with molecular masses of 1-3 kDa was effective for enhancing calcium binding capacity and inhibiting the formation of insoluble calcium phosphate. Jiang and Mine (2001) reported that 1-3 kDa fragments of these peptides derived from partially dephosphorylated phosvitin by tryptic digestion showed a higher ability than did commercial casein phosphopeptides to solubilize calcium in a calcium phosphate precipitate, while Feng and Mine (2006) reported that phosvitin phosphopeptide from partially dephosphorylated phosvitin increased iron uptake in a Caco2 cell monolayer model. Choi et al. (2005) demonstrated high Ca solubilization in the presence of phosvitin or its tryptic peptides when incubated under conditions simulated those of the ileum, while Choi et al. (2005) found that phosvitin peptides are improved the bioavailability of Ca and thus increased incorporation of Ca into the bones of rats.

### *1.5.1 Production of Phosvitin Hydrolysates (PVH)*

The production of PVH was studied with various enzymes such as pepsin, trypsin, and α-chymotrypsin (Goulas et al., 1996). Pepsin produced three peptides of Glycine (Gly) 4-Glutamine (Glu) 41 (38 residues), Asparagine (Asn) 44–Leucine (Leu) 193 (150 residues), and a C–terminal fragment of Leu 193-Glu 214 (21 residues). Trypsin produced two major peptides: Ala 1-Arg 35 (35 residues) and Gln 49- Arg 212 (164 residues). α-chymotrypsin digestion produced two major peptides: Gly 4-Gln 49 (46 residues) and Ala 50-Trp 210 (161 residues). These peptides were evaluated by SDS-PAGE.

All three enzymes more efficiently digested the N-terminus than the C-terminus of the protein. Shainkin and Perlmann (1971) attempted phosvitin hydrolysis using pronase from *Streptomyces griseus* to isolate the peptide bound to the carbohydrate chain (Shainkin and Perlmann 1971). This study was successful at isolating the glycopeptides, but phosvitin digestion was not completed, because a significant amount of phosphoserine residues blocked the enzyme's access to the cleavage sites. Although the phosvitin was saturated with iron to neutralize the effect of the negative charge, fragmentation was not improved. It anticipated that polynuclear complexes could block the formation of the enzyme-substrate complex (Gray 1972).

The dephosphorylation of phosvitin by alkaline treatment and incubation of phosvitin at 37 ºC improved the PV's digestibility. This treatment made phosvitin more susceptible to enzyme accessibility and generated short peptides (1 to 3 kDa) after trypsin treatment, and these phosphopeptides still showed calcium-binding ability (Jiang and Mine 2000). Some researchers have attempted to isolate and enrich PVH without first purifying phosvitin. One such study by Zhang et al., (2012) utilized immobilized metal ion affinity nanoparticles (IMANs) to separate phosphopeptides without using organic solvents or ion exchange chromatography. Sun et al., (2013) showed similar results using magnetite carboxymethyl chitosan nanoparticles and demonstrated the purity of the PVH.

Czernick et al., (2013) were able to generate a significant number of PVH. Phosvitin was digested with trypsin for six hours, followed by a second addition

of trypsin and a second incubation of 18 hours. This led to approximately 25% complete digestion. The partially digested fragments could be further digested by an additional 24-hour trypsin digest. Mass spectrometric analysis was used to identify the PVH sequences and the precise phosphorylation sites. Although the process was slow, Czernick et al., (2013) were able to generate 68 PVH 1 and map 89 phosphorylation sites, and 35 PVH 2 and map 62 phosphorylation sites. PV could be valuable to develop new functional bioactive peptides derived from egg yolk by controlling the phosphate residues and peptides chain length.

### *1.5.2 Functional Properties of Phosvitin Hydrolysates*

#### *1.5.2.1 Metal Chelating Activity of Phosvitin Hydrolysates*

PVH that was partially dephosphorylated by tryptic digestion showed a higher ability than did commercial casein phosphopeptides to solubilize calcium in a calcium phosphate precipitate (Jiang and Mine 2001). Jiang and Mine (2001) found that small peptides (1-3 kDa) containing about 35% of the phosphorus content of native PV showed the highest inhibition effect on calcium phosphate precipitate. This result implies that the PVH has a higher potential to be used as mineral binding agents than native phosvitin (Jiang and Mine 2001). The native PV decreased mineral absorption and digestibility in *in vitro* and *in vivo* experiments (Ishikawa et al., 2007).

Phosphoryl serine blocks $(Ser\text{-}P)_n$ with $n \geq 4$ were isolated from PV under an acid hydrolysis condition (Donella et al., 1976) and found to have $Fe^{3+}$ binding activity but with less activity than phosvitin. However, these findings

indicated that PV-derived phosphoryl serine blocks played a significant role in the Fe-binding activity.

Choi et al. (2005) studied the effect of the PVH tryptic digest in enhancing the Ca absorption and accumulation in Sprague-Dawley rat bones. The PVH-ingestion group showed higher levels of Ca/bone weight and Ca/bone ash ratios in rat bones compared to the control group. It indicated that a greater amount of Ca was absorbed in intestinal tract and Ca accumulation in the bones significantly increased ($p < 0.05$) compared to the control group.

Feng and Mine (2006) observed that at a low concentration of PVH (0.78-3.125 ug/ml) prevent the formation of iron- PVH precipitation and improved iron uptake in Caco-2 cells, however, an excess amount of PVH would bind iron too tightly and reduce absorption (Feng and Mine 2006).

#### *1.5.2.2 Antioxidant Activity of Phosvitin Hydrolysates*

PVH can be exploited as a potential inflammatory response-suppressing agent, oxidative stress, and lipid peroxidation inhibitor in living cells (Katayama et al., 2006). In a study conducted by Xu et al., (2007), PVH was produced from the tryptic digestion of partially dephosphorylated PV. This PVH showed stronger antioxidant activity in the linoleic acid system and radical-scavenging activity on 2,2-diphenyl-1-picrylhydrazyl(DPPH) free radicals compared to intact PV. Xu et al., (2007) reported that PVH showed significantly higher antioxidant activity even at very low concentrations (10 μg/ml) than vitamin E at higher concentration (100 μg/ml).

Although the PVH possesses only 35% phosphorus content of intact PV, the result indicated greater antioxidant activity. This result implied that not only the number of phosphorus groups is important in antioxidant activity, but some other factors also play roles. These factors include other amino acid residues (e.g. histidine, methionine, and tyrosine) and peptides structure (Hegenauer et al., 1979; Xu et al., 2007; Yamashoji 1979).

Recently, antioxidant phosphopeptides were produced from egg yolk using a combination of alcalase and protease N hydrolysis (Young et al., 2011). These peptides exhibited antioxidative properties in an *in vitro* hydrogen peroxide-induced Caco-2 cell culture (Young et al., 2011). *In vivo*, egg yolk phosphopeptides induced glutathione synthesis increased antioxidative enzymes' activities and reduced lipid and protein oxidation (Young et al., 2011). Further studies have been conducted to elucidate and sequence pepsin- and pancreatin-digested PVH. Limited proteolysis of PVH was observed after simulated gastrointestinal digestion. The contiguous serine and phosphoserine sequences may influence bioactivity (Young et al., 2011).

The PVH antioxidant activity in biological systems has also been investigated. The three ion-exchange chromatographic fractions of partially dephosphorylated PVH treated by trypsin have been studied in the Caco-2 cells to relieve the $H_2O_2$-induced oxidative stress *in vitro* (Katayama et al., 2006). Katayama et al., (2006) found that PVH showed significantly reduced production of oxidative stress indicators, such as Interleukin 8 (IL-8), a proinflammatory mediator, compared to the control and intact phosvitin-treated group. Also, those

PVH remarkably suppressed lipid peroxidation and improved the production of glutathione (GHS) by Caco-2 cells treated with $H_2O_2$. These PVH fractions consist of more than 50% serine residues and basic amino acids such as Arg, Lys, and His. It implies the importance of phosphorylated serine moieties in PVH that play a significant role in mitigating oxidative stress.

#### *1.5.2.3 Antimicrobial Activity of Phosvitin Hydrolysates*

The lower antibacterial activity of PV hydrolyzed by α-chymotrypsin compared to native phosvitin was reported by Khan et al., (2000). When the hydrophobic N- and C-terminus were degraded, phosvitin lost its bactericidal activity. This result suggests that both the hydrophilic phosphate groups and hydrophobic termini are necessary for antibacterial activity because the hydrophobic termini are possibly the anchoring parts to the lipopolysaccharides (LPS) (Khan et al., 2000). Lactoferrin is a well-known source of antimicrobial bioactive peptide through its Fe-binding activity (Kontoghiorghes 1986; Rainard 1986; Samuelsen et al., 2005). Since phosvitin's Fe-binding capacity is higher than that of lactoferrin, it could be a better candidate than lactoferrin to produce the antimicrobial bioactive phosphopeptides.

#### *1.5.2.4 Emulsifying Activity of Phosvitin Hydrolysates*

Partially dephosphorylated phosvitin digested by different enzymes such as pepsin, trypsin, and α-chymotrypsin showed lower emulsifying ability and stability compared to that of intact PV (Khan et al., 1998). Thereby, Khan et al., (1998) concluded that N- and C–terminal moieties of phosvitin are essential for its emulsifying properties to anchor the oil droplets. Nakamura et al. (1998) found

that conjugating PV with galactomannan can remarkably increase phosvitin's emulsifying activity and stability (Nakamura et al., 1998).

### *1.6 High hydrostatic pressure-enzymatic hydrolysis (HHP-EH) biotechnology*

High hydrostatic pressure-enzymatic hydrolysis (HHP-EH) Biotechnology is a combination of cold isostatic pressure (100 MPa) and enzymatic hydrolysis. The high hydrostatic pressure technology known as a 'cold isostatic pressure over 300 MPa' is widely used in the food industry to extend shelf life of food products (Aertsen et al., 2009). Recently this system was modified to control pressure and temperature (Toyo Koatsu Co Ltd, Hiroshima Japan), making it applicable to changing functionality of food components (Sunwoo et al., 2013; Sunwoo et al., 2014). High pressure processing changes protein structure by altering non-covalent molecular interactions (Zeece MG 2008) and partially unfolding the protein.

Of particular importance, partially unfolded proteins under high pressure conditions are susceptible to enzyme cleavage at more sites along the protein chain than under ambient conditions. Depending upon the enzymes, pressure conditions and substrates involved, HHP-EH can increase the rate of protein hydrolysis dramatically (Aertsen et al., 2009; Sunwoo et al., 2014). Not only protein but also plant structures can be hydrolyzed by HHP-EH, depending on use of various commercial enzymes. In our preliminary study, HHP-EH process (100 MPa, 50$^{o}$C, cellulase enzyme) effectively hydrolyzed carbohydrates in cellulose, pectin, hemicellulose that are tightly linked with ginsenosides from fresh ginseng roots. This was demonstrated for α- and β-amylase isolated from barley malt; their

catalytic activities are notably higher (25 and 16%, respectively) at an optimum of 60°C and 100 MPa when compared with the control at ambient pressure (Sunwoo et al., 2014).

HHP-EH technology is unique in applying pressure and enzyme hydrolysis simultaneously. Partially unfolded proteins under HHP-EH are more susceptible to enzyme cleavage. Moderate hydrostatic pressure (50–200 MPa) promotes enzyme activity by conformational changes in substrate proteins and breakdown of cellular compartments (Hendrickx M 1998). For some enzymes, pressures and substrates, HHP-EH dramatically increases the rate of protein hydrolysis through more effective enzyme–substrate contact (Aertsen et al., 2009; Buckow et al., 2007; Hendrickx M 1998; Vanhoutte et al., 2009; Zeece MG 2008). Most published research uses high pressure as a protein denaturing pre-treatment before hydrolysis (Chicon R 2008; Dong X 2011; Hoppe A 2013; Sunwoo et al., 2014). With HHP-EH we apply moderate hydrostatic pressure (100 MPa) during hydrolysis to improve enzyme efficiency without fully denaturing enzymes or protein substrates.

Enzymatic hydrolysis of proteins is the starting point for producing bioactive peptides from diverse sources of animal and plant proteins. The major drawbacks of enzymatic hydrolysis include high enzyme cost, low digestibility of some proteins, and enzyme instability, restricting large-scale industrial applications. The main advantages of HHP-EH are higher enzyme efficiencies, lower production costs, and improved functionality of hydrolysates. Our preliminary trials show that half the amount of enzyme is needed in HHP-EH

compared to hydrolysis under atmospheric pressure, and HHP–EH processing is twice as fast. HHP-EH also has potential to generate smaller peptides that are more easily absorbed by the digestive system and have better bioactivity. Batch-to-batch consistency of HHP-EH is better than at atmospheric pressure. HHP-EH also improves hydrolysis rates for proteins resistant to enzymatic attack, such as in waste animal protein (Hendrickx M 1998). A key advantage of HHP-EH is the destruction of microorganisms in the reaction vessel, resulting in lower risk of microbial growth during long operation times. Table 1.6 shows functional properties of PV and PVH.

Table 1.6 Functional properties of Phosvitin and Phosvitin Hydrolysates(PVH)

| Metal chelating activity | Antioxidant activity | Antimicrobial activity | Emulsifying activity |
|---|---|---|---|
| Phosvitin | | | |
| Binding with 95% of the iron ions (Greengard et al., 1964). | $Fe^{2+}$ and $Cu^{2+}$mediated phospholipid antioxidation (Lu and Baker 1986). | Effect on elimination of *E. coli* and S. aureus (Khan et al., 2000, Zhou 2014). ★★ | Emulsifying activity among bovine serum albumin, β-casein, and soy protein at pH 7 (Chung and Ferrier 1991; Khan et al. 1998). ★★ |
| Binding with bivalent cations (Ca, Fe, Mg) (Taborsky 1963, Grizzuti and Perlmann 1973). | Inhibited lipid peroxidation induced by UV irradiation (Ishikawa et al., 2004). ★ | | |

| | | | |
|---|---|---|---|
| ★★ | | | |
| Phosvitin Hydrolysates (PVH) | | | |
| Inhibit the calcium phosphate precipitation (1-3kDa of PVH) (Jiang and Mine, 2001).<br><br>Fe3+ chelation activity (Donella et al. 1976)<br>★★ | Greater antioxidant activity 35% phosphorus content of PVH than PV (Xu et al., 2007).<br>★★ | Low antibacterial activity of PVH (Khan et al., 2000).<br>Δ | Reduced both the emulsifying ability and stability (Khan et al., 1998).<br>Δ |

★★ Excellent activity
★ Good activity
Δ Poor activity

***1.7 Rationale***

Due to the high proportion of phosphates, the PV is a major phosphoprotein in egg yolk with metal chelating, antioxidant, emulsifying and antimicrobial capacities. Although PV possesses versatile functions, its use in nutra- or pharmaceutical application has been restricted mainly due to the formation of insoluble complexes with divalent metal ions (e.g. $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$ and $Fe^{2+}$) in the gastrointestinal tract. The PV, thus, may cause an anti-nutrient effect (Ishikawa et al., 2007). On the other hand, the PV hydrolysates (PVH) that are hydrolyzed by proteases may solve the problem and increase their applications without compromising the PV's functional properties. There are many attempts to

produce the PVH. However, the yield of the PVH is low due to the PV's negative charges causing the resistance to digestive enzymes. Further studies are needed to increase the yield of PVH and enhance their functional properties. As an alternative processing procedure, high hydrostatic pressure combined with enzymatic hydrolysis (HHP-EH) is a new method to augment enzyme efficiency. However, there is limited information available for the production of PVH by HHP-EH.

### *1.8 Hypothesis*

We hypothesize that the application of HHP-EH increases the degree of hydrolysis (DH) of PV. The PVH produced by HHP-EH shows stronger antioxidant and antimicrobial properties compared to the intact PV, and PVH treated under atmospheric pressure (AP).

### *1.9 Objectives*

The objectives of this study are to 1) to increase the yield of PVH by using an HHP-EH under optimal conditions of pressures, enzyme:substrate (E:S) ratios, temperatures and incubation times, 2) to determine the effect of iron-chelation and antimicrobial properties of PVH, and 3) to quantitate the PV in eggs and liquid media by DAS-ELISA.

### *1.10 Specific Objectives*

#### *1.10.1 Production of PVH*

The aim of this study was 1) to determine optimum condition of pressures, enzyme: substrate (E:S) ratios, various temperature and pH for producing PVH; 2) to measure degree of hydrolysis of PVH with 2,4,6-trinitrobenzene sulfonic acid (TNBSA) method; 3) to measure the molecular weight of the PVH by SDS-PAGE, matrix-assisted laser desorption/ionization (MALDI-TOF); 4) to monitor PVH by high performance liquid chromatography (HPLC).

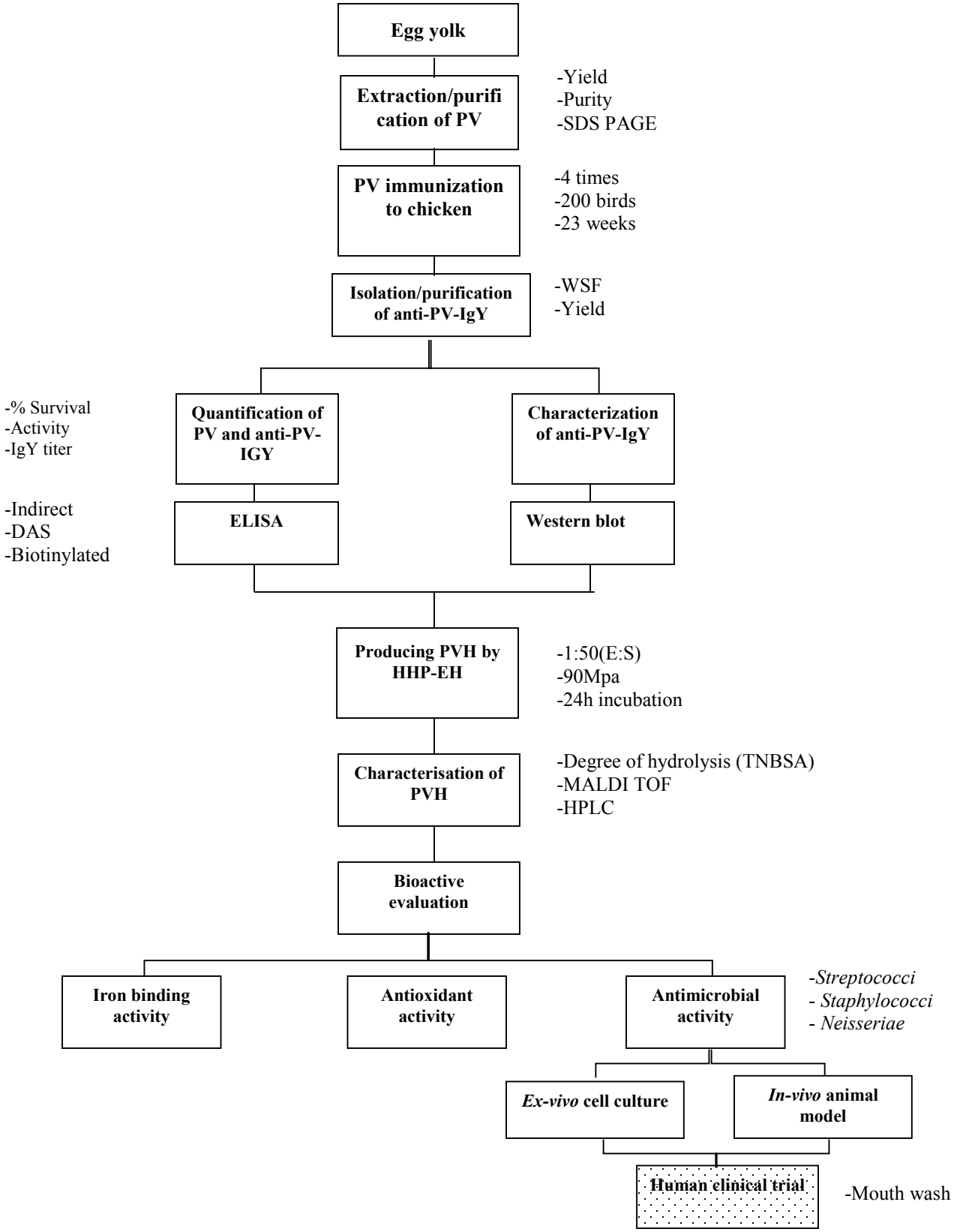
#### *1.10.2 Iron-chelation*

The aim of this study was 1) to determine the iron binding ($Fe^{II}$) capacity of PVH; 2) to measure the ferric ($Fe^{III}$) reducing ability of plasma (FRAP) assay of PVH.

#### *1.10.3 Anti-microbial property*

The aim of this study was 1) to evaluate the PVH's antimicrobial activity *in vitro;* 2) to develop antimicrobial formulation for the prevention of diarrhea in the animal by a combination of PVH and/or IgY antibodies.

### *1.11 Study Plan*



## Chapter 2 (Study 1): Sensitive Double Antibody Sandwich ELISA for the Quantification of Phosvitin

### *2.1 Introduction*

Phosvitin (PV), one of the most highly phosphorylated proteins in nature, is produced by proteolytic cleavage of chicken vitellogenin (a metal-binding protein) which is disseminated in the hen blood and picked up by developing oocytes (Richards 1997). Vitellogenin is cleaved into lipovitellin and PV yolk proteins, sometime during or after transport into the oocyte (Yamamura et al., 1995).

PV accounts for 60% of total egg yolk phosphoproteins and bears about 90% of the egg yolk phosphorus (Xu et al., 2007). The numerous phosphorylated serine residues in PV exhibits strong affinity for divalent metals such as iron, calcium, and magnesium (Grizzuti and Perlmann 1973). The PV prevents lipid oxidation in lipid-containing systems such as food and biological systems also by its ability to bind iron and copper (Lu and Baker 1986). PV has also displayed strong antimicrobial and emulsifying properties (Khan et al., 2000; Khan et al., 1998). It has been considered that the emulsifying activity of PV is better than that of bovine serum albumin, β-casein and soy protein (Chung and Ferrier 1992; M. S. Khan et al., 1998).

In recent years PV has drawn attention of researchers scientists and industries due to its phosphopeptides content for food and nutraceutical formulations (Samaraweera et al., 2011). PV is considered to be an efficient

source of bioactive phosphopeptides but the high cost of PV along with complicated detection and production procedures hinders its application efforts particularly in the food industry. Also, there is a huge potential of PV application in the cosmetics industry as naturally derived antioxidant and anti-aging copper-binding peptides. To the best of our knowledge, there is no well-established method to detect or quantify PV. Accordingly, an efficient, precise and reproducible method for quantitation of PV is necessary for the development of extraction and purification techniques.

Antibodies, being highly specific in nature, are persuasive candidates for PV protein identification (Jensenius et al., 1981). Antibodies are typically produced by immunization of animals, mostly mammals. However, mammalian antibodies are produced by invasive means in limited quantities and their purification is time-consuming as well as expensive. The uses of avian antibodies have increased spectacularly to replenish mammalian antibodies. A major lead of avian antibodies is ease of harvest from their eggs instead of serum. Also, an egg-laying hen antibody productivity is much higher than that of a similar sized mammal (Hau and Hendriksen 2005). Today, hens are recognized as an expedient and economical source of antibodies.

Therefore, in this study, we aim to develop an accurate ELISA based on monoclonal and chicken egg yolk IgY polyclonal antibodies for the quantitation of microgram to nanogram levels of PV. This quantification method developed, may be applied in studies on PV for its accurate quantification and further improved for detection of other challenging proteins.

## *2.2 Materials and Methods*

### *2.2.1 Materials*

Phosvitin (from chicken egg yolk), phosphatase, Freund's incomplete adjuvant, purified chicken IgG, rabbit anti-chicken IgG, rabbit anti-chicken IgG conjugated with horseradish peroxidase and streptavidin-HRPO were purchased from Sigma (St. Louis, MO, USA). Biotinaimdohexanoic acid-3-sulfo-N-hydroxysuccinimide ester (NHS-$PEO_4$-Biotin), Slide-A-Lyzer® and Tetramethylbenzidine (TMB) were purchased from Thermo Fisher Scientific (Burlington, ON, Canada) and KPL (Gaithersburg, MD, USA), respectively. Anti-PV (PPP-6) monoclonal antibody was obtained from Myungji University (Yongin, Korea). Bio-Rad protein assay kit, glycine, Precision Plus Protein Standard, sodium dodecyl sulfate (SDS), Mini-PROTEAN® TGX™ Any kD precast gels Precast Gels in Tris-Glycine-SDS buffer, anion exchange chromatography, Gel Doc™ EZ Imager and BioLogic LP Chromatography system were purchased from Bio-Rad Laboratory (Mississauga, ON, Canada). Sephacryl S-300 gel filtration column was purchased from GE Healthcare Bio-Sciences Corp (Piscataway, NJ, USA). The ELISA $V_{max}$ kinetic microplate reader was obtained from Molecular Devices Corp (Sunnyvale, CA, USA). All analytical grade chemicals and solvents were from commercial sources.

### *2.2.2 Production of Anti-PV Polyclonal IgY*

Production of polyclonal IgY directed against PV was followed by chicken immunization. The Sigma PV was dephosphorylated with acid phosphatase as described by Miller et al. (1982) (Miller et al., 1982). The dephosphorylated PV

was suspended in sterilized PBS at a concentration of 100 µg/mL and emulsified with an equal volume of Freund's incomplete adjuvant. Twelve 23-weeks-old Single Comb White Leghorn chickens were intramuscularly injected with the emulsified saline with or without PV at four different sites (0.25 mL per site) of breast muscles (two sites per left or right breast muscle). Two booster immunizations were given after 2 and 8 weeks of the initial immunization. Eggs were collected daily and stored at 4°C until the extraction of the antibodies.

### *2.2.3 Purification of Anti-PV IgY*

The egg yolk from immunized hens was physically separated from egg white by mixing gently with 8 volumes of cold distilled water (acidified with 0.1 M HCl to give a pH of 4.0) to avoid possible disruptions of egg yolk granules due to the presence of high concentrations of acid. Cold acidified distilled water (pH 2.0) was then added to make the final dilution of 1:10. After mixing well, the mixture was adjusted to a pH of 5.0 – 5.2 and incubated at 4°C for 12 h. Water soluble fraction (WSF) was obtained by centrifugation (3125 × g) at 4°C for 20 min. The supernatant was collected as the IgY rich WSF and titrated by indirect ELISA (described in the following section) using Sigma PV as coating antigen. The fractions found to have high titers, particularly the fractions collected during weeks 4 to 10 of the immunization period were further purified by ammonium sulfate (40%) followed by Sephacryl S-300 gel filtration column (Ishikawa et al., 2007). Control IgY antibody was extracted from eggs obtained from non-immunized chickens.

### *2.2.4 Titer of Anti-PV IgY by Indirect ELISA*

Microtiter plates were coated with 100 µL per well of PV in carbonate-bicarbonate buffer (0.05 M, pH 9.6) at a concentration of 8 µg/mL and incubated at 37°C for 1 h. Throughout the experiment, the plates were then washed four times with PBS containing 0.05% Tween 20 (PBS-Tween) between each step. After washing, 100 µL of 1% BSA solution (w/v) in PBS-Tween was added to each well, and the plates were incubated at 37°C for 30 min. The BSA solution was then discarded, and the wells were washed. Egg yolk WSF (diluted 1:1000 in PBS-Tween) containing specific IgY against PV or non-immunized egg yolk IgY antibody (as a control) was added to each well (100 µL per well), and the plates were further incubated at 37°C for 1 h. After washing the plates, 100 µL of rabbit anti-chicken IgG conjugated with horseradish peroxidase (HRPO, diluted 1:5000 in PBS-Tween) was added to each well and incubated at 37°C for 1 h. The plates were washed with PBS-Tween, followed by the addition of 100 µL of freshly prepared substrate solution, tetramethylbenzidine (TMB). $OD_{650}$ was taken after 30 min using an ELISA $V_{max}$ kinetic microplate reader. The optical density value of antibody activity was determined by subtracting the value of the control antibody from that of specific antibody.

### *2.2.5 Total IgY Concentration*

To quantify the total IgY concentration purified from egg yolks, ELISA was performed as mentioned above. A microtiter plate was coated with 100 µL per well of rabbit anti-chicken IgG at a final concentration of 2 µg. Samples of the PV-specific IgY were diluted 1:10,000 in PBS. Specific and non-specific IgY

were reconstituted and serially diluted in PBS. Two-fold serial dilutions of purified chicken IgG in PBS (0.5 to 0.031 μg/mL) were used as the reference antibody to prepare a standard curve. Total and specific IgY concentrations were determined using the standard curve.

### *2.2.6 Total Protein Assay*

The Bio-Rad protein assay, based on the method of Bradford, was performed using purified chicken IgY (1 mg protein/mL) as the reference protein. Serial dilutions of the purified IgY, and the reference protein in PBS (0.5 to 0.0625 mg/mL) were analyzed using the microtiter plate. $OD_{595}$ was taken after 5 min using an ELISA $V_{max}$ kinetic microplate reader.

### *2.2.7 Electrophoresis and Western Blot*

Sigma PV samples were run on Mini-PROTEAN® TGX™ Any kD Precast Gels in Tris-Glycine-SDS buffer and stained with a solution of 0.05% Coomassie Blue R-250, 0.1M aluminum nitrate, 25% isopropanol, 10% acetic acid, and 1% TritonX-100. Gels were destained with 7% acetic acid. Bio-rad Precision Plus Protein Standards were loaded as a molecular weight marker at a constant voltage (100 V). Images of gels were scanned in the Bio-Rad Gel Doc™ EZ Imager.

For Western blot assay, the gel was electrophoretically transferred to nitrocellulose membrane in Tris-borate electrophoresis buffer without sodium dodecyl sulfate at 40 V for 2 h. Nitrocellulose membrane was then soaked in 2% BSA in PBS (pH 7.2) for 1 h at room temperature. After washing with PBS (three times for 5 min each), the nitrocellulose membrane was incubated with anti-PV IgY antibodies (in PBS containing 1% BSA) for 1 h at room temperature. The

incubation was followed by washing in PBS and 1 h incubation with rabbit anti-chicken IgG conjugated with HRPO (diluted 1:3000 in PBS-Tween). Color was developed by incubation in 0.05% diaminobenzidine tetra-hydrochloride in PBS containing hydrogen peroxide (0.01% w/v) and cobalt chloride (0.033% w/v) for 5 min. Stained blots were then washed several times with water and dried.

### *2.2.8 Double Antibody Sandwich ELISA (DAS-ELISA)*

A microtiter plate was coated with 100 µL of anti-PV mAb (1 µg/mL) in 0.05M carbonate buffer, pH 9.6, at 4°C overnight. Nonspecific binding sites were blocked with 120 µL of 3% BSA for 45 min. The PV was serially diluted from 0.7 to 180 µg/mL in PBS, pH 7.0 (containing 0.05% Tween 20) and added to each well, and then the plate was incubated at 37°C for 1 h. The plate was washed, and 100 µL of the anti-PV IgY (1 µg/mL) was added to each well and incubated at 37°C for 1 h. After incubation, the plate was washed, and streptavidin-HRPO was added and incubated at 37°C for 45 min. TMB substrate was added and $OD_{650}$ was taken after 10 min using an ELISA $V_{max}$ kinetic microplate reader. The antibody titre was determined by subtracting the value of the control antibody from that of specific antibody. Throughout the experiment, four times washing was conducted by PBS-Tween between each step.

### *2.2.9 Biotinylated DAS-ELISA*

Anti-PV IgY polyclonal antibodies were biotinylated by using long arm NHS-$PEO_4$-Biotin. One mg of anti-PV IgY in PBS (pH 7.2) were added to 20 µL of long chain biotin (3 mg/mL) and then incubated at room temperature for 1 h. A 10 µL glycine (100 µg/µL) was added and the solution kept on a shaker for 10

min. The solution was then dialyzed in a Slide-A-Lyzer against PBS (pH 7.2) overnight at 4°C.

Except otherwise indicated, four times washing was conducted by PBS-Tween between each step. A microtiter plate was coated with 100 µL of anti-PV mAb (PPP-6, 1 µg/mL) in 0.05M carbonate buffer, pH 9.6, at 4ºC overnight. Nonspecific binding sites were blocked with 120 µL of 3% BSA for 45 min. The Sigma PV was serially diluted from 0.3 to 160 ng/mL in PBS, pH 7.0, containing 0.05% Tween 20 and added to each well then the plate was incubated at 37°C for 1 h. The plate was washed, and 100 µL of the biotinylated anti-PV IgY (1 µg/mL) was added to each well and incubated at 37°C for 1 h. After incubation, the plate was washed and streptavidin-HRPO was added and incubated at 37°C for 45 min. TMB substrate was added and $OD_{650}$ was taken after 10 min using an ELISA $V_{max}$ kinetic microplate reader. The antibody titre was determined by subtracting the value of the control antibody from that of specific antibody.

### *2.2.10 Validation of the Assay*

The accuracy of the two ELISA methods was evaluated by performing recovery studies. A saline solution was spiked with Sigma PV Standard at levels of 1 µg, 10 µg, 50 µg and 100 µg/mL sample in a total volume of 10 mL. For the determination of recovery rates, the extractions were done in triplicates and analyzed by the DAS-ELISA and biotinylated DAS-ELISA. Intra-assay (within plate) precision was determined as the mean coefficients of variation (CV) based on 10 replicates. Inter-assay (between plates) precision was also determined as the mean CV by triplicate analyses on 10 plates.

The limit of detection (LOD) was calculated from 3.3 times standard error of the predicted Y-value for each X in a regression divided by slope. Additionally, the LOD in three saline solutions was determined in the same way, on the basis of five experiments. Linearity and range of detection were determined from serially diluted Sigma PV standard curve plot.

### *2.2.11 Statistical analysis*

The student t-test (one-tailed t-test) was used to analyze the significant difference ($p < 0.05$) between the groups.

## *2.3 Results*

### *2.3.1 Production of Anti-PV IgY*

The Anti-PV IgY titer of hyperimmunized egg yolks against PV was determined by indirect ELISA method during the immunization period (Figure 2.1). A primary antibody response was determined by slight IgY titer increase after the first PV immunization. Following the first booster a rapid secondary response was expressed with high PV specific IgY activity at a peak of week 5 ($p < 0.05$), thereafter the titer decreased, but reached at a peak again after the second booster at week 8 ($p < 0.05$). As a result, a high antigen-binding specificity of anti-PV IgY could be obtained from the egg yolks collected from immunized eggs during weeks 4 to 10 of the immunization period.

The crude WSF of egg yolk was isolated, further purified by ammonium sulfate precipitation, followed by Sephacryl S-300 gel chromatography. The content of total IgY in the egg yolk was found to be constant (9.0 ± 1.1 mg/mL)

among the chickens immunized with PV during the experimental period, as previously reported (Sunwoo et al., 2011). The total content of IgY in the hyperimmunized egg yolks, was approximately 1.1 times greater than that of the yolks from non-immunized chickens. As expected, the PV-specific IgY was not detected in the non-immunized control group, while the proportion of PV-specific IgY in the total egg yolk IgY was 6.2% in the immunized egg yolks (Table 2.1).

The Coomassie Blue accompanied by aluminum nitrate stained gel electrophoretogram of PV is shown in Figure 2.2 Sigma PV showed high purity with a dominant 37 kDa band (Figure 2.2A, Lane 2). Sigma PV was stained with specific IgY antibodies (Figure 2.2B, Lane 2), to confirm the specificity of IgY antibodies to PV by Western blot analysis

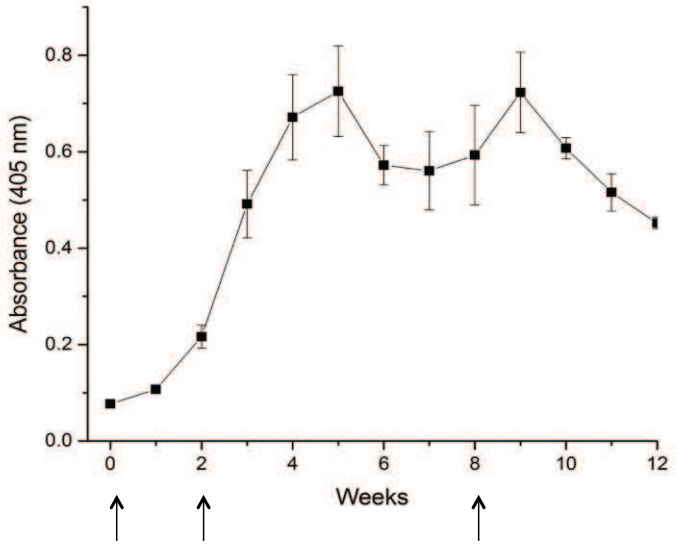



Figure 2.1: The anti-PV IgY antibody activities in the WSF of egg yolk (at 1:1000 dilution) measured by Indirect ELISA and expressed as absorbance at 405 nm. Values are the mean of quadruple samples. Vertical bars indicate the standard deviation. X axis indicates the weeks of immunization. Arrows indicate immunization time points.

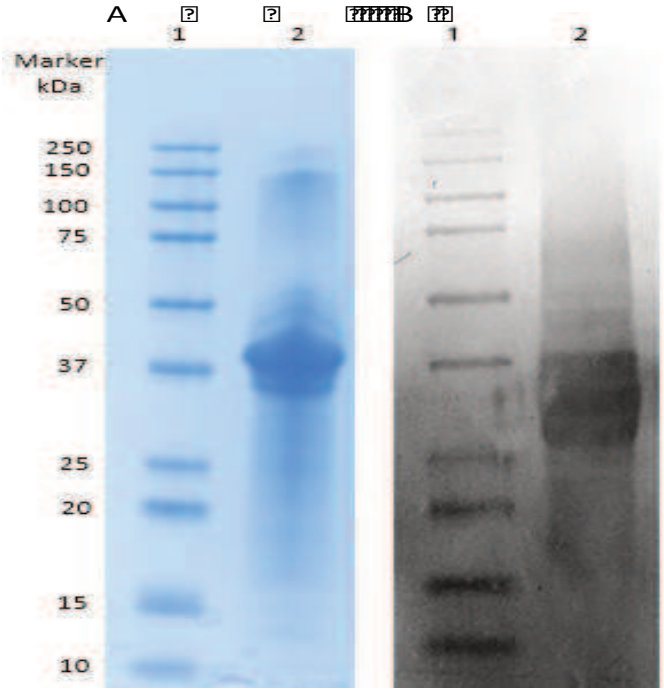



Figure 2.2: SDS-PAGE analysis of Sigma PV (A); Western blot of Sigma PV developed by anti-PV IgY as primary antibody (B). Lanes 1: standard molecular marker; Lane 2: Sigma PV.

Table 2.1 Concentration of total IgY and specific IgY purified from the hyperimmunized and non-immunized egg yolks. Values are the mean of quadruple samples ± SD.

| Egg yolks | Content (mg/mL) | |
|---|---|---|
| | Total IgY | Anti-PV Specific IgY |
| Hyperimmunized | 9.0 ± 1.1 | 0.56 ± 0.04 |
| Non-immunized | 8.2 ± 1.1 | N/A |

### *2.3.2 Double Antibody Sandwich ELISA*

Sigma PV was used to prepare a standard curve of DAS-ELISA using PV-specific mAb as capture and IgY as detection antibodies. The sensitivity and

lower LOD of PV was determined to be 5.6 µg/mL. Figure 2.3 illustrates the working linear range of PV detection determined from five-point calibration curve of 5.6 – 90 µg/mL. The working range of the assay was the linear part of the curve with a squared correlation coefficient ($R^2$ = 0.99). At lower or higher concentrations a reproducibility and repeatability of PV content was not validated.

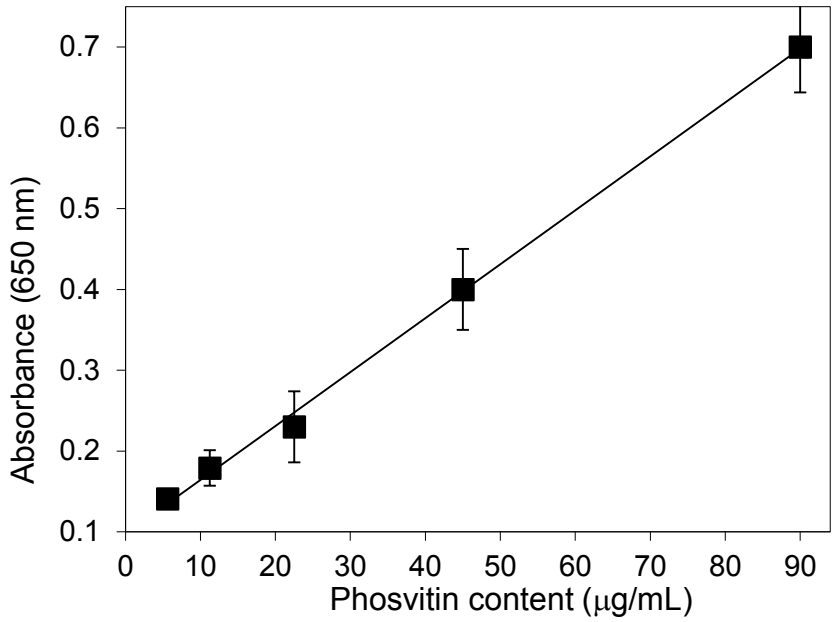



Figure 2.3: Standard curve for PV detection by double antibody sandwich ELISA with a working linear range of 5.6 – 90 µg/mL. (Y=0.0971+0.0067X)

### *2.3.3 Biotinylated Double Antibody Sandwich ELISA*

Sigma PV was used to prepare a standard curve of biotinylated DAS-ELISA using PV-specific mAb as capture and biotinylated IgY as detection antibodies. The sensitivity and LOD of PV was determined to be 2.5 ng/mL. Figure 2.4 illustrates the working linear range of PV detection determined from five-point calibration curve of 2.5 – 40 ng/mL. The working range of the assay was the

linear part of the curve with a squared correlation coefficient ($R^2$ = 0.99). At lower or higher concentrations a reproducibility and repeatability of PV content was not validated.

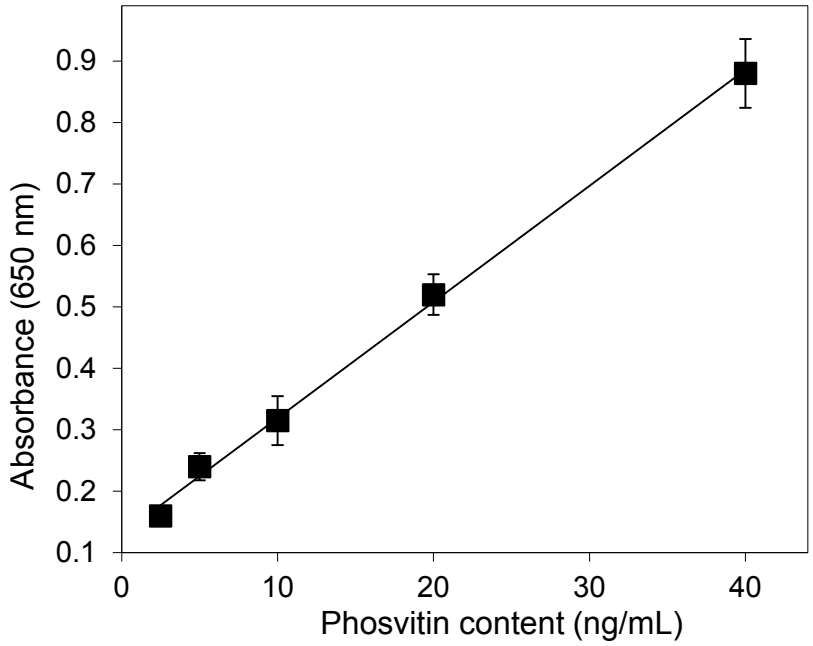



Figure 2.4: Standard curve for PV detection by biotinylated double antibody sandwich ELISA with a working linear range of 2.5 – 40 ng/mL. (Y=0.1302+0.0189X)

### *2.3.4 Accuracy*

Blank saline solutions, spiked with four different amounts of sigma PV, were used to determine the recovery rates as shown in Table 2.2. The recovery of the Sigma PV in the saline solutions determined by DAS-ELISA and biotinylated DAS-ELISA was between 80 – 92%, and 88 – 97%, respectively, independent of spiked PV level. The biotinylated DAS-ELISA being a more accurate assay was selected for the further validation study.

Table 2.2 Recovery (%) of PV from saline solution sample spiked with 1 – 100 µg/ mL Sigma PV

| ELISA Method | Recovery (%) of Sigma PV spiked in µg/mL | | | |
|---|---|---|---|---|
| | 1 | 10 | 50 | 100 |
| DAS-ELISA | 80 ± 17 | 90 ± 15 | 92 + 14 | 92 +11 |
| Biotinylated-ELISA | 88 ± 14 | 92 ± 12 | 94 + 10 | 97 ± 8 |

Values represent the average of three spiking experiments and are reported as mean ± SD.

### *2.3.5 Intra- and Inter-assay Precision*

Five saline solutions were spiked with the different contents of the sigma PV and analyzed by the developed biotinylated DAS-ELISA to determine the intra-assay and inter-assay precision (Table 2.3). The PV content in the saline solutions ranged from 0.05 to 68.9 µg/mL. The intra-assay expressed as the percentage of coefficients of variation (%CV) was 7.6% average of five saline solutions. The inter-assay precision was 9.8 % in the saline solutions.

Table 2.3 Intra- and inter-assay variances (%CV) of biotinylated DAS ELISA for PV quantification in 5 different saline solutions.

| Samples | PV (µg/ mL) | Intra-assay variance (% CV) | Inter-assay variance (% CV) |
|---|---|---|---|
| Saline solution 1 | 2.25 | 7 | 9 |
| Saline solution 2 | 4.5 | 8 | 11 |
| Saline solution 3 | 0.05 | 6 | 8 |
| Saline solution 4 | 18 | 8 | 10 |

| | | | |
|---|---|---|---|
| Saline solution 5 | 68.9 | 9 | 11 |

### *2.4 Discussion*

Egg yolk is a mixture of lipids, lipoproteins, PV, and water-soluble proteins (WSP). The WSP fraction, including α-, ß-, and γ-livetins, was separated from the lipids and granular proteins using the acidified water (pH 5.0-5.2) dilution method (S. K. Lee et al., 2002). The remaining granules includes yolk micelles that contain lipovitellin and PV (Ren et al., 2015). PV is one of the most highly-phosphorylated proteins in nature which contains more than 80% of the phosphorous present in the egg yolk (Joubert and Cook 1958). The high density of polar residues results in a substantial number of charged side chains in physiological pH which leads to different functionalities such as metal chelating, antioxidant, and antimicrobial activities (Li-Chan 2008).

There is limited information available on the quantification of PV. Since PV is a highly phosphorylated protein it cannot be accurately assessed by normal colorimetric assays such as Bradford, BCA and Lowry protein assays due to the interference of their phosphate groups (Hatta et al., 1997). Therefore, this study introduces, for the first time, a simple and reliable method to obtain protein-specific IgY antibodies for quantification of PV in combination with mAb antibodies.

Chicken was used to generate antibody employed in development of PV detection system. It has been reported that the avian maternal antibodies are transferred from serum to egg yolk to confer passive immunity to their offsprings

in the form of IgY antibody. This process of accumulating antibodies in the egg yolk by transfer of IgG from serum to egg yolk has been exploited, leading to increased use of chicken egg yolk for production of antibodies to various antigens (Gujral et al., 2012; E. N. Lee et al., 2002; Palaniyappan et al., 2012). The high amount of chicken IgY generated within a short period of time is an advantage to develop significant antigen-specific IgY compared to mammalian serum antibodies (Schade et al., 1994). Egg yolk IgY, therefore, can be simply produced in large quantities and high titers, which may replace other sources of polyclonal and monoclonal antibodies conventionally used in ELISA or other immunological assay without compromising accuracy of the assay. The use of chickens in antibody production is more ethical and would negate the need for blood collection from animals in the laboratory and considerably reduce research cost (Schade et al., 1994).

Chickens appear to be highly efficient producers of anti-PV IgY (Figure 2.1). It has been reported that percentage of specific antibody in total IgY ranged from 5 (anti-insulin antibody) to 28% (anti-mouse IgG antibody (Hatta et al., 1997). In this study, 6.2% of the PV-specific IgY in total IgY was obtained (Table 2.1). The Western blot result revealed that anti-PV IgY has distinctive antigenic epitopes specific to PV (Figure 2.2). Correspondingly, anti-PV IgY did not cross-react with other egg yolk proteins, determined by ELISA (data not shown).

Two ELISA systems (DAS-ELISA and biotinylaed DAS ELISA) were developed and validated via mAb and egg yolk IgY. The biotinylated ELISA ensured a detection range of 2.5 to 40 ng/mL (Figure 2.4) and is superior in terms

of accuracy of 88 – 97% (Table 2.2). This biotinylated ELISA system is also highly sensitive with over 2000 times lowered LOD as compared to the DAS-ELISA (Figure 2.3). Precision studies were also carried out to validate the arrogant assay, revealing remarkably low intra- and inter-assay variation coefficients (Table 2.3). This expounded detection system has the potential as a valuable research tool in monitoring PV, a promising protein for use in food, nutraceutical and pharmaceutical industries.

### *2.5 Conclusion*

The present study indicates that the IgY-based biotinylated DAS-ELISA developed provides a specific, sensitive, accurate and precise PV detection system for PV, with a working detection range of 2.5 – 40 ng/mL.

# Chapter 3 (Study 2): Phosvitin Hydrolysates with Iron Chelating Capacity Produced by High Hydrostatic Pressure Combined with Enzymatic Hydrolysis

## *3.1 Introduction*

Phosvitin (PV) is a phosphoprotein produced by proteolytic cleavage of chicken vitellogenin, a metal-binding protein in the chicken blood. It is utilized by the developing oocytes as a metal transporter (Richards 1997). This phosphorylated protein, present in the egg yolk, contains 10% phosphorus (Hatta et al., 1988), 56% serine, 15% basic and 10% acidic amino acid residues (Byrne et al., 1984; Taborsky 1963; Taborsky and Mok 1967; Xu et al., 2007). Due to the high proportion of phosphorylated serine residues, PV shows metal chelating (Choi et al., 2005), antioxidant (Xu et al., 2007; Katayama et al., 2006), anti-inflammatory (Xu et al., 2012), emulsifying (Chung and Ferrier 1992) and antimicrobial (Khan et al., 2000) properties. However, PV is considered nutritionally undesirable due to the formation of insoluble iron complex, hindering iron absorption in the digestive tract (Guerin-Dubiard and Anton 2007; Sato et al., 1987). Also, poor dissolution and stability of PV restrict its applications (Lee et al., 2014). PV hydrolysates (PVH) produced by enzymes (Goulas et al., 1996), had enhanced functional properties, although the DH of PVH was low due to the resistance of phosphoserine clusters to enzymatic digestion.

Hydrolysis under harsh condition of pH and temperature increases the degree of hydrolysis (DH) and generates higher generation of short peptides. However,

undesirable and uncontrolled chemical reactions cause side chain modification which suppresses peptide bioactivities (Tornvall 2010). An efficient hydrolysis method is required to produce PV–derived peptides without compromising their bioactivities. Some pre-treatments such as heating (Samaraweera 2014; Sakanaka and Tachibana 2006) and alkaline dephosphorylation (Ren et al., 2015; Huang et al., 2014) have been employed to improve the hydrolysis degree of PV. Several studies have also reported the use of extremely high-pressure, 400–800 MPa (Toldra et al., 2011; Garcia-Mora et al., 2015) as pre-treatment before enzyme hydrolysis (Zeece and Kelly 2008; Chicon et al., 2008; Dong et al., 2011; Hoppe et al., 2013), which resulted in unsatisfactory protein denaturation.

As an alternative PVH processing method, enzymatic hydrolysis under high hydrostatic pressure (HHP-EH) is introduced. HHP is used to augment enzyme efficiency and therefore increase the DH. In the HHP-EH method, moderately high hydrostatic pressure (90 MPa) is applied during hydrolysis to improve enzyme efficiency without denaturation of enzymes or protein substrates. High-pressure processing modifies protein structure by alteration of non-covalent molecular interactions leading to partially unfolded proteins with more susceptibility to enzymatic hydrolysis (Volk et al., 2012). Furthermore, iso-hydrostatic pressure (50-200 MPa) promotes enzyme activity due to higher rates of mass transfer and more effective enzyme-substrate contact (Ludikhuyze et al., 2003). Although enzyme stability and functionality are uninfluenced by pressures lower than 100 MPa, elevated pressures above normal atmospheric level may increase enzyme reaction rates (Weingand-Ziade et al., 2001). A possible

explanation for the acceleration of enzymatic activity at high-pressure could be the breakdown of cellular compartments and compression of solution which increases the concentration and movement of the solvated species (Schettino and Bini 2007).

The main objective of this study was therefore to compare the degree of hydrolysis of PVH obtained from various enzyme treatments under optimal conditions of HHP and atmospheric pressure (AP). The resulting hydrolysates can be used in protein-rich readily absorbed food supplement formulas without the need for further fractionation. Five different enzymes were selected for PV hydrolysis and determined for optimal enzyme:substrate (E:S) ratio, temperatures and incubation period. The PVH was then subjected to evaluation of structural characteristics (i.e., degree of hydrolysis and molecular weights ($M_w$) distribution pattern) and functional characteristics (iron-chelating and reducing capacity). The developed one-step methodology may be applied for other hydrolysis resistant proteins, in order to reduce the processing time, avoid the use of harsh chemical or solvents and conserve their biofunctions.

## *3.2 Materials and Methods*

### *3.2.1 Materials*

Eggs were purchased from the local market. Enzymes were obtained from commercial suppliers (Sigma-Aldrich, MP Biomedicals and Novozymes). Mini-PROTEAN Tris-Tricine Precast Gels and Precision Plus Dual Xtra Protein Standard (15-250 kDa and 2-250 kDa) were obtained from Bio-Rad (Mississauga, ON, Canada). The Greiss reagent, phosphoprotein phosphate assay and Lowry

assay kits were obtained from Sigma (St. Louis, MO, USA), Pierce (Rockford, IL, USA), and Biorad (Mississauga, ON, Canada), respectively. All other reagents were of analytical grade.

### *3.2.2 Apparatus*

A portable scale high-hydrostatic pressure processor (TFS-2L, Toyo-Koatsu Co. Ltd., Hiroshima, Japan) was used for HHP-EH treatment. Chromatographic analysis was performed on a Shimadzu 10 AVP HPLC system equipped with a Biosuite$^{TM}$ 125/5 mm HR-SEC column (7.8 × 300 mm, Waters Corp., Milford, MA, USA). A Mini-Protein III electrophoresis kit and Bio-Rad Gel Doc$^{TM}$ EZ Imager were purchased from BioRad (Mississauga, ON, Canada). The VersaMax ELISA microplate reader was obtained from Molecular Devices Corp (Sunnyvale, CA, USA). All mass spectra were obtained using a Bruker Ultraflex MALDI-ToF/ToF (Bruker Daltonic GmbH, Bremen, Germany).

### *3.2.3 IgY separation from egg yolk*

Eggs were manually broken; yolks were carefully separated from egg white and chalazae by rolling on a Whatman filter paper (No. 1). The yolk membrane was punctured with a needle and the content was collected in a beaker cooled in iced water. The temperature was maintained at 4°C throughout the process. Egg yolk was added to nine volumes of pre-chilled acidic water (final pH 5.0-5.2) and incubated at 4°C for 12 h without agitation. After centrifugation at 3200 × g for 30 min, the supernatant (containing IgY antibody) was separated from the precipitate. The precipitate, containing egg yolk granules, was used for PV isolation.

### *3.2.4 Phosvitin extraction and purification*

Phosvitin was isolated from intact egg yolk and from egg yolk residue after IgY isolation according to the method presented by McBee and Cotterill with slight modifications. The precipitate was diluted with two volumes of distilled water and stirred for 1 h. After centrifugation (3200 ×g, 30 min at 4°C), the pellet (containing egg yolk granules) was collected and dissolved in nine volumes of 10% (w/v) NaCl solution, then centrifuged at the same condition. The supernatant was filtered through Whatman No. 1 filter paper to remove floating particles. NaCl in the solution was removed by dialysis against water using 12 kDa $M_w$ cut-off dialysis membranes. The dialyzed sample was freeze-dried and then stored at -20°C until analysis. The protein content of phosvitin purified from egg yolk was determined using modified Biorad Lowry assay kit. The phosphate content in purified PV was measured by Phosphoprotein Phosphate assay kit.

### *3.2.5 Enzymatic hydrolysis*

Purified PV, dissolved at 10 mg protein/mL in 10% NaCl, was mixed with enzymes at enzyme/substrate ratio of 1:50. The enzymes used for PV hydrolysis include Elastase (Ela), Alcalase (Alc), Savinase (Sav), Trypsin (Try) and Thermolysin (Ther). The enzyme added samples were then subjected to either AP (0.1 MPa) or HHP (90 ± 2 MPa) treatments for 24 h at optimal pH, and temperatures of each enzyme (Table 3.1). The above procedures were also performed in the absence of enzymes to serve as experimental controls. After the treatment, enzymes were heat-inactivated (90°C, 10 min) and centrifuged (5000 ×g, 10 min) to remove the insoluble fractions. The supernatants were filtered

through a 0.22 μm filter, followed by all enzymes removal using Amicon ultracentrifuge filter unit (10 kDa Mw cut-off) and then stored frozen prior to further analysis.

Table 3.1: Enzymes applied in HHP-EH and atmospheric hydrolysis and operational conditions

| Enzyme Treatments | Sources | Unit | pH | °C |
|---|---|---|---|---|
| Alcalase (Alc) | Protease from Bacillus licheniformis | 2.4 U/g | 7 | 50 |
| Elastase (Ela) | Elastase from hog pancreas | 1 U/mg | 8 | 37 |
| Thermolysin (The) | Bacillus thermoproteolyticus | 14 U/mg | 7 | 50 |
| Trypsin (Try) | Protease derived from porcine pancreas | 30 U/g | 7 | 37 |
| Savinase (Sav) | Protease from Bacillus sp. | 0.32 U/g | 7 | 55 |

### *3.2.6 Degree of hydrolysis (DH)*

Hydrolysis degree was analyzed by measuring free primary amines using 2,4,6-trinitrobenzenesulfonic acid (TNBSA) method with L-leucine as standard (Adlernissen 1979). Total content of amine groups expressed as leucine equivalent (μg/mL), was obtained from a standard curve prepared from different concentrations of L-leucine. DH was calculated by the following equation:

$DH = (h/h_{tot}) \times 100$

Where, hydrolysis equivalents (h) is the amount of peptide bonds cleaved during hydrolysis, expressed as millimole leucine equivalents per gram protein (mmol/g protein); $h_{tot}$ is the total amount of peptide bonds in the protein substrate determined from the protein sample totally hydrolyzed with 6 N HCl at 110°C for 24 h.

### *3.2.7 Phosphate content determination*

The phosphate content of PV and PVH samples was determined using phosphoprotein phosphate assay kit. In this assay, phosphate groups are hydrolyzed from serine and threonine residues under alkaline condition and then, quantified through reaction with Malachite green and ammonium molybdate (Ekman and Jager 1993). The absorbance was measured at 650 nm by UV/Vis spectrophotometer.

### *3.2.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

Gel electrophoresis was performed according to the method of Schaegger in Mini-PROTEAN Tris-Tricine Precast Gels (Bio-Rad, Mississauga, ON, Canada) with 12% polyacrylamide gels. Precision Plus Dual Xtra Protein Standard (15-250 kDa and 2-250 kDa, Bio-Rad, Mississauga, ON, Canada) was used as molecular weight marker. The gels were stained with a solution of 0.05% Coomassie Blue R-250, 0.1M aluminium nitrate, 25% isopropanol, 10% acetic acid and 1% TritonX-100, destained with 7% acetic acid and imaged.

### *3.2.9 Size Exclusion High Pressure Liquid Chromatography (SE-HPLC)*

The average $M_w$ of the PVH samples was determined by SE-HPLC using a Shimadzu 10 AVP HPLC system equipped with a Biosuite$^{TM}$ 125/5 mm HR-SEC column (7.8×300 mm, Waters Corp., Mass., USA). Phosphate buffer (100 mM) containing 100 mM NaCl was used as mobile phase at a flow rate of 0.5 mL/min at 25 ºC and the protein was monitored at the UV wavelength of 220 nm. A calibration curve was made from the log $M_w$ of the standard markers and their respective elution times ($R^2 = 0.99$).

### *3.2.10 MALDI-TOF*

MALDI-TOF-MS analysis was performed at the Alberta Proteomics and Mass Spectrometry Facility (Faculty of Medicine and Dentistry, University of Alberta, AB, Canada). One µL of sample was mixed with 1 µL of sinapic acid (10 mg/mL in 50% acetonitrile/water + 0.1% trifluoroacetic acid). One µL of the sample/matrix solution was then spotted onto a stainless steel target plate and allowed to air dry. Ions were analyzed in positive mode and external calibration was performed by use of a standard protein mixture.

### *3.2.11 Iron chelating activity*

The chelation of ferrous ions by PVH was estimated according to the method of Dinis *et al.* with slight modifications. EDTA, a strong metal chelator, was used as a positive control. The ferrous ion chelating ability was calculated by the following equation:

% Iron chelating ability (%) = $(B_c - B_s)/ B_c \times 100$

Where, $B_s$ and $B_c$ represent the absorbances of the sample and the control (everything except the protein hydrolysate), respectively.

### *3.2.12 Ferric Reducing Antioxidant Power (FRAP) assay*

The antioxidant capacity of PVH samples was estimated according to the procedure described by Fogarasi, *et al*. Trolox (1.0-200 µM) were used as positive control. The results were corrected for dilution and expressed as Trolox equivalent (TE) (µM TE/mg sample).

### *3.2.14 Statistical analysis*

All experiments were performed at least in three independent trials and the results were reported as mean ± SD. Results were subjected to the analysis of variance using the SAS software (SAS Institute, Inc., Cary, NC) and statistical significance of differences ($p < 0.05$) was evaluated by the least significant difference procedure.

## *3.3 Results*

### *3.3.1 Isolation of phosvitin*

Figure 3.1 illustrates the electrophoretic pattern of PV staining with regular Coomassie Blue (Fig 3.1a) and Coomassie Blue accompanied by aluminum nitrate (Fig 3.1b). Sigma PV and purified PV showed no band with regular Coomassie Blue staining but strong bands were observed when aluminum nitrate was added. Purified PV isolated from egg yolk residue after IgY extraction showed a higher purity with a dominant 48 kDa and a minor 37 kDa bands (Fig 3.1b, Lane C) compared to PV isolated from whole egg yolk which had other proteins sized 30 kDa (major band) and 55 kDa (minor band) (Fig 3.1b, Lane B). The result indicates that pre-isolation of IgY from egg yolk removes unwanted protein during PV purification. The phosphate content of purified PV, measured by phosphoprotein phosphate assay kit, was 3.47 μmoles/mg protein.

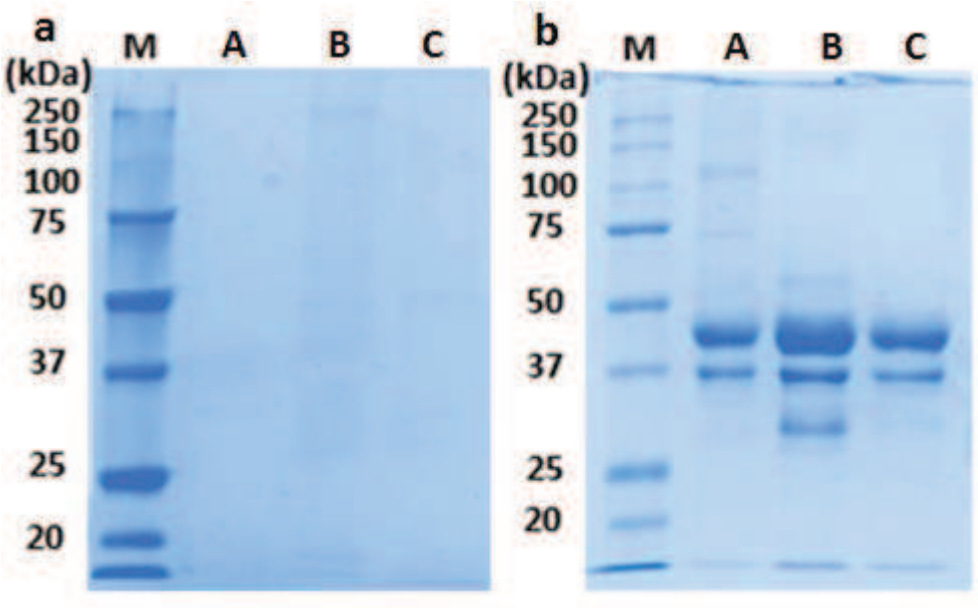



Figure 3.1: SDS-PAGE pattern of commercial and purified PV stained with regular Coomassie Blue (1a) and Coomassie Blue + Aluminum nitrate staining (1b). Lanes; M; molecular weight marker. A, Sigma PV; B, purified PV from whole egg yolk; C, purified PV from IgY pre-isolated egg yolk.

### *3.3.2 Enzymatic hydrolysis of purified PV*

Figure 3.2 illustrates the SDS-PAGE pattern of PVH obtained from various enzyme treatments at both atmospheric pressure (AP, 0.1 mPa) and HHP (90 ± 2 MPa), 1:50 (E:S), 24 h incubation time, optimal pH and temperature. All the enzyme treated PVH under both AP and HHP showed a wide $M_w$ distribution range (2-27 kDa), except Ther-treated PVH which showed smaller bands under AP (Fig 3.2a and b). The enzymatic hydrolysis of PV did not show noticeable differences between HHP and AP conditions. However, different peptide patterns were observed in PVH obtained from various enzymes. Since the electrophoretic bands of small peptides ($M_w$ < 10 kDa) were not clearly visible on SDS-PAGE, further analyses including the degree of hydrolysis, HPLC and MALDI-TOF were

examined.

The phosphate content of PVH samples showed that all PVH had 1.0 ±0.2 µmoles phosphate/mg protein. The concentration of intact PV and PVH was adjusted based on similar phosphate contents of 1.0 µmoles phosphate/mg protein.prior to further studies. Therefore, MALDI-TOF, metal chelating and reducing capacity results of all samples were compared based on the same level of phosphate content.

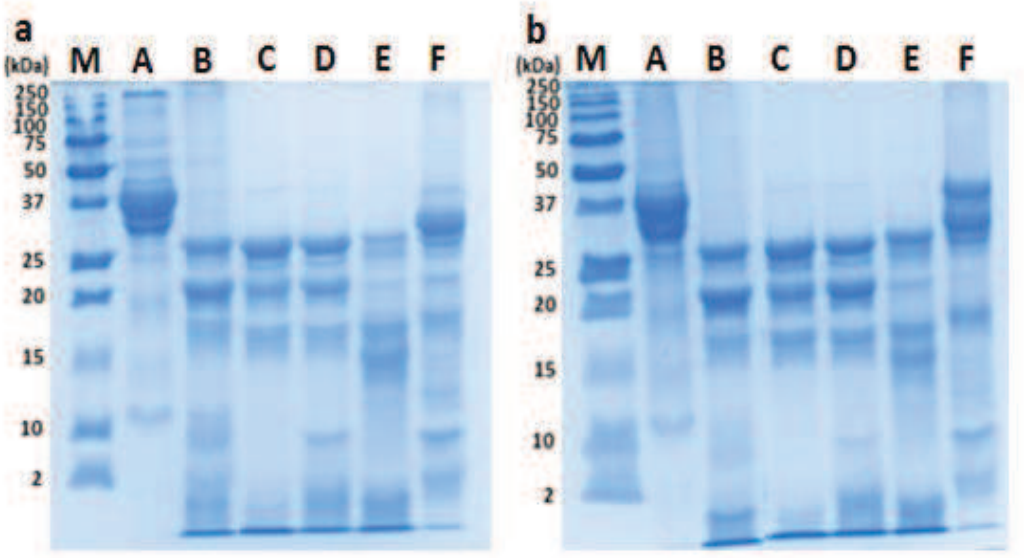



Figure 3.2: SDS-PAGE pattern of PVH produced by different enzymes under atmospheric pressure (0.1 MPa, 2a) and high hydrostatic pressure (90 MPa, 2b). Conditions of PV hydrolysis include E:S (1:50), 24 h incubation time, at optimal pH and temperature of enzymes. Lanes: M, molecular weight marker; A, purified PV; B, Ela; C, Alc; D, Sav; E, Try; F, Ther.

### *3.3.3 Degree of PV hydrolysis*

The DH of PV under HHP and AP was determined, using TNBSA method to measure the concentration of free amino groups. All the PVH samples showed

higher DH under HHP condition compared to AP (Fig 3.3). There is a significant increase in the DH of PVH treated by Ela, Alc and Sav under HHP compared to AP ($p < 0.05$). Among all enzymes, Alc treatment led to the highest DH of 27.5 and 31.3% under AP and HHP, respectively (Fig 3.3). Therefore, PVH-Alc samples were selected for further evaluation on $M_w$ distribution and biological activities compared to PVH-Try.

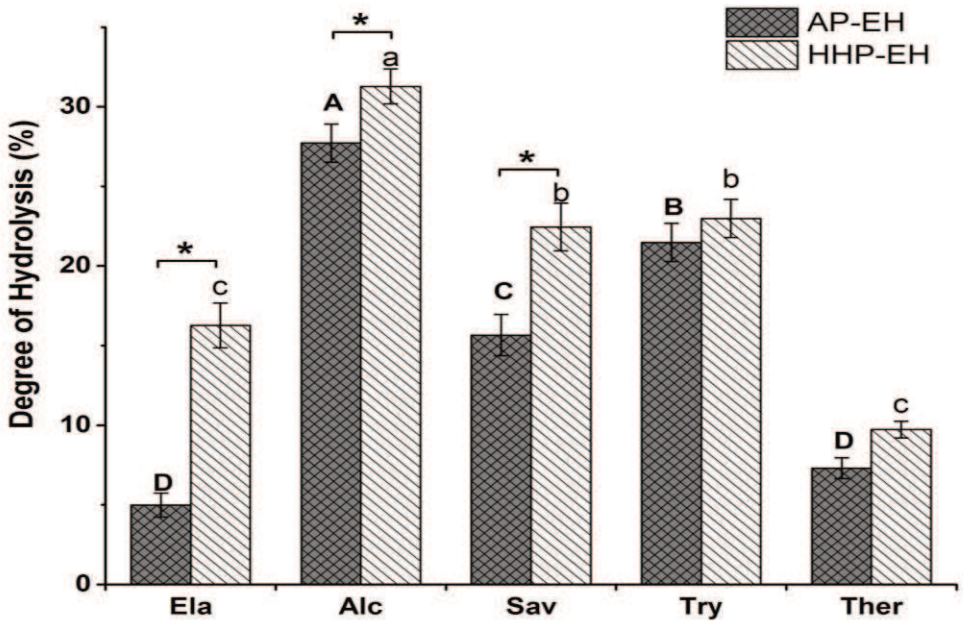



Figure 3.3: Degree of hydrolysis of PVH produced under high pressure and atmospheric condition. Values with different lowercase letters in similar hydrolysis condition differ significantly ($p < 0.05$). Statistically significant differences between HHP-EH and AP-EH for each enzyme are indicated by stars( ★ $p < 0.05$).

### *3.3.4 Size exclusion high performance chromatography (SE-HPLC)*

The SE-HPLC chromatograms of intact PV and HHP-PVH produced by Alc treatment are presented in Fig 3.4. Intact PV chromatogram was identified by four peaks eluted at 13.7, 16, 22 and 24 min time points. The two first peaks at 13.7 and 16 min correspond to the PV aggregates formed during extraction steps because they have significantly higher $M_w$ compared to PV subunits. In PVH-Alc chromatogram, the first aggregate peak was digested but the peak eluted at 16 and 22 min still remained. New peaks of PVH with $M_w$ range lower than 3 kDa were eluted after 25 min.

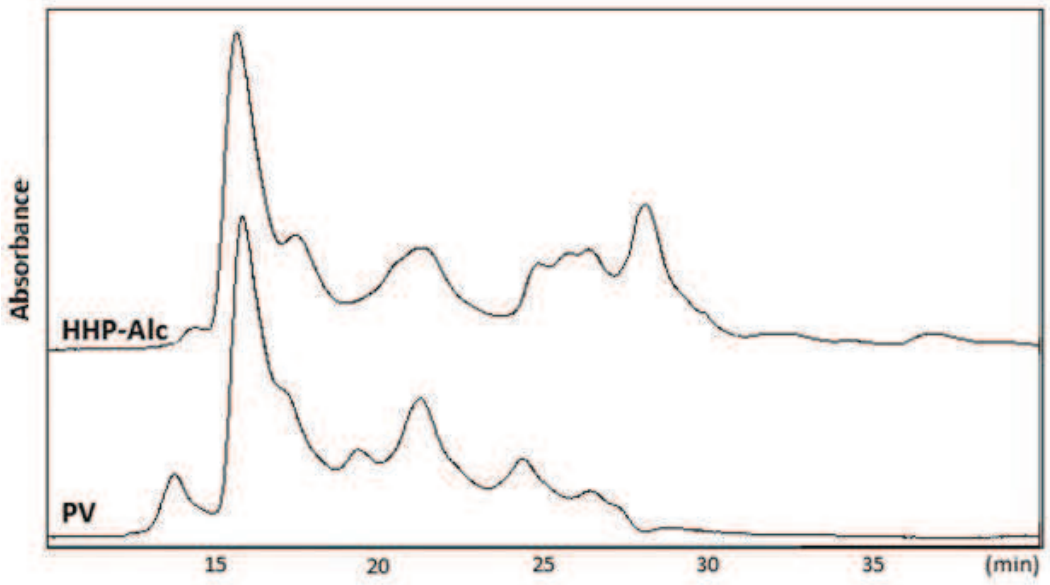



Figure 3.4: Size exclusion chromatograms of PV hydrolysates using Biosuite$^{TM}$ HR-SEC column (7.8 × 300 mm).

### *3.3.5 MALDI-TOF Analysis*

MALDI-TOF spectra of PVH-Alc revealed the $M_w$ of short peptides in a range of less than 3 kDa (Fig 3.5). Alc treated PVH under HHP generated a

greater number of very short peptides (500-2,500 Da) compared to AP (Fig 3.5a and b), indicating a higher hydrolysis efficiency of Alc under elevated pressure. The result indicates that HHP can promote the enzymatic activity to generate smaller fragments of PVH, containing more peptides with $M_W$ < 3 kDa.

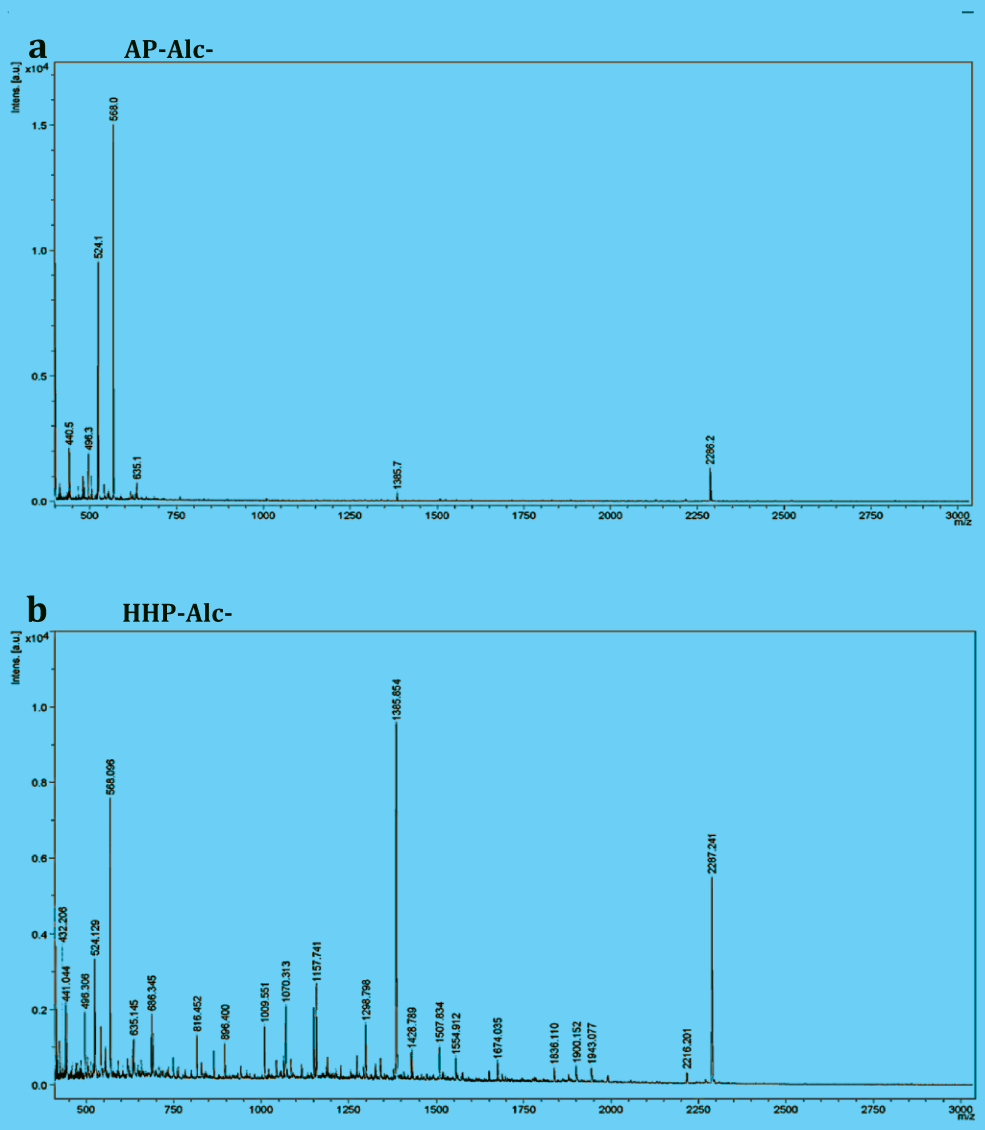



Figure 3.5: MALDI-TOF spectra of PV hydrolysates. PV hydrolyzed by: Alc under atmospheric (5a) and high pressure (5b).

### *3.3.6 Iron chelating activity*

Figure 3.6 illustrates that iron chelation capacity of PVH-Alc and PVH-

Try was significantly higher than intact PV (19%) at the same content of phosphate groups. The PVH treated by Alc and Try showed an iron chelation capacity ranged from 69.7 to 72.7% at 0.5 mg/mL (Fig 3.6), compared to positive control EDTA (73% at 10 μg/mL).

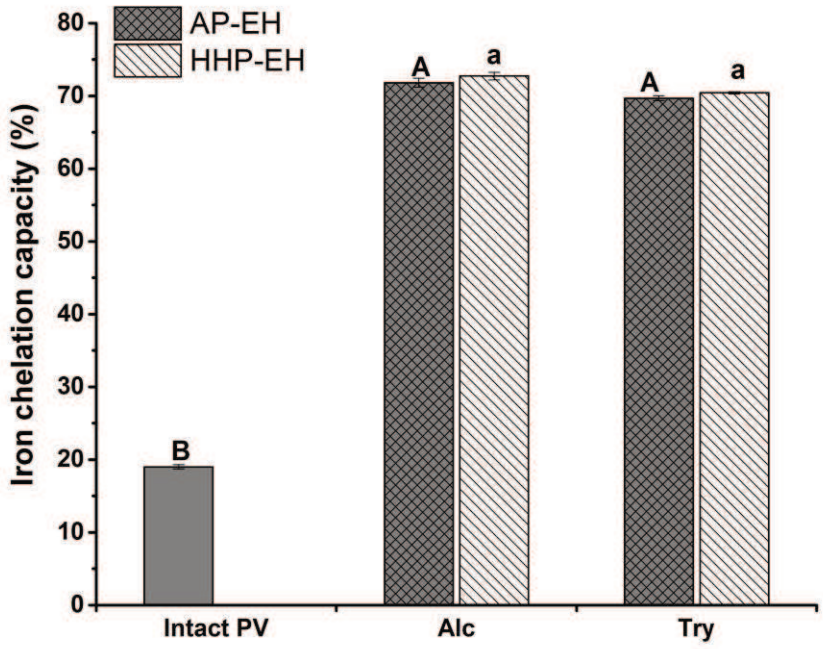



Figure 3.6: Iron chelation capacity of intact PV and PVH at the same phosphate content. Values with different lowercase letters in the same hydrolysis time differ significantly ($p < 0.05$).

### *3.3.7 FRAP assay*

Figure 3.7 illustrates a wide range of FRAP values (0.52-3.5 μM Trolox equivalent (TE) at 0.5 mg/mL) of intact PV and PVH samples. Intact PV showed very low FRAP value (0.52 μM TE/mg sample) while enzyme treated PVH exhibited FRAP value ranged from 1.3 to 3.5 μM TE/mg sample (Fig 3.7). A

significant difference was observed between FRAP values of HHP and AP treated PVH-Alc ($p < 0.05$).

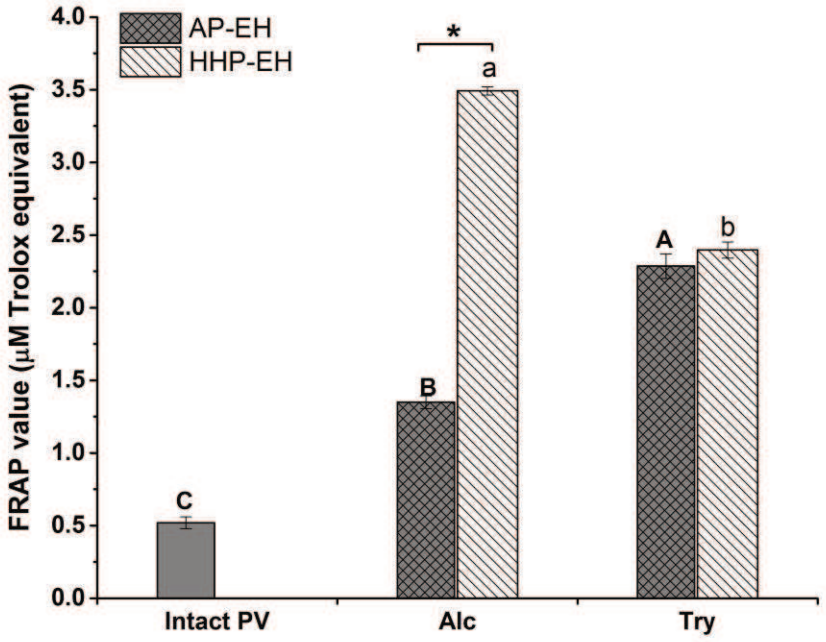



Figure 3.7: FRAP values of intact PV and PVH at the same phosphate content. Values with different lowercase letters in the same hydrolysis time differ significantly ($p < 0.05$). Statistically significant differences between HHP-EH and AP-EH for each enzyme are indicated by stars ( ★ $p < 0.05$).

### *3.4 Discussion*

Egg yolk represents an excellent source of functional proteins and peptides, which can be transformed into value-added agri-food, nutraceutical, and pharmaceutical products. Egg yolk consists of fluids called plasma and suspended particles called granules (Romanoff and Romanoff 1949). Egg yolk antibody (IgY) present in the plasma has drawn great attention of research mainly for the treatment and detection of pathogens. IgY antibody can be easily isolated from

egg yolk by water dilution method, leaving behind egg yolk granules containing lipovitellin and PV(Burley and Cook 1961; Mackenzie and Martin 1967). Our protein of interest PV can be further isolated from the granules by ethanol and NaCl extraction (Lee et al., 2014; Sunwoo et al., 2006).

Mecham and Olcotte reported, for the first time, that egg yolk PV displayed two bands in electrophoresis, called α- and β-phosvitin with $M_w$ of 36 and 40 kDa, respectively (Taborsky and Mok 1967; Clark 1970). Later, SDS-PAGE enabled better separation of PV into several bands with $M_w$ of 37-45 kDa (Abe et al., 1982). In this study, SDS-APGE was used to compare the purity of PV extracted from intact egg yolk and egg yolk residue after IgY isolation (Fig 3.1b). A higher phosphorylated PV purity of 90% was obtained from egg yolk granules after IgY extraction, showing two main electrophoretic bands at 37 and 48 kDa (Lane C), similar to that of Sigma PV (Lane A). PV extracted directly from egg yolk showed an additional major protein band corresponding to lipovitellin sized 30 kDa (Byrne and Gruber 1989) which stains as a phosphoprotein in SDS–PAGE experiments (Meininger et al., 1984). The increased purity of PV can be explained by removal of IgY antibody and other proteins from water soluble fraction during the extraction of IgY. Although other purification steps such as anion exchange chromatography are also available (Fitzgerald 1998), extraction of IgY prior to PV isolation is economical and therefore increases the value of egg components from a commercial perspective.

The PV extracted post-IgY extraction represented high phosphate content of 3.47 μmoles/mg protein which was very similar to the PV phosphate content

previously reported in literature (3.06 μmoles/mg PV) (Zhang et al., 2011). The SDS-PAGE of PV stained with regular Coomassie Blue solution showed no protein bands (Fig 3.1a), confirming the phosphorylated nature of PV. This is explained by repulsion forces between highly negatively charged phosphate of PV and sulfate groups of Coomassie Blue dye (Ahmed et al., 1975). Addition of aluminum nitrate ($Al^{3+}$) to the staining solution facilitates the connection between Coomassie Blue dye and PV phosphoserine residues (Hegenauer 1977) by chelating negative charges, rendering PV bands visible (Fig 3.1b).

The dense phosphopeptides side chains make PV resistant to enzymatic digestion (Hiramatsu et al., 2002), which is a major drawback in PVH production. A study showed that digestive enzymes (pepsin, trypsin, and α-chymotrypsin) produced relatively large peptide digests (Goulas et al., 1996). Czernick, *et al* (Czernick et al., 2013). also reported that only 25% digestion of PV was obtained by two-step 24 h trypsin hydrolysis. Subjection to high pressure of 100 and 200 MPa have been reported to increase 7- and 45-fold activity of α-chymotrypsin and thermolysin, respectively (Kunugi et al., 1997; Mozhaev et al., 1996) compared AP. Similarly, α- and β-amylases from barley malt showed enhanced catalytic activities (25% and 16%, respectively) at 100 MPa compared to their activities at AP (Buckow et al., 2007). In addition to enhanced enzymatic activity, another key advantage of HHP is destruction of microorganisms in the reaction chamber which results in lowered risk of microbial growth during the long hydrolysis period and improved final product shelf-life.

Therefore, in the current study, PV was hydrolyzed using HHP-EH

method with 5 different commercial protease enzymes at their optimal conditions. SDS-PAGE results revealed that enzymes under HHP effectively digested the main PV bands (Fig 3.2b) as compared to AP (Fig 3.2a). The effect of hydrolysis was more optimal when a lower amount of enzyme was used at an E:S ratio of 1:50 under HHP. The higher efficiency of PV hydrolysis from HHP-EH method was confirmed by the DH values (Fig 3.3). The DH of Ela-, Alc-, and Sav treated PVH appeared significantly higher in HHP than AP (3.3, 1.13, and 1.5-fold increased DH value, respectively). Among the 5 enzyme treatments, PVH-Alc showed the highest DH (27.5 and 31.3% under HHP and AP, respectively). In most published studies PV hydrolysis was performed using Try with very low digestion degree reported (Czernick et al., 2013; Ren et al., 2015). Therefore, in this study, PVH-Alc was selected for further characterization in comparison to PVH-Try. SE-HPLC chromatograms of PVH also confirmed extensive hydrolysis of PV (Fig 3.4) under HHP. As presented in MALDI-TOF spectra (Fig 3.5), HHP resulted in PVH-Alc with higher content of short peptides (< 1000 Da).

Although some studies have reported biofunctional properties of PV phosphopeptides, but there are some inconsistencies in metal ion chelation results since the level of phosphorylation were not mentioned. To avoid such inconsistencies, in this study PV and PVH concentrations were adjusted based on similar phosphate contents of 1.0±0.2 μmoles phosphate/mg protein prior to iron chelation and FRAP studies. Figure 3.6 demonstrated higher iron chelation capacity of both PVH-Alc and PVH-Try as compared to intact PV. The iron chelation capacity of both PVH-Alc and PVH-Try were not influenced by HHP

treatment. Considering that iron chelation capacity of PVH was evaluated at similar phosphate contents, it can be concluded that phosphate groups are the major functional groups involved in metal chelation capacity of peptides. In contrast to our result, Xu *et al*. reported lower iron chelation capacity of PVH by trypsin than intact PV, since no adjustment of phosphate content was performed. Electrostatic interaction of charged groups on peptide chain with the metal ions is considered the main mechanism of peptide-metal ion chelation while physical entrapment of metal ions was also reported for larger peptides (Bamdad et al., 2015; Zhang et al., 2009). Although similar iron chelation capacity was observed under HHP and AP, PVH samples exhibited higher iron chelation capacity than casein (Dong et al., 2011), milk-derived peptides (De Gobba et al., 2014) and corn zein hydrolysates (Kong and Xiong 2006).

To determine the reducing power of PVH, we used FRAP assay which measures the ability of PVH samples from different enzyme treatments in reducing $Fe^{3+}$ to $Fe^{2+}$. In this assay, PVH-Alc displayed significantly higher FRAP values under HHP compared to AP (Fig 3.7). Reducing capacity of peptide is achieved by phosphate groups and donor side chains of some amino acids such as tyrosine and tryptophan (Pihlanto 2006). Alcalase is an endopeptidase that cleaves the peptide bonds of non-terminal amino acids. Therefore, it produces mainly small and medium-size peptides (Adlernissen 1979). It has a broad spectrum substrate specificity in peptide bond cleavage, preferring bonds that involve aromatic amino acids such as Phe, Try and Tyr (Doucet et al., 2003). Therefore, the increase in reducing capacity of PVH-Alc can be explained by

more exposure of polar and charged residues. Amino acid sequence of PV is rich in serine (55%) and charged residues (24%). Phosphorylation of serine residues also provides an additional source of electrons to have a high redox potential.

### *3.5 Conclusion*

The present study provides an important insight that the bioactive properties of PVH is closely related to the enzyme used, due to the effect of enzymes on amino acid profile and digestibility nature of peptide hydrolysates. To overcome the challenge of PV hydrolysis, in this study Alcalase enzyme was used under HHP to prepare bioactive PVH. The HHP-EH method produced PVHs containing shorter bioactive peptides with strong iron chelation and reducing capacities. PVH-Alc can used as natural mineral chelating, antioxidant and anti-inflammatory agents in food, nutraceutical and pharmaceutical products. Further investigation is required to identify the amino acid sequence of effective peptides and confirm the correlation between metal chelation and antimicrobial capacity of these peptides.

## Chapter 4 (Study 3): Growth Inhibitory Effects of Combination of IgY and Phosvitin to Enterotoxigenic *Escherichia coli* K88 and K99 *in vitro*

### *4.1 Introduction*

Enterotoxigenic *Escherichia coli (*ETEC) is the main cause of fatal diarrhea in piglets (Moxley et al., 1998), during the first week of life and over the time of weaning (Butler 1994; Vaandrager et al., 1992). The strains of ETEC associated with porcine intestinal colonization are those that express the K88, K99, P987 and F41 fimbrial adhesions (Garabal et al., 1997). Pathogenesis of ETEC-causing diarrhea involves intestinal colonization mediated by fimbriae and hypersecretion of water and electrolytes caused by heat-stable or heat-labile enterotoxins, or both (Holmgren 1985; Saeed et al., 1983; Parry S H 1984). On account of the incidence, severity and large economic loss, ETEC has been the subject of intensive study.

While feeds primarily serve as sources of nutrients for animals, they can also be important vehicles for the transmission of infectious diseases to and from animals. Protective strategies for infections are not simple, since they include hygiene improvement and effective ETEC vaccines development. Although antibiotics have been routinely used in an animal feed to promote growth and prevent diseases in pig farms (de Lange 2006) the emerging problem of multiple antimicrobial resistance affect the efficacy of drugs used. Moreover, due to public concerns about healthy foods and pressure to discontinue use of antibiotics, alternatives therapies to achieve similar goals are in dire need.

Antibodies are ideal candidates to replace the use of antibiotics in the animal feed industries. Chickens have been known as a potent and economic egg yolk antibody producers, termed as immunoglobulin Y (IgY) (Leslie and Clem

1969), in substitute of mammalian antibodies. The IgY accumulated in chicken egg yolk can be more largely produced and more simply purified in comparison to the mammalian IgG. In addition to these advantages, IgY is relatively stable under various conditions, including heat, pressure, acid, alkali, and proteolytic enzymes (Otani 1991). These characteristics of IgY have made it possible to achieve a broad range of applications such as passive immunization and diagnosis (Sim 2000).

Both experimental and clinical studies indicate that orally administered IgY has strong immune modulating properties including anti-toxin (Lee et al., 2015), anti-microbial (Wang et al., 2014; Sui et al., 2011) and anti-allergen effects (Gujral et al., 2015). In particular, IgY has been found to be imperative to the health and function of the gastrointestinal tract, and greatly reduces systemic and intestinal inflammation (Vega et al., 2011; Sarker et al., 2007).

Another glycoprotein egg yolk component, phosvitin (PV, 36-40 kDa), is produced by proteolytic cleavage of chicken vitellogenin, and functions as a metal-binding protein in the egg yolk (Richards, 1997). Due to the high proportion of phosphorylated serine residues, PV shows iron uptake regulation (Feng et al., 2006), metal chelation (Choi et al., 2005), antioxidation (Katayama et al., 2006; Xu et al., 2007), emulsification (Chung et al., 1992) and antimicrobial (Khan et al., 2000) properties.

Although, both IgY and PV in egg yolk possess anti-microbial activity, their dual use to prevent feed contamination have not been explored. A combination of IgY specific for ETEC and metal-chelating PV may have a

synergistic effect in reducing the risk of feed contamination with ETEC by inhibiting bacterial proliferation and stipulating protection against ETEC infection. To support IgY and PV application in the feed industry, this study is aimed to produce and investigating their effect on ETEC *in vitro* growth inhibition.

### *4.2. Materials and Methods*

#### *4.2.1 Materials*

Eggs were purchased from a local market. A local strain of ETEC K88 and K99 (Em 88-1604) were obtained from Center for Nutraceutical and Pharmaceutical Materials, Myongji University, Yongin, Korea. Phosvitin, Freund's incomplete adjuvant, purified chicken IgG, rabbit anti-chicken IgG and rabbit anti-chicken IgG conjugated with horseradish peroxidase (HP), Alcalase, and Savinase (Sigma-Aldrich, Oakville, ON, Canada), Elastase and Thermolysin (MP Biomedicals, Santa Ana, Ca, USA) and Trypsin (Gibco, Burlington, ON, Canada) were obtained from commercial sources. Sephadex G-200 column (2.5 x 119 cm) was purchased from GE Healthcare (Piscataway, NJ, USA). Microtiter 96-wells plates were purchased from Costar Inc. (Corning, NY, USA). 2-2'-azino-bis (3-ethylbenzthiazoline-6- sulphonic acid) (ABTS) substrate was purchased from KPL (Frederick, MD, USA). Pierce™ modified Lowry protein assay and Pierce™ phosphoprotein phosphate estimation assay kits were obtained Thermo scientific (Ottawa, ON, Canada). Precision Plus Dual Xtra Protein Standard, Bradford protein assay and electrophoresis reagents were obtained from BioRad (Missisauga, ON, Canada). Membrane filter 0.22 μm-pore-size was obtained from

Millipore (Bedford, MA, USA). Columbia agar was from Oxoid (Basingstoke, Hampshire, England) and TSB agar plates from Difco (Detroit, MI, USA). All other reagents were of analytical grade.

### *4.2.2 Apparatus*

A portable scale high-hydrostatic pressure processor (TFS-2L, Toyo-Koatsu Co. Ltd., Hiroshima, Japan) was used to apply HHP during enzyme treatment. The BioLogic DuoFlow chromatography system and Mini-Protein III electrophoresis kit (BioRad, Mississauga, ON, Canada) was used for gel chromatography and electrophoretic techniques, respectively. Bio-Rad Gel Doc™ EZ Imager (BioRad, Mississauga, ON, Canada) was used to image gels. The VersaMax ELISA microplate reader (Molecular Devices Corp, Sunnyvale, CA, USA) was used to obtain sample UV/Vis optical reading. Beckman Coulter centrifuge (Allegra X-30R, Brea, CA, USA) was used in all centrifugation steps. The Innova 42 incubator (New Brunswick Scientific, Edison, NY, USA) was used for culture plate incubation. The Nikon Eclipse TS 100 (Nikon Canada Inc., Mississauga, ON, Canada) was used to perform microscopic procedures.

### *4.2.3 Anti-ETEC IgY Antibody Preparation*

#### *4.2.3.1 Bacteria and Culture Conditions*

Strains of ETEC K88 and K99 bacteria were cultured in E-media broth (0.1 mM $MgSO_4 7H_2O$, 0.01 M Citric acid, 0.06 M $K_2HPO_4$, 0.02 M $NaNH_4HPO_4H_2O$, 0.002% chloroform (v/v), 0.5% dextrose (v/v)) at 37°C for 48 hr with shaking. After incubation, cells were harvested by centrifugation at 5,000 ×*g* for 15 min and were treated with 3.7% formalin overnight. The inactivated

cells were washed three times, suspended in sterile saline and then freeze-dried. Lyophilized whole cell cultures were stored at -20°C until used.

#### *4.2.3.2 Immunization of Chickens*

All chickens were cared for in accordance with the Canadian Council on Animal Care guidelines of animal welfare. Immunization of hens was carried out as described by Sunwoo et al., (1996). Lyophilized ETEC K88 and K99 whole cells were suspended in sterilized phosphate buffered saline (PBS, 500 μg of cell/mL; 15.6 μg of protein/mL) and emulsified with an equal volume of Freund's incomplete adjuvant. Single Comb White Leghorn (SCWL) 23-week-old chickens were divided into 3 groups (n=8) and immunized intramuscularly at four different sites (0.25 mL per site) of breast muscles with ETEC K88 and K99 or without cell emulsion as a control. Two booster immunizations of each antigen were given at week 2 and 4 after the initial immunization in the same manner. Eggs were collected daily and stored at 4°C until used.

#### *4.2.3.3 IgY Separation from Egg Yolk*

The water soluble fractions (WSF) containing anti-ETEC K88, anti-ETEC K99 and control egg yolk antibody (IgY) were prepared from egg yolk using the water dilution method (Gujral et al., 2015). Eggs were manually broken, and yolks were carefully separated from egg white and chalazae by rolling on Whatman filter paper (No. 1). The yolk membrane was punctured with a needle and the content was collected in a beaker cooled in iced water. Temperature was maintained at 4°C all through the process. Egg yolks were added to nine volumes of pre-chilled acidic water a (final pH of 5.0-5.2) and incubated at 4°C for 12 hr

without agitation. After centrifugation at 3200 × *g* for 30 min, supernatant (containing IgY antibody) was separated from the precipitate. The WSF was freeze-dried and analyzed for protein content, total IgY, and specific IgY content. The precipitate, containing egg yolk granules, was also used for the isolation of PV.

### *4.2.4 Protein Assay*

The Bio-Rad protein assays based on the method of Bradford was performed using the purified chicken IgG (1 mg/mL of protein) as a reference protein. The powder of WSF (diluted 1:100 in phosphate buffered saline, PBS) and two-fold serial dilutions of the reference protein in PBS (0.5 to 0.0625 mg/mL) were analyzed on the microtiter plate. Absorbance at 595 nm after 5 min reaction was measured using a kinetic microplate reader. The protein concentrations of reconstituted ETEC-K88, -K99 specific and non-specific IgY were measured by using the same procedures.

### *4.2.5 Specific IgY Concentration*

The concentration of ETEC K88 and K99-specific IgY in the WSF powder was measured by using the ELISA method as described by Gujral et al., (2012). Unless indicated otherwise, all incubations were performed at 37°C with four times washing by PBS-Tween 20 (PBST) in each step. Wells of a microtiter plate were coated with 150 μL of rabbit anti-chicken IgG and lyophilized whole cells (ETEC K88 or K99) at concentrations of 3.75 μg/mL and 0.5 mg/mL in carbonate-bicarbonate buffer (0.05 M, pH 9.6), respectively, for 90 min. Non-specific binding sites were blocked with 200 μl 1% Bovine serum albumin (BSA)

in PBS and incubated for 45 min. After washing, three dilutions of reconstituted specific (25, 12.5 and 6.25 μg/mL) and non-specific (4.5, 2.25, and 1.13 mg/mL) IgY powder in PBS were added to wells (150 μl/well) coated with ETEC K88 whole cells. Wells coated with rabbit anti-chicken IgG were filled with two-fold serial dilutions of purified chicken IgG (1 mg/mL) in PBS (0.5 to 0.008 μg/mL). The plates were subsequently added with rabbit anti-chicken IgG conjugated with horseradish peroxidase (diluted 1:1,000 in PBS) and incubated for 90 min, followed by addition of 150 μl of freshly prepared substrate solution, ABTS in 0.05 M phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide. The reaction was continued for 30 min. Absorbance of mixture was read at 405 nm using a kinetic microplate reader. The ELISA value of antibody activity was determined by subtracting the value of control antibody from that of specific antibody. Specific IgY concentration was calculated using the quantitative standard curve determined by the titration between rabbit anti-chicken IgG and purified chicken IgG.

### *4.2.6 Total IgY Concentration*

The ELISA was performed as described above, except the microtiter plate was coated with 150 μl of rabbit anti-chicken IgG at a final concentration of 3.75 μg/mL. The WSF samples were diluted 1:90,000 with PBS. Specific or non-specific IgY powder was reconstituted and serially diluted with PBS (2 to 0.125 μg/mL). Two-fold serial dilutions of purified chicken IgG (1 mg/mL) in PBS (0.5 to 0.031 μg/mL) were used as the reference antibody to prepare the standard curves.

### *4.2.7 Phosvitin Hydrolysates Preparation*

#### *4.2.7.1 Phosvitin Extraction and Purification*

The PV was isolated from egg yolk granule after the IgY extraction according to the method presented by Lee et al., 2014, with slight modifications. The precipitate was diluted in two volumes of distilled water and stirred for 1 hr. After centrifugation at 3,200 × *g* for 30 min at 4°C, the precipitate was collected and dissolved in four volumes of 85% ethanol to remove lipids. The fat-free precipitate was collected and dissolved in nine volumes of 10% (w/v) NaCl solution at pH 4, then centrifuged at the same condition. The supernatant was filtered through Whatman No.1 filter paper to remove floating particles, and desalted by using a membrane filtration of 12 kDa molecular weight cut-off membrane.

#### *4.2.7.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

SDS-PAGE was carried out on Tris-glycine acrylamide gels with Tris-glycine buffer system and 12% polyacrylamide gels. Ten µL of PV extracted directly from egg yolk and PV extracted post-IgY extraction samples (10 mg protein/mL) and 10 µL of Precision Plus Dual Xtra Protein Standard, as a molecular weight marker, were loaded and electrophoresed on gels at 100 V. The gels were stained with a solution of 0.05% Coomassie Blue R-250, 0.1M aluminum nitrate, 25% isopropanol, 10% acetic acid and 1% TritonX-100 and destained with 7% acetic acid (Hegenauer et al., 1977). Images of gels were obtained using Bio-Rad Gel Doc$^{TM}$ EZ Imager. The SDS-PAGE was used to

estimate the purity of both PV isolation.

#### *4.2.7.3 Phosvitin Enzymatic Hydrolysis*

Purified PV, dissolved at 10 mg protein/mL in 10% NaCl, was mixed with enzymes Alcalase (Alc), Elastase (Ela), Thermolysin (Thr), Trypsin (Try), or Savinase (Sav) at E:S ratio of 1:50 for 12 hr under atmospheric pressure (AP) or high hydrostatic pressure (HHP). The treatments of enzymes were performed under their optimal conditions: Alc (2.4 U/g; pH 7, 50°C), Ela (1 U/mg, pH 8, 37°C), Thr (14 U/μg; pH 7, 50°C), Try (30 U/g, pH 7, 37°C), and Sav (0.32 U/g; pH 7, 55°C). After the treatment, enzymes were inactivated by heating (90°C, 10 min) and centrifuged at 5000 × *g* for 10 min to remove the insoluble fractions. The supernatants were filtered through a 0.22 μm filter and stored frozen before analysis.

### *4.2.8 Degree of Hydrolysis (DH)*

The DH of PV and phosvitin hydrolysates (PVH) were subjected to enzymes mentioned above, and were analyzed by measuring free primary amines using 2,4,6-trinitrobenzenesulfonic acid (TNBSA) method with L-leucine as a standard (Adlernissen 1979). Briefly, samples of purified PV and PVH were mixed with phosphate buffer (pH 8.5) and 0.01% TNBS solution, incubated at 37°C for 2 hr. After incubation, reaction was terminated by adding 1 N HCl and 10% SDS. Amine content of samples was measured by reading absorbance at 335 nm. Total content of amine groups, expressed as leucine equivalent (μg/mL), was obtained from a standard curve prepared from different concentrations of L-leucine. DH was calculated by the following equation:

$DH = (h/h_{tot}) \times 100$

Where, h (hydrolysis equivalents) is the amount of peptide bonds cleaved during hydrolysis, expressed as millimole leucine equivalents per gram protein (mmol/g protein); $h_{tot}$ is the total amount of peptide bonds in the protein substrate determined from the protein sample totally hydrolyzed with 6N HCl at 110°C for 24 hr.

### *4.2.9 Lowry Protein Assay*

PV samples (40 µL) were mixed with 200 µL of a Modified Lowry Reagent (Bio-rad) and 20 µL of Folin-Ciocalteu Reagent in 96-well microplates. The assay mixture was conducted in a dark room and incubated at room temperature for 30 min followed by absorbance measurement at 750 nm using a kinetic microplate reader. Standard curve was prepared using Sigma PV as a reference protein.

### *4.2.10 Growth Inhibition of PV and IgY*

#### *4.2.10.1 Preparation of Bacteria*

The same strains of ETEC K88 and K99 used as antigens for immunizing chickens were separately subcultured on blood agar plates (5% defibrinated sheep blood in Columbia agar) at 37°C overnight and then suspended in E-media broth. The suspensions were adjusted to an optical density of 0.05 at 600 nm, corresponding to a cell density of about $2.7 \times 10^7$ CFU/mL. The same volume of 20% glycerol in E-media broth were added and stored at -70°C until used.

Two milliliters of prepared bacteria culture were mixed with 2 mL of E-media broth and incubated at 37°C with shaking. The turbidity of culture (OD at

600 nm) was measured by a spectrophotometer at 1 hr intervals. The growth curve was plotted until reaching a stationary phase.

#### *4.2.10.2 ETEC K88 K99 Growth Inhibition*

The ETEC specific IgY powder was reconstituted to 100, 200, and 400 μg/mL with E-media broth. Purified PV and PVH were diluted in E-media broth at concentrations of 0.5, 1 and 2 mg protein/mL. Non-specific IgY powder reconstituted to 0.5 mg/mL was used as a control. The mixtures were centrifuged at 1,500 × *g* and 4°C for 20 min, and supernatants collected were sterilized using a 0.22 μm-pore-size membrane filter. Two milliliter of specific IgY, non-specific IgY, PV or PVH was added to the same volume of prepared ETEC K88 and K99 cultures. The mixture of bacteria and treatment were incubated at 37°C with a continuous gentle shaking. Aliquots of samples (100 μL) were taken at 0, 2, 4, and 8 hr of incubation time. Plate counts were performed by the spread plate method on TSB agar plates in duplicate. The inoculated plates were incubated at 37°C overnight. The number of colony-forming units (CFU) per plate was counted to determine the total number of bacteria CFU per mL of sample.

#### *4.2.10.3 Statistical Analysis*

All experiments were performed at least in three independent trials, and the results were reported as mean ± SD. Results were subjected to the analysis of variance using the SAS software (SAS Institute, Inc., Cary, NC), and statistical significance of differences ($p < 0.05$) was evaluated by the least significant difference procedure.

## *4.3 Results*

### *4.3.1 Concentrations of Protein, total and specific IgY in the WSF*

The ETEC K88 and K99 specific IgY activities in the WSF was monitored by the ELISA method every week during the immunization period (Figure 4.1). The both ETEC K88 - and K99 - specific IgY increased gradually after the first immunization followed by an exponential rise after the first booster immunization. The level of anti-K88 and K99 IgY increased continuously to a maximum of $OD_{405}$ 0.72 and 0.78, respectively, 2 weeks after the second booster immunization. Subsequently, the level of IgY remained relatively high showing no considerable decline throughout the period that the eggs were monitored (up to 8 weeks). Eggs collected during 6 to 8 weeks expressing relatively high ETEC K88 - and K99 - specific IgY titre were stored for further use.

Table 4.1 shows that both protein and total IgY concentrations were relatively constant in ETEC specific and non-specific WSF ($p > 0.05$). The percent ratio of total IgY to total protein of all 3 WSF samples was approximately 18%. The antigen specific IgY concentration of the control sample was significantly lower than the experimental sample containing antigen specific IgY ($p < 0.05$). The proportion of ETEC K88- and K99-specific IgY in total IgY concentration was 7.8% and 8%, respectively.

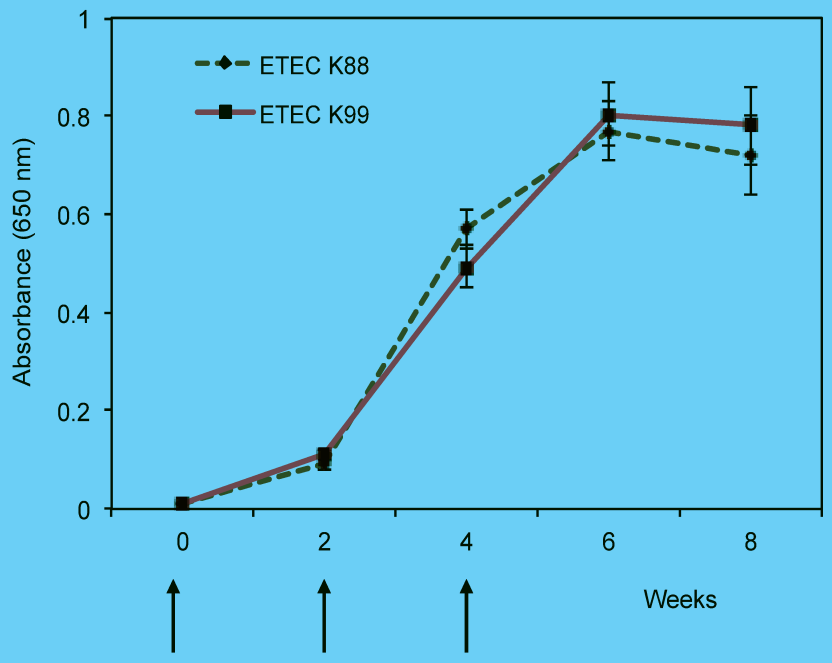



Figure 4.1: Specific IgY activity in the WSF obtained from chickens immunized with Enterotoxigenic *Escherichia coli* (ETEC) K88 and K99 during the immunization period measured by Indirect ELISA method. Values are the mean of quadruple samples. Vertical bars indicate the standard deviation. Arrows indicate time points of immunization.

Table 4.1 The concentrations of protein, total IgY and specific IgY in WSF powder containing Enterotoxigenic Escherichia coli (ETEC) K88 and K99-specific and non-specific IgY. Values are the mean ± standard deviation.

| | Concentration (mg/g) | | |
|---|---|---|---|
| Lyophilized WSF | Protein | Total IgY | Specific IgY |
| ETEC K88-specific | 500 ± 25 | 90 ± 16 | 7 ± 0.7 |
| ETEC K99-specific | 510 ± 19 | 93 ± 12 | 7.4 ± 0.5 |
| Non-specific | 456 ± 42 | 88 ± 14 | < 0.001 |

### *4.3.2 Phosvitin Extraction*

Figure 4.2 illustrates the protein distribution patterns of Sigma PV in comparison with purified PV fractions, being stained with added Coomassie Blue aluminum nitrate solutions. Purified PV post IgY isolated egg yolk showed high PV purity with a dominant 48 kDa and a minor 37 kDa bands (Figure 4.2. Lane B).

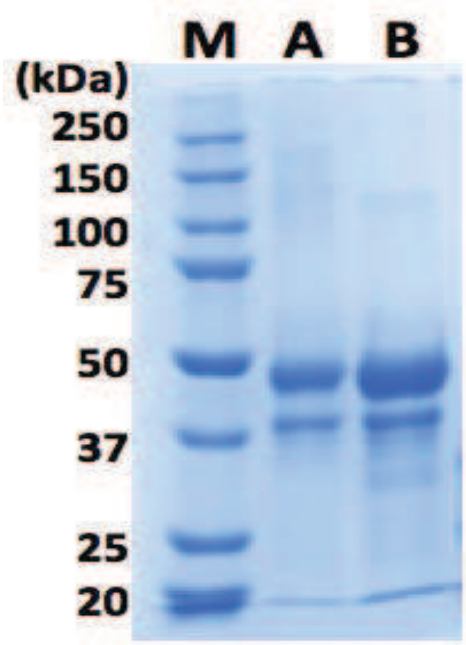



Figure 4.2: SDS-PAGE stained with aluminum nitrate added Coomassie blue added. Lanes: M, molecular weight marker; A, Sigma PV; B, purified PV from IgY pre-isolated egg yolk.

### *4.3.3 Enzymatic Hydrolysis of Phosvitin*

Table 4.2 exemplifies the DH of PV and PVH obtained from various enzyme treatments under AP and HHP. The DH was determined by TNBSA method to measure the concentration of free amino groups. All the PVH samples

showed higher DH under HHP condition compared to AP. The DH of PVH treated with Ela, Alc and Sav were significantly increased under HHP (100 MPa) compared to AP ($p < 0.05$). Among all enzymes, Alc treatment led to highest DH of 28.7 and 38.9% under HHP and AP, respectively. PVH obtained from Alc treatment under HHP (PVH-Alc-HHP) with the highest DH values was therefore selected for further evaluation on antimicrobial activity in comparison with intact PV.

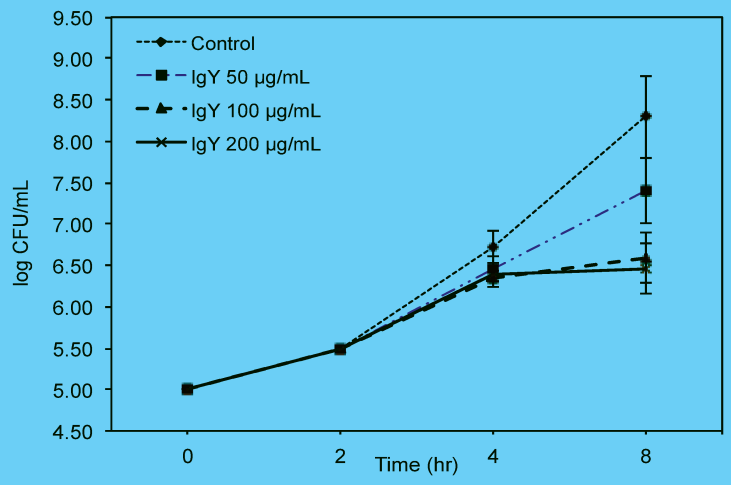
Table 4.2 Degree of hydrolysis of PVH produced under high pressure and atmospheric pressure conditions over a period of 24 hr. Values with different lowercase letters in similar hydrolysis condition differ significantly ($p < 0.05$). Statistically significant differences between HHP-EH and AP-EH for each enzyme are indicated by stars (*, $p < 0.05$).

| Enzyme Treatments | AP | HHP |
|---|---|---|
| Alcalase (Alc)* | $28.7 \pm 3.3^{A}$ | $38.9 \pm 4.2^{a}$ |
| Elastase (Ela)* | $5.5 \pm 0.9^{B}$ | $18.2 \pm 2.5^{c}$ |
| Thermolysin (The) | $7.5 \pm 1.1^{D}$ | $9.9 \pm 0.5^{b}$ |
| Trypsin (Try) | $22.4 \pm 2.1^{E}$ | $23.7 \pm 1.6^{e}$ |
| Savinase (Sav)* | $17.8 \pm 3.8^{C}$ | $25.4 \pm 1.7^{e}$ |

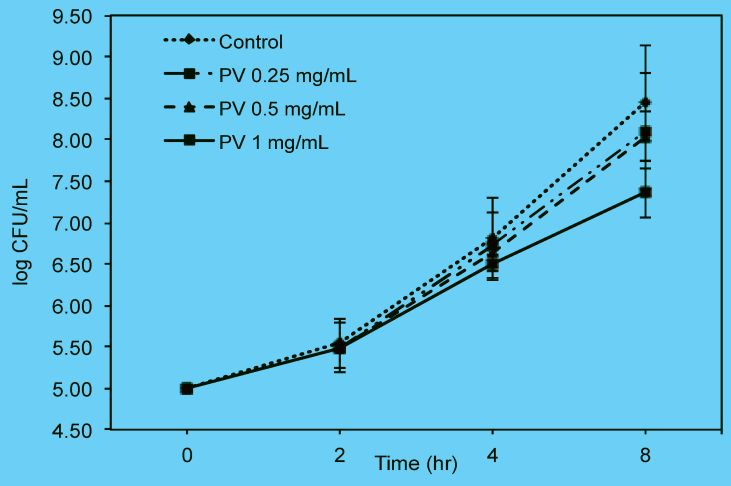
### *4.3.4 Growth Inhibition Assay of IgY and PV*

The growth of ETEC K88 and K99 consists of a lag phase (0 to 2 hr of the incubation time), followed by an exponential growth (2 to 8 hr) and then a stationary phase (> 8 hr). The growth inhibition curve of ETEC K88 is demonstrated when subjected to IgY (Figure 4.3a), PV (Figure 4.3b) and PVH-Alc-HHP (Figure 4.3c) treatments at varying concentrations.

a)


Control
IgY 50 µg/mL
IgY 100 µg/mL
IgY 200 µg/mL
log CFU/mL
Time (hr)
9.50
9.00
8.50
8.00
7.50
7.00
6.50
6.00
5.50
5.00
4.50
0
2
4
8

b)


Control
PV 0.25 mg/mL
PV 0.5 mg/mL
PV 1 mg/mL
log CFU/mL
Time (hr)
9.50
9.00
8.50
8.00
7.50
7.00
6.50
6.00
5.50
5.00
4.50
0
2
4
8

c)

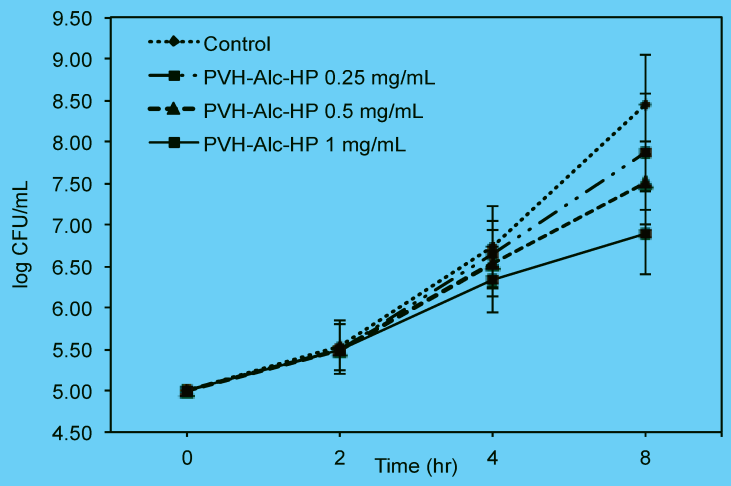
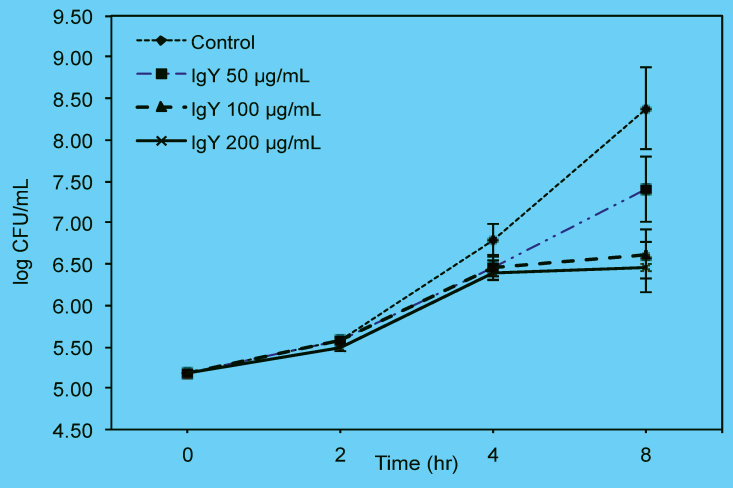

Control
PVH-AIc-HP 0.25 mg/mL
PVH-AIc-HP 0.5 mg/mL
PVH-AIc-HP 1 mg/mL
log CFU/mL
Time (hr)
9.50
9.00
8.50
8.00
7.50
7.00
6.50
6.00
5.50
5.00
4.50
0
2
4
8

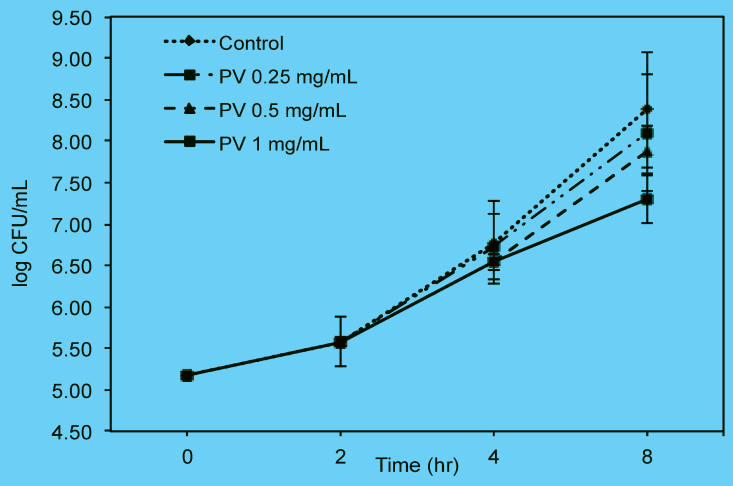
Figure 4.3: Enterotoxigenic *Escherichia coli* (ETEC) K88 growth inhibition effects of IgY (a); PV (b); PVH (c). Values are the mean of quadruple samples. Vertical bars indicate the standard deviation.

Under the same treatment conditions, figure 4.4 illustrates the growth inhibition curve of ETEC K99. The result shows no significant difference between the growth of ETEC K88 and K99 when subjected to their specific IgY antibodies (50 µg/mL) and non-specific IgY (control group) WSF powder ($p > 0.05$). In contrast, the bacterial growth was significantly suppressed in the presence of higher concentrations of specific IgY. Anti-ETEC K88 and anti-ETEC K99 IgY (100 µg/mL) were considered the optimal concentration for both ETEC K88 and K99 growth inhibition since no additional benefits were observed at higher concentrations.

a)




b)




c)

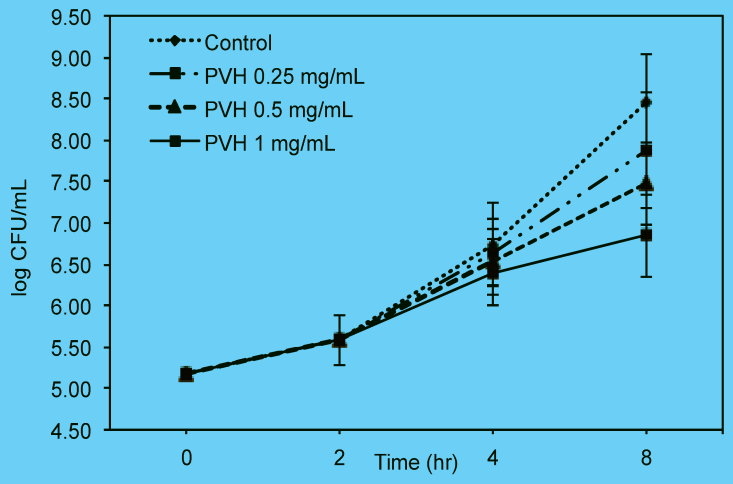
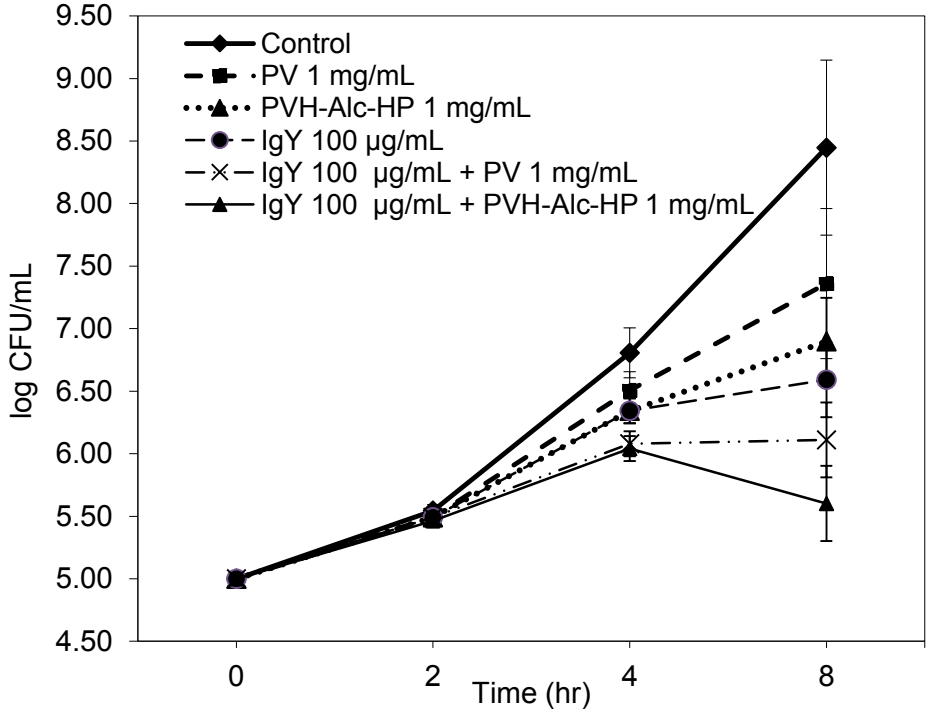



Figure 4.4: Enterotoxigenic *Escherichia coli* (ETEC) K99 growth inhibition effects of IgY (a); PV (b); PVH (c). Values are the mean of quadruple samples. Vertical bars indicate the standard deviation.

PV and PVH-Alc-HHP at 0.25, 0.5 and 1 mg/mL were also subjected in the same manner to determine the optimal concentration for the aforementioned bacterial growth inhibition. The best results for both PV and PV-Alc-HHP were observed at 1 mg/mL in both the cultures. Therefore, IgY, PV, and PV-Alc-HHP

at their optimal concentrations were compared when used solely and in combinations in ETEC K88 (Figure 3.5a) and ETEC K99 (Figure 4.5b) cultures.

a)




b)

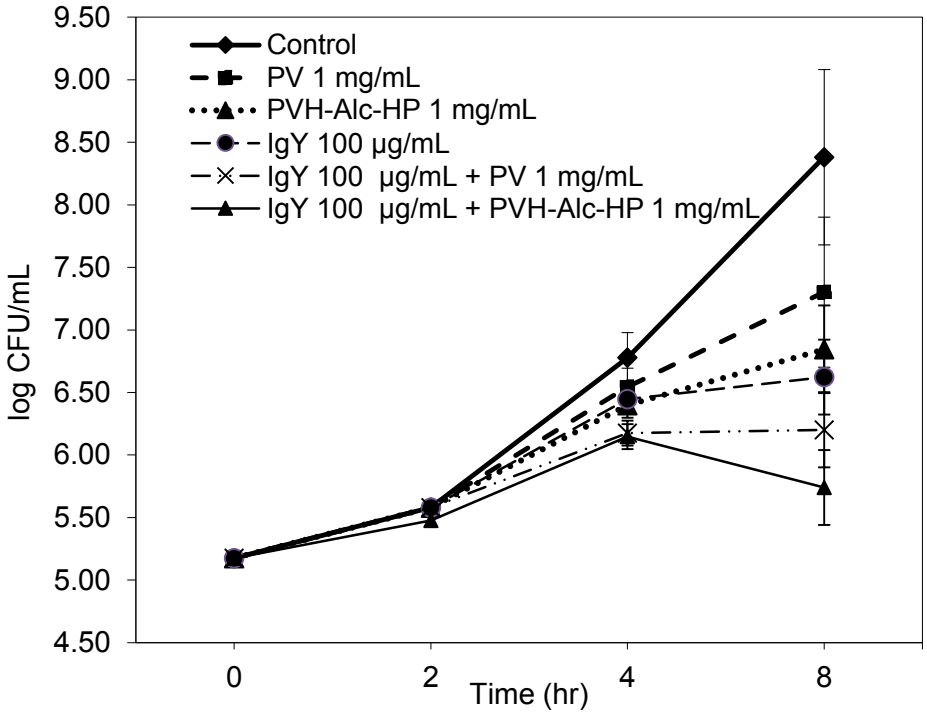



Figure 4.5: The effects of IgY, PV, PVH and their combinations on the growth of Enterotoxigenic *Escherichia coli* (ETEC) K88 (a), ETEC K8 (b). Values are the mean of quadruple samples. Vertical bars indicate the standard deviation.

All treatments had no effect on the growth of ETEC K88 and K99 during the lag phase ($p > 0.05$). During the exponential phase, the increase in a number of ETEC K88 and K99 incubated with its specific IgY, PV and PVH-Alc-HHP were less than that of the control group ($p < 0.05$). Cell counts of the ETEC K88 and K99 control group were increased by 2.9 and 2.8 log CFU/mL, respectively, during the exponential phase. A significant decrease in bacterial cell counts was observed from all treatment groups on both cultures as compared to control group. The cell count of ETEC K88 in treatment groups: PV, PVH-Alc-HHP, IgY, IgY+PV, and IgY+PVH-Alc-HHP increased by 1.87, 1.41, 1.10, 0.62 and 0.14 log CFU/mL, respectively. In a similar pattern, the cells count of ETEC K99 in

treatment groups: PV, PVH-Alc-HHP, IgY, IgY+PV, and IgY+PVH-Alc-HHP increased by 1.72, 1.27, 1.04, 0.62 and 0.26 log CFU/mL, respectively.

### *4.4 Discussion*

In recent years, consumers are in demand of natural food antimicrobials to extend the shelf life and to enhance the safety of foods. These include antimicrobial substances from a microbe, plant or animal source (Gálvez et al., 2014). In this study, we produced and explored the antimicrobial ability of two egg-derived compounds: IgY and PV in ETEC K88 and K99 media culture systems.

To implement IgY as an animal feed supplement, modest and practical antibody production is required. In this study we produced anti-ETEC K88 and K99 IgY antibodies in SCWL chickens by intramuscular immunization of ETEC K88 and K99 whole cells, respectively, with Freund's incomplete adjuvant. Both antibodies produced contained high titre of specific IgY antibodies during weeks 6-8 of the immunization period (Figure 4.1), indicating that SCWL chickens expressed relatively strong immune response against ETEC K88 and K99 whole cells.

IgY (aka γ-livetin) exists in egg yolk together with two other water-soluble proteins (α-, and β-livetin) and lipoprotein (McCully KA et al., 1962). Therefore, separation of IgY requires extraction of the water-soluble proteins from yolk lipoprotein, followed by separation from other livetins (Polson et al., 1980). The WSF containing IgY was isolated from ETEC K88 and K99 hyperimmunized eggs using the water dilution method. The freeze-drying being a suitable method

for preparation of dried concentrated product with high yield and lower moisture content (Yokoyama et al., 1992), was used to prepare IgY powder of WSF containing specific and non-specific IgY. The concentration of protein and total IgY was subsequently determined, as shown in Table 4.1, which is relatively similar to previous studies (Sunwoo et al., 2010). In literature, the percentage of specific in total IgY antibody ranged from 5 (anti-insulin antibody) to 28% (anti-mouse IgG antibody) (Hatta et al., 1997). Our results showed 7.8% and 8% of the ETEC K88 and ETEC K99-specific IgY concentration in total IgY, respectively, determined by ELISA.

PV is another attractive egg yolk component with functional phosphopeptides for metal ion binding, which possesses remarkable bactericidal property (Khan et al., 2000). In this study, PV purification performed using IgY extracted egg yolk precipitate, demonstrated higher purity as compared to intact egg yolk (Figure 4.2). The result was confirmed by gel chromatography indicating that pre-isolation of IgY from egg yolk removed unwanted proteins. This method was therefore carried out for PV purification.

Due to the negatively charged phosphorylated PV with serine residues, PV causes insoluble complex in gastrointestinal tract (Ishikawa et al., 2007). Hydrolysis of PV into PVH may restrain the intact PV from the formation of insoluble complexes, therefore assisting the absorption of calcium and iron in the gut (Choi et al., 2005). However, the enzymatic hydrolysis of PV is extremely difficult, due to negative charges of the phosphate group surrounding the PV molecule, which prevents enzymes from access to the peptide bonds (Gray, 1971).

HHP is reported to accelerate enzyme activity (Sunwoo et al., 2014), hence, in this study, HHP treatment is used to ease enzyme access into PV structure during enzymatic treatment to produce higher yield of smaller PVH fragments. Since different classes of protease differ in their peptide specificity, several commercial protease enzymes were used to determine the most appropriate protease for PVH production. Most published studies on PV hydrolysis used Try and reported very low digestion degree (Czernick et al., 2013; Ren et al., 2015). It has also been reported that the activity of α-chymotrypsin and thermolysin increased 7- and 45-fold at 100 and 200 MPa, respectively (Kunugi et al., 1997; Mozhaev et al., 1996), compared to their activity at AP. Similarly, α- and β-amylases from barley malt showed enhanced catalytic activities (25% and 16%, respectively) at 100 MPa compared to their activities at optimum catalytic temperatures and AP (Buckow et al., 2007). Furthermore, a key advantage of using HHP is the destruction of microorganisms in a reaction chamber which results in lower risk of microbial growth during long hydrolysis time and improved shelf-life of the final product.

The DH of PVH revealed that enzymes under HHP digested PV to a higher extend as compared to AP condition (Table 3.2). The DH of Ela-, Alc-, and Sav treated PVH appeared significantly higher in HHP than AP (3.3, 1.13, and 1.5-fold increase in DH value, respectively). Among all enzyme treated PVH, PVH-Alc-HHP showed the highest DH of 38.9%. Therefore, PVH-Alc-HHP is selected for ETEC growth inhibition study in comparison with intact PV.

There have been several reports on the use of IgY in the prevention and control of diarrhea causing infections in animal. IgY against Salmonella spp. proved useful in preventing the infection (Chalghoumi et al., 2009; Lee et al., 2002). IgY against ETEC 987P whole cells resulted in *in vitro* growth inhibition of effect ETEC 987P (Sunwoo et al., 2010), and *E. Coli* O157:H7 (Sunwoo et al., 2002). Specific IgY against K88 and K99 fimbrial adhesins of ETEC prevented neonatal piglets from fatal enteric colibacillosis (Yokoyama et al., 1992). Weaned pigs challenged with F18+ ETEC were fully protected by oral administration of IgY against the same fimbriae (Zuniga et al., 1997). *In vitro* studies showed that the inhibition of ETEC adhesion to piglet intestinal cells or mucosa by IgY reduced the virulence of the pathogen (Yokoyama et al., 1992; Jin et al., 1998; Jungling et al., 1991).

Khan et al., (2000) found that PV reduced the growth of *E. coli* K12 strain at 50°C for 20 min. The effect of PV is believed to be due to its strong capacity to bind iron, thereby barring microbial proliferation by limiting the iron supply (Greengard et al., 1964). As an iron carrier, the role of PV is also the protection against oxidation of iron-based free radical reactions (Taborsky 1963).

In this study, the dose-effect relationship of specific IgY, PV and PVH-Alc-HHP in ETEC K88 (Figure 4.4) and K99 (Figure 4.5) cultures was determined for an incubation period of 8 hr. A non-significant difference in bacterial growth inhibition pattern was observed between ETEC K88 and K99 under all treatment conditions. After 8 hr incubation in ETEC K88 and K99 cultures, the specific IgY antibodies at the optimal concentration (100 μg/mL)

inhibited 62% and 62.7% of bacterial growth, respectively. Intact PV and PVH-Alc-HHP at a concentration of 1 mg/mL were considered optimal for both ETEC K88 and K99 growth inhibition. In ETEC K88 and K99 cultures, intact PV inhibited 35.6% and 38.5%, while PVH-Alc-HHP inhibited 51.5% and 54.8%, of ETEC K88 and K99, respectively. Even though IgY has higher potency as compared to PVH-Alc-HHP in terms of lower optimal concentration and higher bacterial inhibition ability, neither did IgY nor PVH-Alc-HHP provide satisfactory bacterial growth inhibition when used as a sole. When used in combination, the synergistic effect of IgY and PVH-Alc-HHP on ETEC K88 (Figure 4.6a) and K99 (Figure 4.6b) growth inhibition was satisfactorily expressed at 90.6% and 95.2%, respectively.

### *4.5 Conclusions*

The egg-derived antimicrobial compounds: specific IgY (100 μg/mL) and PVH-Alc-HHP (1 mg/mL) proved to be the most efficient combination for controlling the foodborne ETEC K88 and K99 pathogens used in the present study. The synergistic anti-microbial activities of IgY and PV may support their potential application in feed supplementation to prevent microbial contamination and infectious diseases.

## Chapter 5: Conclusions, implications and future work

The phosvitin (PV) has a great potential to produce functional bioactive peptides with mineral chelating, antioxidant and antimicrobial properties. However, the challenge to produce phosvitin hydrolysates (PVH) is a resistance of PV to proteolytic digestion due to the unique amino acids sequence and high content of phosphate groups. The present results revealed that PV can be effectively hydrolyzed using high hydrostatic pressure (HHP) combined with enzymatic hydrolysis (HHP-EH) method to produce PVH containing more short peptides with strong iron chelation, reducing capacity and antimicrobial activity compared to PVH hydrolyzed by enzymes under atmospheric pressure (AP).

The objective of the first study was to boost the yield while maintaining the functional properties of PVH under suitable conditions of HHP and AP. Five different enzymes were selected for PV hydrolysis to find the optimal enzyme:substrate (E:S) ratio, temperature and incubation period. The PVH was then evaluated for iron-chelating and reducing capacity, as well as structural characteristics (i.e., degree of hydrolysis and molecular weights ($M_w$) distribution pattern). Our data indicated that enzymatic digestion produced PV hydrolysates (PVH) with improved iron-chelating function, although the PVH yield was low due to resistance of phosphoserine clusters to enzymatic digestion. HHP-EH produced PVH with higher degree of hydrolysis containing more peptides with $M_w$< 3 kDa compared to AP. PV hydrolysis with Alcalase (Alc) under HHP resulted in the highest degree of hydrolysis (31.3%). Iron chelation and reducing

capacity of Alc-PVH were compared to trypsin-PVH at similar phosphate content. The Alc- and Try-PVH obtained from both HHP and AP treatments showed superior iron chelation capacity (69-73%). It indicates the critical role of phosphate groups in metal chelation. Alc-PVH produced by HHP-EH displayed significantly greater reducing power (3.5 μM Trolox equivalent/mg) than AP-PVH (1.3 μM Trolox equivalent/mg). This study revealed that HHP produced PVH with greater proportion of short peptides, and stronger iron chelating and reducing capacity. It demonstrates the great potential of HHP-EH in production of short bioactive peptides from hydrolysis-resistant protein sources. Further studies need to be conducted to determine identify the amino acid sequence of effective PVH and confirm the relation between metal binding and antimicrobial capacity. These peptides could use as food products in nutraceutical and pharmaceutical applications. PV-derived hydrolysates showed great iron chelation and reduction capacity which can be used as natural mineral chelating agents for food, nutraceutical and pharmaceutical applications.

The objective of the second study is to produce and investigate the effect of PVH or a combination of IgY antibody on Enterotoxigenic *Escherichia coli* (ETEC) *in vitro* growth inhibition. The antimicrobial efficacy of egg-derived IgY antibodies or phosvitin (PV) alone, or combined against ETEC K88 and K99 in liquid culture media was investigated. Although, both IgY and PV in egg yolk possess anti-microbial activity, their dual use to prevent feed contamination have not been explored. A combination of IgY specific for ETEC and metal-chelating PV may have a synergistic effect in reducing the risk of feed contamination with

ETEC by inhibiting bacterial proliferation and stipulating protection against ETEC infection. ETEC, a major cause of fatal diarrhea in piglets, is associated with porcine intestinal colonization expressing K88 and K99 fimbrial adhesions. Anti-ETEC K88 and K99 IgY were isolated from the egg yolks of 23-week-old Single Comb White Leghorn chickens immunized with ETEC K88 and K99 whole cells, respectively. High titres of specific IgY against ETEC K88 and K99 were found during 6 to 8 weeks of the immunization period. The proportion of specific IgY against ETEC K88 and K99 in total IgY concentration was 7.8% and 8%, respectively. During the exponential phase, the increase in a number of ETEC K88 and K99 incubated with its specific IgY, PV and PVH-Alc-HHP were less than that of the control group ($p < 0.05$). Among all treatment conditions, specific IgY (100 μg/mL) plus PVH-Alc-HHP (1 mg/mL) exhibited the greatest inhibitory effect against both pathogens with a difference of 2.85 and 2.64 log CFU/mL, respectively, compare to control. The combination of the two is highly effective in bacterial growth inhibition and has great potential for improving the microbial safety in the animal industry. In recent years, consumers are in demand of natural food antimicrobials to extend the shelf life and to enhance the safety of foods. These include antimicrobial substances from a microbe, plant or animal source. The egg-derived antimicrobial compounds: specific IgY (100 μg/mL) and PVH-Alc-HHP (1 mg/mL) proved to be the most efficient combination for controlling the foodborne ETEC K88 and K99 pathogens used in the present study. The synergistic anti-microbial activities of IgY and PV may support their

potential application in feed supplementation to prevent microbial contamination and infectious diseases.

The objective of the third study is to develop a sensitive ELISA based on monoclonal and chicken egg yolk IgY polyclonal antibodies for the quantitation of microgram to nanogram levels of PV. An effective double antibody sandwich ELISA based on monoclonal (mAb) and chicken egg yolk IgY antibodies were developed to determine phosvitin (PV) content in therapeutic and functional products. Leghorn laying hens were immunized with the dephosphorylated PV to produce anti-PV IgY antibody in the egg yolk. High anti-PV IgY titre obtained from the egg yolks collected during 4 to 10 weeks of the immunization period contained approximately 6.2% of specific anti-PV IgY in total IgY. The double antibody sandwich ELISA (DAS-ELISA) and biotinylated DAS-ELISA developed has a PV detection range of 5.6 – 90 μg/mL and 2.5 – 40 ng/mL, respectively. However, biotinylated DAS-ELISA is a superior method for PV quantification regarding accuracy and sensitivity. This highly efficient PV detection method may recuperate the performance of the existing protein assay methods as well as facilitate future research on PV bioactivities and applications. There is limited information available on the quantification of PV. Since PV is a highly phosphorylated protein it cannot be accurately assessed by normal colorimetric assays such as Bradford, BCA and Lowry protein assays due to the interference of their phosphate groups. Therefore, this study introduces, for the first time, a simple and reliable method to obtain protein-specific IgY antibodies for quantification of PV in combination with mAb antibodies.

In summary of this study is, the purified PV from IgY extracted egg yolk showed higher purity compare to whole egg yolk. The DAS-ELISA and the biotinylated DAS-ELISA showed phosvitin quantitation detection ranges of 5.6 – 90 μg/mL and 2.5 – 40 ng/mL, respectively. After quantitation of PV, it was hydrolyzed to determine optimal parameters of pressures (0.1 vs. 90 Mpa), proteases, enzyme: substrate (E:S) ratios, incubation time, temperatures and pH. The result showed that Alc-PVH-HHP showed highest DH (31.3%) when the PV hydrolyzed under the optimal condition of 90 Mpa, pH 7, 50°C, 24 h incubation, and 1:50 (E:S). Also, PVH-Alc-HHP showed significantly greater reducing power (3.5 μM Trolox equivalent/mg) than Alc-PVH-AP (1.3 μM Trolox equivalent/mg) while both treatments had similar iron chelation capacity (69-73%). Intact PV inhibited 35.6% and 38.5% of ETEC K88 and K99 growth, respectively, while PVH-Alc-HHP inhibited 51.5% and 54.8%. A synergistic effect was observed when IgY and PVH-Alc-HHP were used in combination, showing the ETEC K88 and K99 growth inhibition of 90.6% and 95.2%, respectively. The combination of the two has proved to be highly effective in bacterial growth inhibition. The PVH as natural mineral chelating and the anti-microbial agent may have high potential nutraceutical and pharmaceutical as well as veterinary biologic values.

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# Appendix

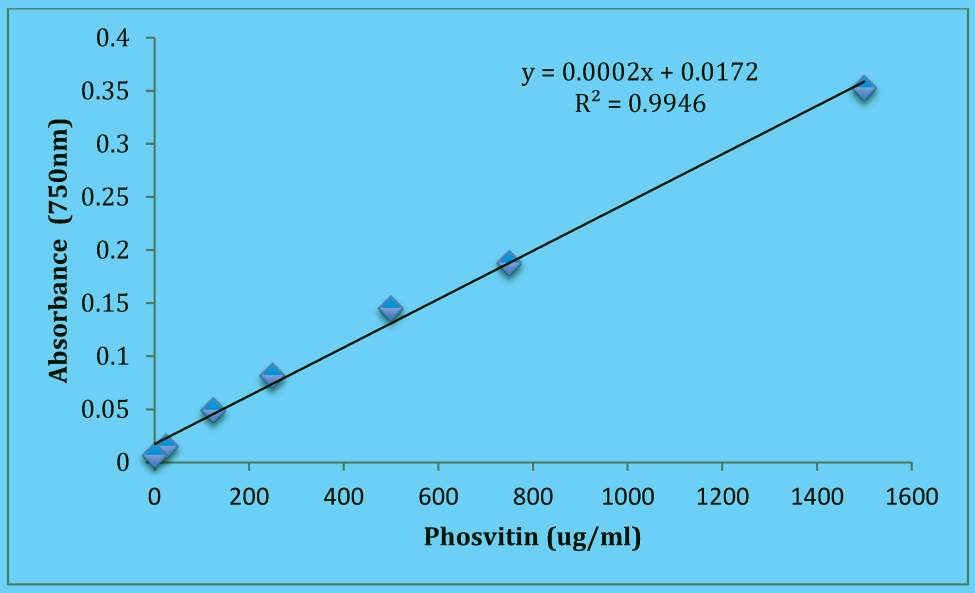


Figure A-1: Lowry Protein Assay Standard Curve. Sigma Phosvitin was used as standard protein at various concentrations.

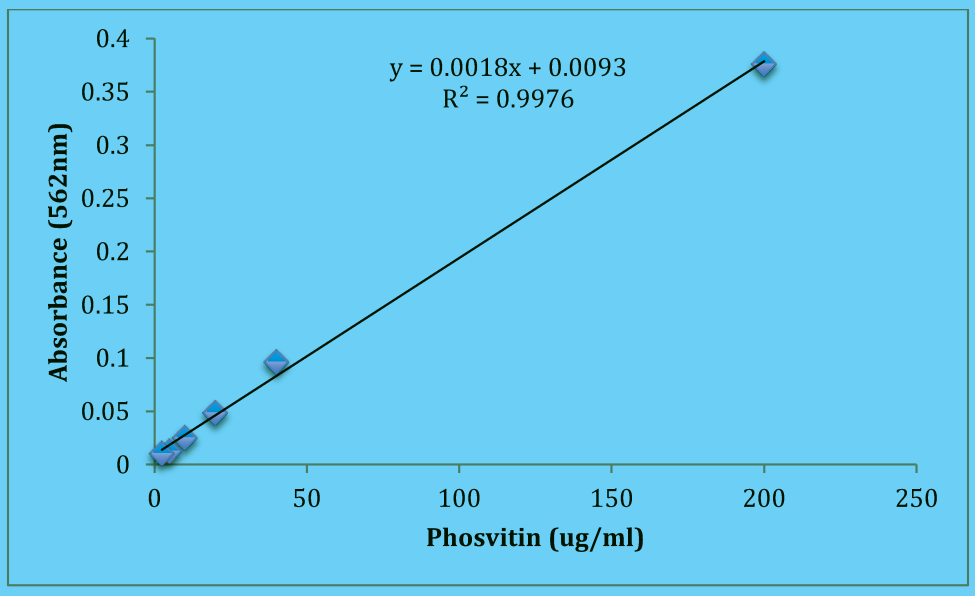


Figure A-2: BCA Protein Assay Standard Curve. Sigma Phosvitin was used as standard protein at various concentrations.

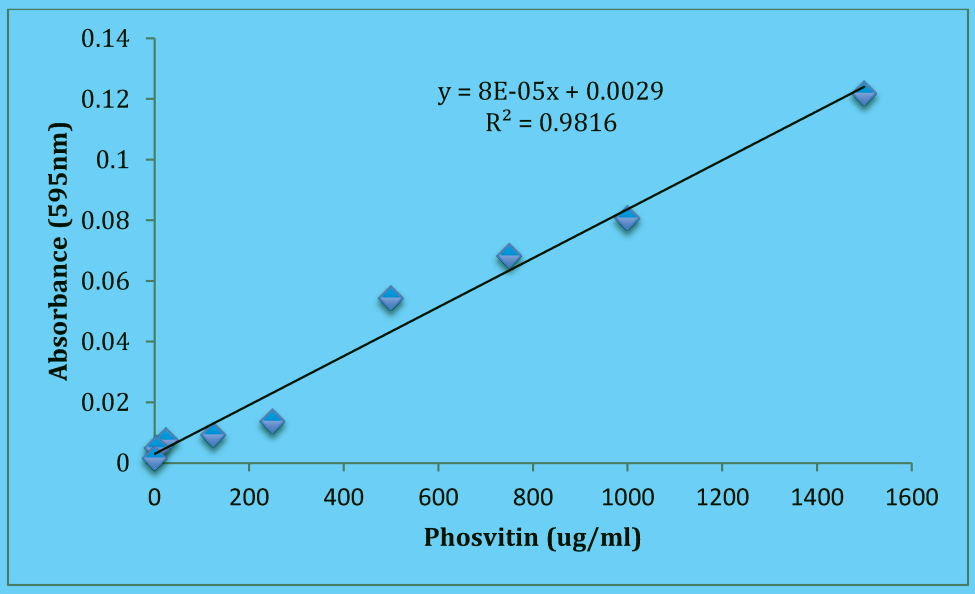


Figure A-3: Bradford Protein Assay Standard Curve. Sigma Phosvitin was used as standard protein at various concentrations.

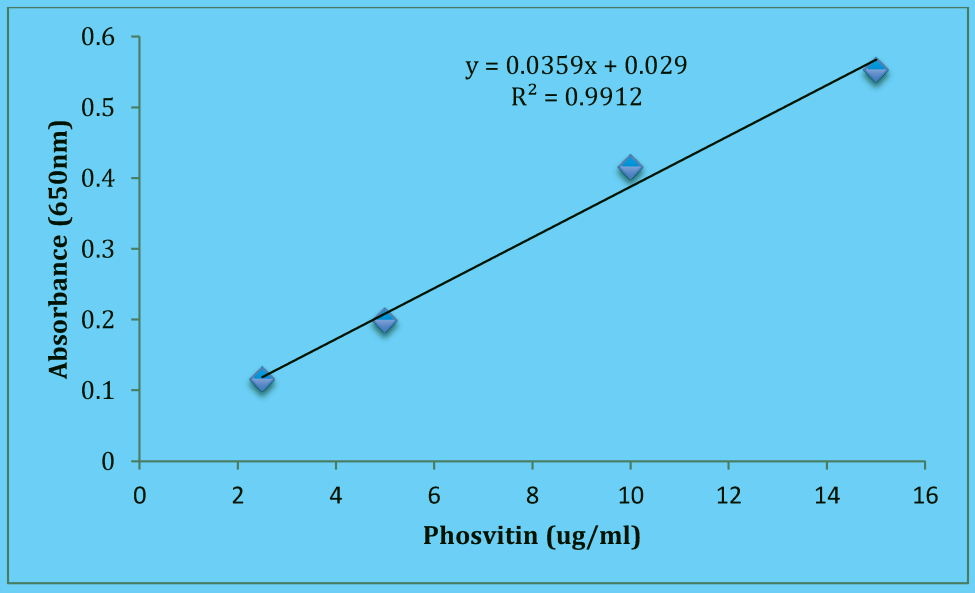


Figure A-4: Phosphate Assay Standard Curve. Sigma Phosvitin was used as standard protein at various concentrations.

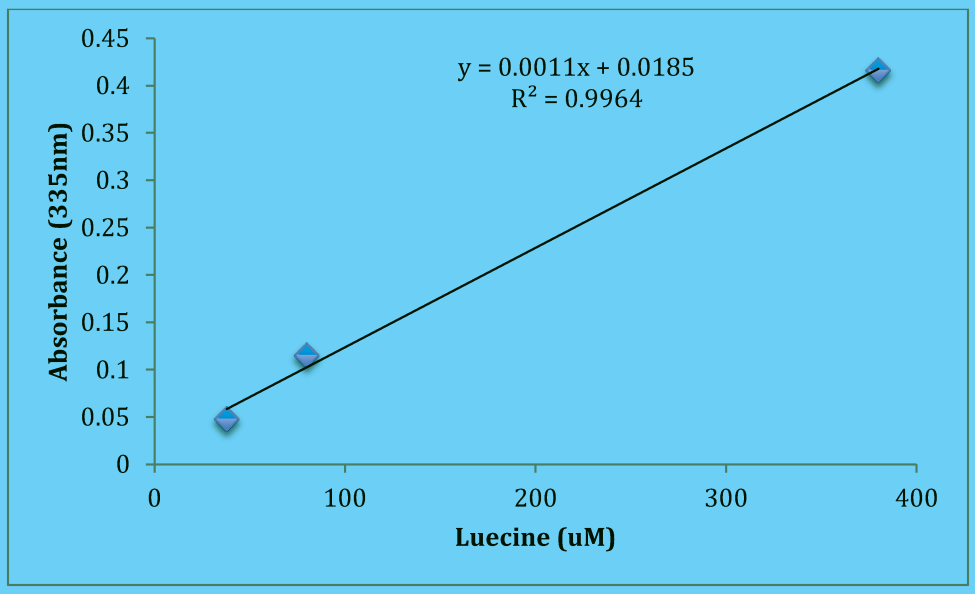


Figure A-5: Degree of hydrolysis (TNBS) Standard Curve. Luecine was used as standard at various concentrations.

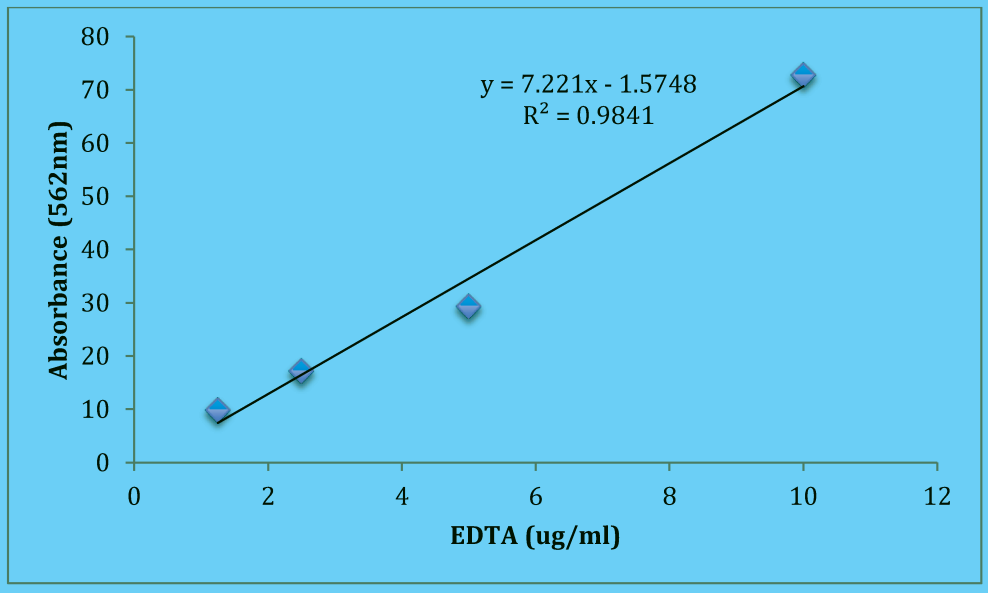


Figure A-6: Iron Chelation Assay Standard Curve. EDTA was used as standard at various concentrations.

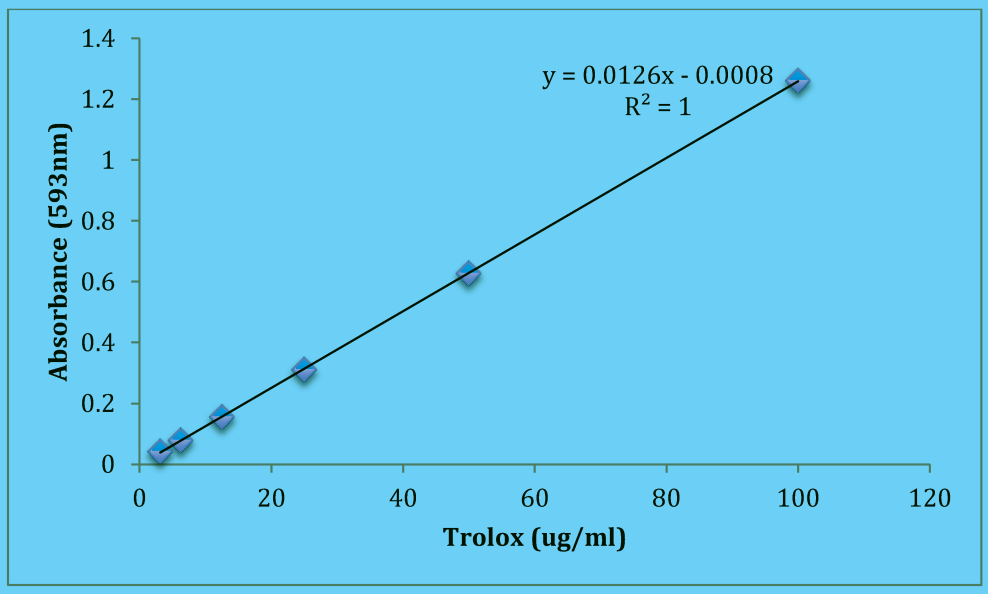


Figure A-7: FRAP Assay Standard Curve. Trolox was used as standard at various concentrations.

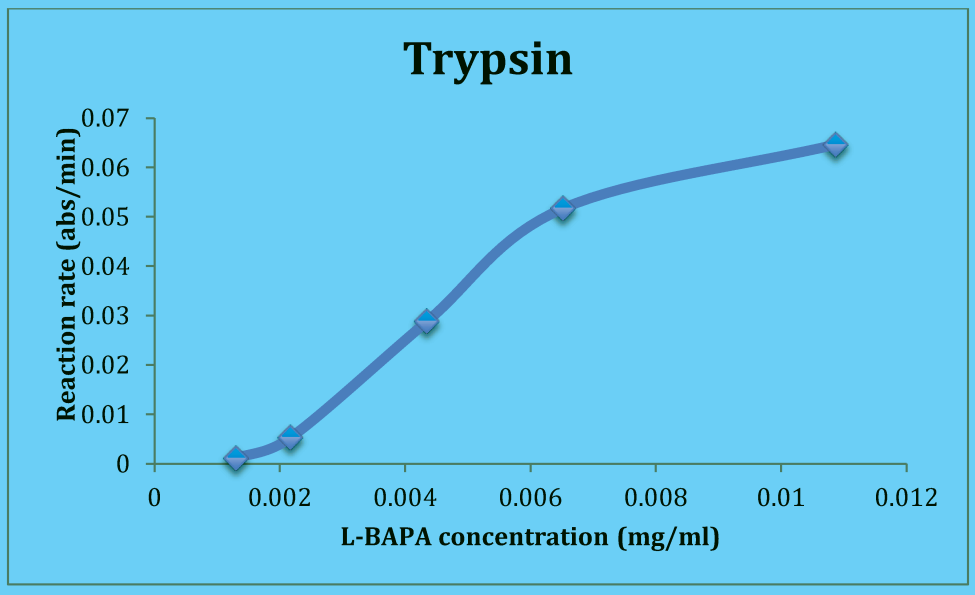


Figure A-8: Enzyme Kinetics for Trypsin. $N_{\alpha}$-Benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPA) was used as substrate. Absorbance was measured at 385nm.

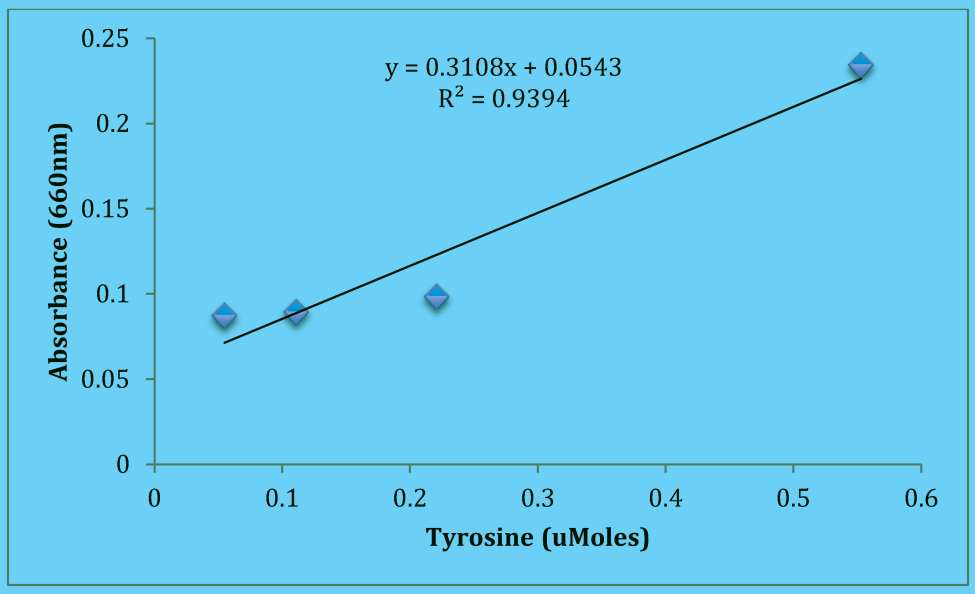


Figure A-9: Standard curve for Alcalase Enzyme kinetics. Tyrosine was used as standard at various concentrations.

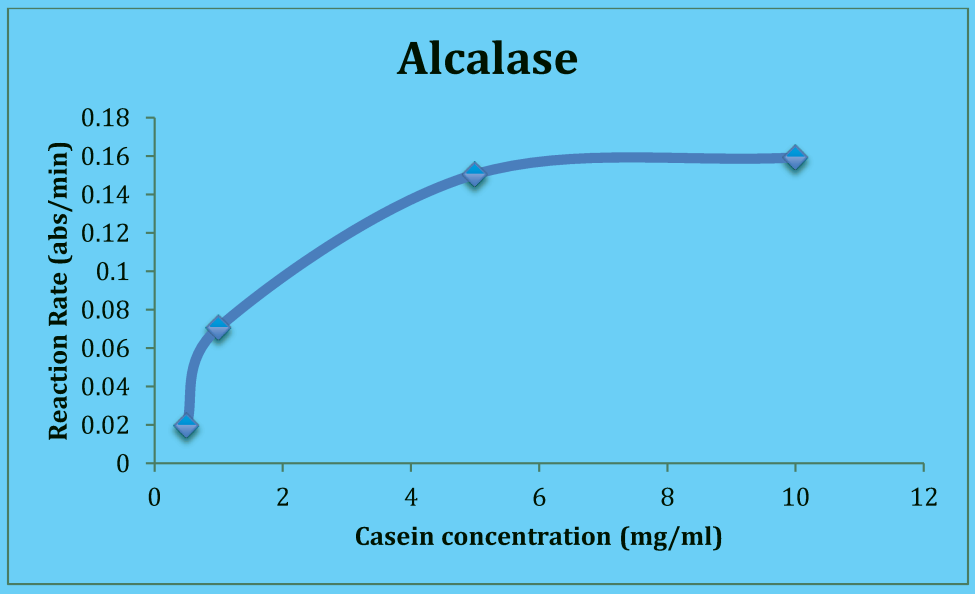


Figure A-10: Enzyme kinetics for Alcalase. Casein was used as standard at various concentrations. Absorbance was measured at 660nm.