THE RECOVERY OF PROTEIN FROM EGG YOLK PROTEIN EXTRACTION GRANULE BYPRODUCT

A Thesis

presented to

the Faculty of California Polytechnic State University,

San Luis Obispo

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Agriculture

with a Specialization in Dairy Products Technology

by

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May 2017

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ABSTRACT

The Recovery of Protein from Egg Yolk Protein Extraction Granule Byproduct

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In addition to proving an excellent source of nutrients, eggs are used in the food, cosmetic, and biotechnology industries for their rheological and bioactive properties. Much of the potential for the added value is in individual components of the egg, rather than the whole egg. At low speed centrifugation, yolk separates into two distinct fractions—granules and plasma. It is becoming increasingly popular in the industry to remove the plasma fraction of the egg yolk to use for its livetins, particularly immunoglobulin Y, leaving behind a granule by-product ("yellow cake"). Previous research has shown potential added-value from the granule fraction, especially from its phosvitin and phospholipids. Granules are protein aggregates with complexes of phosvitin and high density lipoproteins linked by phosphocalcic bridges. In their native form, the proteins are mostly insoluble, however previous studies have shown the links can be broken by alterations in pH, ionic strength, and mechanical treatments. This thesis project seeks to find potential uses for the egg yolk by product after the removal of the livetin fraction by means of further fractionation with mechanical treatment (filtration). Two variables were tested to extract more proteins from the yellow cake. Salt was added to 10% solids solution of yellow cake in water before filtration at four different NaCl levels: 0%, .05%, 1%, and 2.5%. Additionally pH was tested at four different levels: 4.6, 4.8, 5.0, 5.2. The samples were also tested for antibacterial properties against *Escherichia*

coli with a minimum inhibitory concentration assay (MIC). Analysis with BCA showed salt concentration had a significant effect on the yield of protein. The highest concentration of salt tested, 2.5%, had the highest protein yield. Additionally, SDS-PAGE showed 2.5% salt had the most unique protein bands. This could be to the disruption of the phosphocalcic links between the phosvitin and HDL by NaCl, allowing the protein to solubilize. pH did not have a significant effect on the yield or types of proteins in the range tested in this experiment. There is no conclusive evidence of antibacterial properties against *E. coli* from the protein extract. The MIC assay had growth show up in all wells with the protein extract, however there was a visible decrease in turbidity with higher concentration of the protein extract. This could mean that the protein extract does have some antibacterial properties, but needs testing at higher concentrations or with isolated proteins/peptides. The SDS-PAGE revealed bands showing phosvitin present, which has known antibacterial properties. Overall, improvements to the methods for further protein extraction from egg yolk by-products will help lead the industry to finding novel uses and product applications.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Rafael Jimenez-Flores, for his guidance and support, all the way from San Luis Obispo, CA to Indianapolis, IN to Columbus, OH. Thank you to Dr. Vincent Yeung, Dr. Marie Yeung, and Dr. Amy Lammert for your advice, knowledge, and time.

I would also like to thank Dr. Jennifer VanderKelen for all of the support in the Baker lab. Thank you to Andrea Laubscher for the endless patience, advice and troubleshooting with me.

Thank you to Sean Vink, Paul Lyons, Matt Arnold, and Kristy Murray for always being available to answer a million questions, help with the processing and fix problems in the DPTC. Thank you to Ivan Filipi from Nestle Purina, for your insight on the initial extraction process.

Thank you to all of my fellow graduate students for the sense of community, support, friendship and advice. A special thank you to Marcela Morales, who helped with every step of the project that physically needed more than two hands, as well as bringing the most positive attitude to any and all work. Thank you to my friends back at home for the constant encouragement.

Thank you to my parents and my sister for the endless love and patience throughout this process. Thank you to my step family and my in-laws for supporting me and always cheering me on. Lastly, thank you to my husband, Michael, for always believing in me.

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CHAPTER 1: INTRODUCTION

Hen eggs have long been acknowledged as a good source of nutrition in the food industry. They provide an excellent source of essential protein, fat, vitamins and minerals. Eggs have also been touted for their use as an emulsifier in foods, as well as cosmetics. In more recent years, eggs are being recognized for their benefits beyond basic nutrition and emulsification. Eggs, and their components, are found to contain health benefits and bioactive properties (Anton et al., 2006). Some of these beneficial properties are found in individual components of the egg, rather than the whole egg.

While egg yolks are generally sold as whole, there is more potential added value in the applications of its fractions. At low speed centrifugation, yolk separates into two distinct fractions—granules and plasma. The plasma is comprised of low-density lipoproteins and livetin. Granules are composed of high-density lipoproteins, phosvitin, and low-density lipoproteins (Anton and Gandemer, 1997). It is becoming increasingly popular to remove livetin from the water-soluble fraction (plasma) of the egg yolk. The three types of livetin include α-livetin, β-livetin, and γ-livetin (Mine, 2008). Of these, γ-livetin, referred to as IgY, is of particular interest because of its potential immunotherapeutic and immunodiagnostic properties. IgY from egg is analogous to the mammalian immunoglobulin IgG, but has many benefits, including cost, and a reduction and refinement in animal use (Karlsson et al., 2004). As the use for egg yolk fractions expands, including commercial scale IgY extractions, there has been an increase in leftover yolk product after removal of livetin. Use of granules has not been as extensively studied, but has added- value potential, especially in its phospholipids and phosvitin.

Granules are tightly compact in their structure, with complexes of phosvitin and HDL linked by phosphocalic bridges. Untreated, the proteins are largely insoluble, but can be broken by alterations in pH, ionic strength, and mechanical treatments.

The purpose of this thesis is to look to recover and characterize the proteins leftover in the by-product of a large IgY extraction representative of many of the current models of extraction. Specifically, this thesis seeks to understand the effect of mechanical treatments, pH, and NaCl concentration on protein recovery. Furthermore, it explores the potential anti-microbial activity of the recovered proteins.

CHAPTER 2: LITERATURE REVIEW

2.1 Egg Yolk Introduction

2.1.1 History

Hens' eggs are important in the embryonic stage of development of the chick, serving as a source of energy and protection. The unique egg components designed for development are also what make them a good source of nutrition, functional activity, and biotechnological agents (Anton, 2006). Eggs have been consumed by humans for as long as we have existed. Birds and eggs predate humans, and evidence has found that wild fowl were domesticated since 3200 B.C. Descendants of chickens used for egg laying today are believed to have been brought to America with Columbus' ships in 1493. In the mid-late 1900's major improvements to sanitation and technology were made, shifting small family flocks to large commercial egg operations. Continued improvement in caging, automation, and hen health has since led to higher production, better hen health, uniform quality, and lower costs. (AEB, 2017). Since the 1960s, advances include efficient egg breaking machines, better technology in pasteurization, improved freezing methods, and better spray driers (Mine, 2008). The average laying hen produced 160 eggs in 1960, and from all the improvements has increased to an average of 325 eggs in 2009 (Purdue Agriculture: Food Animal Education Network, 2008).

With improved technology in processing eggs, there has been an increase in further processed egg products. Examples of egg products are hard boiled pre chopped eggs, omelets, quiches, precooked scrambled eggs, precooked egg patties, and scrambled

egg mixes. Some processed egg products are used in the form of ingredients, including liquid whole egg yolk, whites, frozen yolk, and dried yolk or whites (Mine, 2008). Egg components are also being used in the cosmetic, medical, and nutraceutical industries (Anton, 2006).

The yolk of the chicken egg serves the biological function of sustaining the developing chicken embryo with lipids, proteins, vitamins and minerals, as well as preparing the first immune defense for the embryo. The aggregate of antibodies from the mother provide the embryo with this immunity (Mann, 2008). As well as its reproductive purposes, the yolk has uses in industry, including food, cosmetics, and biotechnology. The yolk provides a rich source of dietary lipids, proteins, vitamins and minerals (Anton, 2006) Yolk's unique properties make it a good food emulsifier, often used in the case of mayonnaises, salad dressings, and cream (Anton, 2013).

2.1.2 Nutritive Value

Eggs are known worldwide as an exceptional source of nutrition, especially providing a low cost, high quality source of protein. Whole egg protein holds the highest biological value, 94, of any of the major food protein sources (Mine, 2008). Though protein is found throughout the entire egg, most of it is found in the egg white (50%) and egg yolk (40%) with the remaining found in the shell and shell membranes. (Abdou, 2013). Eggs contain all nine essential amino acids that humans require in our diets. Additionally, eggs are a good source of lipids, vitamins and minerals. The yolk contains vitamins A, D, E and K, as well as a good source of iron and

phosphorous. B1 and B12 are deposited in the albumen. The yolk and albumen both equally contain B2 and B9 (Anton, 2006). Song and Kerver (2000) found that the daily nutrient intake of people that consumed eggs was significantly higher than that of those who do not consume eggs. The study included fat (saturated and polyunsaturated), cholesterol, fiber, sodium, Vitamins E, A, C, B6, B12, and folate. They found all of these nutrients except fiber and B6 were higher in the egg consumers. Additionally, there was no association with high serum cholesterol concentrations (Song et al., 2016). Further, the food industry can fortify eggs with beneficial nutrients through the diet of the hen. Omega-3 fatty acids, lutein, and Vitamin E are commonly added to eggs to enhance nutrition (Mine, 2008).

2.1.3 Beyond Nutrition

Health benefits of food today are not solely assessed on their macronutrient and micronutrient content. Scientific research is finding that certain foods have physiologically active components that add value besides the established nutrients they contain; these foods are called functional foods. The International Food Information Council (IFIC) explains functional foods as "foods that provide health benefits beyond basic nutrition." These foods include whole foods, as well as fortified, enriched, or enhanced foods, and can possibly lead to the reduction of disease (ADA, 2004).

Eggs yolks are mostly made up of lipids (65%) and consist of triglycerides, phospholipids (PL), cholesterol, and free fatty acids. The omega 3 fatty acids and

phosphatidylcholine (PC) found in the yolk are of special interest due to their importance of in brain development and function, particularly for infants. PC and other phospholipids are being studied as a potential treatment for neurological disorders (Kullenberg, 2012). Egg yolk phospholipids have three times more PC than from natural soy phospholipids (Anton, 2006). Research in dietary PC has showed some evidence of cancer prevention. Suppression of nodule formation related to hepatic cancer and preneoplastic liver lesions were found in a study with PC supplementation in rats. It has also been found that the PLs could reduce the negative gastrointestinal side effects of non-steroidal anti inflammatory drugs when paired together (Kullenberg, 2012).

2.2 Structure and Composition

2.2.1 Whole Egg Structure

The main components of a hen's egg are the ovum (yolk), albumen (white), shell membranes, and shell. The shell consists of three layers; the outermost layer is the cuticle, then a calcium carbonate layer, and an innermost mammillary layer.

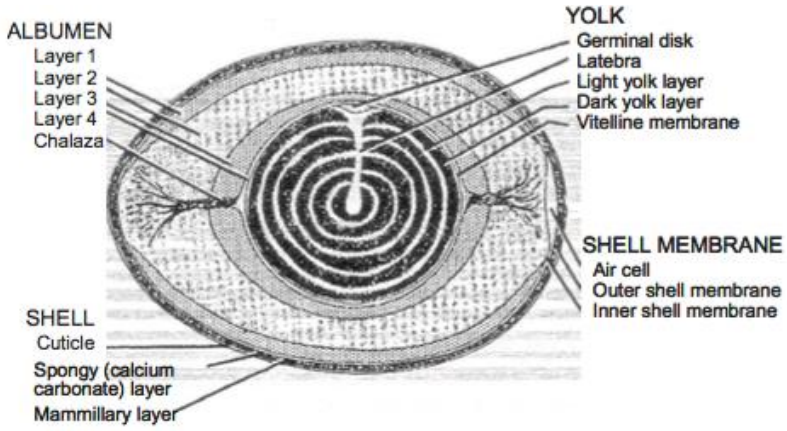

Figure 2.1 Structure of whole egg (Mine, 2008)

2.2.1.1 Shell and Shell Membrane

The shell makes up ~11% of the egg (USDA, 2000). Eggshells' main purpose is to protect the egg against physical damage and microorganisms. (Mine, 2003) The shell membrane is just below the inner surface of the shell and encompasses the albumen. It consists of two membranes, inner and outer, that together are only 0.00609 mm thick. The inner membrane is made up of 3 layers of fibers that run parallel to the shell, while the outer membrane consists of 6 layers in different directions. The main purpose of the shell membrane is to contribute to the strength of the shell (Mine, 2008).

2.2.1.2 Albumen and Ovum

The albumen, often referred to as the egg white, is made up of four layers. Total it represents ~58% of the egg. (USDA, 2000) Its main function is to deliver nutrients to the chick embryo during development. The albumen also serves to protect the yolk from harmful microorganisms (Abdou et al., 2013). There are two outer layers, one thick and one thin, and two inner layers. The outer thin layer next to the membrane is ~23.3%, followed by the outer thick white layer at ~57.3%. The thick layer is more viscous because of its high concentration of ovomucin. The inner thin layer that makes up ~16.8% of the white, and innermost to the yolk is the calaziferous layer, ~2.7% (Mine, 2008). The chalaziferous layer, attached to the chalazae, encompasses the egg yolk. Although thin, it is a gelantinous strong layer that holds the yolk in place (USDA, 2000).

The ovum, most often referred to as the yolk, consists of about 31% of the total weight an egg (USDA, 2000). It is composed of a three layer membrane called the vitelline membrane surrounding the yolk. Most of the yolk is made of yellow yolk, with only 2% white yolk (Mine, 2008). The more complex microstructure of the yolk will be covered in more detail.

2.2.2 Whole Egg Composition

The majority of the egg by weight is water, totaling 75%. Proteins and lipids constitute the majority of the rest of the egg, along with small amounts of carbohydrates and minerals (Mine, 2008). While these are the main contribution to human nutrition, there are also other minute components that are being studied for their biological activities and functional properties.

Table 2.1 Composition of Egg (adapted from USDA, 2000)

	Percent	Water	Protein	Fat	Ash
Whole egg	100	65.5	11.8	11.0	11.7
White	58	88.0	11.0	0.2	0.8
Yolk	31	48.0	17.5	32.5	2.0
		Calcium carbonate	*Magnesium carbonate*	*Calcium carbonate*	*Organic matter*
Shell	11	94.0	1.0	1.0	4.0

By weight the shell is ~94% calcite, with the remaining components mostly glycoprotein, proteoglycans, and minerals (Mine, 2003). The shell membranes are composed largely of 90% protein, with small amounts of ash and glucose. Water

makes up most of the albumen, with proteins making up the highest amount of solids, about 10% of the total weight of the white. Glucose, lipids, and minerals are also present in smaller amounts (Mine, 2008). There are ~40 different proteins in the albumen, the majority composed of ovalbumin. About half of the total protein found in eggs is found in the albumen, 40% in the yolk, with the remainder in the shell and shell matrix (Abdou, 2013). The yolk is 50% solids. Lipids make up ~70% of the yolk (solid matter) and proteins ~30% (Mine, 2008). Ovovitellin makes up the majority of the yolk protein. Of the lipid content, triglycerides make up 65.5%, phospholipids 28.3%, and cholesterol 5.2%. The pH of the yolk when freshly laid is ~6.0, and after storage rises to ~7.0 (USDA, 2000). The yolk is composed of plasma and granule fractions, which will be covered in more detail in following sections.

Table 2.2 Major Albumen Proteins (Adapted from Awade, 1996)

Protein	Amount of total protein (%)
Ovalbumin	54
Ovotransferrin	12-13
Ovomuvoid	11
Lysozyme	3.4-3.5
Ovomucin	1.5-3.5
G2 ovoglobulin	1.0
G3 ovoglobulin	1.0
Ovoflavoprotein	0.8
Ovostatin	0.5
Cystatin	0.05
Avidin	0.05

Table 2.3 Major Yolk Constituents (Adapted from Mine, 2008)

Constituent	Major Component	Relative %
Proteins	Apovitellenin I-VI	37.3
	Lipovitellin apoproteins	
	α-Lipovitellin	26.7
	β-Lipovitellin	13.3
	Livetins	
	α-Livetin	2.7
	β-Livetin	4.0
	γ-Livetin	2.7
	Phosvitin	13.3
	Biotin binding protein	Trace
Lipids	Triglyceride	65
	Phosphatidylcholine	26
	Phosphatidylethanolamine	3.8
	Lysophosphatidylcholine	0.6
	Cholesterol	4
	Sphingomyelin	0.6

2.2.3 Yolk Composition

Composition of egg yolk can vary depending on the diet of the hen. Yolk mainly consists of proteins and lipids. Supplementation of various minerals or foods high in omega-3 fatty acids have been reported as changing the protein and lipid content, and have been used in industry to fortify the yolk for enhanced nutrition (Mine, 2008). The yellow pigment of the yolk, xanthophyll, comes from greens and corn in the hen's diet (USDA, 2000). Light centrifugation will separate the yolk into a plasma supernatant that makes about 77-81% of the yolk dry matter and a granule precipitate that makes up 19-23% (Anton, 2013). Plasma is composed of 85% low-density lipoproteins (LDL) and 15% livetins. Granules are composed of 70% high-density lipoproteins (HDL), 16% phosvitin, and and 12% LDL. (Anton, et al., 2000) As previously mentioned, numerous vitamins and minerals are present in the egg yolk. There is also a small amount of carbohydrate present in yolk, about 0.7-1.0%, mostly

bound as glycoproteins and glycolipids (Mine, 2008). Figure 2.2 breaks down the plasma and yolk components and their relative proportions.

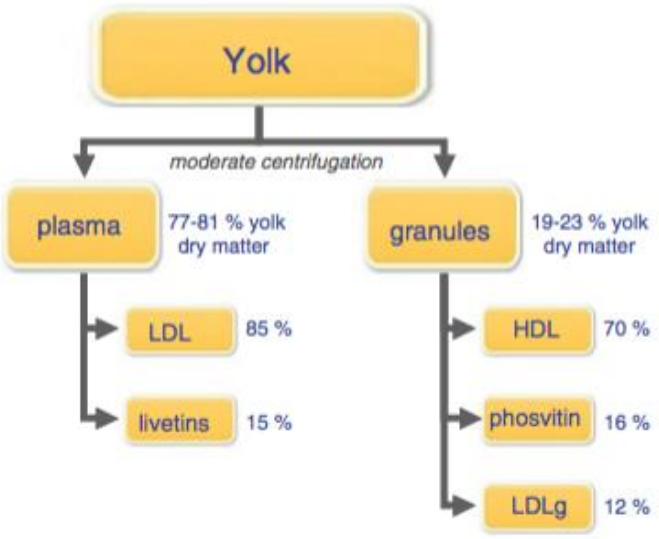

Figure 2.2 Composition of Yolk (Anton, 2013)

2.2.3.1 Lipids

Yolk lipid is made up of ~65% triglycerides, 28-30% phospholipid (PL), and 4-5% cholesterol. Composition of lipids can be affected by the hen's age, genotype, and diet (Mine, 2008). Research has suggested there are human health benefits to eggs fortified with PUFAS by means of the hen's diet. The essential fatty acid ratio in untreated egg yolk is about 9:1 n-6 PUFA to n-3 PUFA, while the healthy recommended proportion associated with reduced risk of cardiovascular disease is 5:1 (Marcet et al., 2014). A study by Milinsk et al. fed hens diets with different oils containing different amounts of fatty acids. They determined that the addition of oils into the diets were able to produce eggs with higher n-3 to n-6 ratio in the yolk lipids, while not affecting cholesterol content (2003).

The lipids in yolk are a majority triacylglycerols, or triacylglycerides (TG). Saturated palmitic acids and stearic acids make up ~30-38% of the fatty acids in yolk lipids, and monounsaturated and polyunsaturated fatty acids make up another ~30% (Mine, 2008). A study by Brady et al., 2002 suggests that the TG released from the inner core of the lipoprotein is likely responsible for the antibacterial effect of egg yolk lipoprotein (Brady, 2002) Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major egg yolk PLs. Respectively, they make up ~81% and 12% of egg yolk lecithin. Lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and sphingomyelins are also present in smaller amounts in yolk PL (Mine, 2008).

Sphingomyelins are only a minor constituent of yolk phospholipids. Although they only make up ~2% of yolk lipids, they are important. Sphingomyelin may have chemoprotective properties, as well as therapeutic preventative uses (Barrenetxe et al., 2006). It also plays an important role in the cell membranes of mammalian tissues, particularly in the brain and nervous tissues, working to regulate cholesterol (Slotte et al., 2007). Cholesterol makes up ~1.6% of total egg yolk, and ~5% of the egg yolk lipids (Mine, 2008).

Table 2.4 Composition of yolk, granules and plasma (g/100g dried matter) (Adapted from Anton and Gandemer, 1997)

Constituent	Yolk	Granules	Plasma
Proteins	33.2	63.8	24.6
Lipids	63.4	30.7	72.6
Triglycerides (TG)	43.2	19.6	51.7
Phospholipids (PL)	17.7	11.1	18.2
Phosphatidylcholine (PC)	15.7	10.0	16.0
Phosphatidylethanolamine (PE)	2.0	1.1	2.1
Cholesterol	2.6	1.3	2.7
Lipids/Proteins	1.9	0.5	3.0
PL/TG	0.41	0.61	0.35
PC/PE	7.5	9.1	7.6

2.2.3.2 Proteins

LDL accounts for about two thirds of the yolk's total protein. It has a low density of .98 because its high lipid to protein content. It is responsible for much of the emulsifying functionality of egg yolk. LDL has at least two subfractions, called differing names such as LDL1 and LDL2, LDF1 and LDF2, and LDP1 and LDP2 (Mine, 2008). A study by Jolviet et al. (2006) showed there were nine major protein bands by SDS-PAGE. Two bands were a monomer and dimer of apovitellin I, and all other bands were associated with fragments of apolipoprotein B (2006).

HDL has a higher protein content of 80%, to a lipid content of 20% (Mine, 2008). The high protein content of HDL accounts for granules' density of 1.190 g/mL. (Strixner and Kulozik, 2013). HDL is composed of α- and β-lipovitellins, which vary in amino acid composition (Mine, 2008). HDL makes up about a sixth of the yolk

solids in the granular fraction. It exists as a complex with phosvitin (Abdou et al., 2013).

Phosvitin is a phosphoglycoprotein with a high phosphorous content of ~10%. It is one of the most phosphorylated proteins in nature. Although it is insoluble in water in untreated conditions, with low ionic or acidic conditions, it can become soluble and has an ability to form a complex with metal ions (Abdou et al., 2013) Phosvitin is responsible for 60% of total egg yolk phosphoproteins and ~90% of the phosphorous in yolk (Samaraweera et al., 2011).

Livetin makes up 30% of the plasma proteins. It is made of α-livetin, β-livetin, and γ-livetins, which are analogous to the mammalian plasma proteins serum albumin, α2 glycoprotein, and γ-globulin, respectively (Mine, 2008). Most research has been done on the immunoglobulin IgY because of its promising uses. The γ-livetins, referred to as IgY, are the major antibodies found in hen's eggs. The IgY comes from the hen serum IgG, but has a different name to distinguish it from mammalian IgG (Mine, 2008). There are about 100-400 mg IgY in an egg, with 1.23 times more in the yolk than in the serum concentration (Abdou et al., 2013).

Table 2.5 Properties of Yolk Proteins (Adapted from Mine, 2008)

Protein	Percent	Localization	Molecular Weight	Characteristics
Low-density lipoprotein (LDL)	65	Plasma and granules	10,300 kDa (LDL_1) 3,300 kDa (LDL_2)	Lipid content ~90% Apovitellenins I-VI known as lipoproteins
Lipovitellin; high-density lipoprotein (HDL)	16	Granules	400 kDa (α, β-lipovitellin complex)	Lipid content ~25%
Livetin	10	Plasma	80 kDa α-livetin 40, 42 kDa β-livetin 180 kDa γ-livetin	Serum albumin Fragment of*C*-terminus in vitellogenin IgY (hen's serum IgG)
Phosvitin	4	Granules	33, 45 kDa	Most phosphorylated protein in nature
Egg yolk riboflavin-binding protein (RBP)	0.4	Plasma	36 kDa	Similar to flavoprotein in egg white and to serum RBP
Others	4.6	Mainly plasma		

2.2.4 Yolk Microstructure

Egg Yolk is composed of non-soluble protein aggregates dispersed in a clear solution of soluble proteins and low-density lipoproteins. The non-soluble aggregates are called granules, and the soluble solution is called plasma. Centrifugation can easily separate the yolk into these two fractions (Anton, 2013). Plasma and granules exhibit different functionality, due to their differing compositions and structures.

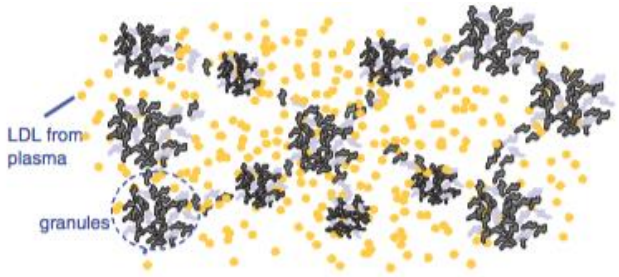

Figure 2.3 Egg Yolk Structures (Anton, 2013)

2.2.4.1 Plasma

Plasma consists mainly of LDL and livetins, and is known to be soluble in its native form. Plasma is known as a useful emulsifying agent. The emulsifying properties of plasma behave similarly to yolk, while granules behave differently. This suggests that the plasma fraction is responsible for the yolk's emulsifying capability. Specifically, the LDL has been attributed with the excellent emulsifying properties (Jolviet et al., 2006).

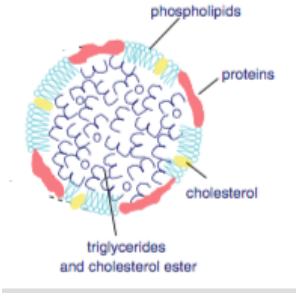

Figure 2.4 LDL Structure (Anton, 2013)

LDL are spherical nanoparticles that range from 17nm to 60 nm in diameter. They have a lipid core made of triglycerides and cholesterol esters surrounded by a monofilm of phospholipids and proteins. LDL proteins are non-soluble in water or aqueous buffer, making direct adsorption difficult, shown in Figure 2.5. The

nanostructure of the LDL is what allows the transport through the aqueous phase in a soluble form to the interface to then be released (Anton, 2013). Jolviet et al. (2006) identified two apoproteins from purified LDL—Apovitellenin I and Hen Apolipoprotein B (2006). Their study suggests that it is the apoproteins that serve as the initial anchorage at the interface, then initiate disruption of the structure and denaturation of the protein, leading to the spread of LDL at the interface.

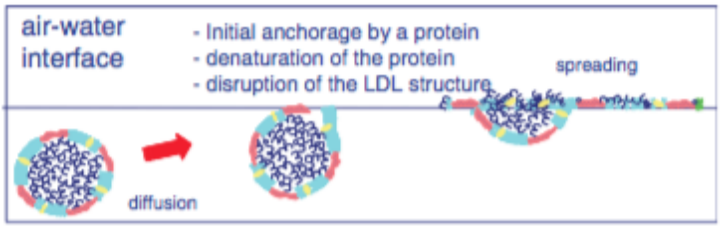

Figure 2.5 LDL Adsorption (Anton, 2013)

Livetin is a water-soluble, non-lipid, globular glycoprotein. Immunologically it is analogous to mammalian protein plasma (Abdou et al., 2013). The structure of IgY and mammalian IgG are similar; both have two heavy chains and two light chains, but also come with differences. The molecular mass of IgG is 150 kDa, and 180 kDa for IgY. It has been proposed that IgY is more hydrophobic than IgG, favorable to the high lipid environment in egg yolk. The different amino acid sequences and structure cause different functionality in the immunoglobulins (Karlsson et al., 2004). Research has shown that IgY is accompanied by a polypeptide complex called yolkin. Yolkin is an immunostimulatory heterogeneous set of proteins that range in size from 1-35 kDa (Zablocka et al., 2014).

2.2.4.2 Granules

Granules are made up of protein aggregates ranging from 0.3μm to 2μm in diameter. Granules are mainly insoluble in their native form, as the HDL and phosvitin form complexes linked by phosphocalcic bridges (Anton, 2013). The HDL and phosvitin both have high amounts of phosphoserin amino acids that are able to bind calcium. These phosphocalcic bridges make the structure of the granule compact, poorly hydrated, and weakly accessible to enzymes. This leads to an efficient protection against thermal denaturation and heat gelation. The heat stability attributes of the granule fraction makes them a better choice for emulsion stabilization over plasma or yolk (Sirvente et al., 2007). Phosphopeptides have a molecular structure that allow that usually contain clusters of phosphoserines, which is responsible for binding calcium and inhibiting the formation of insoluble calcium phosphates. The phosvitin in egg yolk is of particular interest because a molecule of it has ~120 phosphoserine residues, as opposed to a casein subunit with only 1-13 residues. This could produce calcium binding and releasing capabilities (Samaraweera et al., 2011).

2.3 Bioactive Proteins

Many foods contain proteins that have reported biological activities. Such activities include antimicrobial, anti hypertensive, anti oxidative, vitamin and mineral binding, immunoregulatory and immunosuppressing, and enzyme inhibitory properties. Often it is the peptides released during food fermentation or from enzymatic proteolysis during digestion that possess the biological activities (Walther and Sieber, 2011). Eggs are a valuable source of such proteins and peptides with biological properties.

These active molecules potentially provide use in the medical, pharmaceutical, cosmetic, nutraceutical and biotechnological industries (Anton et al., 2006). The use of peptides and proteins with antimicrobial activity is of particular significance to medicine, pharmacology, and the food industry because of recent microbial resistance to antibiotics. Such proteins and peptides are able to inhibit or attack pathogenic microorganisms, but leave the host cells unharmed. Greater insight on antimicrobial peptides could help a better understanding of the resistance mechanisms in bacteria and a potential replacement of antibiotics as a therapeutic agent against pathogenic diseases (Coutinho et al., 2007).

While egg white bioactive proteins and peptides have been more extensively researched and used in the food industry, yolk proteins are currently less described. Though many components of the egg white contain biological activity, this section will briefly describe a few of the most abundant and used bioactive protein applications in the egg white, but this thesis will focus on the utilization of the bioactive proteins and peptides in the yolk portion.

2.3.1 Egg White Bioactive Proteins

2.3.1.1 Lysozyme

Lysozyme is a widely used enzyme across the world in the foods and pharmacological industries. It is used to preserve fresh produce and meats by coating the surfaces, added to cheeses to control undesirable bacteria, and added to some wines to replace SO_2 to inhibit malolactic fermentation. More than 100 tons of

lysozyme are used annually (Mine et al., 2004). The antimicrobial activity of lysozyme is effective against Gram-positive bacteria. It breaks down peptidoglycan, the structural component of Gram-positive bacterial cell walls, by splitting its β-(1,4) linkages between N-acetylmuramic acid and N-acetylglucosamine (Xu et al., 2007). Although lysozyme is commonly found in nature, including humans' secretions and tissues, hens eggs are a richer and easily available source of the enzyme (Abdou et al., 2013).

Table 2.6 Properties of Egg White Proteins (Adapted from Mine, 2008)

Protein	Proportion of Albumen (%)	Molecular Weight (kDa)	Characteristics
Ovalbumin	54	45	Enzyme inhibitor; binds Fe, Mn, Cu
Ovotransferrin	12	76	Binds metallic ions
Ovomucoid	11	28	Inhibits trypsin
Ovomucin	3.5	110	Inhibits viral Hemagglutination
Lysozyme	3.4	14.3	Lyses some bacteria
Ovoglobulin G2	1	33-49	Good foaming agent
Ovoglobulin G3	1	33-49	Good foaming agent
Ovoinhibitor	1.5	49	Inhibits serine proteases
Cystatin	0.05	12.7	Inhibits thioproteases
Ovoglycoprotein	1.0	24.4	Sialoprotein
Ovoflavoprotein	0.8	32	Binds riboflavin
Ovomacroglobulin	0.5	760-790	Strongly antigenic
Avidin	0.05	68.3	Binds biotin
Other proteins	4.2		

2.3.1.2 Ovalbumin

Ovalbumin is often used as a reference protein in biochemistry as a carrier, stabilizer, blocking agent, and standard (Abdou et al., 2013). Two peptides, ovokinin and ovikin

(2-7) are derivatives of ovalbumin that have been associated with preventing hypertension. Ovokinin is obtained via peptic digest of ovalbumin, and ovokinin (2-7) is obtained from chymotryptic digest of ovalbumin (Anton et al., 2006). Research has also has shown antioxidant, anticancer, and antimicrobial properties (Mine 2008).

2.3.1.3 Ovotransferrin

Ovotransferrin is an iron-binding protein. It can be used as a nutritional supplement in products like protein supplements, iron supplements, and other iron fortified foods and beverages (Abdou et al., 2013). While lysozyme is effective at inhibiting Gram-postitive bacteria, ovotransferrin can inhibit Gram-negative bacteria by depriving the bacteria of iron, which is essential to its growth (Anton et al., 2006).

2.3.2 Egg Yolk Bioactive Proteins

The egg yolk contains bioactive components mostly in the form of proteins (17% of yolk) and lipids (34%). The most researched physiological functions of the egg yolk are their antibacterial, antiviral, anticancer, antioxidant, anti-inflammatory, and immunological properties (Mine, 2008). The lipoproteins, phosvitin, and livetins are the main bioactive proteins, as well as precursors to bioactive peptides, in the yolk. The hen's liver synthesizes the egg yolk protein, secretes it into the blood stream, transfers it to the ovary, and then finally to the oocyte to accumulate yolk (Mine, 2008). Often it is the case that the bioactive peptides within the protein are inactive in its native form, and released upon proteolysis during digestion or processing

(Samaraweera et al., 2011). It is worth noting that the lipid portion of the egg yolk contains many bioactives, although will not be mentioned in detail this review.

2.3.2.1 Lipoproteins

Although LDL is commonly known for its emulsifying abilities, it also has bioactive properties. One study by Shinohara et al. (1993) looked at the effects of lipoproteins on the growth and production of IgM in a human-human hybridoma cell. The LDL portion was shown to promote the production of the IgM (Abdou et al., 2013). Egg yolk has also been used to preserve mammalian spermatozoa against cold shock in frozen semen stored for artificial insemination. It has been suggested that the LDL is responsible for the resistance against cold shock, as well as improved motility after storage. The mechanism proposed for this property could be that the LDL adheres to cell membranes during the freeze-thawing, protecting the spermatozoa membranes (Anton et al., 2006). While there is not extensive research on this cryoprotective property, it could be useful beyond sperm preservation.

The HDL portion of egg yolk is important for antimicrobial activity. Kassaify and Mine (2004) studied the effect of non-immunized egg yolk powder on the pathogens *S. enteritidis*, *S. typhimurium*, and *E. coli* O157:H7 when included in the hens' feed. At powder concentrations between 5-10%, colonization of all mentioned pathogens were either eliminated or reduced in the intestines, as well as elimination or reduction in the internal organs. The results showed inhibited colonization, as well as invasion, implying anti-adhesive properties of egg yolk. A later study by Kassaify et al. in

2005 announced that this anti-adhesive effect is associated with the HDL and their peptides (Mine and Kovacs-Nolan, 2006).

The lipoproteins in egg yolk are important protein precursors to biologically active peptides. When vitellenin, an apoprotein of yolk lipovitellenin (LDL), is digested with pronase, it forms glycopeptides A and B. Glycoprotein A has a high content in sialic acid, a carbohydrate known for blood protein half-life regulation, toxin neutralization, regulation of cellular adhesion, and inhibition of cell cytosis. It is proposed that these peptides could be used as carriers of sialic acid, aiding in its adsorption and bioavailability (Zambrowicz, et al., 2014).

2.3.2.2 Phosvitin

Due to its structure, phosvitin has a high ability to chelate metals, particularly calcium, magnesium, and iron. The majority of iron present in the yolk is bound to phosvitin. Even under thermal stress, phosvitin maintains its high ability to bind iron when compared to other phosphoproteins (Samaraweera et al., 2011) Xu et al. (2007) found that phosvitin also has a higher ability to bind iron that its phosphopeptide derivatives. This metal binding capacity is partly responsible for certain bioactive traits, including antibacterial and anti-oxidative, as well as its emulsifying properties. Research has shown antibacterial activity against *E. coli* under thermal stress, as it disrupts the cells and DNA of the bacteria. This effect is caused by a combination of the metal-chelating abilty and high surface activity (Mine and Kovacs-Nolan, 2006). Though the phosphopeptides from phosvitin exhibit higher antioxidant properties, the

phosvitin itself also shows to have antioxidant activity. In a phospholipid emulsion system with Fe^{2+}, Cu^{2+}, and hemin, the phosvitin successfully inhibited Fe^{2+} and Cu^{2+} mediated oxidation of lipids, though not effective against hemin-mediated phospholipids oxidation. This antioxidant activity went down after autoclaving, however was maintained at pasteurization temperatures (Samaraweera et al., 2011).

Phosphopeptides from milk caseins are used in industry for their antioxidant, mineral-binding, and anti-microbial effects. Egg yolk phosvitin has not yet received as much attention for the production of phosphopeptides. Egg yolk phosvitin is the most phosphorylated protein in nature and could be a better source of phosphopeptides but could because it contains more phosphates in the molecule than casein (Samaraweera et al., 2011). A study by Xu et al. (2007) examined antioxidant activity and iron binding capability of tryptic digests of hen egg yolk phosvitin. They found that phosvitin, and phosvitin oligophosphopeptides (PPP) both inhibited linoleic acid oxidation, however the PPP were significantly more effective, even at a lower concentration than the phosvitin. Since PPP's only contain 35% the amount of phosphorous compared to phosvitin, they concluded that the ability of PPP to inhibit linoleic acid oxidation is not solely from its metal ion-binding capability. PPPs antioxidant effect could also be from the amino acid composition and/or sequence.

2.3.2.3 Immunoglobulin Y

Immunoglobulin Y technologies have been increasingly gaining attention for all of their potential applications, including disease prevention in humans, medical

diagnoses, and an alternative to antibiotics in animal feed. There are many advantages to using immunoglobulin Y (IgY) from hen's egg yolk over traditionally used mammalian IgG. There are benefits to animal welfare, cost effectiveness, and no problematic interactions. Antibiotics are used in the livestock industry for growth and disease prevention for over 50 years. However, in recent years, the industry has been under pressure to find an alternative because of the growing rate of antibiotic resistance in animals and humans. The E.U. banned antibiotics for growth production in 2006, and the U.S. FDA started phasing out non-medical antibiotic use in livestock at the end of 2013 (Li et al., 2015).

IgY passive immunization therapy could be an effective alternative to antibiotics. In passive immunity, the antibodies produced from one individual are transferred to another, like in the case of a mother to her newborn via the placenta or a hen to her egg. In contrast, active immunization is where the animal has to produce its own antibodies, after acquiring a disease or receiving a vaccination. (CDC, 2017) Because hens transfer their antibodies to their chick in the yolk, immunizing the hen will produce eggs composed of the specific antibodies. This could easily be done orally as a feed additive, usually as a freeze- or spray-dried powder (Marcq et al., 2013).

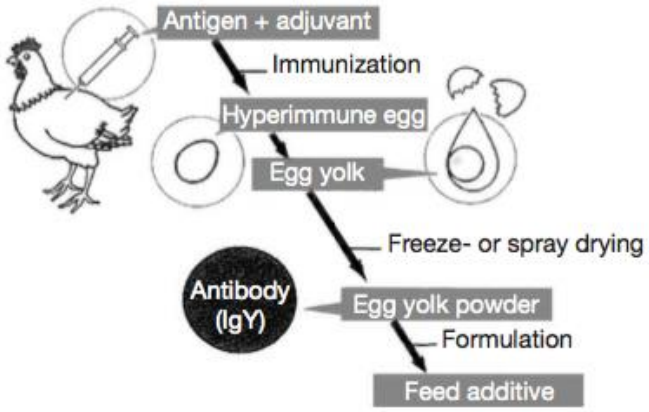

Figure 2.6 Production of antigen-specific IgY from laying hens for passive immunization via animal feed (Marcq, et al., 2013)

Oral administration of IgY has shown it could be a valuable treatment for numerous enteric infections, including rotaviruses, enterotoxigenic *E. coli*, *Salmonella*, and *H. pylori*. Studies with oral treatment of pathogen specific IgY have been successful in treating rotavirus, *E. coli* and *Salmonella* in studies done on animals (Karlsson et al., 2004).

Antibodies specific to certain pathogens can effectively be orally administered to humans to build protective immunity. Unless someone has an allergy to eggs, IgY from eggs can be given orally with virtually no risk of side effects because eggs are a normal dietary ingredient (Anton et al., 2006). *Heliobacter. pylori* is a pathogen that resides in the gastric and duodenal muscosa that causes gastritis and ulcers and infects over half the population worldwide. Antibiotics are usually administered to patients with H. pylori, but are sometimes ineffective or have adverse side effects. When *H. pylori* colonizes in the gastric mucosa, it produces urease enzyme to degrade urea into ammonia. *H. pylori* uses the ammonia to neutralize the microenvironment in gastric

muscosa. Subsequently, urease specific IgY has ben created, and has proved to work to suppress the *H. pylori* colonization. In Japan yogurt fortified with IgY urease made from egg yolk was developed to decrease *H. pylori*. Clinical studies in Japan, Korea, and Taiwan all showed significant results in a decrease in the bacteria in volunteers who ate the yogurt twice daily for 12 weeks (Anton et al., 2006).

Although specific antibodies have been prepared and collected from the blood serum of small mammals such as rabbits, goats, and guinea pigs, there are many advantages to using chicken IgY as an alternative. It is a far less invasive procedure to procure IgY from eggs than it is to procure IgG from mammals. For eggs, the chicken is vaccinated and the eggs collected. The yolk and white are separated, and the IgY is extracted from the yolk from a choice of extraction processes. It is a much more invasive procedure to extract IgG from mammalian serum because the animals must be bled to obtain the antibodies. Also, the egg yolk only has IgY to begin with, while mammalian serum additionally has IgA and IgM that need to be separated out (Mine, 2008). In addition to animal welfare, egg yolk is a more cost effective source than mammals. One hen can produce the same amount of antibodies in a year that would take 4.3 rabbits to produce. The cost is also lower to maintain laying hens than mammals (Li et al., 2015). Functionally, chicken IgY is advantageous to use in humans over mammalian serum IgG because there is no cross reactivity between chicken IgY and mammalian IgG. Mammalian IgG activates with Fc and complement receptors, which is not a problem with chicken IgY. Not only does this make IgY a better choice for immune responsiveness in humans, but also reduces interference

problems when used in immunological assays (Karlsson et al., 2004). Another study by Nilsson et al. (2012) looked at the stability of IgY stored at room temperature, above 4°C. Over a six month period, they found no reduction in antibody activity. This means that eggs could potentially stored for at least six months at room temperature without decline in antibodies, allowing increased batch sizes in production.

Table 2.7 Egg yolk IgY compared to mammalian IgG (Adapted from Mine, 2008)

Egg Yolk IgY	Mammalian IgG
Molecular weight of ~180,000 kDa	Molecular weight of ~150,000
H-chain region contains 4 domains	H-chain region contains 4 domains
Isoelectric point= ~6.0	Isoelectric point =~7.0
Thermal denaturation= 73.9°C	Thermal denaturation=77.0°C
Does not activate complement of mammals	
Does not combine with proteins A and G (IgG binding proteins)	
Does not combine with a rheumatic factor	
Does not combine with Fc receptor of mammalian a cell	

2.4 Effects of processes on structure and functionality of yolk fractions

When egg yolk is fractionated, the plasma and granules behave differently than the native yolk. By using the fractions, as opposed to the whole yolk, there is more of an opportunity to maximize the functionalities of the yolk, as well as by products that are

produced from processes like protein extractions. The microstructure of the egg is what gives it much of its functionality, and can be exploited by different treatments. Modifications of the pH, ionic conditions, and presence of mineral cations all closely effect the structure and function (Causeret et al., 1991).

2.4.1 Salt

Ionic strength greatly affects the solubility of the granule fraction, but not the plasma. At ionic strength below 0.1M NaCl, complete yolk is 25% solubilized. For plasma, it is about 80% solubilized, and granules only 10%. The solubility for the yolk and granules rose to ~80% with an increase to 0.3M NaCl, while the plasma remained at 80% solubility. When raised to 0.5 M NaCl, these values remained the same (Anton and Gandemer, 1997). The granules reach complete disruption at 1.71M NaCl, releasing the HDL and phosvitin from their complexes. The solubility increase with addition of NaCl is because phosvitin is a soluble protein at low levels of salt, and HDL behaves like soluble proteins (Anton, 2013). The reason for low solubility of granules below 0.3M NaCl, is due to the structure; specifically the HDL-phosvitin complexes. When NaCl is increased to above 0.3M, the phosphocalcic bridges linking HDL and phosvitin are disrupted by monovalent sodium (Anton and Gandemer, 1997). Emulsions made with yolk, plasma and granules all showed no difference at 0.5M NaCl, however, in distilled water the granules have a lower emulsifying activity than plasma. It is suggested that the granules have a similar emulsifying capability at 0.3M NaCl that plasma has in its native form (Strixner and Kulozik, 2013).

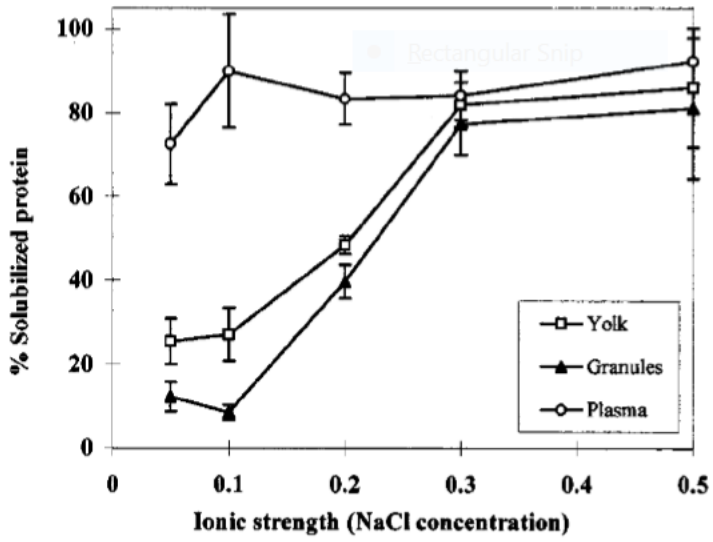

Figure 2.7 Effect of NaCl on protein solubility at pH 7 (Anton and Gandemer, 1997)

2.4.2 pH

Previous findings about the pH of the granule fraction has shown a mixed relationship with functionality of the product. Causeret (1991) observed that when the original pH, in the case of the study 6.3, that both an increase and decrease in pH could disrupt the granules. At a pH less than 4.2 or above 6.3, the solubility and viscosity of the mixture increased. The acidification or alkalization both break up the phosphocalcic bridges that link the HDL and phosvitin. However, when brought back to original pH, the solubilization was reversed and returned to the original low solubility. Research by Anton (2013) shows that pH does not have a strong effect on the solubility and emulsifying properties of plasma, but a strong effect on granules. His study found that granules are insoluble aggregates and were essentially unusable at too low of a pH, but become soluble micelles at a pH ~7.

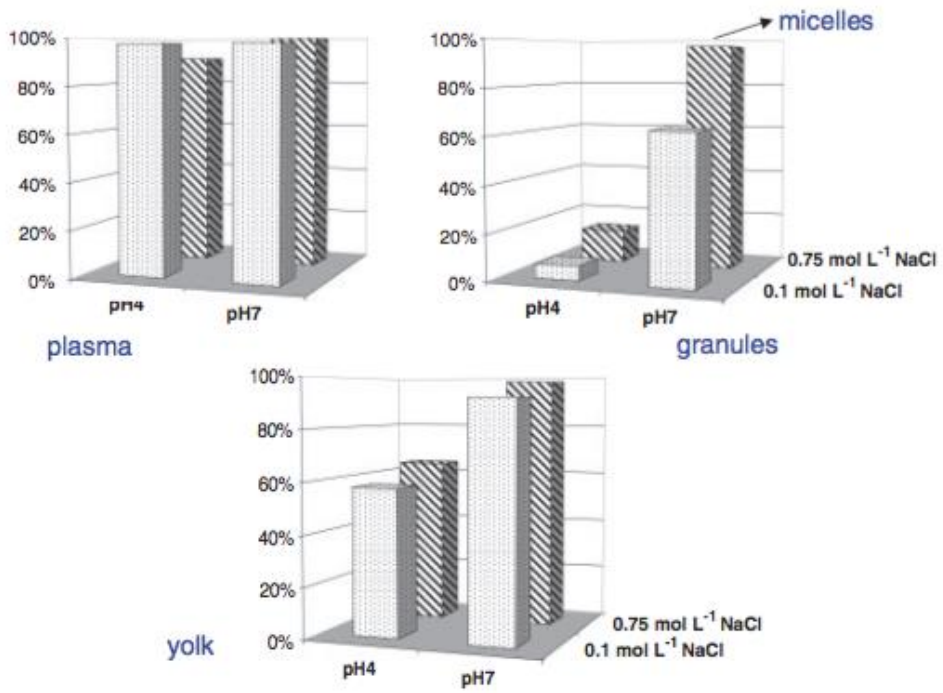

Figure 2.8 Effect of pH and NaCl on protein solubility (Anton, 2013)

2.4.3 Temperature (Freeze/Thaw)

The effect of temperature on egg yolk products is particularly important because egg products are often pasteurized to a temperature between 60-68°C microbial safety. Even so, pasteurization temperatures do not completely remove all pathogenic organisms, and egg products must be kept at 4°C with a limited shelf life. Studies have shown that granules are resistant to heat treatment, while plasma is sensitive. This is largely due to the denaturation that occurs in the plasma proteins, y-livetins, a-livetins, and LDL (Denmat et al., 1999). The granule proteins' abilities to resist heat was originally thought to be because of the phosphocalcic bridges, but newer research suggests otherwise. A study by al Anton et al. (2000) showed that although β phosvitin and β HDL were not affected by heating, α-HDL was. Since both forms of HDL are linked by the phosphocalcic bridges with phosvitin, this complex is not what

protects the proteins from denaturation. Rather, it could be that difference in protein composition is responsible for its heat resistance. Yet, the native structure of the granules does restrict aggregation of the HDL and phosvitin, avoiding gelation and decreased protein solubility. This implies that the granules could be heated to pasteurization temperatures without losing emulsifying capabilities (Anton, 2013) A study by Denmat et al. (1999) showed the emulsifying stabilization of both granules and plasma are mostly unaffected by heat, the emulsifying activity went down for plasma above 72°C, while granules remained unaffected.

Freezing is a way to preserve yolk and yolk products in storage over longer periods of time. Yolk undergoes gelation during freeze thawing, likely from the LDLs (Anton, 2013). Under a scanning electron microscope it was shown that an unfrozen sample of egg yolk had a close-knit structure with small voids, compared to the frozen-thawed yolk. The frozen-thawed sample had a much coarser network appearance. They also saw a more open network when the samples were diluted, and conversely, an even closer knit structure upon concentration. This could mean that the addition or removal of water could result in lipoprotein destabilization and aggregation (Hasiak et al., 1972).

2.5 Impact on Industry

The American Egg Board (AEB) reports that today the United States produces roughly 75 billion eggs per year, at 10 percent of the world's supply. Of the supply, 60 percent are used by consumers, 9 percent used by foodservice industry, and about

30 percent used for food manufacturing industry. As specific egg components are becoming used more and more for their functional properties in both the food and cosmetic industries, there is a growing demand for industrial scale extraction and isolation processes that are safe for consumers.

2.5.1 Large Scale Protein Extractions

The development of antibodies for immunotherapeutic and immunodiagnostic therapies is expanding. Because of the many advantages of chicken IgY over mammalian IgG, eggs as a source of antibodies is increasingly important. This poses the problem of isolating the IgY because of the high concentrations of lipids present in the egg yolk (Nilsson et al., 2008). Early publications of methods for IgY extraction were not efficient at the industrial scale, and often were not food-safe, as they relied on the use of organic solvents. Methods since have become more cost efficient and safer, utilizing salt, adjusted pH, temperature, and food gums.

Multiple methods for extracting and isolating IgY based on a simple water dilution have been studied and published. Akita and Nakai (1992) compared four different purification methods of IgY: water dilution, polyethylene glycol, dextran sulphate, and xanthan gum. The study concluded that the water dilution was the most efficient method because the procedure was simple, produced high yields of active IgY, and can be easily adapted for large scale production. In the water dilution method described by Akita and Nakai, they used a 9:1 water to yolk dilution, incubated it at 4°C for 6 hours, and then centrifuged it. Upon centrifugation, the granules formed a

compact sediment on the bottom, and the supernatant could be easily decanted off for further purification treatments.

Modifications to the basic water dilution methods have been able to improve efficiency of the purification method. Akita and Nakai (1992) used a sixfold water dilution with an adjusted pH of 5.0-5.2 incubated at 4°C for 6 hours to obtain a recovery of IgY at 93-96%. Another study by Hodek et al. (2012) utilized pH, salt, and freeze thawing in their water dilution method. They used a 1:7 water to yolk dilution at pH 5.0, and then froze the contents to -20*C and thawed before additional adjustments to the water soluble fraction. Following thawing, they adjusted the pH to 4.0 and added NaCl to a concentration of 8.8% before centrifugation. They obtained a purity of 97% IgY recovery.

2.5.2 By Products

Eggs are becoming an important source of phospholipids and immunoglobulins, and their extractions leave large quantities of by-products behind. Many of these by-products are full of valuable bioactive and functional elements. As the demand for these large scale extractions has been recognized over recent years, there has been a growing interest to utilize the by-products left behind.

2.5.2.1 Enzymatic Proteolysis

The by-products of egg extractions are potentially full of proteins and peptides with bioactivities. The bioactive peptides are inactive in their precursor sequence, but once

released, interact with receptors in the body, regulating functions of systems such as nutrient uptake, immune defense, antioxidant, antimicrobial, antiadhesive, and ACE-inhibitory. A common method to obtain the bioactive peptides is to use enzymatic proteolysis with enzymes from animals, plants, or microbes (Eckert et al., 2014).

A study by Eckert et al. (2014) examined the use of an unconventional proteinase from Asian pumpkin (*Cucurbita ficifolia*) to obtain bioactive peptides from the by-product of a phospholipid extraction from egg yolk. They found that the serine protease from *C. ficifolia* successfully recovered peptides with ACE-inhibitory properties. Another study by Zambrowicz et al. (2012) investigated the antioxidant and antimicrobial activity of hydrolysates from egg yolk by-product after lecithin removal. They prepared hydrolysates from trypsin, chymotrypsin, and pepsin. Higher antioxidative effects were found in hydrolysates treated by trypsin and chymotrypsin than those of pepsin. They also found that the trypsin treated hydrolysates showed weak antimicrobial activity against some *Bacillus* species. Park et al. (2013) also looked at the antioxidative properties of peptides prepared from protein hydrolysate of lecithin-free egg yolk with the enzyme Alcalase. They found antioxidative properties present, and varied among different molecular weights.

2.5.2.2 Rheological Properties

The by-products from industrial processes have also been considered for their rheological properties in foods. After removal of livetins, the physiochemical properties of egg yolk are altered, but have potential to be salvaged as an emulsifier.

Emulsions using this by-product are less stable than egg yolk. They have a higher viscosity and viscoelastic moduli, likely because of an increase in hydrogen bonds and hydrophobic interactions in this fraction (Zambrowicz et al., 2012). Marcet et al. (2014) considered egg yolk granules for a low-cholesterol replacer of whole egg yolk in gluten-free muffins. They found that the bakery product made with the granules was similar to the one made with whole egg yolk. However, they also found that the viscoelastic behavior of the batter was altered. The effect on the baked product was a harder muffin and a change in color. There are confirmed differences in the rheological properties of egg yolk fraction by-products compared to whole egg, and more research needs to be done with consumer testing to see if using yolk by-product as a replacement for egg is a viable option.

2.6 Summary

With increasing novel uses for specific egg portions in the food, cosmetic and pharmaceutical industries, there is more of a need for large scale commercial extractions. The food industry produces substantial amounts of "waste" by-products from different egg processing. These by-products are known to be rich in bioactive proteins, antibodies, and functional phospholipids (Schmidt et al., 2006). Further, Granules are often a by-product from defatted lipid and IgY extractions (Eckert et al., 2014). With the recognition of IgY as an important source of antibodies, there is a significant need to find uses for the granule by-product of industrial scale IgY extractions. While previous literature has focused on techniques for such extractions, the utilization of the protein by-products has not been widely studied and reported on.

By adapting techniques used on whole egg yolk extractions for the granule by-product portion, there is potential for separation of more proteins. Addition of salt is a known way to disrupt the granule fraction. Further, the change of density with addition of salt inverts the granules above the water soluble (WS) layer. This adaptation could optimize the processing and collection of protein by eliminating unnecessary steps and equipment used to siphon the WS layer from the top, and instead collect it from the bottom of the tank.

Consequently, this project seeks to find potential uses for protein by-products of industrial IgY extractions by further treatment (filtration, salt addition, and pH adjustment). Based on previous literature, the following hypotheses were developed:

1) The egg yolk granule by-product can further fractionate and recover more water soluble proteins from lipid fraction by mechanical treatment (filtration and centrifugation).
2) The amount of crude protein salvaged from egg yolk granule by-product can be increased/optimized with changes in pH and salt concentration.
3) Recovered protein and protein hydrolysates can have antibacterial properties against *Escherichia coli (E. coli)*

The objectives of this study include:

1) To characterize the recovered protein after the different treatments (mechanical, salt, pH) in yolk by-product.
2) To determine the protein concentration at which antibacterial properties are effective against *E. coli*

3) To use salt to attempt to invert the fractionation of the insoluble yellow layer on top and water soluble layer on bottom for ease of collection.

CHAPTER 3: MATERIALS AND METHODS

3.1 Yellow Cake Collection and Standardization

3.1.1 Collection

A by-product from an experimental pilot plant IgY extraction, which will be called yellow cake, was obtained from lipoprotein fraction of a 3 part water soluble protein extraction from a project run by Nestle. Pasteurized, non-homogenized, egg yolk product used for original Nestle extraction was prepared by La Belle Associates, Inc. Sixty pounds of egg yolk were added to stainless steel conical tank. After a 10 fold water egg yolk dilution was made, stirred for six hours, and allowed to settle for 12 hours, a yellow lipoprotein sedimentation formed on the bottom of the tank, showed in Figures 3.1 and 3.2 A lobe pump was used to siphon out the water soluble IgY fraction from the tank. The sediment was diluted again following the same steps twice more. The water soluble fraction was used for further isolation and purification for an industry project, leaving behind a yellow lipid/protein sedimentation on the bottom of the tank. This waste layer, the yellow cake, was collected in containers by opening the valve at the bottom of the tank. After collection, the yellow cake was frozen in sanitized plastic tubs and stored at -20°C degrees. Three independent replicates of yellow cake samples were obtained from different weeks.

Figure 3.1 Phase Separation of egg yolks during IgY extraction. Water soluble layer is visible over lipid layers

Figure 3.2 Yellow cake solids in tank before removal (work of this thesis project)

3.1.2 Standardization

Frozen samples of yellow cake were given forty-eight hours to thaw at 4°C. Once thawed, the yellow cake was gently stirred continuously for one minute to ensure a homogenous sample. The CEM LabWave9000 Microwave Moisture/Solid Analyzer was then used to measure the percent total solids of the yellow cake under the settings for cream. The percent solids readings were taken three times and then averaged. The results were used to dilute the yellow cake to a solution of five percent solids in tap water. The samples were prepared in seven 10 gallon, stainless steel milk cans at 15 pounds of solution each.

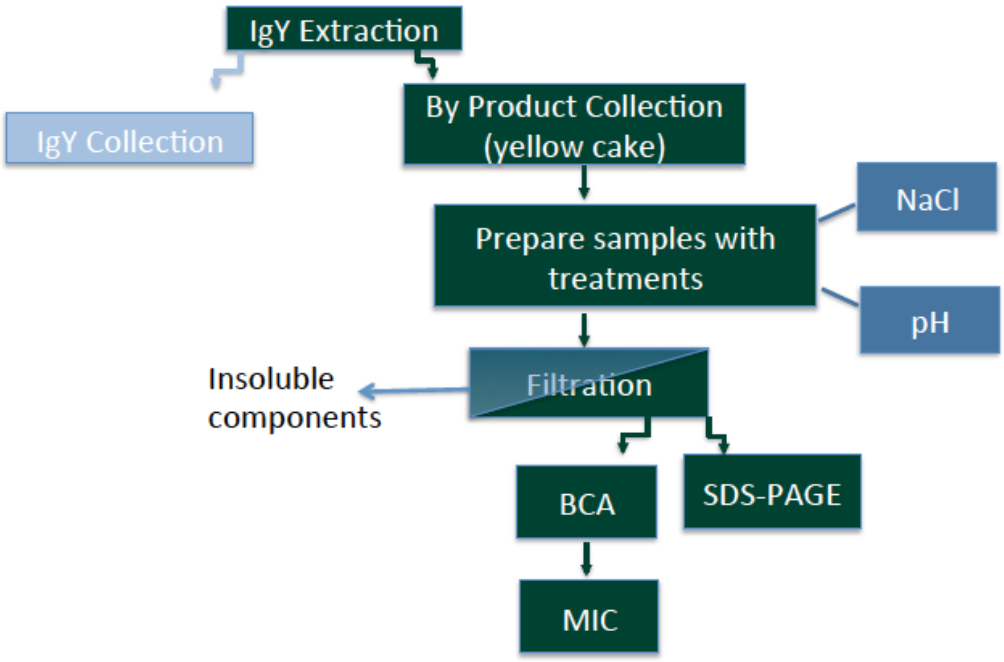

Figure 3.3 Flow diagram indicating the procedure to generate the samples and treatments in this work.

3.2 Sock Filtration

3.2.1 Sample Preparation

The five percent solids yellow cake solutions were prepared at four different pHs: 4.6 ± 0.05, 4.8 ± 0.05, 5.0 ± 0.05, and 5.2 ± 0.05. Experimental treatmeants are shown in Figure 3.3. A 10% Lactic Acid solution was added to the fifteen pounds of yellow cake solution to adjust pH. These four solutions at different pHs had no salt added. PerpHecT digital LogR Advanced Benchtop pH/mV/ORP/ Temperature Meters Orion 350 was used for pH measurements.

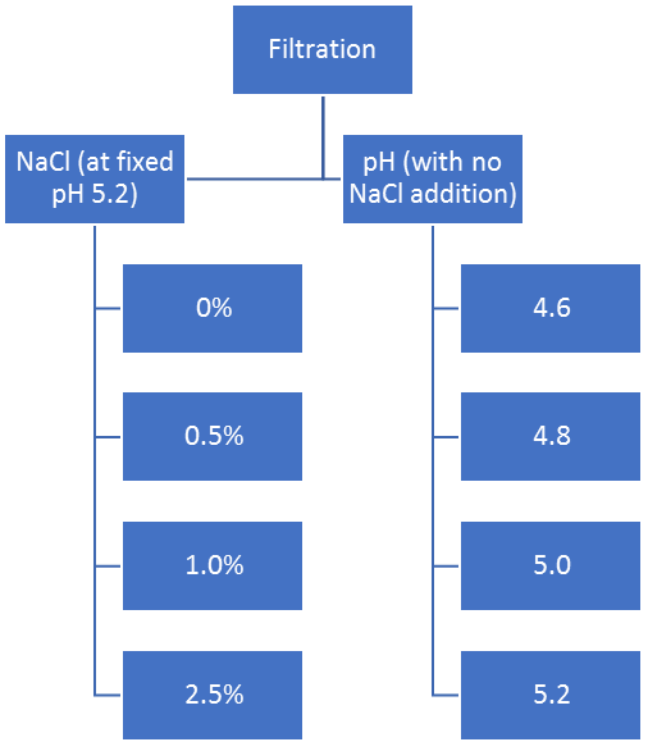

Figure 3.4 Experimental Design for Sock Filtration Experiment

The five percent solids yellow cake solutions were prepared at four different NaCl concentrations by weight: 0%, 0.5%, 1%, and 2.5%. NaCl was added to milk cans

with 15 lbs of yellow cake solution and mixed vigorously for two minutes before pH adjustment.

3.2.2 Filtration

Tri Clover Mainstream filter, shown in Figure 3.5, was used with a 100 micron mesh sock filter to pass through the yellow cake solutions through the first pass. The solution was pumped through the filtration system. A hose was used to pass the treated yellow cake samples through the pump, which connected to the filter system via a plastic hose, and passed through the 100 micron filter and out of stainless steel piping. Tap water was run through the filter first for thirty seconds, and then the yellow cake solution was pumped through, and filtrate collected into clean milk cans for a second pass. A second pass was repeated with the filtrate through a 10 micron felt sock filter. Filters were cleaned and changed, and system was emptied and cleaned in between each sample can.

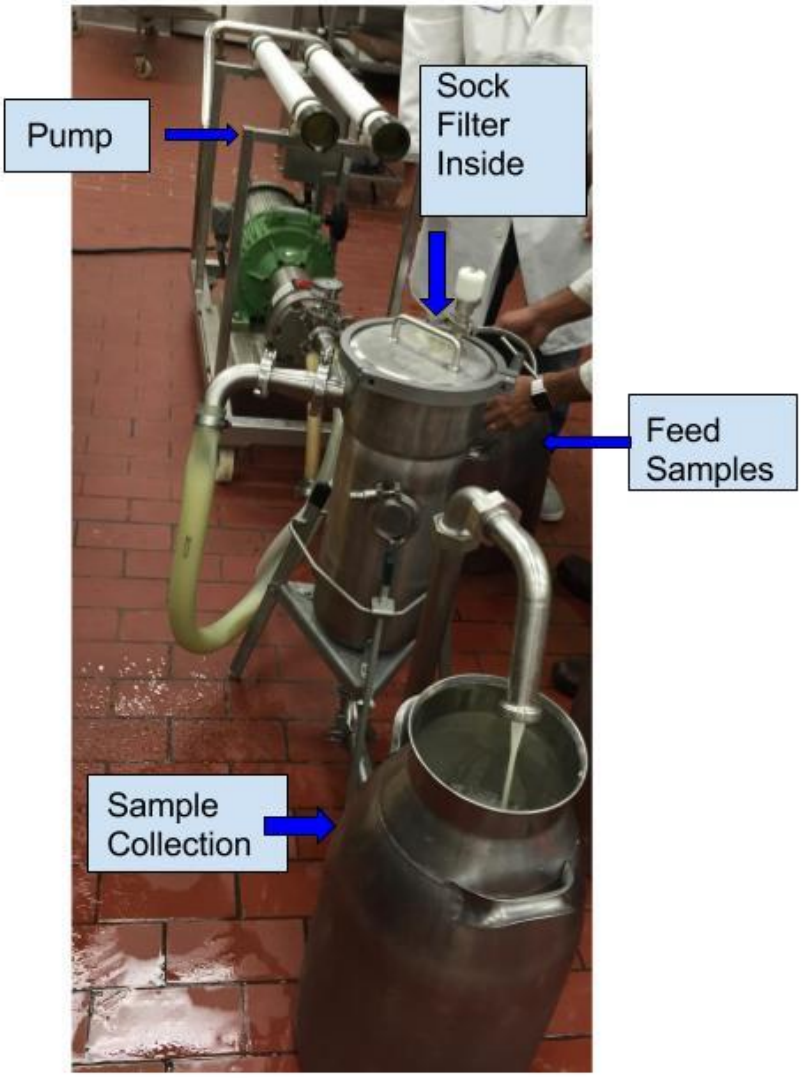

Figure 3.5 Sock Filtration Set up

Figure 3.6 Filter after running samples through

Figure 3.7 Filtrate samples collected after passing through filter system

3.2.3 Sampling

After pushing water through the filter, the samples were pushed through and after 30 seconds the filtrate started to come through yellowish in color. After another 30 seconds upon color change, samples were collected in milk cans until all of the sample was pushed through, shown in Figure 3.6 and 3.7. Additional water was pushed through after all of the sample solution was taken up. Thirty seconds after the water was added, sample collection stopped and the remainder filtrate was discarded. This was to ensure a consistent sampling procedure to optimize a most pure sample undiluted with the initial water and final water. Yellow lipid solids left in filter were discarded. The collected protein solution was kept for analysis.

3.2.4 Filtrate Storage

Two complete sample sets of filtrate (protein) for each of the three trials were stored in Falcon 50 mL Conical Centrifuge tubes for analysis. Remaining samples were stored in 7 oz Whirl-Pak write on bags used for the precipitation portion of the project. Upon collection and bagging, they were immediately frozen at -20°C degrees until analysis.

3.3 Precipitation

3.3.1 Sample preparation

Frozen samples of yellow cake solids were thawed and standardized to a five percent solids solution in water with the same procedure as 3.1.2 at a lab scale. 500 mL of

solution were prepared in 1000 mL glass beaker. Yellow cake solution was distributed into ten Falcon 50 mL Conical Centrifuge tubes, with 30 mL in each tube. Preliminary experimentation showed that a minimum of 2.5% NaCl yellow cake solution was needed in order to make the lipid layer start to separate and float separation after centrifugation. NaCl concentration of each tube was adjusted to 3%,4%,5%,6%, and 7%. Appropriate weights of NaCl were measured and added to the solutions. They were then dissolved by vigorously shaking the tubes by hand. Two tubes were made of each concentration. Figure 3.8 shows a flow of this experiment.

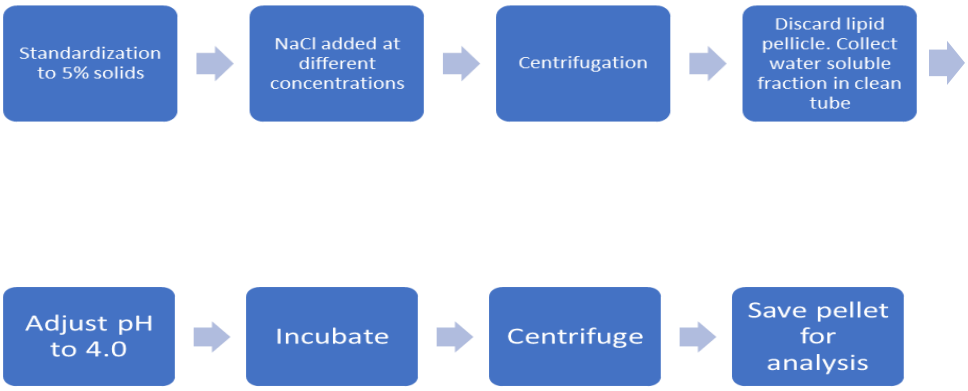

Figure 3.8 Precipitation Design Flow

3.3.2 Centrifugation

Eppendorf Centrifuge 5810 R was used to centrifuge the samples in the 50 mL falcon tubes samples were prepared in at 4000 RPM for 20 min at 4°C. After centrifugation, a gelatinous yellow layer of lipid was on the top with a clear layer on the bottom. The clear water soluble layer from each sample was extracted using a 50 mL pipette and put into a new 50 mL falcon tube. The lipid layers were discarded.

3.3.3 Acidification and incubation

Hydrochloric acid .5M (HCl) was used to acidify all of the remaining water soluble portion of the samples to pH 4±0.05. One sample tube of each salt concentration was left to incubate at room temperature for 1.5 hours, while the other sample tube for each salt concentration was incubated at 4°C for 1.5 hours. After the incubation period, samples turned to a milky white color, and started to have sediment settle at the bottom. All sample tubes were centrifuged a second time at 4000 RPM for 20 minutes at 4°C.

3.3.4 Collection and storage

After second centrifugation, white pellets formed on the bottom of each falcon tube, with a distinct clear layer on the top of all but two tubes. The tubes with 3% NaCl had some yellow particles still suspended in the water. The water layer was decanted for each tube and discarded. The pellets were each dissolved in 5mL of DI water and stored at 4°C for one day until analysis.

3.4 Protein Analysis

3.4.1 Protein quantification

3.4.1.1 Preparation

The frozen treated samples of yellow cake were taken out of the freezer to thaw overnight for protein analysis. Samples were balanced and put in Falcon 15mL Conical Centrifuge tubes. An Avanti J-E Centrifuge (Beckman Coulter) was used to

centrifuge samples at 4,500 RPM for 15 minutes at 4° C with a JS-5.3 rotor. Supernatent was analyzed.

3.4.1.2 BCA

A bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL) was used to detect and quantify the total protein. To prepare the protein standard for the appropriate working range for the Microplate Procedure (Pierce Biotechnology Rockford, IL), a 1 mL ampule containing bovine serum albumin (BSA) at 2mg/mL in 0.9% saline and 0.05% sodium azide was diluted into mini eppendorf tubes (.5 mL) using DI water as the diluent. The samples were prepared in two different dilutions to assure the samples would fall into the working range. Preliminary studies showed that all samples would have best results undiluted or with a 1:9 dilution. Samples were run undiluted, as well as 1:9 dilution. The following formula was used to calculate the total volume of working reagent (WR) required:

The WR was prepared by mixing 50 parts of BCA Reagent A from the kit with 1 part of BCA Reagent B. Reagent A contained of sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide. Reagent B contained 4% cupric acid. WR was prepared immediately before use. Twenty five µl of each sample were pipetted into separate wells in a 96 well microplate (Pierce Biotechnology Rockford, IL). 25 µL of each standard were also pipetted into separate wells. Each sample and standard were run in duplicate and averaged. 200 µL of the WR was added to each well and mixed on a shaker plate for 30 seconds. The plate

was covered with parafilm and incubated at 37°C for 30 minutes. Following incubation, the plate was cooled to RT. The absorbance was measured at 562 nm on a plate reader. A molecular devices SpectraMax M2e device was used to read absorbances.

3.4.2 Protein characterization

3.4.2.1 Preparation

SDS-PAGE was used to characterize the proteins in different treatments of pH and salt concentration in the filtration experiment and for the different concentrations of salt and temperature in the precipitation experiment. Samples analyzed with SDS-PAGE were from the same preparation and sample pool as section 3.4.1.1. The samples were then diluted in a 1:1 ratio with Lammeli buffer. To prepare the sample buffer, 2x Lammeli buffer (Bio-Rad Laboratories, Hercules, CA) was mixed in a 19:1 dilution with β-mercaptoethanol (ME) (Fisher Scientific). 1.9 mL of 2x Lammeli buffer was mixed with 100 uL of ME. 50 µL of buffer were mixed with 50 µL of sample in mini eppendorf tubes. Tubes were boiled for 5 minutes. After cooling to room temperature, samples were centrifuged in a minispin plus eppendorf centrifuge (Sigma-Aldrich) for 60 seconds at 12,000 RPM. For the precipitation experiment, samples from section 3.3.4 were shaken on a vortex for 30 seconds each before gel preparation same as above. However, the precipitation samples were additionally prepared in a 1:10 dilution with buffer.

3.4.2.2 Gel Electrophoresis

Mini-PROTEAN TGX Precast 4-20%, 10 comb, 50 uL well Gels (Bio-Rad Laboratories, Hercules, CA) were assembled into running module cells and chambers were filled with 1X Tris-Glycine-SDS buffer(Bio-Rad Laboratories, Hercules, CA). 10 uL of Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories, Hercules, CA) and 10 uL of each prepared sample were loaded into each well. The gels were run with a Bio Rad PowerPac Basic power supply at 90 V until the protein bands entered the resolving gel. The voltage was then increased to 110 V until the bands reached the reference lines and power turned off. The process took approximately one hour and forty five minutes.

Gels were stained for 48 hours in Coomassie Brilliant Blue with shaking. Following staining, gels were destained with destaining solution(40% methanol, 10% acetic acid, 50% deionized water). Gels sat in destain solution with shaking for 20 minutes, then the destain solution was changed out for fresh and remained destaining for 24 hours. After 24 hours, the gels were rinsed with DI water. The gels were imaged on a Molecular Imager Gel Doc XR+ with Image Lab 4.1 Software (Bio-Rad laboratories, Hercules, CA) with Trans white light.

3.5 Antibacterial Function of bioactives

3.5.1 Bacteria and broth preparation

3.5.1.1 *E. coli*

E. coli strain ATCC 25922 was grown on LB media using the streak plate method from pure culture. Plate was incubated overnight at 37°C. The next day the *E. coli* was inoculated in liquid media. 3 mL of sterile Mueller Hinton broth were added to sterile test tube. Bacteria sample was scooped from plate into test tube and then incubated for 24 hours at 37°C. Prior to MIC procedure, turbidity of *E. coli* was adjusted to 1-2 x 10^8 cfu/mL at OD 600. A blank cuvette of 1mL of MHB broth was run at 600 nm in the SpectraMax M2e, followed by a 1:10 dilution of the bacterial culture. The results were a concentration of 1.7 x 10^9 cfu/mL.

Equation 1. To calculate 5 mL of 10^8 cfu/mL of *E. coli*
(1.7 x 10^9)/mL (X) = (1 x 10^8)/mL x (5mL)
X=0.294 mL of *E. coli* in 4.7 mL of MHB

200 uL of adjusted bacterial suspension was added to 19.8 mL of sterile MHB to prepare 20 mL of a 1:100 dilution of inoculum.

3.5.1.2 Broth

Mueller Hinton Broth was prepared by adding DI water to a flask with MHB powder. It was stirred with heating until dissolved. It was then autoclaved for 15 minutes, and cooled and stored until use.

3.5.2 Minimal Inhibitory Concentration (MIC)

The MIC procedure used was adapted from the method described in Nature Protocols by Wiegand et al. (2008). This procedure was mostly used to find the MIC for antibiotic drugs, but was adapted for proteins and peptides with bacteriostatic properties.

3.5.2.1 Protein standardization and preparation

BCA was run (Section 3.4.1) on filtration protein samples immediately prior to MIC procedure. Results from BCA were used to standardize solution concentrations of 256 µg/mL of crude protein in MHB. Protein samples were diluted in MHB for MIC plates without TrypZean. Protein samples were diluted in PBS (Bio-Rad Laboratories, Hercules, CA) for proteolyzed plates. 1 mL of each sample was prepared.

3.5.2.2 Controls

Each MIC microtiter plate had 3 individual columns controlling for three different variables. The unhydrolyzed plates each had one column with a mixture of *E. coli* and MHB with no protein to ensure adequate bacteria growth from culture. The proteolyzed plates had this column with PBS as well, to ensure the growth in the presence of PBS. Each plate had one column of the standardized 256 µg/mL protein samples with broth and no bacteria to control for contaminated protein. The non proteolyzed plates also had a column with just broth to control for contaminated broth. The proteolyzed plates had this column with MHB and PBS.

3.5.2.3 Dilutions

For each trial, 50 uL of MHB were pipetted into columns 2-10, and 12 of independent sterile 96 microtiter plate. 100 uL of MHB was added into column 11. For each row 100 uL of standardized 256 µg/mL peptide solution was pipetted into column 1. Hydrolyzed plates used the digested samples to be described in the next section. For each row, 50 uL from column 1 was withdrawn and pipetted into column 2 with a multichannel micropipette. This step was repeated for dilution up until column 9. Fresh tips were changed for each withdrawl, and lids were kept close on microtiter plates were on whenever not in use to minimize chance of contamination.

3.5.2.4 Inoculation

Each well containing the peptide solution and growth control well were inoculated with 50 µL of the bacterial suspension. This resulted in the desired inoculum of 5x 10^5 cfu/mL. The microtiter plates were then incubated at 37°C for 20 hours. After the 20 hours, the plates were observed and minimum inhibitory concentrations determined by growth/no growth across the plates. Figure 3.9 visualizes the plate.

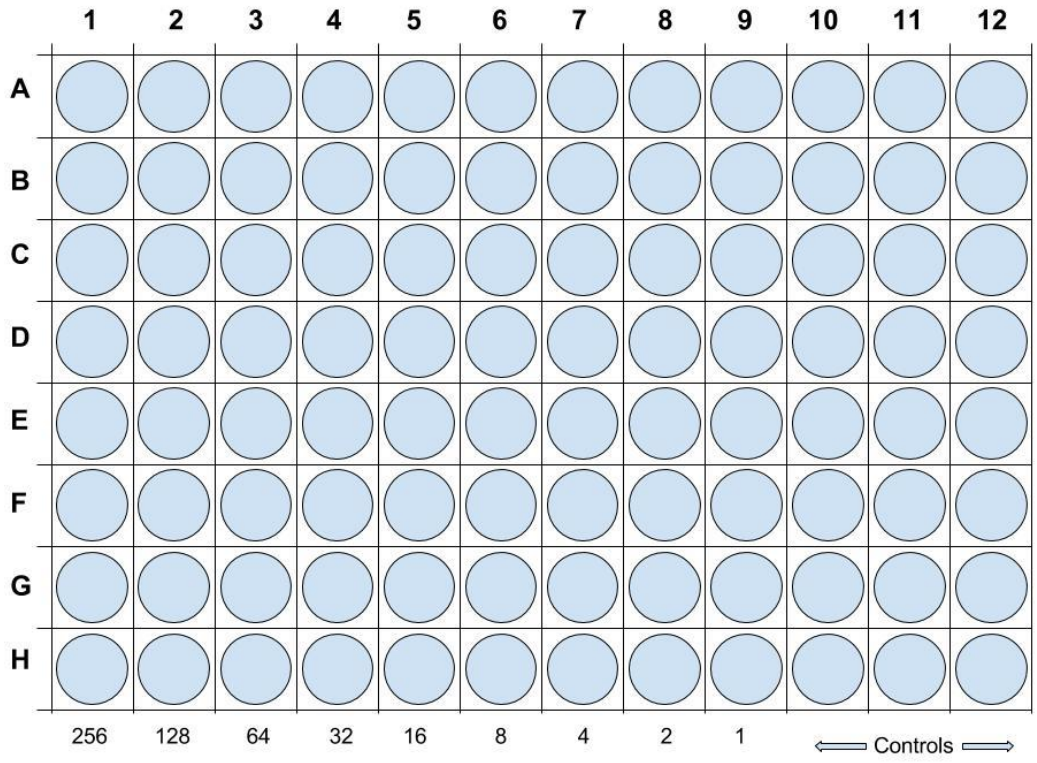

Figure 3.9 Example of MIC plate. Protein across rows is in µg/mL

3.6 Enzyme Digest

3.6.1 TrypZean preparation

Sample of TrypZean from Cal Poly with a specific activity of 4538 USP units/mg was used to digest protein samples from the filtration experiment. To prepare a 20mg/mL concentration of TrypZean, 10 mg of lyophilized dessicated TrypZean powder was mixed into 500 uL of 1mM HCl. To make the 1mM HCl, 100 uL of 1M HCl were added into 99.9 mL of DI water.

3.6.2 Proteolysis

The MIC assay was run both with and without proteolysis on the samples. Each trials 1-3 had their own plate for an MIC assay without proteolysis performed, and each

had a separate plate for proteolysis without an endpoint. Trial 2 had 2 additional plates to test proteolysis with an endpoint.

2.56 uL of prepared TrypZean were added to each standardized protein sample from trials 1-3 and incubated for 2 hours at 37°C. For trial 2 samples this portion was run an additional 2 times to test different endpoints. After incubation period, one set of trial 2 samples were boiled for 5 minutes. One set from trial 2 samples were heated to 50°C for 5 minutes. All other samples had no endpoint. These samples were then pipetted into wells on microtiter plate for serial dilution and inoculated according to the same procedure in 3.5.2.3 and 3.5.2.4.

3.7 Statistical Analysis

JMP pro 13 was used for analysis in the sock filtration experiment. Response for protein amount was analyzed using a two factor ANOVA. Results were considered significant with a p-value < 0.05. Statistical analysis and graphs for the raw data can be found in the appendix as noted.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Sock Filtration

4.1.1 Protein Content

SDS-PAGE was used to profile the proteins contained in the yellow cake after filtration. Samples were subjected to and analyzed under seven different conditions, considering two variables: NaCl content and pH. After diluting samples, gels were run accordingly, taking care that the tracking dye did not travel farther than the bottom of the gels. Gels were stained using Coomassie Brilliant Blue to view the bands.

Figures 4.1, 4.2, and 4.3 illustrate the protein content in the three replicated trials for the sock filtration. For all all three figures: Lane one represents the protein standard. Lane 2 represents 0% NaCl, pH of 5.2. Lane 3 represents 0.5% NaCl, pH of 5.2. Lane 4 represents 1% NaCl, pH of 5.2. Lane 5 represents 2.5% NaCl, pH 5.2. Lane 6 represents 0% NaCl, pH of 4.6. Lane 7 represents 0% NaCl, pH of 4.8. Lane 8 represents 0% NaCl, pH of 5.0.

All three trials showed the same migration patterns. Most bands appeared in the 37-75 kDa range, though some very light. In all seven treatments, two most distinct bands appeared at 75 kDa and ~45kDa. There were also lighter, but distinct bands among all samples at 37 and ~65 kDa, although barely detectable in trial 3 image. In the image for trial 2, lanes 4 and 8 have extra bands appearing at 125 and ~119 kDa. In all images, lane 5 had much darker, distinct bands, as well as more bands. These bands

appeared at 250 kDa, ~30(absent in trial 1) ~8, ~13, and a darker diffuse band between 100-130 kDa (faint in trial 1). In general, bands were most pronounced in trial 3, and faintest in trial 1.

Bands were compared to known weights of expected proteins in previous literature. Protein identities were estimated by comparison with mobilities of standards. The prominent band at 75 kDa appeared across all trials and treatments, as expected, because it corresponds with one of the major apoproteins apparent in all fractions of egg yolk in previous literature (Itoh et al., 1986). The other prominent band that appeared on trials and treatments at ~45kDa confirmed the presence of β-phosvitin (Abe et al., 1982; Itoh et al., 1986, Ko et al., 2011) The 37 kDa band is estimated to be α-phosvitin or riboflavin binding protein (RFB) (Abe et al., 1982; Awade, 1995). The bands at 65 kDa and 125 kDa, and 250 kDa could be attributed to one of the main apoproteins of LDL (Itoh et al., 1986).

Lane 5 had 2.5% NaCl and a pH of 5.2. It had 5 unique and distinct bands that the other samples at 5.2 pH with less salt concentrations did not have. These bands were at ~225, ~13, 7~, ~30, and ~100. Additionally, these bands were unique among the other pH samples with 0% NaCl concentrations. Bands at ~7 and ~13 were likely apolipovitellenins. (Awade, 1995; Zambrowicz et al., 2014; Anton and Gandemer, 1997) The band at ~30 corresponds to an apoprotein of HDL (Mine, 2008). It is likely that the higher salt concentration solubilized more proteins out of the yellow cake solution by disruption of the granule structure. At full disruption of granules, the

presence of more unique proteins is likely due to the broken HDL-phosvitin complexes allowing those proteins to be released and solubilized in this environment. In salt concentrations under 2.5%, there are still different proteins and peptides that are bound in the phosvitin-HDL complexes.

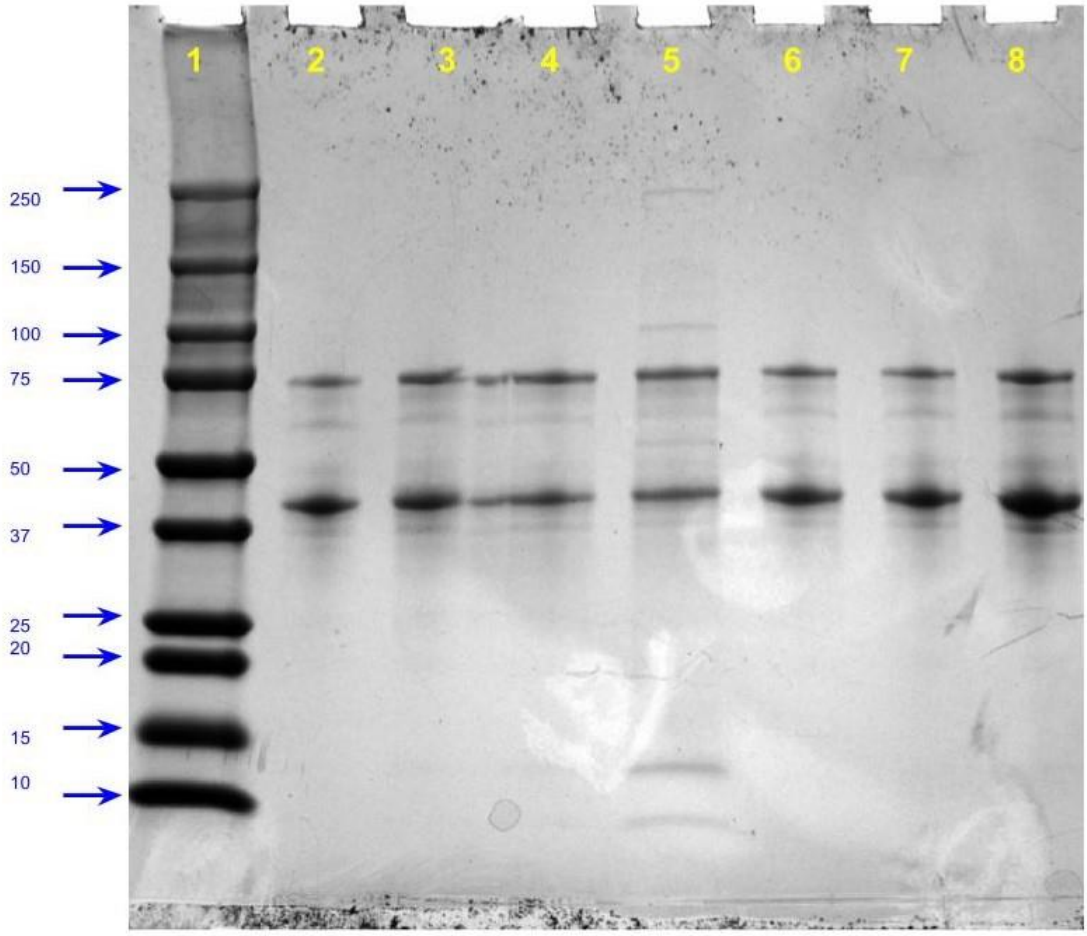

Figure 4.1. Trial 1 filtrate samples run on SDS-PAGE (4-20% gel) visualized with Coomassie staining. Lane 1. Protein Standard; Lane 2. 0% NaCl, pH of 5.2; Lane 3. 0.5% NaCl, pH of 5.2; Lane 4. 1% NaCl, pH of 5.2; Lane 5. 2.5% NaCl, pH 5.2; Lane 6. 0% NaCl, pH of 4.6; Lane 7. 0% NaCl, pH of 4.8; Lane 8. 0% NaCl, pH of 5.0. The molecular sizes of the marker are given in kDa.

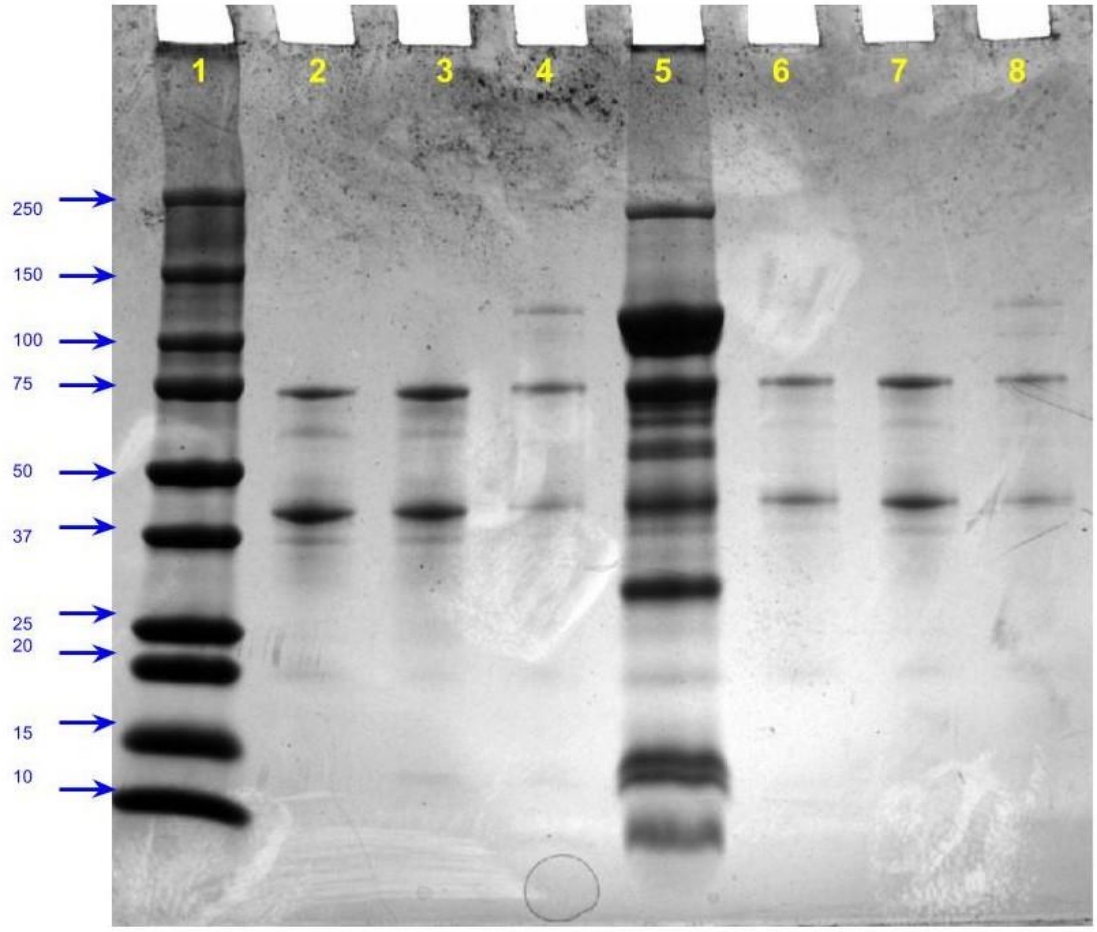

Figure 4.2. Trial 2 filtrate samples run on SDS-PAGE (4-20% gel) visualized with Coomassie staining. Lane 1. Protein Standard; Lane 2. 0% NaCl, pH of 5.2; Lane 3. 0.5% NaCl, pH of 5.2; Lane 4. 1% NaCl, pH of 5.2; Lane 5. 2.5% NaCl, pH 5.2; Lane 6. 0% NaCl, pH of 4.6; Lane 7. 0% NaCl, pH of 4.8; Lane 8. 0% NaCl, pH of 5.0. The molecular sizes of the marker are given in kDa.

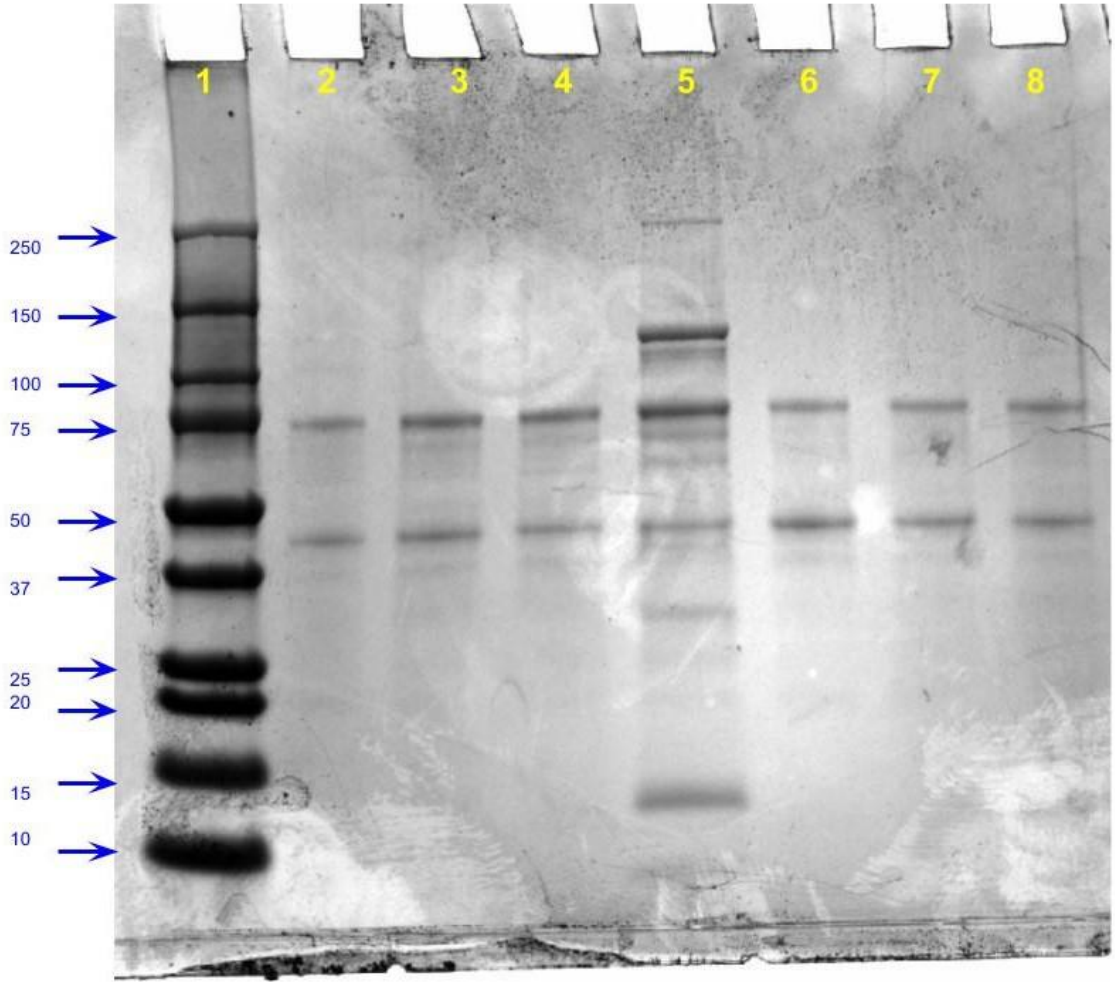

Figure 4.3. Trial 3 filtrate samples run on SDS-PAGE (4-20% gel) visualized with Coomassie staining. Lane 1. Protein Standard; Lane 2. 0% NaCl, pH of 5.2; Lane 3. 0.5% NaCl, pH of 5.2; Lane 4. 1% NaCl, pH of 5.2; Lane 5. 2.5% NaCl, pH 5.2; Lane 6. 0% NaCl, pH of 4.6; Lane 7. 0% NaCl, pH of 4.8; Lane 8. 0% NaCl, pH of 5.0. The molecular sizes of the marker are given in kDa.

4.1.2 Protein Quantification

Total amounts of crude protein in the yellow cake samples after filtration and preparation (with centrifugation) were analyzed using a Thermo Scientific Pierce BCA Protein Assay kit (Rockford, IL). The BCA method was chosen over the Biuret, Lowry, and Bradford methods because of its higher sensitivity and lower variability. Sample preparations from trials 1-3 were run both with a 1:9 dilution and undiluted. The undiluted data all fell within the working range and was subsequently used for analysis, as well as for standardization for the MIC procedure.

After centrifugation, a small milky yellow sediment formed on the bottom of the falcon tubes, with the exception of the sample with 2.5% NaCl. This tube formed a yellow pellicle on the top of the tube. The tubes with 1% NaCl had small floating light yellow particles suspended in solution. The supernatant was then prepared and analyzed with BCA.

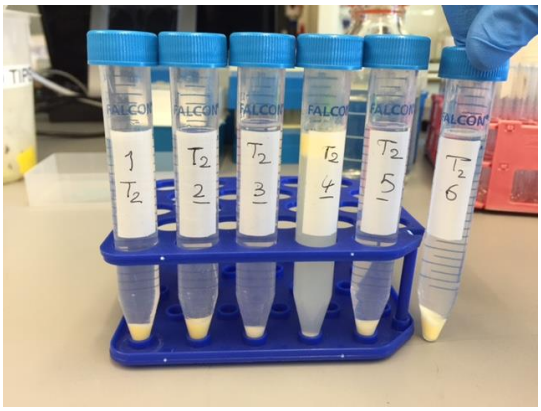

Figure 4.4 Sediment formed on bottom of centrifuged samples. From right to left: pH 5.2: 0% NaCl, 0.5% NaCl, 1% NaCl, 2.5% NaCl. 0% NaCl: pH 4.6, pH 4.8. Pellicle formed on trial with 2.5% NaCl

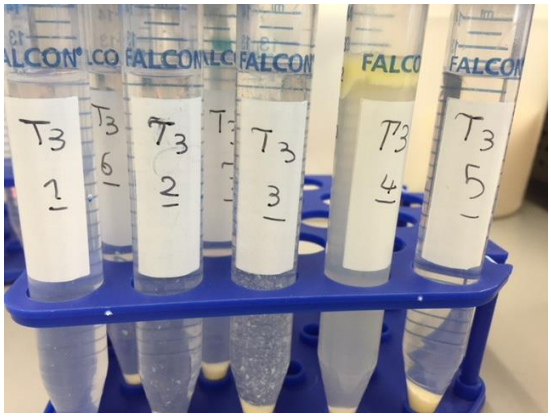

Figure 4.5 From right to left: pH 5.2: 0% NaCl, 0.5% NaCl, 1% NaCl, 2.5% NaCl. 0% NaCl: pH 4.6 Floating particles in tube with 1% salt

A protein standard of bovine serum albumin (BSA) was used as reference to determine unknown protein amounts. The microplate procedure was used to run the samples and standards. After running the samples in the spectrometer at 562 nm, the unknown sample protein amounts were determined based on the standard curve.

Table 4.1 Averaged protein concentration from BCA after treatments

Trial number	Salt Conc %	pH	Avg Protein Conc in ug/ml
1	0	5.2	692
1	0.5	5.2	776
1	1	5.2	532
1	2.50%	5.2	985
1	0	4.6	730
1	0	4.8	691
1	0	5	1265
2	0	5.2	669
2	0.5	5.2	763
2	1	5.2	388
2	2.50%	5.2	3051
2	0	4.6	306
2	0	4.8	537
2	0	5	221
3	0	5.2	283
3	0.5	5.2	518
3	1	5.2	401
3	2.50%	5.2	1468
3	0	4.6	429
3	0	4.8	281
3	0	5	284

The results for the different levels of NaCl effect on protein concentration did show that NaCl concentration had a statistical significance, as salt concentration had a p-value <0.05. Overall, there was a noticeable trend of higher protein concentrations with the addition of salt. However, data showed there was a small decrease with 1% salt, which could be due to a few reasons. It is likely the suspended particles present in the 1% salt samples interfered with the BCA analysis, giving an inaccurate reading. If there was no interference, there could be a salting in effect on the protein. The increase at 2.5% is likely due to the disruption of the granules, solubilizing phosvitin

and HDL (Huopalahti et al., 2007). At this concentration, the phosphocalcic bridges would break with sodium replacement of calcium. The free phosvitin would solubilize because of the salt concentration. At a concentration at 2.5% NaCl, the most protein is extracted, as well as more types of proteins, as shown from the previous section in the gels. Figure 4.6 visualizes the effect of NaCl on protein concentration.

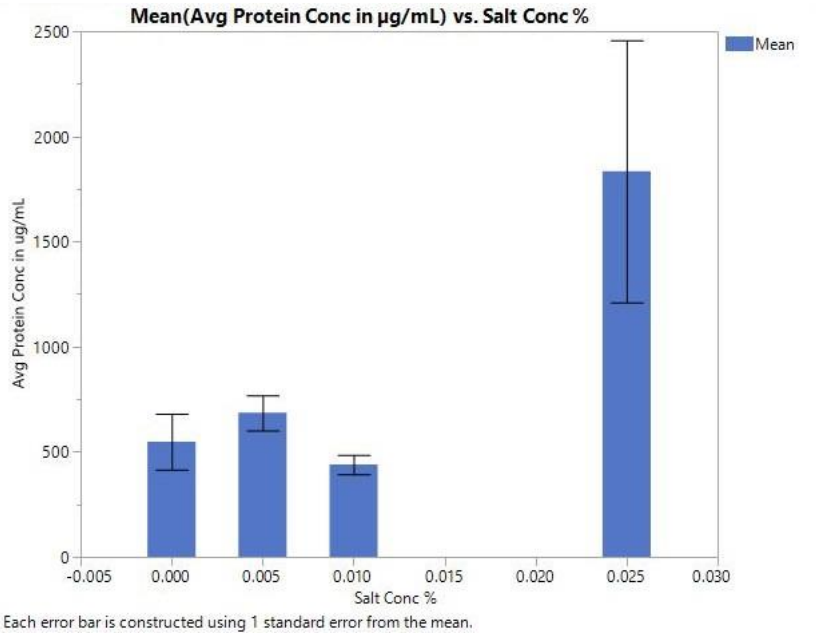

Figure 4.6 Effect of NaCl on protein concentration

The results in the pH range of 4.6-5.2 did not prove to be statistically significant. There is no evidence that pH adjustments within this range have an effect on the protein concentration. Figure 4.7 visualizes the effect of pH on protein concentration.

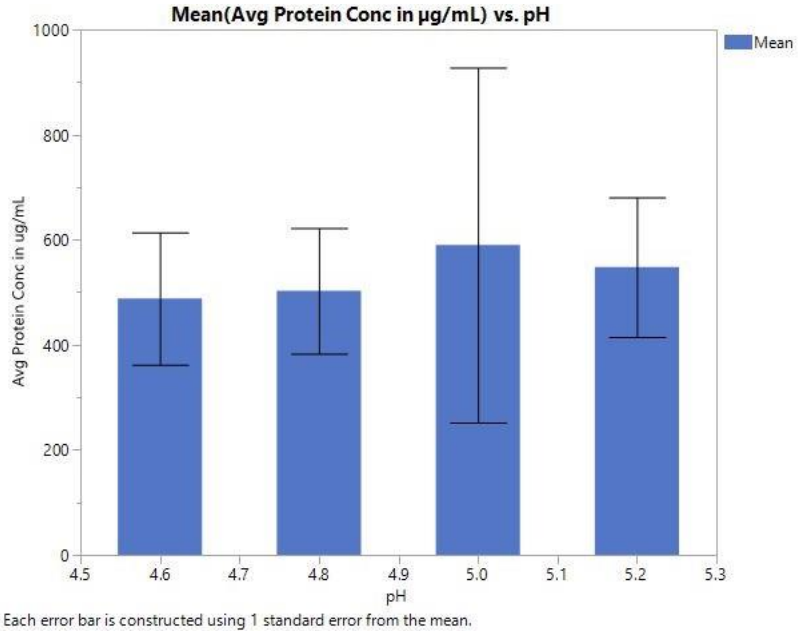

Figure 4.7 Effect of pH on protein concentration

4.2 Precipitation

4.2.1 Observations of addition of NaCl

Lab scale samples of yellow cake were diluted in water to make a 5% solids solution. After adjusting the solution to samples of 5 different salt concentrations and the first round of centrifugation, a gelatinous pellicle formed on the top of the solution. At 3% NaCl content, there were visible puffy light yellow clumps and strands floating just below the pellicle. At 4% and 5% NaCl, there were less clumps hanging attached to the bottom of the pellicle layer. By 6% NaCl, there appeared to be complete separation, as well as 7%. This is shown in figures 4.8-4.12.

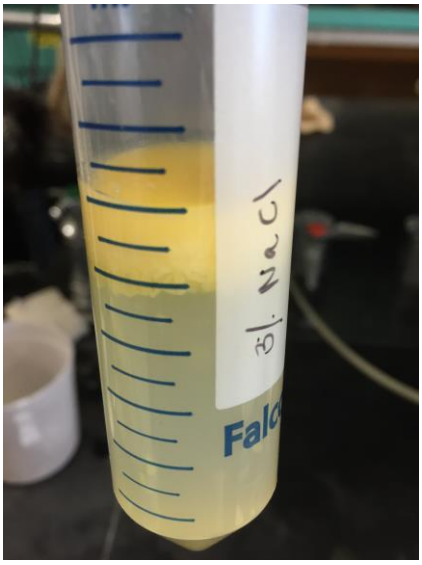

Figure 4.8 3% NaCl Solution

Figure 4.9 4% NaCl Solution

Figure 4.10 5% NaCl Solution

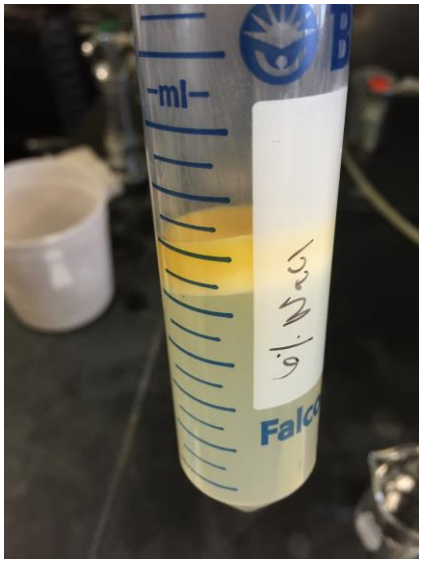

Figure 4.11 6% NaCl Solution

Figure 4.12 7% NaCl Solution

The adjustment of density with addition of salt allowed the insoluble yellow layer to invert and form a pellicle on top. At 3% NaCl, the pellicle was less compact with strands attached to it, as well as a more turbid water-soluble portion. By the concentration of 6% NaCl, the pellicle on top was compacted with no floating particles beneath it.

After addition of HCl and centrifugation, firm white pellets precipitated on the bottom of the falcon tubes. In an industry scale extraction, this simple inversion, followed by precipitation could make the process more efficient. When collecting the water soluble protein fraction, it could be easily emptied from the bottom of the tank, then run through a centrifuge or separator, and then the protein pellet collected. This eliminates a more complicated process of siphoning and pumping the water soluble fraction out when it is the floating layer on top.

4.2.2 Protein Content

SDS-PAGE was used to profile the proteins contained in the reconstituted pellet from the precipitation experiment. Samples were subjected to and analyzed under ten different conditions, considering two variables: NaCl content and temperature. After diluting samples, gels were run accordingly, taking care that the tracking dye did not travel farther than the bottom of the gels. Gels were stained using Coomassie Brilliant Blue to view the bands.

Figure 4.13 illustrates the protein content in the supernatant from the precipitation experiment. These samples are the result of the procedure illustrated in diagram 3.7. Lane 1 represents the protein standard. Lane 2 represents 3% NaCl incubated at room temperature (RT). Lane 3 represents 4% NaCl incubated at RT. Lane 4 represents 5% NaCl incubated at RT. Lane 5 represents 6% NaCl incubated at RT. Lane 6 represents 7% NaCl incubated at RT. Lane 7 represents 4% NaCl incubated at 4°C. Lane 8 represents 5% NaCl incubated at 4°C. Lane 9 represents 6% NaCl incubated at 4°C. Lane 10 represents 7% NaCl incubated at 4°C.

The same migration pattern appeared across all sample treatments for the precipitation treatments. Bands ranged from 10 kDa to 250 kDa. The largest, prominent band appeared at ~125 kDa as diffuse band. Smaller bands appeared under it in the 100-125 kDa range. There were more prominent bands at ~74, ~45, ~35,and ~29 kDa. The addition of salt at this range did not affect the different proteins

solubilized. It is likely that the granules are already completed disrupted at a concentration at 2.5%.

The band at 125 kDa is likely apoprotein of LDL (Awade 1995; Zambrowicz et al., 2014). At 100-125, 74, 28 and 45 kDa, the bands are likely polypeptides of HDL (Awade 1995; Zambrowicz et al., 2014; Strixner and Kulozik, 2013). It is possible the ~35 marker is β-livetin, if there was some leftover from the plasma fraction, but not likely.

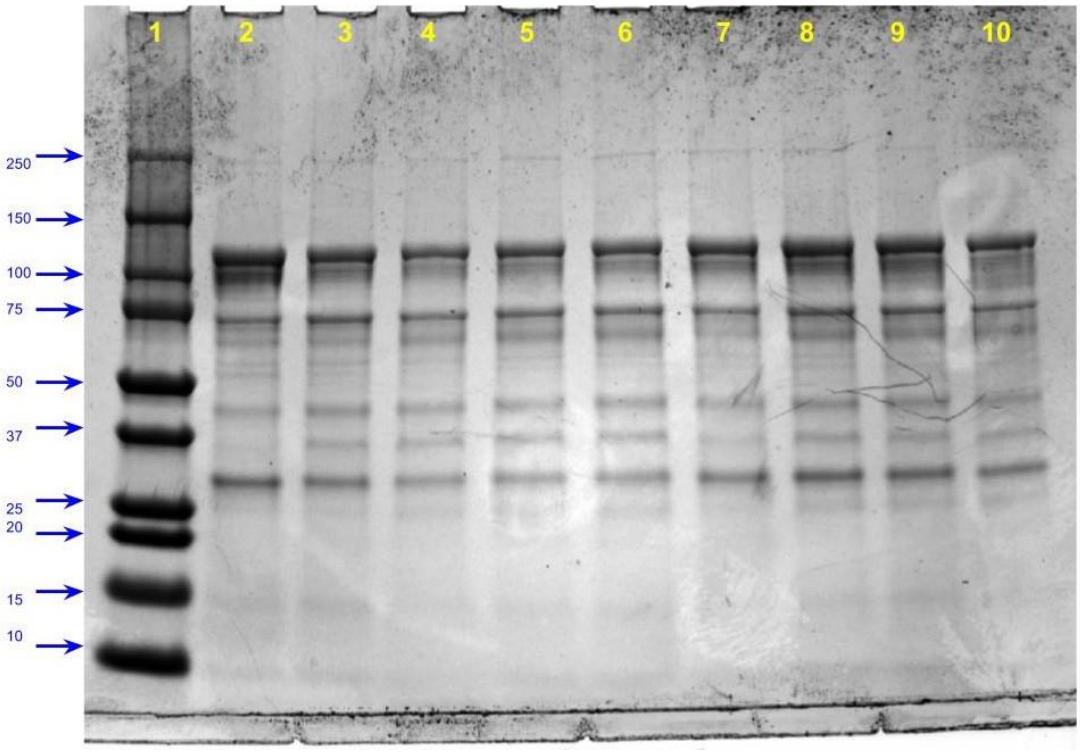

Figure 4.13 Precipitation samples run on SDS-PAGE (4-20% gel) visualized with Coomassie staining. Lane 1. Protein Standard; Lane 2. 3% NaCl, room temperature (RT); Lane 3. 4% NaCl, RT; Lane 4. 5% NaCl, RT; Lane 5. 6% NaCl, RT; Lane 6. 7% NaCl, RT; Lane 7. 4% NaCl, 4°C; Lane 8. 5% NaCl, 4°C; Lane 9. 6% NaCl, 4°C; Lane 10. 7% NaCl, 4°C. The molecular sizes of the marker are given in kDa.

4.3 MIC

Three plates, one from trial 1, 2, and 3, were assayed without proteolysis. Crude protein was standardized to 256 µg/mL in MHB and diluted according to procedure. After 20 hours of incubation at 37°C, growth/no growth was observed. The test results for the MIC procedure are based on a growth/no growth response to find the lowest concentration of proteins to inhibit the growth of bacteria. Although the results did not conclusively show a minimum inhibitory concentration, the patterns are worth examining for potential insight about the possible antibacterial properties of the protein extract.

4.3.1 No Proteolysis

For Trial 1, there was no growth in any of the wells in the control columns 11 and 12. For column 10 with just *E. coli* and broth, there was growth in all cells in the column. This confirms the MHB and the protein were not contaminated, and that the *E. coli* was present in sufficient amount. There were no other wells with zero growth, however all wells in columns 1 and 2 with higher protein concentrations showed less turbidity, implying less growth.

For Trial 2, there was no growth in all wells in column 12 or in column 11 wells B,C, and D. This shows there may have been some contamination of the protein samples for this trial. There was were no other wells with zero growth. Like Trial 1, columns 1 and 2 were less turbid, as well as wells 5C and 5D.

For Trial 3, there was slight growth in column 11, and no growth in column 12. There may have also been some slight contamination in the protein from this trial. All other wells in all columns had similar turbidity throughout.

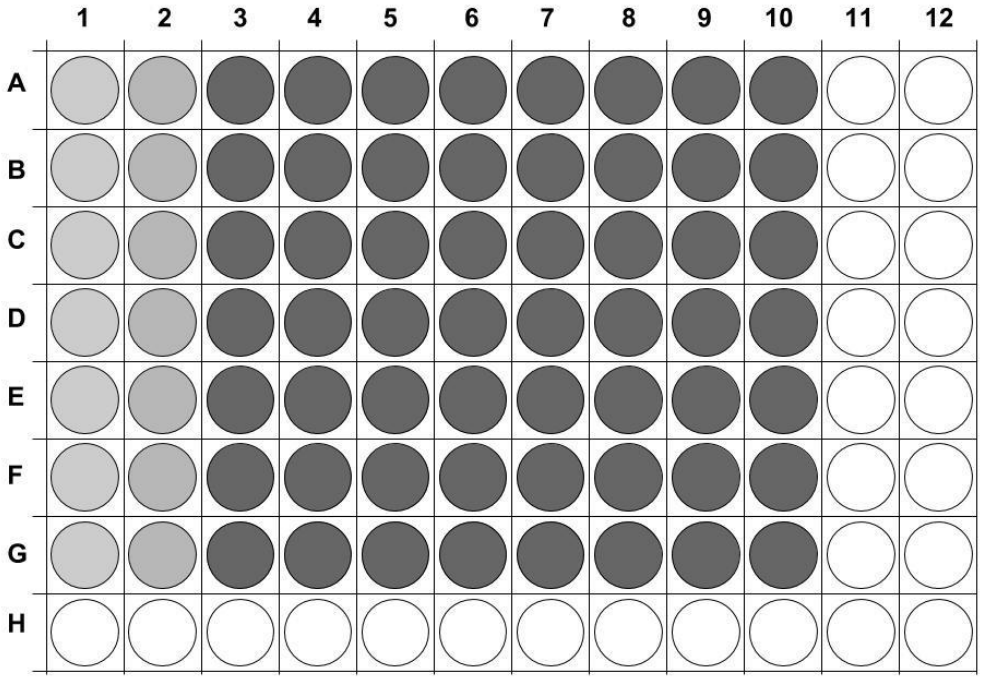

Figure 4.14 Trial 1 MIC with no proteolysis

Row A: 0.0% NaCl, pH=5.2
Row B: 0.5% NaCl, pH=5.2
Row C: 1.0% NaCl, pH=5.2
Row D: 2.5% NaCl, pH=5.2
Row E: 0.0% NaCl, pH=4.6
Row F: 0.0% NaCl, pH=4.8
Row G: 0.0% NaCl, pH=5.0
Row H is empty
Protein Concentrations in MHB inoculated with *E. coli*:
Column 1: 256 µg/mL
Column 2: 128 µg/mL
Column 3: 64 µg/mL
Column 4: 32 µg/mL
Column 5: 16 µg/mL
Column 6: 8 µg/mL
Column 7: 4 µg/mL
Column 8: 2 µg/mL
Column 9: 1 µg/mL
Controls:
Column 10: *E. coli* and MHB
Column 11: 256 µg/mL protein solution in MHB
Column 12: MHB

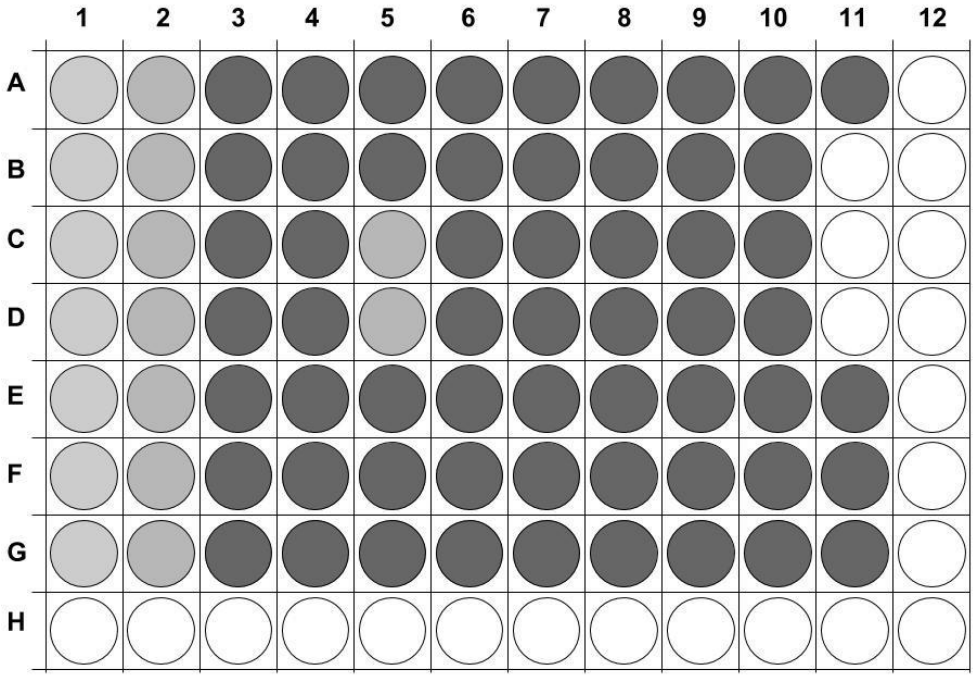

Figure 4.15 Trial 2 MIC with no proteolysis

Row A: 0.0% NaCl, pH=5.2
Row B: 0.5% NaCl, pH=5.2
Row C: 1.0% NaCl, pH=5.2
Row D: 2.5% NaCl, pH=5.2
Row E: 0.0% NaCl, pH=4.6
Row F: 0.0% NaCl, pH=4.8
Row G: 0.0% NaCl, pH=5.0
Row H is empty
Protein Concentrations in MHB inoculated with *E. coli*:
Column 1: 256 µg/mL
Column 2: 128 µg/mL
Column 3: 64 µg/mL
Column 4: 32 µg/mL
Column 5: 16 µg/mL
Column 6: 8 µg/mL
Column 7: 4 µg/mL
Column 8: 2 µg/mL
Column 9: 1 µg/mL
Controls:
Column 10: *E. coli* and MHB
Column 11: 256 µg/mL protein solution in MHB
Column 12: MHB

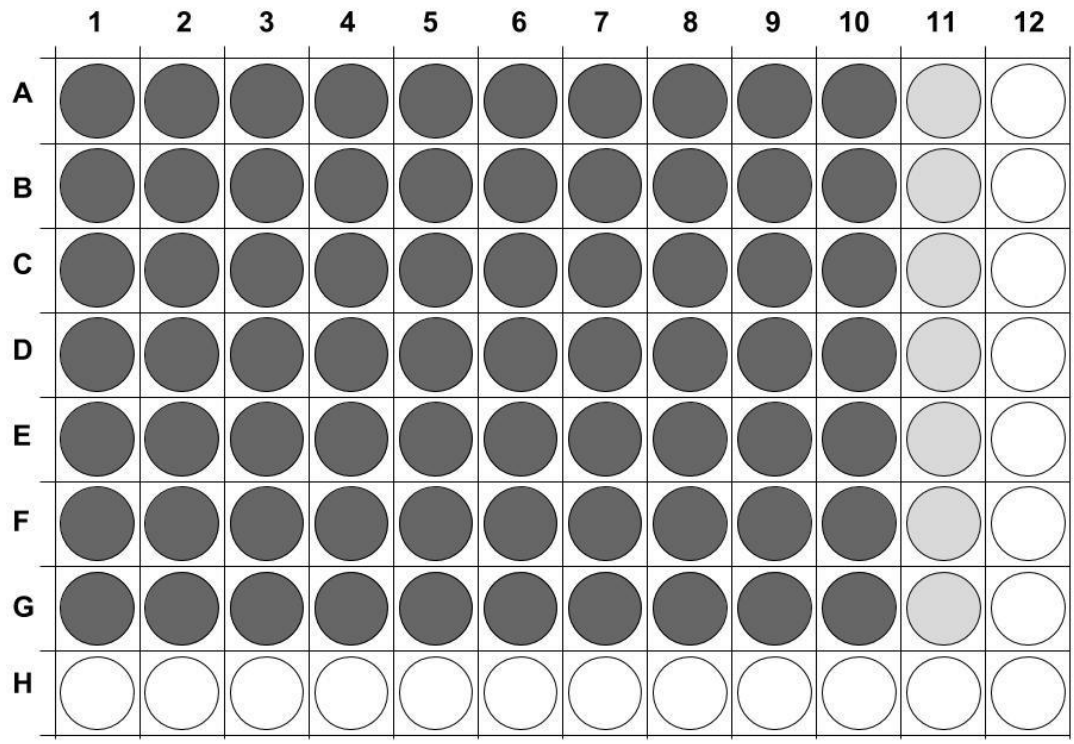

Figure 4.16 Trial 3 MIC with no proteolysis

Row A: 0.0% NaCl, pH=5.2
Row B: 0.5% NaCl, pH=5.2
Row C: 1.0% NaCl, pH=5.2
Row D: 2.5% NaCl, pH=5.2
Row E: 0.0% NaCl, pH=4.6
Row F: 0.0% NaCl, pH=4.8
Row G: 0.0% NaCl, pH=5.0
Row H is empty
Protein Concentrations in MHB inoculated with *E. coli*:
Column 1: 256 µg/mL
Column 2: 128 µg/mL
Column 3: 64 µg/mL
Column 4: 32 µg/mL
Column 5: 16 µg/mL
Column 6: 8 µg/mL
Column 7: 4 µg/mL
Column 8: 2 µg/mL
Column 9: 1 µg/mL
Controls:
Column 10: *E. coli* and MHB
Column 11: 256 µg/mL protein solution in MHB
Column 12: MHB

4.3.2 Proteolysis no endpoint

Three plates, one from trial 1, 2, and 3, were assayed with proteolysis without an endpoint. Crude protein was standardized to 256 μg/mL in a solution of PBS and enzyme solution and then diluted according to procedure. 2.56 μL of prepared TrypZean were added to each well of sample and incubated for 2 hours at 37°C. After 20 hours of incubation at 37°C, growth/no growth was observed. Figure 4.17-4.19 illustrates the growth patterns.

For Trial 1, there was no growth in column 12, and there was a very small button appeared down wells in column 11. Wells in column 1 had less turbidity than all other columns. All other columns showed similar turbidity throughout.

For Trial 2, there was no growth in column 12, with a small button in column 11. Wells in column 1 had less turbidity than all other columns. There also appeared to be a gradient of turbidity across the rows. Well D5 also showed hardly any turbidity.

For Trial 3, there was no growth in column 12. Column 11 had no growth in wells D and E, with small button in the rest of the column. The turbidity increased across rows. The gradient that appeared in plates from trial 2 and 3 show a possibility of some bacteriostatic property. Although there is no conclusive evidence in this assay, this gradient appeared most prominently in the plates that had proteolysis without an endpoint. There is a possible release of peptides after addition of TrypZean that may inhibit some *E. coli* growth. The samples with proteolysis with an endpoint did not

show this pattern, implying that the enzymatic reaction needed more time for digestion. Although this is only speculative because MIC is a growth/no growth test, there are implications for potential bacteriostatic activity in the peptides.

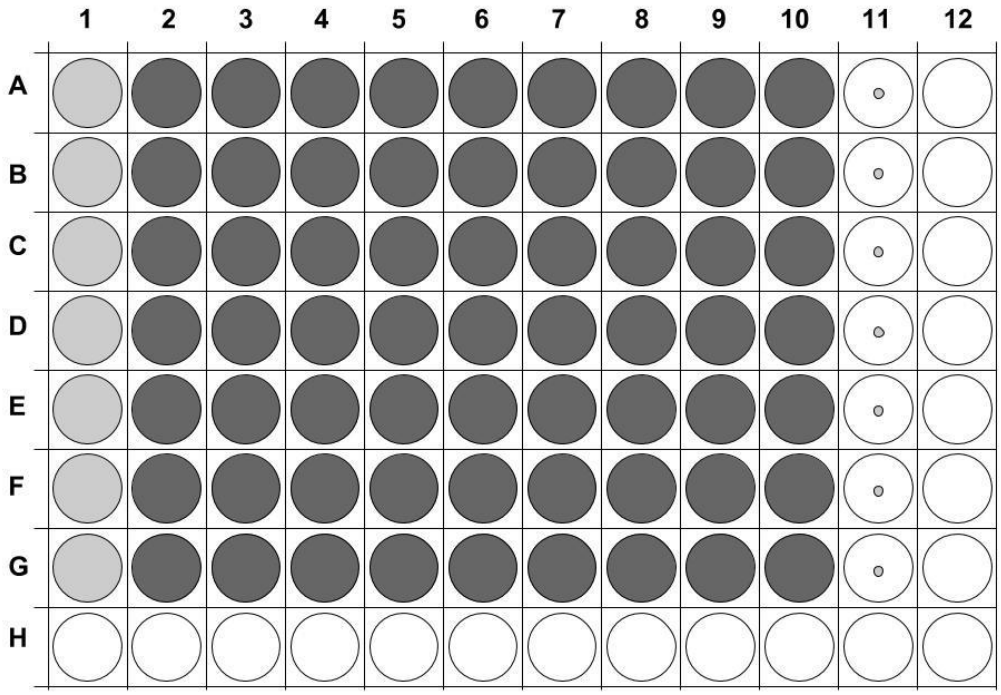

Figure 4.17 Trial 1 MIC with proteolysis with no endpoint

Row A: 0.0% NaCl, pH=5.2
Row B: 0.5% NaCl, pH=5.2
Row C: 1.0% NaCl, pH=5.2
Row D: 2.5% NaCl, pH=5.2
Row E: 0.0% NaCl, pH=4.6
Row F: 0.0% NaCl, pH=4.8
Row G: 0.0% NaCl, pH=5.0
Row H is empty
Protein Concentrations in MHB inoculated with *E. coli*:
Column 1: 256 µg/mL
Column 2: 128 µg/mL
Column 3: 64 µg/mL
Column 4: 32 µg/mL
Column 5: 16 µg/mL
Column 6: 8 µg/mL
Column 7: 4 µg/mL
Column 8: 2 µg/mL
Column 9: 1 µg/mL
Controls:
Column 10: *E. coli* and MHB + PBS
Column 11: 256 µg/mL protein solution in MHB/TrypZean
Column 12: MHB and PBS

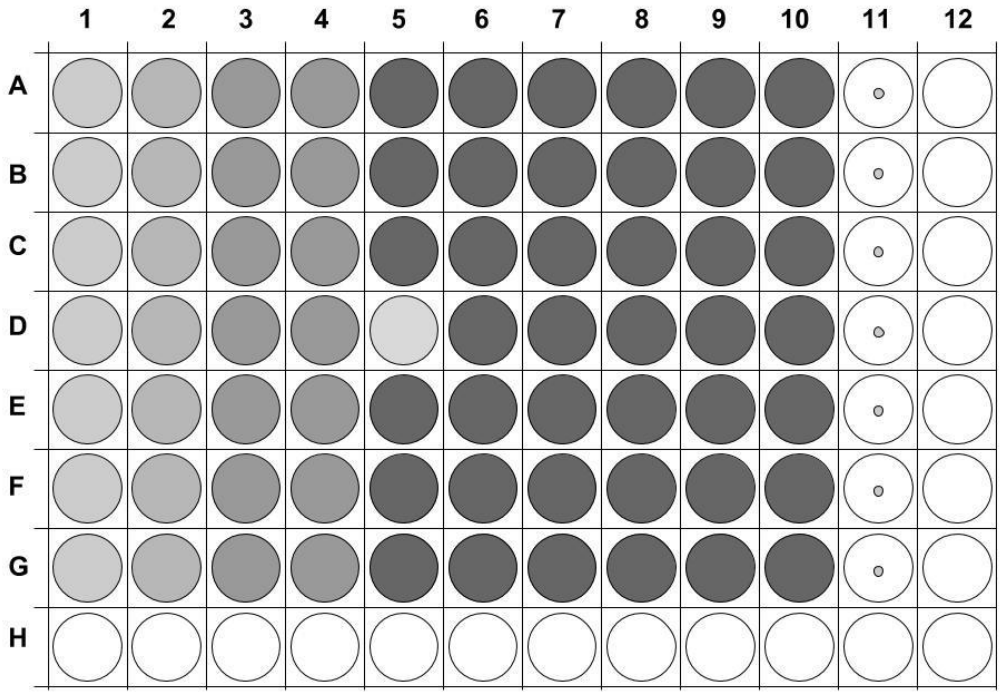

Figure 4.18 Trial 2 MIC with proteolysis with no endpoint

Row A: 0.0% NaCl, pH=5.2
Row B: 0.5% NaCl, pH=5.2
Row C: 1.0% NaCl, pH=5.2
Row D: 2.5% NaCl, pH=5.2
Row E: 0.0% NaCl, pH=4.6
Row F: 0.0% NaCl, pH=4.8
Row G: 0.0% NaCl, pH=5.0
Row H is empty
Protein Concentrations in MHB inoculated with *E. coli*:
Column 1: 256 µg/mL
Column 2: 128 µg/mL
Column 3: 64 µg/mL
Column 4: 32 µg/mL
Column 5: 16 µg/mL
Column 6: 8 µg/mL
Column 7: 4 µg/mL
Column 8: 2 µg/mL
Column 9: 1 µg/mL
Controls:
Column 10: *E. coli* and MHB + PBS
Column 11: 256 µg/mL protein solution in MHB/TrypZean
Column 12: MHB and PBS

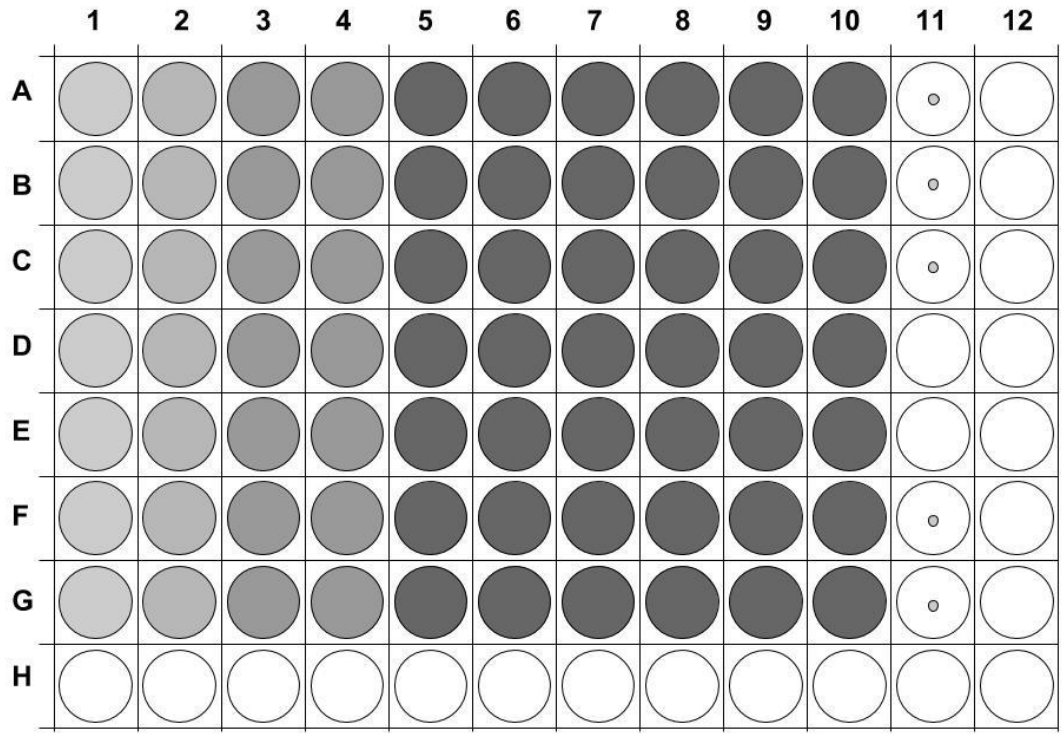

Figure 4.19 Trial 3 MIC with proteolysis with no endpoint

Row A: 0.0% NaCl, pH=5.2
Row B: 0.5% NaCl, pH=5.2
Row C: 1.0% NaCl, pH=5.2
Row D: 2.5% NaCl, pH=5.2
Row E: 0.0% NaCl, pH=4.6
Row F: 0.0% NaCl, pH=4.8
Row G: 0.0% NaCl, pH=5.0
Row H is empty
Protein Concentrations in MHB inoculated with *E. coli*:
Column 1: 256 µg/mL
Column 2: 128 µg/mL
Column 3: 64 µg/mL
Column 4: 32 µg/mL
Column 5: 16 µg/mL
Column 6: 8 µg/mL
Column 7: 4 µg/mL
Column 8: 2 µg/mL
Column 9: 1 µg/mL
Controls:
Column 10: *E. coli* and MHB + PBS
Column 11: 256 µg/mL protein solution in MHB/TrypZean
Column 12: MHB and PBS

4.3.3 Proteolysis with endpoints

Two additional plates run with samples from trial 2 were assayed with proteolysis with different endpoints. Crude protein was standardized to 256 µg/mL in a solution of PBS and enzyme solution and then diluted according to procedure. 2.56 µL of prepared TrypZean were added to each well of sample and incubated for 2 hours at 37°C. One set was boiled for 5 min, and one set was incubated at 50°C for 5 minutes. After an additional 20 hours of incubation at 37°C, growth/no growth was observed. Figures 4.20 and 4.21 illustrate the growth patterns.

The plate with 5 minutes of boiling showed no growth in columns 11(except for 11D) or 12. Wells D5 and C3 showed only slight turbidity. Otherwise, there was similar growth throughout.

The plate heated to 50°C for 5 minutes showed no growth in column 12, and no growth in column 11 wells C and D. Otherwise column 11 showed a slight turbidity. F8 had no growth. Columns 1 and 2 were less turbid than other columns.

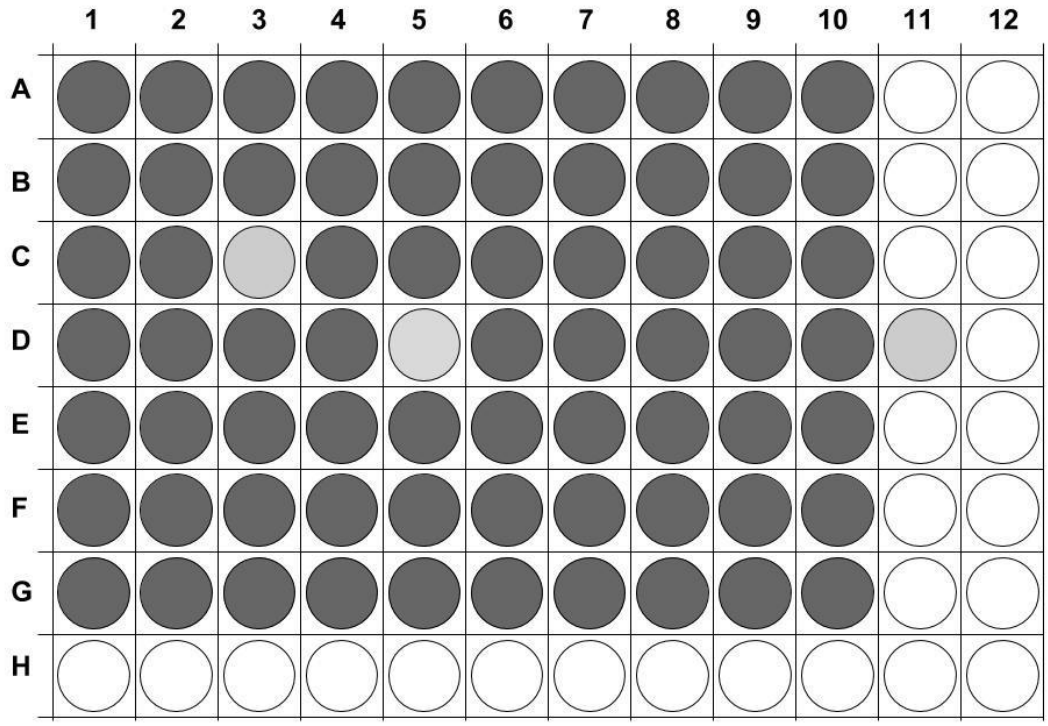

Figure 4.20 Trial 2 MIC with proteolysis with boiling as endpoint

Row A: 0.0% NaCl, pH=5.2
Row B: 0.5% NaCl, pH=5.2
Row C: 1.0% NaCl, pH=5.2
Row D: 2.5% NaCl, pH=5.2
Row E: 0.0% NaCl, pH=4.6
Row F: 0.0% NaCl, pH=4.8
Row G: 0.0% NaCl, pH=5.0
Row H is empty
Protein Concentrations in MHB inoculated with *E. coli*:
Column 1: 256 µg/mL
Column 2: 128 µg/mL
Column 3: 64 µg/mL
Column 4: 32 µg/mL
Column 5: 16 µg/mL
Column 6: 8 µg/mL
Column 7: 4 µg/mL
Column 8: 2 µg/mL
Column 9: 1 µg/mL
Controls:
Column 10: *E. coli* and MHB + PBS
Column 11: 256 µg/mL protein solution in MHB/TrypZean
Column 12: MHB and PBS

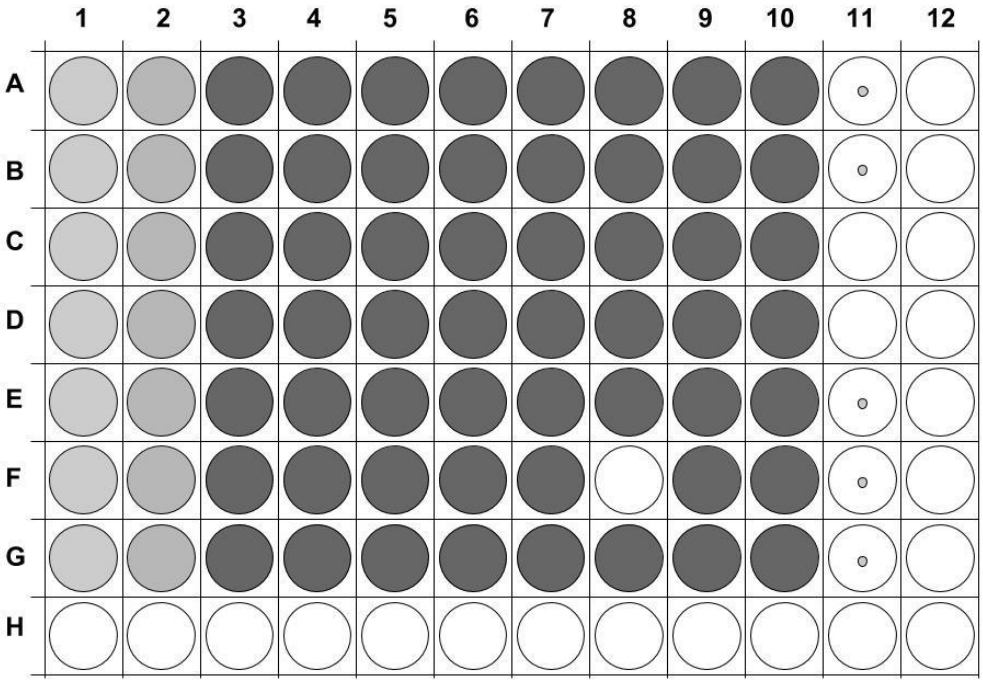

Figure 4.21 Trial 2 MIC with proteolysis heated to 50°C as endpoint

Row A: 0.0% NaCl, pH=5.2
Row B: 0.5% NaCl, pH=5.2
Row C: 1.0% NaCl, pH=5.2
Row D: 2.5% NaCl, pH=5.2
Row E: 0.0% NaCl, pH=4.6
Row F: 0.0% NaCl, pH=4.8
Row G: 0.0% NaCl, pH=5.0
Row H is empty
Protein Concentrations in MHB inoculated with *E. coli*:
Column 1: 256 µg/mL
Column 2: 128 µg/mL
Column 3: 64 µg/mL
Column 4: 32 µg/mL
Column 5: 16 µg/mL
Column 6: 8 µg/mL
Column 7: 4 µg/mL
Column 8: 2 µg/mL
Column 9: 1 µg/mL
Controls:
Column 10: *E. coli* and MHB + PBS
Column 11: 256 µg/mL protein solution in MHB/TrypZean
Column 12: MHB and PBS

CHAPTER 5: CONCLUSIONS AND LIMITATIONS

The main purpose of this thesis was to find a use for an industrial egg yolk by-product from IgY extraction that has previously been treated as waste. This objective included exploring techniques for separating proteins from lipids from the by-product, as well as quantifying and characterizing recovered proteins. Furthermore, it was hypothesized that the protein extract would show anti-microbial properties against *E. coli*.

This study showed that the by product of the IgY extraction is rich in proteins that have potential uses. The results proved that addition of NaCl to the yellow cake significantly affected the amount of protein recovered. The greatest amount of protein recovered was demonstrated at the highest NaCl level tested, 2.5%, suggesting the disassociation of the HDL-phosvitin complexes. The increased amount of salt also increased the variety of proteins recovered. At a level of 2.5% NaCl, additional unique bands were revealed on the SDS-PAGE gels.

Moreover, as salt addition increased, the water-soluble portion inverted to the bottom layer, forming a lipid pellicle on top. With additional pH adjustment and centrifugation, a protein pellet formed on the bottom. This is important for an industrial scale separation because it will make collection of the protein fraction from the bottom of a tank a much easier process. At a concentration of 5% NaCl, the two fractions inverted completely. At pH of 4, salt concentration between 3-7% did not have an effect on the variety of proteins extracted.

There was no evidence that pH has an effect on the amount of protein recovered in the range that was tested in this project. It also did not change the variety of proteins that were recovered.

There was no conclusive evidence on the anti-bacterial properties of the crude protein extract against *E. coli*. Although there was no quantifiable evidence from the MIC assay, the results showed an interesting trend. Some of the plates showed increased turbidity across the plates at lower concentrations of the protein extract. This suggests that the crude protein extract may have some bacteriostatic abilities. Some of the individual proteins and peptides in the extract may harbor these properties.

Due to the nature of projects utilizing by-products, it needs to be considered that there are variations in results caused by inconsistencies of the processes upstream. The project was confined to three trial replicates because of limited time and resources, but more trial replicates would help minimize deviations in the data.

CHAPTER 6: DIRECTIONS FOR FUTURE RESEARCH

This project provides an opening for further research on how to best utilize by-products from commercial scale IgY extractions. The separation methods laid out are a practical solution for the food and cosmetic industries for further separation of water soluble fractions because they successfully utilize common equipment and food grade materials. It is therefore pragmatic to continue to explore optimization of by-products of similar nature. Some ideas for future research include:

- Purification of the peptides in the crude protein extract by means of highly accurate techniques, such as HPLC.
- Expand anti-bacterial study by testing effectiveness of crude protein extract, as well as isolated peptides, against a variety of different bacterial organisms, including gram-positive and gram-negative.
- Broaden study to include effect of different types of salt on protein recovery
- Test for different functionalities of proteins, including antioxidative properties

Additionally, there are many opportunities for research containing the lipids present in the by-product. This would include characterizing and isolating the lipids to use for their bioactive and rheologic potential.

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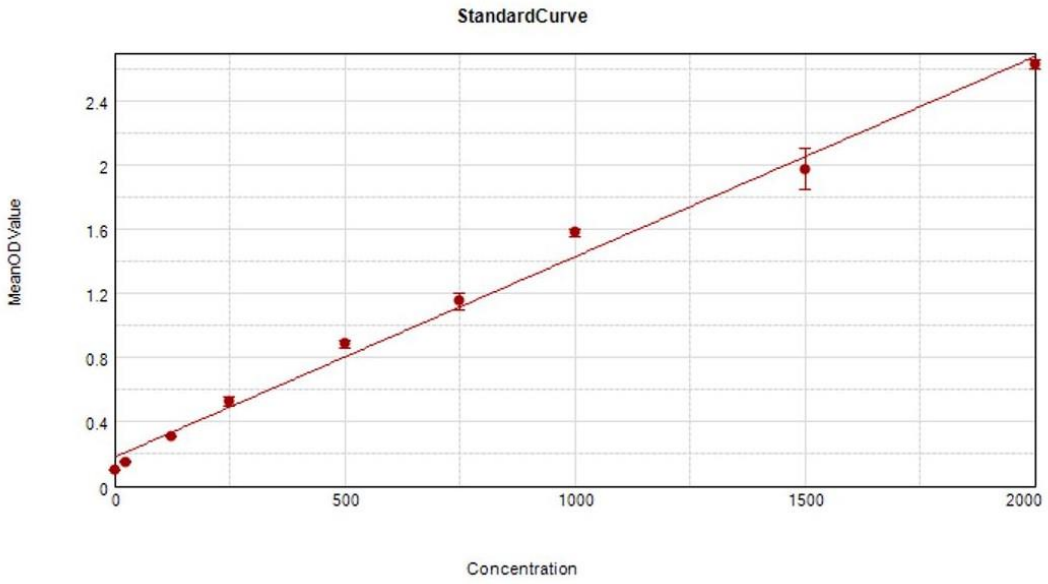
APPENDICES

Appendix A: BCA output

1. Standard Curve



2. BCA output

Unknowns

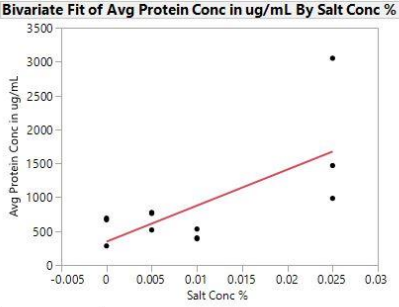
Sample	Wells	OD_Values	R	Concentration	MeanConc	SD	CV
O2	E12	0.137		-35.798	-35.798	0.000	0.0
O4	G10	0.146		-27.867	-43.810	19.649	44.9
	G11	0.099	R	-65.762			
	G12	0.134		-37.801			
T1.1	C1	1.059		702.957	692.342	15.013	2.2
	C8	1.032		681.727			
T1.2	C2	1.126		756.876	776.665	27.986	3.6
	C9	1.175		796.454			
T1.3	C3	0.808		502.506	532.149	41.922	7.9
	C10	0.882		561.792			
T1.4	C4	1.367		950.358	985.529	49.740	5.0
	C11	1.455		1020.700			
T1.5	C5	1.035		683.970	730.077	65.205	8.9
	C12	1.150		776.184			
T1.6	C6	1.010		664.101	691.541	38.806	5.6
	D8	1.079		718.981			
T1.7	C7	1.703		1218.909	1265.897	66.452	5.2
	D9	1.820		1312.886			
T2.1	D10	1.023		674.115	669.349	6.741	1.0
	E1	1.011		664.582			
T2.2	D11	1.136		764.808	763.005	2.549	0.3
	E2	1.131		761.202			
T2.3	D12	0.696		412.695	388.940	33.594	8.6
	E3	0.637		365.186			
T2.4	E4	3.981	R	3044.448	3051.979	10.650	0.3
	E8	4.000	R	3059.510			
T2.5	E5	0.550		295.324	306.380	15.636	5.1
	E9	0.577		317.436			
T2.6	E6	0.873		554.341	537.597	23.680	4.4
	E10	0.831		520.852			
T2.7	E7	0.454		218.412	221.376	4.192	1.9
	E11	0.461		224.340			
T3.1	F8	0.544		290.917	283.867	9.971	3.5
	G1	0.527		276.817			
T3.2	F9	0.857		541.442	518.889	31.895	6.1
	G2	0.801		496.337			
T3.3	F10	0.698		414.377	401.438	18.298	4.6
	G3	0.666		388.499			
T3.4	F11	2.106		1542.100	1468.352	104.295	7.1
	G4	1.922		1394.605			
T3.5	F12	0.718		429.840	429.840	0.000	0.0
	G5	0.718		429.840			
T3.6	G6	0.522		272.811	281.584	12.407	4.4
	G8	0.544		290.357			
T3.7	G7	0.522		273.131	284.148	15.579	5.5
	G9	0.550		295.164			

Appendix B: BCA raw data table

Trial number	Salt Conc %	pH	Avg Protein Conc in ug/ml
1	0	5.2	692
1	0.5	5.2	776
1	1	5.2	532
1	2.50%	5.2	985
1	0	4.6	730
1	0	4.8	691
1	0	5	1265
2	0	5.2	669
2	0.5	5.2	763
2	1	5.2	388
2	2.50%	5.2	3051
2	0	4.6	306
2	0	4.8	537
2	0	5	221
3	0	5.2	283
3	0.5	5.2	518
3	1	5.2	401
3	2.50%	5.2	1468
3	0	4.6	429
3	0	4.8	281
3	0	5	284

Appendix C: JMP output for NaCl study

Bivariate Fit of Avg Protein Conc in ug/mL By Salt Conc %



Linear Fit

Linear Fit

Avg Protein Conc in ug/mL = 345.40476 + 53176.19*Salt Conc %

Summary of Fit

RSquare	0.474725
RSquare Adj	0.422197
Root Mean Square Error	573.1713
Mean of Response	877.1667
Observations (or Sum Wgts)	12

Analysis of Variance

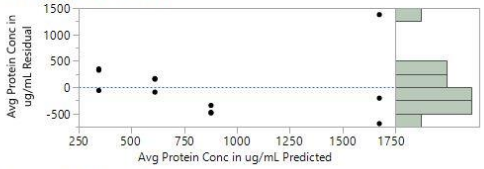
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	2969092.6	2969093	9.0376
Error	10	3285253.1	328525	Prob > F
C. Total	11	6254345.7		0.0132*

Parameter Estimates

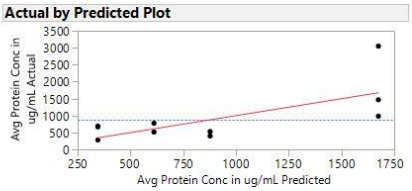
Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	345.40476	242.2091	1.43	0.1843
Salt Conc %	53176.19	17688.45	3.01	0.0132*

Diagnostics Plots

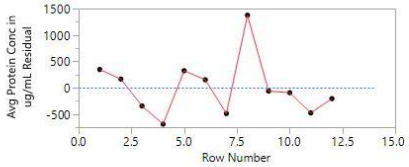
Residual by Predicted Plot



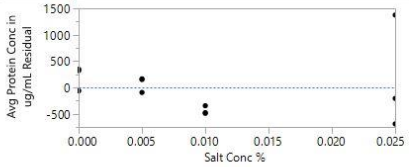
Actual by Predicted Plot



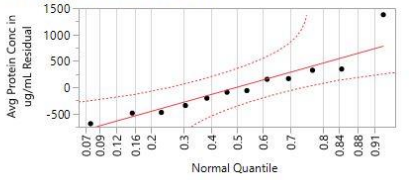
Residual by Row Plot



Residual by X Plot

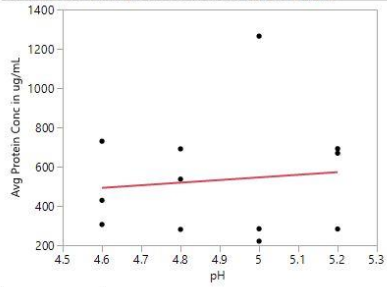


Residual Normal Quantile Plot



Appendix D: JMP output for pH study

Bivariate Fit of Avg Protein Conc in ug/mL By pH



Linear Fit

Linear Fit

Avg Protein Conc in ug/mL = -119.3667 + 133*pH

Summary of Fit

RSquare	0.010709
RSquare Adj	-0.08822
Root Mean Square Error	313.1238
Mean of Response	532.3333
Observations (or Sum Wgts)	12

Analysis of Variance

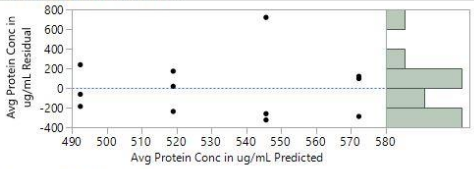
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	10613.40	10613.4	0.1082
Error	10	980465.27	98046.5	Prob > F
C. Total	11	991078.67		0.7489

Parameter Estimates

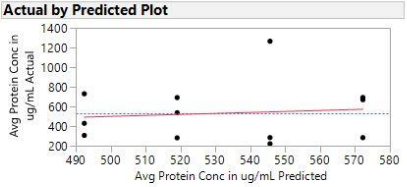
Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-119.3667	1982.843	-0.06	0.9532
pH	133	404.2411	0.33	0.7489

Diagnostics Plots

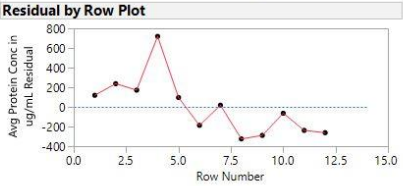
Residual by Predicted Plot



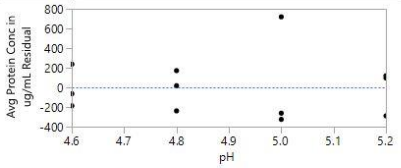
Actual by Predicted Plot



Residual by Row Plot



Residual by X Plot



Residual Normal Quantile Plot