CHICKEN EGGSHELL MEMBRANE AND CUTICLE: INSIGHT FROM BIOINFORMATICS AND PROTEOMICS

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Authorization

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Megan Rose-Martel is a Ph.D. candidate in Dr.Hincke's lab and she wrote the Chapter III-Proteomic analyses provide new insight into the chicken eggshell cuticle.

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The first project that I worked on is the bioinformatics analysis of the over-expressed genes in the white isthmus during the eggshell membrane formation (Chapter II). For the proteomic study of the cuticle layer, I was working on the cuticle extraction by SDS-method (Chapter III). In addition, to extract the cuticle protein and study the protease inhibitor activity of cuticle extracts were another tasks of the proteomic project.

Abstract

The chicken eggshell possesses physical and chemical barriers to protect the embryo from pathogens. The avian eggshell cuticle is the outmost layer of the eggshell whose protein constituents remain largely unknown. Since eggs with incomplete or absent cuticle are more susceptible to bacterial contamination, we hypothesize that cuticle protein components play an important role in microbial resistance. In our study, at least 47 proteins were identified by LC/MS/MS in the non-calcified cuticle layer. Similar to Kunitz-like protease inhibitor (also annotated as ovocalyxin-25, OCX-25) and ovocalyxin-32 (OCX-32) were two of most abundant proteins of the cuticle proteins. Some proteins that have antimicrobial activity were also detected in the proteomic results, such as lysozyme C, ovotransferrin, ovocalyxin-32, cystatin, ovoinhibitor. This study represents the first comprehensive report of the cuticle proteome. Since the sequence similarity of the kunitz motif in OCX-25 is similar to that of BPTI, it is predicted that it will have the same trypsin inhibitory and antimicrobial activity against Gram-positive and/or Gram-negative bacteria. In order to test the antimicrobial property and trypsin inhibitor activity of OCX-25, cuticle proteins were extracted by 1N HCl. Antimicrobial activity was monitored using the Bioscreen C instrument; and antimicrobial activity was identified primarily against *Staphylococcus aureus*. Trypsin inhibitor activity was studied by using a specific trypsin assay, and the assay indicated that the cuticle proteins could inhibit the reaction of trypsin and substrate. Therefore, the current research has provided some insight into the antimicrobial and enzymatic aspects of the cuticle proteins, and its function for egg protection.

Eggshell membranes are another important component of the chicken eggshell.

Due to its insoluble and stable properties, there are still many questions regarding formation and constituents of the eggshell membranes. The purpose of our study was to identify eggshell membrane proteins, particularly these responsible for its structural features, by examining the transcriptome of the white isthmus during its formation. Bioinformatics tools were applied to analyze the differentially expressed genes as well as their encoded proteins. Some interesting proteins were encoded by the over-expressed genes in the white isthmus during the formation of eggshell membranes, such as Collagen X, and similar to spore coat protein SP75. These proteins may have potential applications. Our study provides a detailed description of the chicken white isthmus transcriptome during formation of the eggshell membranes; it could lead to develop the strategies to improve food safety of the table egg.

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List of Abbreviations

BCA	Bicinchoninic acid
BLASTN	Nucleotide basic local alignment search tool
BLASTP (pBLAST)	Protein basic local alignment search tool
BPI	Bactericidal/permeability-increasing
BPTI	Bovine pancreatic trypsin inhibitor
DAVID	Database for Annotation, Visualization and Integrated Discovery
EASE	Expression Analysis Systematic Explorer
emPAI	Exponentially modified protein abundance index
EST	Expressed Sequence Tag
GO	Gene Ontology
ISM	Inner shell membrane
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
OC	Ovocleidin
OCX	Ovocalyxin
OSM	Outer shell membrane
SCPSP75	Similar to spore coat protein SP75
SMF	Eggshell membrane fibers
SERPIN	Serine proteinase inhibitor

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Chapter I General Introduction

1. Overview of Egg Formation

The egg is composed of a yolk surrounded by the vitelline membrane, egg white, eggshell membranes, calcified eggshell and the outmost cuticle layer. The accumulation of egg yolk proteins takes place in the chicken ovary. The other components are formed as the egg passed through different segments of the oviduct. After ovulation, the yolk passes into the oviduct infundibulum to acquire its vitelline membrane. Water, ions and the egg white proteins are secreted in the magnum (2- to 3-h period) to coat the yolk (Hincke et al., 2008), and then in the isthmus, the inner and outer eggshell membranes are formed (1- to 2-h period) (Arias et al., 1993; Nys et al., 2004). Finally, within the uterus (shell gland), the eggshell mineralization occurs (16- to 17-h). The outmost layer of the eggshell is the cuticle layer that is deposited approximately 1.5 to 2 hours before the egg is laid (Nys et al., 2001) (Figure 1)

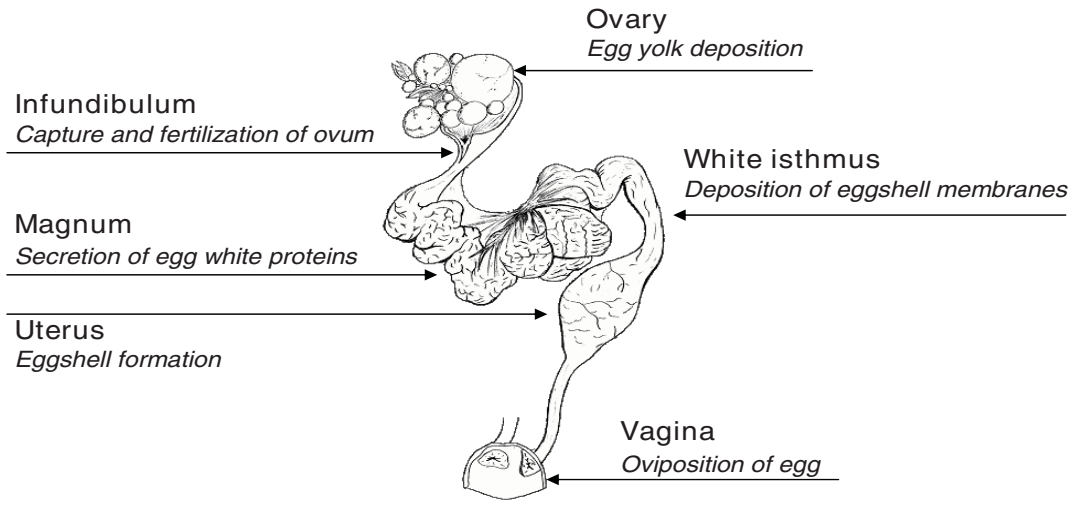

Figure 1: Sequential formation of the egg through the hen reproductive tract. It indicates the phases of egg and formation location (Reprint from Jonchere et al., 2010, with the permission from BMC Genomics)**.**

2. Composition of Eggshell

The eggshell consists of calcified and non-calcified zones; it can be divided into

at least three layers: eggshell membranes, calcified shell and cuticle layer.

2.1 Eggshell Membrane

The innermost layer of the eggshell is the eggshell membrane that are deposited as a highly crossed-linked fibrous meshwork during 1- to 2-h period in the white isthmus (Arias et al., 1993; Nys et al., 2004). It consists of inner and outer eggshell membranes. Its fibers are organized into inner and outer eggshell membranes. According to electron microscopy, the inner and outer membranes are connected by fibers that cross the two layers. These fibers (Figure 2) form a tight meshwork, and the arrangement of these fibers is in alternate layers parallel to the surface (Hincke et al., 2000). The inner membranes remain uncalcified, while at specific sites, the fibers of the outer shell membrane penetrate the mammillary cones of the calcified shell (Figure 2A). This represents the specific nucleation sites of the mammillary cones on the outer eggshell membrane where mineralization is initiated (Arias et al., 1993; Nys et al., 2004).

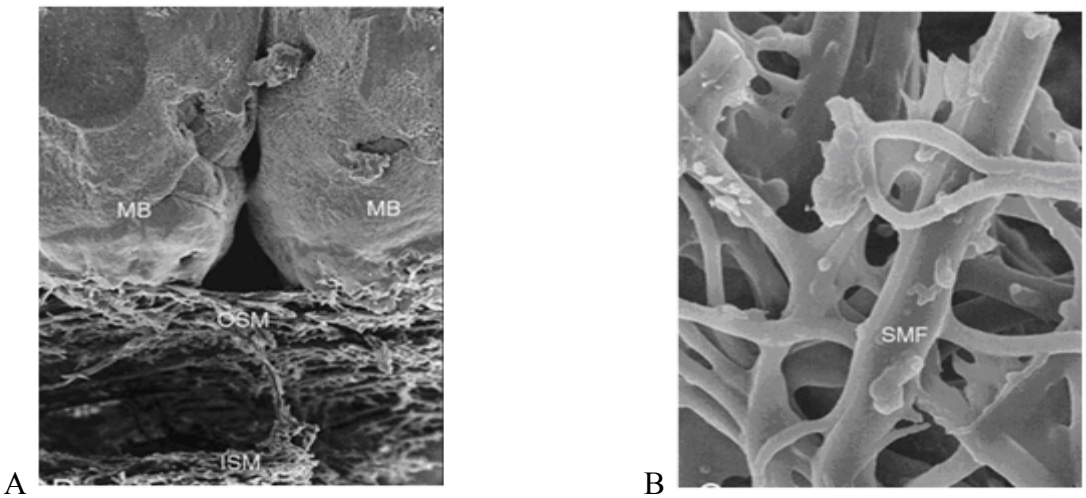

Figure 2: Scanning electron micrographs indicating the morphology of the eggshell membranes. (A) MB: mammillary body (mammillary cones) OSM: outer eggshell membrane ISM: inner eggshell (B) SMF: eggshell membrane fibers (Reprinted from Hincke et al., 2000, with permission from Elsevier).

Previous studies have identified several key proteins in the eggshell membrane using traditional biochemical techniques; more recently, proteomic methods have provided additional information. There are approximately 10% collagens, consisting of types I, V and X. Type X collagen is a cross-linked and insoluble protein that masks type I collagen (Wong et al., 1984; Arias et al., 1991). Type X collagen also contributes to structural integrity of the eggshell membrane (Wang et al., 2002). More recently proteomic analysis identified 29 proteins in the eggshell membrane (Kodali et al., 2011), including a disulfide rich protein, **similar to spore coat protein SP75**, which may be the major structural element. Some proteins that were detected in the eggshell membrane are also known to have the antimicrobial activity. For instance, Ovocalyxin-36 (OCX-36) is a novel and specific chicken eggshell protein that is located in the outer eggshell membrane, and is related to the superfamily of lipopolysaccharide-binding proteins/bactericidal permeability-increasing proteins and Plunc proteins (Gautron et al., 2008). It may take part in natural defense mechanisms that keep the egg free of pathogens. In addition, some of the egg white proteins that are present in the eggshell membranes are ovotransferrin and lysozyme (Hincke et al., 2000; Gautron et al., 2001).

The eggshell membranes are considered to be a waste industrial byproduct that has many potential uses. Isolated eggshell membranes have been exploited in various fields. Eggshell membrane supplement (Natural Eggshell Membrane (NEM®) is a neutraceutical which has been shown in clinical trials to reduce joint pain and stiffness from osteoarthritis of the knee (Ruff et al., 2009a; 2009b). Fresh eggshell membranes are antimicrobial and can help heal certain skin lacerations as an antimicrobial material (Zadik, 2007; Cordeiro and Hincke, 2011). The eggshell is not only being used in the field of

medicine, but recently it is also being used in chemical and industrial fields. For example, the eggshell membrane proteins are able to bind metal ions or other positively charged molecules; therefore, eggshell membranes can remove heavy metals from aqueous solutions (Chojnacka, 2005; Vijayaraghavan et al., 2005; Park et al., 2007; Tsai et al., 2008). In order to fully develop the potential uses of eggshell membranes, it is important to identify the protein constituents of eggshell membrane as the first step to develop and improve the utilization of the eggshell membrane in various fields.

2.2 Eggshell

The next layer of the eggshell is the calcified layer which is the main part of the eggshell. The major component of the mineralized eggshell is calcium carbonate, and it extends from the mammillary cones, through the intermediate palisade layer and the outer vertical crystal layer (Nys et al., 1999; Nys et al., 2004) (Figure 3).

The calcified eggshell offers protection to the egg embryo from physical damage and bacterial infection. Eggshell mineralization occurs during 16 to 17h in the uterus of hen oviduct (Bellairs et al., 2005). The epithelial and mucosal cells that line the lumen of the uterus secrete all components that form the eggshell calcified layer. The incomplete egg bathes in the uterine fluid which contains these components. During eggshell formation, the whole process of calcification can be divided into three stages: initiation (5h), growth or rapid calcification stage (12h) and terminal stage (1.5h) (Nys et al., 1999). The protein and ionic constituents of the uterine fluid progressively change during the process of eggshell mineralization. When the eggshell mineral is deposited on the eggshell membrane, it is accompanied by an organic matrix. This organic matrix is a complicated mixture of proteins, glycoproteins and proteoglycans, which regulate the process of calcification (Nys

et al., 2004; Gautron and Nys, 2007; Rose and Hincke, 2008). The mammillary layer is made of a regular array of cones to the tips of which are embedded the individual fibers of the outer eggshell membrane. The intermediate palisade layer is made up of groups of columns composed of crystallites which the outermost part of the palisade region is the vertical crystal layer, and this layer is continuous with the surface cuticle layer (Nys, 2004).

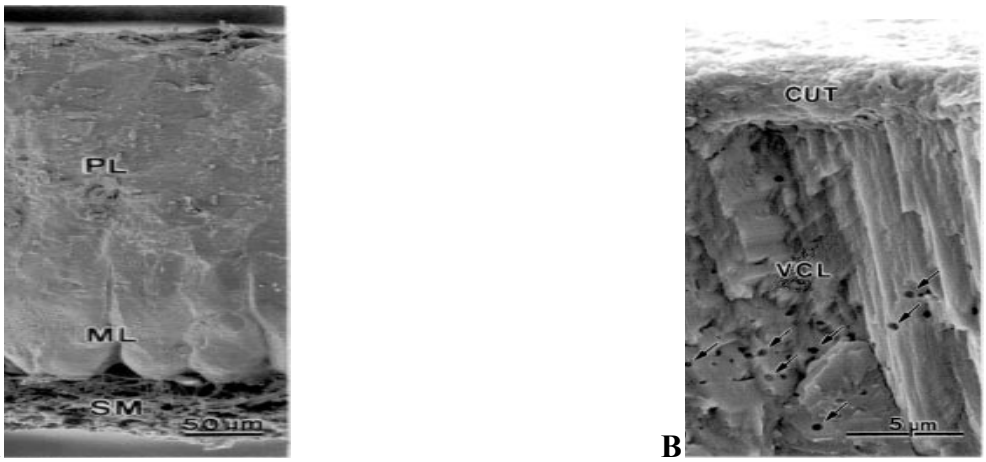

Figure 3: Scanning electron micrographs of eggshell. (A) ML: mammillary layer; PL: palisade layer SM: eggshell membrane. (B) CUT: surface cuticle layer; VCL: vertical crystal layer (Reprinted from Gautron et al., 2001, with permission from American Society for Biochemistry and Molecular Biology)**.**

2.3 Eggshell cuticle

The cuticle layer is composed of an inner calcified and outer non-calcified layer. The non-calcified layer consists of proteins and coats the eggshell. The cuticle layer is produced by secretory cells in the chicken uterus, and forms on the surface of the calcified shell in the last 1.5 hours before oviposition (Wyburn et al., 1973). The structure of the eggshell cuticle layer has been studied by using light and electron microscopy studies (Baker et al., 1962; Dennis et al., 1996). The dry cuticle covers the eggshell cuticle and plugs the pores of the shell. There are two or more layers of cuticle that can be seen clearly in thin sections of the shell, but they do not cover the whole egg calcified layer (Wyburn et

al., 1973). Not all avian eggs have a cuticle layer, for example, pigeon eggs, as well as a small proportion of chicken eggs are lacking the cuticle layer (Board et al., 1973; Board, 1974). The non-calcified cuticle layer contains proteins that are glycoproteins, polysaccharides, lipids and inorganic phosphorus including hydroxyapatite crystals (Dennis et al., 1996; Whittow et al., 2000; Fernandez et al., 2001).

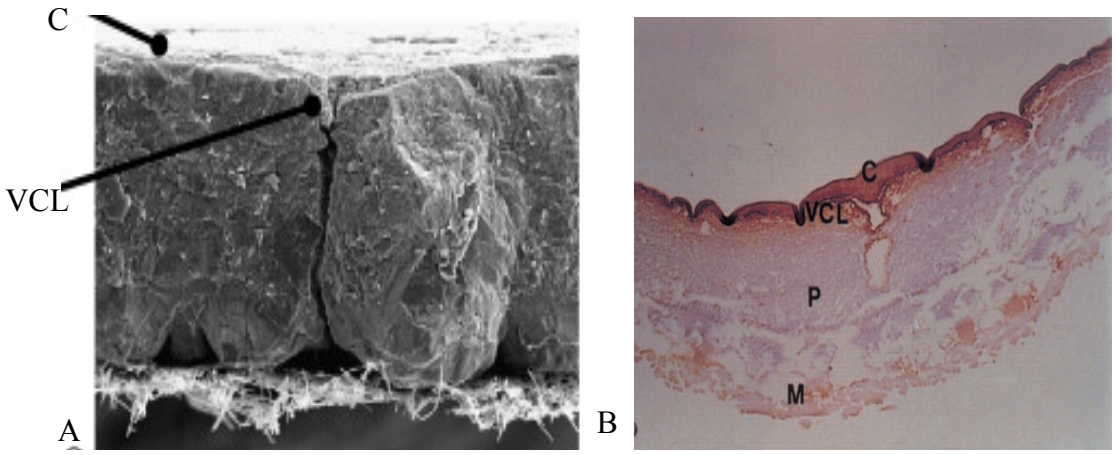

Figure 4: (A) Scanning electron microscope cross-section of eggshell, illustrating the external cuticle layer (Reprinted from Cabeza et al., 2011, with permission from Innovative Food Science and Emerging Technologies) (B) Positive immunostaining of the eggshell layer with anti-32-kDa antiserum. M, membranes and mammillary layers; P, palisade layer; VCL, vertical crystal layer; C, cuticle (Reprinted from Gautron et al., 2001, with permission from American Society for Biochemistry and Molecular Biology)**.**

The function of the cuticle layer is to prevent bacterial infection of the egg through the pores by forming a physical barrier and antimicrobial chemical defense. The cuticle layer participates in controlling the exchange of water/gas and prevents the egg from losing water in the dry terrestrial environment (Board and Tranter, 1986). In our study, we hypothesize that the chemical defense of the cuticle is from its protein composition, and the cuticle layer is an important defense against bacterial contamination.

3. Eggshell Protective Mechanisms

More than 8000 years ago, the jungle fowl was domesticated for the first time in

Southeast Asia, and was later found in northeast China. The chicken was brought to Europe by the Roman conquests and is now found worldwide (Price, 2002). The egg is very nutritious and supplies all the essential amino acids for humans, as well as several vitamins and minerals. The table egg is a very important food for human consumption. Therefore, protection of the chicken egg from the bacterial infection is necessary in order to ensure our continuing quality of food safety and human health.

Bacterial infection of chicken eggs remains a major concern in the poultry industry and the public. It is recognized that infections occur horizontally and vertically. The horizontal infections involve bacteria passing though the shell, via either pores or microcracks, and either remaining in the egg white or infecting the egg yolk. Vertical infection involves a contaminated chicken passing the infection into the egg through its reproductive tissues during the process of egg formation (Omwandho, 2010). Factors that affect bacterial infection of the egg are its shell quality, egg albumen pH, eggshell white and the storage environment (Cox et al., 2000). A number of bacterial strains that can cause visible disease in chicken are *Salmonella enterica serovar Gallinarum* and *Pullorum*, as well as *Mycoplasma gallisepticum* (Omwandho, 2010). The most prevalent bacterial infection of eggs is by *Salmonella enterica serovar Tyhimurium*. The *Salmonella enterica serovar Typhimurium*, the least infectious of the *Salmonella* bacteria, is contained in contaminated eggs, and most healthy adults are able to overcome the infection within a week. However, for young children and elderly adults whom have underdeveloped or compromised immune systems, the infection can be fatal. According to *Statistics Canada 2011* and *Health Canada 2011*, over six thousand to twelve thousand cases of food poisoning occur every year. *Salmonella enterica serovar Enteritidis* is the most common

strain of bacteria found in eggs (Omwandho et al., 2010). The *Salmonella enterica (S.) serovars Typhimurium* and *Enteriditis* infect both poultry and humans, while S. serovar Typhi, can cause typhoid fever, and only infects humans through oral-fecal transmission. S. *serovar Typhimurium* is transmitted through chicken meat mostly, while S. *serovar Enteritidis* is mainly through chicken eggs (Clavijo et al., 2006). Campylobacter bacterial strains are found and transmitted to humans through poultry meat, causing similar symptoms to Salmonella poisoning. Therefore, in order to increase food safety in the poultry industry, isolating novel antimicrobial agents is imperative.

In order to reduce the opportunity for bacterial infection of the table egg during production, the eggs are washed by detergents to remove feathers and feces from the surface of the egg. However, this process is known to introduce microcracks to the surface and can weaken eggs, which allows bacteria to adhere to the shell surface. The washing process also removes a protective layer, the cuticle layer from the exterior of the egg.

For a fertilized ovum to develop into a viable chick after 21 days, two principal defense mechanisms to protect the egg embryo must be in place: the physical defense and chemical protection. The physical defense is the intact eggshell that works as a physical barrier to protect the egg embryo from the infection of bacteria, physical damage and small predators (Gautron et al., 2007b). The chemical protection system is composed of the albumen and eggshell matrix proteins that work as an antimicrobial defense (Seuss-Baum et al., 2007; Rehault et al., 2007; Hincke, 2008; Hervé-Grépinet et al., 2009).

The outer part of the egg is the eggshell cuticle and calcified eggshell, which compose the first physical barrier against bacteria. Pores inside the eggshell allow the

water/gas exchange for the growing embryo. In addition, the inner and outer eggshell membranes separate the eggshell from the egg white and form a mesh to capture bacteria. The egg white contains a variety of antimicrobial proteins such as lysozyme (Pellegrini et al., 2008), ovotransferrin (Clavijo et al., 2006) and ovalbumin (Pellegrini et al., 2004). The process of producing these defenses is entirely linked to the process of egg formation. Each layer of the egg must be formed in the appropriate tissue for the required amount of time, in order to ensure the transfer of the defensive peptides.

A proteomic approach has been used to identify protein components of the egg, such as constituents of the vitelline membrane, egg yolk, egg white and eggshell (Mann et al., 2007; Miksík et al, 2007;Mann 2008a; Mann et al., 2008b; Omana et al., 2011). A total of 137 proteins were identified in the vitelline membrane, 13 of them were known previously to be components of it (Mann, 2008a). Most of the components of the vitelline membrane were also known from the other egg compartments, such as lysozyme C, ovalbumin, ovotransferrin and ovomucin (Mann et al., 2008a). In the egg yolk, 119 proteins were identified by proteomic analysis (Mann et al., 2008b). Among these 119 proteins, there were 86 proteins that were previously found in the egg yolk. Egg white contains a lot of proteins, 78 chicken egg white proteins were identified by LC/MS/MS, and 54 of them were identified in egg white for the first time (Mann et al., 2007; Omana et al., 2011). So far, more than 500 eggshell matrix proteins have been identified by using tandem- mass spectrometry, including some matrix proteins that were already known before (Mann et al., 2006). Among these 520 eggshell matrix proteins, there are ten abundant proteins that have been identified by using the biochemical and molecular techniques and these proteins belong to three categories. First of all, ovalbumin (Hincke, 1995), lysozyme (Hincke et al.,

2000) and ovotransferrin (Gautron et al., 2001) are three **egg white proteins** that are found in the eggshell. Secondly, osteopontin (Pines et al., 1994), and clusterin (Mann et al., 2003) are two important proteins can be **found in many tissues**. Thirdly, ovocleidin-17(OCX-17) (Hincke et al., 1995), ovocleidin-116(OCX-116) (Hincke et al., 1999), ovocalyxin-32(OCX-32) (Gautron et al., 2001), ovocalyxin-36(OCX-36) (Gautron et al., 2007) and ovocalyxin-21(OCX-21) (Gautron et al., 2007) are **egg- specific proteins only secreted in the uterus** where eggshell formation occurs.

4. Hypothesis and Objectives

The avian egg possesses a wide variety of physical and chemical barriers to protect the embryo from bacterial infection. This is not only important for survival of the species, but also protect the consumer against food borne illnesses.

There have been careful studies to identify the proteome of vitelline membrane, egg yolk, egg white and eggshell, but almost nothing has been studied on the cuticle layer of the eggshell. The function of the cuticle layer is to prevent bacterial infection of the egg through the pores by forming a physical barrier. In our study, we hypothesize that the chemical defense of the cuticle is from its protein composition, and the cuticle layer is an important defense against bacterial contamination. Therefore, identification of total proteome of the cuticle layer is the first and necessary step to understanding the antimicrobial mechanisms of the avian eggshell.

Since poultry and eggs are at risk for bacterial contamination, novel antimicrobial agents must be identified as a first step towards inhibition of bacterial growth, and decreases in infection rates. A variety of antimicrobial proteins have been identified in the

egg cuticle layer. One of the most abundant protein, similar to Kunitz-like protease inhibitor (also known as Ovocalyxin-25) in the cuticle layer, is poorly understood. OCX-25 was predicted to be a Kunitz-like serine protease inhibitor. Although its mechanism is unknown, several serpins possess antimicrobial activity. Due to the similarity between OCX-25 and BPTI (Bovine pancreatic trypsin inhibitor), it is hypothesized that OCX-25 is a Kunitz-like protease inhibitor, with antimicrobial properties that function to inhibit Gram-negative and/or Gram-positive bacteria. Although serpin and antimicrobial activities are predicted for OCX-25, it is essential to experimentally measure these properties with the isolated protein. Therefore, one of the objectives of my research is to extract OCX-25 in order to test its enzymatic and antimicrobial activity

The eggshell membranes that line the calcified shell are another natural barrier against bacterial penetration if they are formed properly in the white isthmus of chicken oviduct. Because of the highly cross-linked and stable nature of the membrane fibers, there are many questions about their composition and functional significance. The previous transcriptome study of the chicken uterus during formation of the eggshell provides the first detailed description of the over-expressed transcripts during eggshell formation. The 605 over-expressed uterine transcripts correspond to 469 unique genes, which encode 437 different proteins. There were 54 proteins secreted by the uterus during eggshell formation (Jonchere et al., 2011). In order to identify the properties of the eggshell membrane, we examine over-expressed genes in the white isthmus during the formation of the eggshell membrane, since the transcriptome study can provide a detailed understanding of gene expression. Using bioinformatic analysis the genes over-expressed in the white isthmus can be identified and linked to the eggshell membrane proteins that contribute to the eggshell

membrane physical and chemical features.

Chapter II Identifying specific proteins involved in eggshell membrane formation using gene expression analysis and bioinformatics.

(This chapter is preparing to publish for BMC journal)

Abstract

Background

The avian eggshell membrane provides a structural foundation for eggshell calcification, but is also a natural biomaterial with many potential applications. However, due to its insoluble and stable nature, there are still many questions regarding its formation and constituents. The purpose of this study was to identify eggshell membrane proteins, particularly these responsible for its structural features, by examining the transcriptome of the white isthmus segment of the oviduct, the specialized region which is responsible for the fabrication of the membrane fibres.

Results

A total of 135 transcripts were over-expressed in the white isthmus compared with both adjacent magnum and uterine segments of the hen oviduct (over-expression levels ranged from 1.1- to 156-fold). These white isthmus transcripts correspond to 103 unique genes, which encode 85 annotated proteins. Gene Ontology (GO) analysis was used for interpretation of protein function. Fourteen proteins possess a classic signal peptide that could specify extracellular secretion via the ER / golgi route; another 32 were predicted to be secreted by an unconventional mechanism. Structural proteins that were found to be highly over-expressed in white isthmus were collagen X and **similar to spore coat protein SP75** (annotated as Cysteine Rich Eggshell Membrane Protein by Kodali et al. (2011) PLoS ONE 6: e18187). Moreover, genes encoding collagen-processing enzymes were over-expressed, as were proteins known to regulate disulfide cross-linking, suggesting that coordinated upregulation of gene suites in the white isthmus is associated with eggshell membrane fibre formation.

Conclusions

Genes associated with eggshell membrane formation in the white isthmus have been identified. These results will assist with development of selection strategies to improve eggshell quality and food safety of the table egg.

1. Background

Biomaterials have increasing applications in many useful fields; in order to maximally explore their function it is essential to fully understand their components and individual properties. For instance, current research on spider silk indicates its potential use as an incredibly strong and versatile material (Hagn, 2012). Another interesting biomaterial is the chicken eggshell membrane, which is available in large quantities as waste from the egg processing industry. Due to its unique properties, it has industrial, clinical, cosmetic and neutraceutical applications (Cordeiro and Hincke, 2011). For example, eggshell membranes can selectively adsorb metal ions and other positively charged molecules from industrial water waste (Park et al., 2007). Clinical trials indicate that ingestion of glycosaminoglycan and proteins from eggshell membrane is beneficial for healthy joints and regulation of connective tissues (Ruff et al., 2009). Since the eggshell membrane hydrolysate is composed of hexosamine, chondroitin sulfate, hyaluronic acid, collagens and other proteins, it has been used in fabrication of cosmetics (Long et al., 2008). Eggshell membrane has also been used as a biotemplate for the development of metallic nanostructures (Lee et al., 2010). In order to optimally exploit this biomaterial, it is necessary to fully characterize its constituents.

During egg formation, the accumulation of liver-secreted egg yolk proteins takes place in the chicken ovary. After ovulation, the egg yolk enters the infundibulum of the oviduct where the vitelline membrane surrounding the yolk is deposited. During the subsequent passage of the forming egg though specialized regions of the oviduct, the egg white (magnum), eggshell membranes (white isthmus), eggshell and eggshell cuticle (uterus) are sequentially deposited. The innermost layer of the eggshell is the eggshell

membranes that are deposited as a highly crossed-linked extracellular fibrous meshwork during ≤1.5 h passage through the white isthmus (Arias et al., 1993; Nys et al., 2004). The fibres are organized into inner and outer membranes, which are joined by inter-connecting fibres, and form a highly cross-linked meshwork, arranged in alternating layers parallel to the eggshell surface (Hincke et al., 2000).

Calcification of the eggshell originates at nucleation sites on the surface of the outer eggshell membranes, which are the origins of the mammillary cones of the inner, mineralized, eggshell. The nature of the template for initiation of mineral deposition at this array of nucleation sites is poorly understood. However, this initial calcification is a key step in eggshell mineralization. The texture and many ultrastructural features of the resulting shell such as mammillary cone spacing and their attachment to the underlying membrane fibres are important for shell strength (Bain, 1992; Dunn et al., 2012; Garcia-Ruiz and Rodriguez-Navarro, 1994). Animal studies have demonstrated that disruption of eggshell membrane fibres (by altered collagen cross-linking and/or organization) severely reduces eggshell quality and strength (Chowdhury and Davis, 1995; Arias et al., 1997a; Chowdhury and Davis 1998); the membranes are essential elements for fabrication of a normal eggshell which resists bacterial contamination of the table egg (Chowdhury, 1990).

Due to the stable, insoluble and highly cross-linked nature of the membrane fibres, there are many unanswered questions about their composition and functional significance. Earlier workers identified types I, V and X collagens in the eggshell membranes (Leach, 1982; Wong et al., 1984; Arias et al., 1991; Arias et al., 1997b; Fernandez et al., 1997). Moreover, other proteins have been identified in the eggshell

membranes using traditional biochemical techniques. Egg white proteins, such as lysozyme and ovotransferrin, have been identified in the eggshell membrane (Hincke et al., 2000; Gautron et al., 2001). Ovocalyxin-36 (OCX-36) is a novel chicken eggshell protein that is concentrated in the eggshell membranes; it belongs to the superfamily of lipopolysaccharide-binding proteins/bactericidal permeability-increasing proteins and Plunc proteins (Gautron et al., 2007; Gautron et al., 2011). More recently partial proteomic analysis of eggshell membranes identified a disulfide rich protein, **similar to spore coat protein SP75** (abbreviated SCPSP75) and termed "Cysteine Rich Eggshell Membrane Protein" (Kodali et al., 2011). In addition, the secretion by the isthmus of a keratan sulphate proteoglycan, the core of which remained unidentified, coincides with the formation of the mammillae and is located at the site of nucleation of the first crystals (Fernandez et al., 1997, 2001).

Even though previous studies defined some proteins that are located in the eggshell membrane fibres, we ndo not yet have a complete understanding of all proteins involved in the formation of the eggshell membrane. In the present study, we adopt a different strategy to investigate such proteins, using transcriptional profiling of the hen oviduct to identify genes that are differentially expressed in the white isthmus during the formation of eggshell membrane. Since the entire oviduct originates from the same population of cells (Guioli et al., 2007), we hypothesize that over-expressed genes in each specialized region of the oviduct specify the proteins that are involved in the formation of that particular egg component. Our previous study of the hen uterus transcriptome during formation of the eggshell identified a large number of functional genes that participate in eggshell formation and ion transport for its mineralization (Jonchère et al., 2010; 2012).

Therefore, the comparison of gene expression in the white isthmus, where the eggshell membrane is formed, with two adjacent segments (magnum, where the egg white is formed, and uterus, site of eggshell calcification) is predicted to reveal specific genes encoding proteins involved in the formation of the eggshell membranes. In this study, we used bioinformatics analysis, such as functional annotation and gene ontology enrichment studies, to identify the proteins that are encoded by the over-expressed genes in the white isthmus and are likely to have a key role in the structure and formation of the eggshell membranes.

2. Results

2.1 White isthmus gene over-expression

The Del-Mar 14K Chicken Integrated Systems microarray (GPL1731) was used to analyze gene expression in various segments of the chicken oviduct (Cogburn et al., 2004). Validation by qPCR previously performed (Jonchere et al., 2009, 2012). The comparison of gene expression in the white isthmus, with two other segments of the oviduct (magnum or uterus) was hypothesized to predict genes encoding proteins involved in the formation of the eggshell membranes. When the over-expression threshold was set at 1.1-fold, a total of 582 genes were over-expressed in the white isthmus compared to the magnum, while 1507 genes were over-expressed in the white isthmus compared to the uterus (false discovery rate (FDR) <0.05). However, only 135 over-expressed transcripts were common to the two comparisons (Figure 1); according to our hypothesis, these over-expressed genes are related to white isthmus-specific functions.

2.2 Gene functional annotation

The 135 over-expressed transcripts that were common to two comparisons

(WI/Ut and WI/Ma) were further assessed (Table 1). Two of them do not correspond to any EST sequence. Moreover, eighteen transcripts had no hits in the Entrez Gene database and were annotated as unknown genes. The remaining 115 over-expressed transcripts correspond to 103 unique Entrez Gene IDs. Nineteen sequences have no records in the UniGene database, and the remaining 114 sequences were found to correspond to 101 unique chicken UniGene IDs.

Further review revealed that the over-expressed genes in the white isthmus correspond to 85 proteins possessing an NCBI Reference Sequence ID (Table 1). Among these 85 proteins, 7 proteins were annotated as hypothetical proteins and were dropped from the analysis.

2.3 Gene ontology enrichment analysis

The gene ontology enrichment analysis of gene expression by EASE utilizes GO term groups that are classified according to biological function (Table 2, Figure 2). The intracellular part contains 29 genes that are distributed in overlapping manner between Intracellular (GO:0044424), Cytoplasm (GO:0005737, GO:0044444) and Ribosome (GO:0005840). The Metabolic Process group contains 25 genes that are divided into 5 genes of the Cellular Protein Metabolic Process (GO:0044267) and 20 genes in the cellular carbohydrate metabolism process, protein metabolic process and cellular macromolecular process (GO:0044262; GO:0019538; GO:0044260; respectively). The biosynthetic process group has 13 genes (GO:0009059; GO:0044249; GO:0009058). The Translation group has 11 genes (GO:0005853; GO:0006412). The Molecular activity group has 7 genes; of these Fbn1 is only in the Structural molecule activity group (GO:0005198) while the remaining 6 are also in the Structural constituent of ribosome group (GO:0003735). The last group is

the nucleic acid binding group, with 6 genes in the RNA binding (GO:0003723) group.

2.4 Prediction of extracellular secretion

Our next step was to determine which proteins are predicted to be secreted, since these are candidates to participate structurally or enzymatically in the formation of the eggshell membrane fibres which occurs into the lumen of the oviduct.

The SecretomeP 2.0 server was used to interrogate the protein sequences (UniProt/SwissProt database) of over-expressed transcripts to search for predictions of protein secretion by via classical signal peptide – mediated machanism or the unconventional pathway which is increasingly recognized for leaderless secretory proteins (Dyrløv Bendtsen et al. 2004a; 2004b) (Table 3). Of these, 14 proteins were predicted to have signal peptides that could lead to passage through the classic ER pathway, while another 32 had characteristics predicting unconventional secretion (Table 3). Those with predicted signal peptides included collagen X and two enzymes associated with collagen processing and posttranslational modification (P4HA2, PPIC). Another was gallinacin-10, belonging to the antimicrobial family of avian beta-defensins. The potential functions of these proteins was assessed according to their annotations in the UniProtKB/SwissProt and NCBI protein databases (Table 3). Potential structural proteins that possessed sequence determinants for unconventional secretion included **Similar to Spore Coat Protein SP75** and Fibrillin-1.

3. Discussion

The avian eggshell membrane has many potential uses due to its biomaterial properties. For example, isolated chicken eggshell membranes have been used in clinical, industrial, cosmetic and other fields (Cordeiro and Hincke, 2011). The eggshell membrane

gives good results as a biological dressing for burn (Maeda, 1984), and is also suitable for the adherence of stromal cells in cell culture (Tavassoli, 1983). The eggshell is not only being used in the field of medicine, but recently has been used in chemical and industrial fields. For example, the eggshell membrane proteins are able to bind metal ions or other positively charged molecules, and can be used to remove heavy metals from aqueous solutions (Park et al., 2007). Because of many potential uses for eggshell membranes, identifying the protein components is the first step to develop and improve the utilization of the eggshell membrane in various fields. However, it has been difficult to analyze the components of the eggshell membrane due to its insolubility and highly cross-linked nature. For this reason, a bioinformatics approach is useful to identify the proteins that are encoded by over-expressed genes in white isthmus and are hypothesized to be involved in the formation and fabrication of the eggshell membrane.

Our previous transcriptome study of the chicken uterus during eggshell calcification provided a detailed description of the over-expressed genes likely to be important for eggshell formation (Jonchere et al., 2010). In a related study, the genes encoding proteins responsible for the ion transport necessary for eggshell mineralization were identified (Jonchere et al., 2012). We have now extended this strategy to gain insight into the genes associated with eggshell membrane formation in the white isthmus. Using a threshold of 1.1-fold over-expression, we identified 133 genes and 85 annotated proteins that are over-expressed in white isthmus compared to both the magnum and uterine segments of the oviduct. Of these, 45 proteins were predicted to either possess a signal peptide or determinants for secretion via an unconventional mechanism, which could lead to secretion into the lumen of the oviduct during eggshell membrane formation. A well

known member of this second group is ovalbumin, the most abundant egg white component, which does not possess a signal peptide but is predicted by SecretomeP 2.0 server to be secreted by the unconventional pathway. A number of the other over-expressed genes were predicted by Gene Ontology analysis to be associated with intracellular metabolism, gene transcription, and protein synthesis.

In the next section, we will focus the discussion on the most highly over-expressed genes (Table 4), which we arbitrarily defined as over-expression ≥ 2-fold relative to both magnum and uterus. A relatively small number of genes were in this category. Two of these genes (Collagen X, IgGFc-binding protein-like) were identified by more than one clone, which was a microarray characteristic and underlines the reproducible nature of the over-expression assessment. Collagen X (111.3- and 50.3-fold in WI/Ut, 155.8- and 94.5-fold in WI/Ma) was previously identified as a significant component of the eggshell membrane fibres (Arias et al., 1997). IgGFc-binding protein-like (22.6- and 7.6-fold in WI/Ut, 34.0- and 8.8-fold in WI/Ma) may be involved in the maintenance of mucosal structures as a gel-like component (Harada, 1997) and could contribute to mucosal function in the white isthmus. Another over-expressed gene, **similar to spore coat protein SP75** (abbreviated SCPSP75), was expressed in a strongly white isthmus– specific manner (70.0-fold in WI/Ut; 57.9-fold in WI/Ma). The corresponding protein was recently identified in chicken eggshell membranes as the main source of cysteine-containing peptides and possibly is a major structural component (Kodali et al., 2011). This disulfide-rich protein is currently annotated as **similar to spore coat protein SP75,** which was originally identified in slime moldA number of genes encoding enzymes predicted to be associated with disulfide mediated protein cross-linking were found in our study to be

over-expressed in the white isthmus. Thioredoxin is encoded by TXN (2.6-fold in WI/Ut, 2.1-fold in WI/Ma); it participates in many biological processes as an antioxidant by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Protein disulfide isomerase (PDIA5) catalyzes the formation of disulfide bonds (over-expressed 1.3-fold in WI/Ut and 1.3-fold in WI/Ma). Kodali et al. (2011) showed that reduced **similar to spore coat protein SP75 (SPCSP75)** is an efficient thiol substrate of sulfhydryl oxidase (QSOX1), which can be detected in the egg white and hen oviduct tissue (Hoober et al., 1996; Kodali et al., 2011; Alon et al., 2012). However, our study did not find that QSOX1 was over-expressed in the white isthmus; therefore, we predict that egg white QSOX1 may function by catalyzing the cross-linking of of membrane fibre precursors upon the surface of the egg white compartment as the membrane fibres form.

Other highly up-regulated genes listed in Table 3 do not have an obvious function, MAX interactor 1(MXI1) (14.1-fold in WI/Ut and 9.8-fold in WI/Ma) corresponds to a putative uncharacterized protein (SwissProt: Q5ZMT3_CHICK); in humans, the protein encoded by this gene is a transcriptional repressor thought to negatively regulate MYC function by competing for MAX, another basic helix-loop-helix protein that binds to MYC and is required for its function (Kim, 2010). Another highly over-expressed gene was Gallus R3H domain and coiled containing 1 (79.5-fold in WI/Ut and 136.1 in WI/Ma); the function of the protein encoded by this gene is unknown although it is predicted to bind ssDNA or ssRNA in a sequence-specific manner (Marchler-Bauer et al., 2009). LIM and calponin homology 1 is also highly over-expressed in the white isthmus (11.6-fold in WI/Ut and 15.5-fold in WI/Ma); it contains two domains: the calponin homology domain is actin binding and the LIM domain is a small protein-protein

interaction domain with two zinc fingers (Krause et al., 2004). Sorbitol dehydrogenase is an enzyme of glucose metabolism which produces fructose and is encoded by the SORD gene (1.8-fold in WI/Ut and 2.3-fold in WI/Ma).

3.1 Collagens and related proteins

Previous research found that the eggshell membrane fibres are highly cross-linked, and contain at least 10% collagens (types I, V and X) (Wong et al., 1984; Arias et al., 1991). Since the eggshell membrane fibres provide a template for initiation of calcification at an array of nucleation sites, which specify the organization of the mammillary cones, the eggshell membranes must form properly for optimal mineralization. Inhibition of collagen cross-linking, for example, by feeding β-aminoproprionitrile to laying hens, causes eggshell membrane disorganization and defects in eggshell mineralization (Arias et al., 1997). If the eggshell membrane fibres do not form properly, appropriate nucleation and spacing of mammillary cones do not occur and the resulting eggshell is abnormal. In situ hybridization and immunohistochemistry has indicated that collagen X is mainly expressed in the tubular gland cells of the isthmus segment (Wang et al., 2002). A proteomic study also detected collagen X in trypsin - digested eggshell membranes (Kodali et al., 2011). Collagen X is a short chain collagen which contributes to structural integrity, and is proposed to inhibit generalized mineralization of the membranes by establishing boundaries which are protected from mineral deposition (Arias et al., 1997).

In our study, the microarray contains two independent clones that are specific for different regions of the collagen alpha-1(X) chain-like (GENE ID: 100858979). Collagen X alpha-1 overexpression levels in white isthmus were extremely high versus both magnum (155.8-, 94.5-fold) and uterus (111.3-, 50.3-fold), as detected with clones pgm1c.pk003.b7

and pco1c.pk001.c12, respectively. The Del-Mar 14K microarray does not contain any clones corresponding to the collagen type X alpha-2 chain, so we cannot assess overexpression of that gene.

Type I and V collagens are less abundant constituents of the eggshell membranes, and represent 4mg/g of whole eggshell membrane, or 0.6% of the membrane protein (Wong et al., 1984; Arias et al., 1991). In our study, over-expression of collagen I alpha-1 (expression level: 1.23-fold) and alpha-2 (expression level: 1.19-fold) was detected In the WI/Ma group; however, neither of the type I collagen chain genes were over-expressed in the WI/UT comparison. Type III collagen (alpha-1 chain) was found to be over-expressed (1.16-fold) in the WI/Ma comparison. Collagen type IV (alpha-2 chain) and type V collagen (alpha-2 chain) were slightly under-expressed in the WI/Ma comparison. On the other hand, no overexpression of collagens I – V were detected in the WI/UT group, suggesting that these genes were expressed at equivalent levels in both white isthmus and uterine segments of the oviduct. Collagen proteins [type I (both alpha-1 and -2 chains) and III (alpha-1 chain)] have been detected in avian eggshell matrix (Miksik et al., 2010) (Supplemental table 2). In earlier proteomic studies, peptides derived from collagens type II, VI, and X (alpha-1) were also detected in the tryptic digests of acid-soluble organic matrix of chicken eggshell (Mann et al., 2006). In summary, a significant structural fibrillar proteins, collagen X, was found to be the most highly upregulated gene in our study, while a number of other collagen genes are also significantly over-expressed.

In view of our demonstration that a number of collagen genes were up-regulated in white isthmus, we searched for over-expression of genes encoding collagen processing enzymes. Lysyl oxidase is a copper-sensitive enzyme that is associated with formation of

collagen cross-links. Lysyl oxidase activity is located in the copper-rich region in the isthmus of the hen oviduct (Harris et al., 1980), and can be detected in the eggshell membranes (Akagawa et al., 1999). This enzyme participates in the cross-linking of eggshell membrane protein (Harris et al., 1980). In chicken, there are 5 lysyl oxidase genes (Lysyl oxidase - LOX, and lysyl oxidase homologs 1 – 4); the Del Mar 14K Chicken Integrated Systems microarray contains clones allowing detection of LOX and Homologs -1, -2 and -3. Lysyl oxidase homolog-3 (XP_423667.3) was identified as a constituent of eggshell membranes by proteomic analysis (Kodali et al., 2011). Our results showed that this lysyl oxidase was only over-expressed 1.3-fold in the WI/MA group, but not over-expressed in the WI/UT comparison. However, our data reveals that lysyl oxidase homolog-2 (XP_003642594.1) was highly over-expressed, but only in the WI/UT group (18.3-fold). These results indicate that both lysyl oxidase homolog-2 and -3 are likely to participate in the cross-linking of collagen during formation of the eggshell membranes..

Prolyl 4-hydroxylase subunit alpha-2 is a collagen-associated protein encoded by the P4HA2 gene, which is a component of prolyl 4-hydroxylase, a key enzyme that is required for collagen synthesis. This gene was over-expressed in the white isthmus (1.2-fold and 1.6-fold in the WI/Ut and WI/Ma comparisons, respectively). Prolyl 4-hydroxylase catalyzes the formation of 4-hydroxyproline that is essential to the proper three-dimensional folding of newly synthesized procollagen chains (Bassuk et al., 1989; Annunen et al., 1997). Peptidyl-prolylcis-trans isomerase C is another collagen-associated protein, which catalyzes the cis-trans isomerization of proline imidic peptide bonds. In vitro refolding of denatured type III collagen is rate-limited by cis-trans isomerization of the peptide bond by peptidyl-prolylcis-trans isomerase (Bächinger et al., 1987). Prolyl

3-hydroxylase-1 and peptidyl-prolylcis-trans isomerase B form a key complex which is involved in prolyl 3-hydroxylation of type I procollagen (Pyott et al., 2011). Therefore, genes encoding collagens as well as collagen-processing enzymes are upregulated in white isthmus.

3.2 Similar to spore coat protein SP75 (SCPSP75)

Our present study showed that the expression level of SCPSP75 in the white isthmus is 50.3 times higher than in the uterus (eggshell), and 57.9 times higher than in the magnum (egg white). A previous study related to eggshell membrane composition (Kodali et al., 2011) showed that SCPSP75 peptides were present in the partial tryptic digest of reduced alkylated eggshell membrane. They proposed that SCPSP75 is the main source of cysteine-containing peptides in eggshell membrane and might be a major structural component of the membrane fibres. Our transcriptional profiling results for the white isthmus supported the proteomic study since this gene is highly and specifically overexpressed in the white isthmus, and SecretomeP 2.0 analysis indicates that it is likely secreted via a non-classical pathway.

3.3 Fibrillin-1

Another protein that may be structurally involved in the eggshell membrane is fibrillin-1 (FBN-1). The over-expression level was 45.9 times in the WI/Ut and 2.5 times in the WI/Ma. Many studies show that FBN-1 is an extracellular protein (Robert et al., 2000), and our analysis predicts secretion by the unconventional pathway. Research investigating fibrillin gene expression in a mouse model indicated that fibrillin-1 offers structural support for tissues outside the cell (Sakai et al., 1991; Zhang et al., 1995). The FBN-1 sequence that we identified contains the EGF (epidermal growth factor, cl09941) domain that appears in

membrane-bound and extracellular proteins.

3.4 Protein secretion prediction

It is important to note that the over-expressed genes identified in this report are hypothesized to be involved specifically in the formation of the eggshell membranes. However, proteins that are key components in its formation or its antimicrobial protection could also be highly expressed in the uterus (eggshell) or magnum (egg white) and therefore were eliminated as over-expressed white isthmus transcripts in this comparative analysis. In order to predict whether the 85 over-expressed proteins are secreted during the eggshell membrane formation, the potential for secretion,mediated by signal peptide or by unconventional secretion pathway, was assessed using SecretomeP 2.0 (Table 3). This analysis predicted that almost half of these proteins are possibly secreted. Collagen alpha-1(X) chain encodes the alpha chain of type X collagen. Collagen-associated proteins in this group are prolyl 4-hydroxylase subunit alpha-2 and peptidyl-prolylcis-trans isomerase C. In addition, both lysyl oxidase homolog-2 and homolog-3 are predicted to possess a signal peptide. However, many of them were shown for the first time to be expressed in the white isthmus, and their functions are not clear.

Two of the predicted extracellular proteins are potential antimicrobial components that could contribute to the egg's innate defense mechanism. The first is putative phospholipase B-like 1; the annotation of this protein in the UniProtKB/Swiss-Prot database is from Bovine Q9GL30 (PLBL1_BOVIN). Its function in birds is not clear; however, it may be involved in antimicrobial protection and in the generation of lipid mediators of inflammation in human, rat, cattle and other species (Morgan et al., 2004). The second, gallinacin-10 (Avian beta-defensin 10 (AvBD10)) is encoded by the GAL10 gene

(Lynn et al., 2007). Beta-defensins are a group of cysteine-rich antimicrobial peptides that are effective against Gram-positive and Gram-negative bacteria, fungi and yeast. Proteomic analyses of egg compartments have detected avian beta defensins in multiple egg components (Mann et al., 2006; Mann et al., 2008).

Calcium-binding proteins mediate Ca^{2+} signaling in various cellular activities. Reticulocalbin-2 (Ozawa and Muramatsu, 1993) is a calcium-binding protein that contains six conserved regions similar to the EF-hand motif. The EF-hand is a common structural motif in calcium-binding proteins. Transforming growth factor beta 3 (TGF-β3) is a member of a cytokine family-TGF-β superfamily, and it is involved in cell differentiation, embryogenesis and development as well as anti-inflammatory activities (Herpin A et al., 2004). A disintegrin and metalloproteinase with thrombospondin motifs 1 is an enzyme that is encoded by the ADAMTS1 gene, a metalloprotease with proteoglycan-degrading activity that has a role in the remodelling of extracellular matrix and is involved in mineralised nodule and bone formation (Lind et al., 2005) .

Among 85 predicted proteins, 7 were annotated as hypothetical proteins and their function is unknown. It is intriguing that amongst the 78 annotated proteins identified in our study, only 3 proteins: collagen X, Similar to spore coat protein SP75 and lysyl oxidase homolog-3, were also detected by partial digestion and proteomic analysis of eggshell membrane (Kodali et al., 2011). Thus, quite different results were obtained with two different strategies to identify eggshell membrane constituents. In our study, we identified up-regulated genes in white isthmus during eggshell membrane formation. On the other hand, proteomic analysis only detected abundant components of the eggshell membrane that were susceptible to tryptic digestion. Perhaps this is related to the observation that

some authentic membrane components are actually highly up-regulated in other oviduct segments, such as OCX-36 (uterus, Gautron et al., 2007) and lysozyme (magnum, Hincke et al., 2002) , and become incorporated / concentrated at the membrane fibres by accretion or unknown mechanisms.

4. Conclusions

Gene expression profiling of the chicken oviduct during the formation of eggshell membrane has revealed numerous differentially expressed genes. The function remains unknown for most of the proteins that are encoded by the over-expressed genes identified in this study. In our study, bioinformatics tools were applied to analyze the differentially expressed genes as well as their encoded proteins. This method allowed us to identify 135 over-expressed genes in the white isthmus, corresponding to as many as 85 annotated proteins, which are hypothesized to be involved specifically in the formation of the eggshell membrane fibres.

The detailed annotation of these genes and their encoded proteins, leading to a clear knowledge of their functional properties, will be an important step towards determining their role in eggshell membrane formation.

Eggshell membrane fibres have a highly cross-linked nature and function as a natural filter to defend the egg against bacteria. We have identified potential antimicrobial protein components of the membranes and clearly defined abundant structural elements which may have potential applications. This information may lead to novel strategies to optimize egg antibacterial defenses to reduce the risks of food-borne disease.

5. Methods

5.1 Ethics statement

All experiments, including all animal-handling protocols, were carried out in accordance with the European Communities Council Directives of 24 November 1986 (86/609/EEC) concerning the practice for the care and Use of Animals for Scientific purposes and the French ministerial decree 87848 of 19 October 1987 (revised on 31 May 2001) on Animal experimentation under the supervision of authorized scientists (authorization # 7323, delivered by the DDPP, direction départementale de la protection des populations, d'Indre et Loire). The experimental unit UE-PEAT 1295 where the birds were kept has the agreement for rearing birds and for the euthanasia of experimental animals (decree N° B37-175-1 of August 28th 2012 delivered by the Préfecture d'Indre et Loire following the inspection of the Department Direction of Veterinary Services). The protocol was approved by an ethics committee (comité d'éthique de val de Loire, officially registered under number 19 of the French national ethics committee for animal experimentation).

5.2 Animal Handing and Tissues Collection

Forty-week old brown egg-laying hens (ISA brown strain) were caged individually, with a light/dark cycle of 14 hours light and 10 hours darkness, and controlled humidity and temperature. Each cage was equipped with a device for automatic recording of the oviposition time. The hens were fed a layer mash that was recommended by the Institut National de la Recherche Agronomique (INRA).

Tissue samples were collected from different segments (magnum, white isthmus

and uterus) of the hen oviduct during the phase of eggshell calcification. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until isolation of RNA.

5.3 RNA Isolation and Microarray Hybridization

The Del-Mar 14 K Chicken Integrated Systems Microarray (NCBI GEO: GPL1731) was used to analyze gene expression in different parts of the hen oviduct during the formation of the eggshell membranes. This microarray system represents four major physiological systems, with 9,833 unique cDNA clones from the metabolic and somatic systems, and 7,937 unique cDNA clones from the neuroendocrine and reproductive systems (Cogburn et al., 2004), representing 14,053 unique cDNAs.

RNA extraction, quality controls, cDNAs labeling and hybridization were performed as previously reported (Jonchère et al. 2010). Briefly, RNeasy Mini Kit (Qiagen, France) was used to extract RNA from frozen tissue samples; the samples were also treated with DNase 1 (Macherey-Nagel EURL, France). RNA concentration was determined at 260nm. The intergrity of RNA was analyzed on a 1% agarose gel with an Agilent 2100 Bioanalyzer (Agilent Technologies, France). RNA samples with a 28S/18S ratio > 1.3 was used for labeling and hybridization. The Superscript Plus Indirect cDNA labeling System (Invitrogen) was utilized to label total RNA (20□g). After synthesis and purification, the labeled cDNA sample was assessed with a Nanodrop ND 1000 (Nanodrop, Nyxor Biotech).

A balanced block design was applied to hybridization; half of the samples were labeled with Alexa 555 and the other half were labeled with Alexa 647 (Fisher Scientific). There were two comparisons, White isthmus vs. Uterus (WI/Ut) and White isthmus vs. Magnum (WI/Ma), a total 16 microarray slides were utilized for to hybridization with 32

samples. Only cDNA probes with an incorporation efficiency of >11.4 dye molecules/1000 bases were used for hybridization. Prehybridization was performed with 100ul DIG easy buffer for all microarray slides in a humidified chamber for 1 hour at 42 °C. The slides were washed with distilled water for 10mins at room temperature. The same amount of Alexa 555- and Alexa 647- labelled cDNA probes from two samples was added to the hybridization solution, and then denatured at 100 °C for 2mins. These solutions were placed on the slides, which were cover slipped and placed in the hybridization chamber for 16 hours at 42 °C. The slides were washed with 0.2X SSC buffer and SDS buffer for 15mins at 42 °C, and then washed by 0.2X SSC for 15mins at room temperature. Finally, the slides were washed with distilled water. A GenePix 4000 B scanner was used to scan the microarray slides, Alexa 555 was scanned at 532 nm and Alexa 647 was read at 635 nm. GenePix software was used to analyze the spot intensity raw data.

GenePix report (GPR) files containing spot intensity raw data were stored in the BioArray Software Environment (BASE) of SIGENAE (Système d'Information du projet d'Analyse des Génomes des Animaux d'Elevages) and the microarray data was uploaded in the NCBI Gene Expression Omnibus (GEO) database.

5.4 Statistical data analysis

Gene expression was compared between isthmus and magnum (8 microarrays, 16 samples) and between uterus and isthmus (8 microarrays, 16 samples). For these two comparisons, differentially expressed genes were identified using the 'anapuce' package in R (http://cran.r-project.org/web/packages/anapuce/index.html). Spot intensities were calculated using the median value, which was transformed to log2 value. Normalization consisted of global locally-weighted regression (Lowess) applied on the overall intensity

log2 ratio to remove dye bias due to efficiency of fluorescent dye incorporation. A block effect was corrected by subtracting the median value. Spot intensities were retained when present in at least 50% of samples. Assuming various sources of variance, we estimated the gene variance using a mixture model integrated into the VarMixt method (Delmar et al., 2005). Taking into account gene variance, we performed a unilateral statistical t-test to identify genes over-expressed in the uterus compared to either the magnum or white isthmus. P-values were adjusted by the Benjamini-Hochberg multiple testing procedures (Benjamini et Hochberg, 1995), to control false discovery rate (FDR<0.05).

5.5 Functional Annotation

The clone sequences of the 135 over-expressed transcripts were available from the UID Chick EST and NCBI databases. BlastN analysis of the transcript sequences was performed against the NCBI *Gallus gallus* Refseq RNA and nucleotide databases. BlastP analysis of identified proteins was performed against the NCBI non-redundant / SwissProt *Gallus gallus* protein databases.

5.6 Gene Ontology Enrichment Analysis

Gene Ontology (GO) terms represent the functions of proteins encoded by over-expressed genes that are revealed by microarray analysis. Expression Analysis Systematic Explorer (EASE) software based on the GO database was used to identify biological themes for 135 over-represented genes in white isthmus where the formation of the eggshell membranes occurs (the Expression Analysis Systematic Explorer. http://david.abcc.ncifcrf.gov/ease/ease.jsp). EASE software was used to compare the GO term enrichment of the over-represented white isthmus transcripts to the overall GO terms in the Del-Mar 14K cDNA microarray. Each GO term corresponded to an EASE score (a

modified Fisher Exact P-Value and high enrichment value). Only GO terms with an EASE score ≤0.05 were considered to be significantly enriched.

5.7 Secreted protein identification

Two protein informatic softwares, SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen et al., 1997; Bendtsen et al., 2004a) and SecretomeP 2.0 (http://www.cbs.dtu.dk/services/SecretomeP/) (Bendtsen et al., 2004b), were applied to identify the over-expressed genes in white isthmus that encoded sequences specifying either endoplasmic reticulum – mediated export (Signal P) or protein sequences predictive of unconventional secretion (SecretomeP). For SignalP 3.0 analysis, the proteins were only accepted for secretion if they met two requirements: 1) both the neural network and hidden Markov model identified a signal peptide and a cleavage site; 2) a Markov model probability was higher than 95%. The mature proteins that were predicted to be secreted were examined using proteomic resources (UniProtKB/SwissProt and NCBI protein databases). The SecretomeP cutoff was 0.5 for unconventional secretion. Genes encoding sequences that met the cutoff values for either classic or unconventional secretion are listed in Table 3.

6. Authors' contributions

JD carried out the bioinformatic analyses, interpreted and annotated data, and wrote the first draft of the paper. MH is the supervisor of JD (MSc student). He contributed to the interpretation of data. He was extensively involved in writing of the paper. AB carried out experiments and analyses, interpreted data, annotation and statistical analyses. CHA performed statistical analyses. JG conceived the strategy, designed and carried out experiments, interpreted data and statistical analyses.YN conceived the overall research program. He was involved in the experimental design, data interpretation and in the writing of the paper. All authors have read and approved the final manuscript.

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repository and the data annotation.

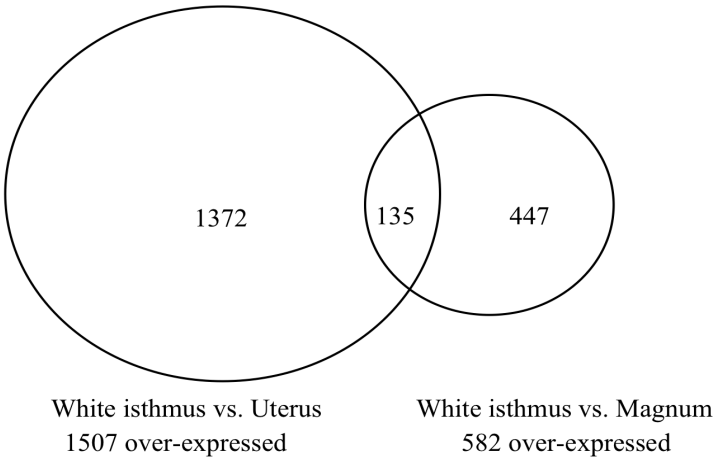

Figure 1. Over-expressed genes in the white isthmus compared with uterus and magnum. There were 1507 and 582 overexpressed genes for the WI/UT and WI/MA comparisons, respectively. The over-expression threshold was set at > 1.1.

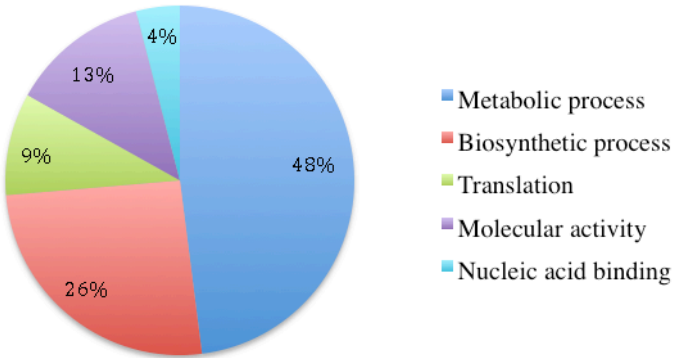

Figure 2. Pie chart showing the distribution of over-expressed white isthmus transcripts, as per their biological function given by the EASE software.

Table 1. Functional annotation of over-expressed WI genes.

Database	Total	Unknown	Unique
NCBI EST and UD Chick EST	135[a]	2	-
Entrez Gene	133	18	103
UniGene	133	19	101
	Total	**Hypothetical proteins**	**Annotated protein**
NCBI (nr database)[b]	85[c]	7	78

a. The **most** highly over-expressed genes are identified in Table 4
b. Non-redundant protein sequence database
c. Analyzed by SignalP 3.0 and SecretomeP 2.0 softwares (Table 3)

Table 2. Gene Ontology analysis.

Description	GO terms	EASE score	Gene ID
Metabolic process	**GO:0044267** Cellular protein metabolic process	2.72E-02	Fbn1, ENO4, LOC395492,GNS, TGFB3
	GO:0044262 Cellular carbohydrate metabolic process	2.87E-02	EEF1B,rpl31,RPS24,PSMB4,DPH5,EIF4A2,ppiC,eprs, RPL3,rpl11,UBE2D3P, Adamts1, BBS12, rps23, rpl21, CTSB, UFM1, MARS2, EEF1D
	GO:0019538 Protein Metabolic process	2.19E-02	EEF1B,rpl31, RPS24, P4HA2, PSMB4, DPH5, EIF4A2, ppiC, eprs, RPL3, rpl11, UBE2D3P, Adamts1, BBS12, rps23, rpl21, CTSB, UFM1, MARS2, EEF1D
	GO:0044260 Cellular macromolecule metabolic process	1.47E-02	EEF1B,rpl31, RPS24, P4HA2, PSMB4, DPH5, EIF4A2, ppiC, eprs, RPL3, rpl11, UBE2D3P, Adamts1, BBS12, rps23, rpl21, CTSB, UFM1, MARS2 ,EEF1D
Biosynthetic process	**GO:0009059** Macromolecule biosynthetic process	5.07E-04	EEF1B,rpl31, rpl21, rps23, RPS24, EIF4A2, MARS2, eprs, RPL3, EEF1D, rpl11
	GO:0044249 Cellular biosynthetic process	1.69E-03	EEF1B,rpl31 rpl21 rps23 RPS24 EIF4A2 NME2 MARS2 eprs RPL3 EEF1D rpl11
	GO:0009058 Biosynthetic process	3.89E-03	EEF1B,rpl31, RPS24, EIF4A2, DPH5, eprs, RPL3, rpl11, rps23, rpl21, NME2, MARS2, EEF1D
Intracellular part	**GO:0044424** Intracellular part	3.80E-02	rpl31,GNB2L1,PSMB4, ybx1, rpl11, rps23, CTSB, EEF1B, MXI1, ENO4, KDELR2, RPS24, Chp, Mbnl1, LAPTM4A, eprs, RPL3, Sh3glb2, TXN, C20orf42, rpl21, NME2, UFM1, MARS2, TPD52, RCN2 TMSB4X HDLBP EEF1D
	GO:0005737 Cytoplasm	4.21E-04	rpl31,GNB2L1,ybx1, rpl11, rps23, CTSB, EEF1B, ENO4 ,RPS24, KDELR2, Chp, LAPTM4A, eprs, RPL3, Sh3glb2, TXN, rpl21, NME2, UFM1, MARS2, TPD52, TMSB4X, RCN2, HDLBP, EEF1D
	GO:0044444 Cytoplasmic part	8.56E-03	EEF1B,rpl31, ENO4, RPS24, KDELR2, LAPTM4A, RPL3, rpl11, rps23, rpl21, CTSB, TPD52, RCN2, TMSB4X, EEF1D
	GO:0005840 Ribosome	4.97E-03	rpl31,rpl21, rps23, RPS24, RPL3, rpl11
Translation	**GO:0005853** Eukaryotic translation elongation factor 1 complex	3.39E-02	EEF1B,EEF1D
	GO:0006412 Translation	5.19E-05	EEF1B,rpl31, rpl21, rps23, RPS24, EIF4A2, MARS2, eprs, RPL3, EEF1D, rpl11
Molecular activity	**GO:0005198** Structural molecule activity	4.82E-02	Fbn1,rpl31, rpl21, rps23, RPS24, RPL3, rpl11
	GO:0003735 Structural constituent of ribosome	4.85E-03	rpl31,rpl21, rps23, RPS24, RPL3, rpl11

Nucleic acid binding	**GO:0003723** RNA binding	2.39E-02	PUM2,EIF4A2, Mbnl1, PABPC1, HDLBP, ybx1	

Table 3. Predicted signal peptides.

Accession No	Gene symbol	Protein name	Secretion
P08125.4	LOC100858979	Collagen alpha-1(X) chain	Classical
XP_001236415.1	LOC776923	PREDICTED: similar to spore coat protein SP75, partial .	Non-classical
XP_428412.2	LOC100859738	PREDICTED: similar to IgG Fc binding protein, partial .	Classical
XP_421393.3	TDRD9	PREDICTED: putative ATP-dependent RNA helicase TDRD9.	Non-classical
XP_422226.2	PHGDH	PREDICTED: d-3-phosphoglycerate dehydrogenase	Classical
XP_413719.1	SORD	PREDICTED: sorbitol dehydrogenase.	Non-classical
XP_417988.2	HSD11B1	PREDICTED: corticosteroid 11-beta-dehydrogenase isozyme 1.	Classical
NP_989745.1	YBX1	Nuclease-sensitive element-binding protein 1.	Non-classical
XP_416206.2	PLBD1	PREDICTED: phospholipase B domain containing 1	Classical
NP_001034378.1	SNX14	sorting nexin-14	Non-classical
XP_417127.1	RPL21	PREDICTED: 60S ribosomal protein L21 isoform 2	Non-classical
CAG28668.1	P4HA2	prolyl 4-hydroxylase alpha-2 subunit .	Classical
XP_417063.2	RCBTB1	PREDICTED: RCC1 and BTB domain-containing protein 1	Non-classical
XP_417267.3	HPX	PREDICTED: hemopexin	Non-classical
NP_001001315.1	TMSB4X	thymosin, beta 4	Non-classical
NP_990232.1	EEF1B2	elongation factor 1-beta	Non-classical
XP_001231333.2	PPIC	PREDICTED: peptidyl-prolyl cis-trans isomerase C	Classical
NP_001006241.1	RPL3	60S ribosomal protein L3	Non-classical
CAG31714.1	EIF3D	hypothetical protein RCJMB04_10a6 .	Non-classical
NP_001026075.1	RPL11	60S ribosomal protein L11	Non-classical
NP_990416.1	ITM2B	integral membrane protein 2B	Non-classical
NP_001007931.1	CHP	calcineurin B homologous protein 1	Non-classical
XP_416214.2	SERHL2	PREDICTED: serine hydrolase-like 2	Non-classical
ADP21276.1;S23734	LOC100859544	envelope protein [Avian leukosis virus]	Non-classical
NP_001006472.1	KDELR2	CD151 antigen	Non-classical
NP_001153187.1	No hits	ER lumen protein retaining receptor 2	Non-classical
XP_001232465.1	TSPAN3	PREDICTED: similar to transmembrane 4 superfamily member 8 isoform 1 .	Non-classical
XP_001232537.2	LOC769292	PREDICTED: mannose-1-phosphate guanyltransferase alpha-A-like	Non-classical
NP_001006223.1	ALDH3A2	fatty aldehyde dehydrogenase.	Non-classical
XP_415246.1	SLC5A1	PREDICTED: rhabdoid tumor deletion region protein 1	Non-classical
XP_422097.1	PDIA5	PREDICTED: protein disulfide-isomerase A5	Non-classical
XP_413734.2	RCN2	PREDICTED: reticulocalbin-2	Classical
XP_420621.2	BBS12	PREDICTED: Bardet-Biedl syndrome 12 protein	Non-classical
XP_421155.2	TMEM87A	PREDICTED: transmembrane protein 87A-like	Classical
NP_990785.1	TGFB3	transforming growth factor beta-3 preproprotein	Classical
NP_997063.1	PIT54	PIT 54 protein	Classical
XP_415554.1	ENTPD8	PREDICTED: ectonucleoside triphosphate diphosphohydrolase 8	Classical
NP_001012592.1	SH3BGRL	SH3 domain-binding glutamic acid-rich-like protein	Non-classical
NP_989528.1	DPYSL4	dihydropyrimidinase-like 4	Non-classical

NP_001006263.1	TRAPPC2	trafficking protein particle complex subunit 2	Non-classical
XP_426247.2	MARS2	PREDICTED: methionyl-tRNA synthetase, mitochondrial	Non-classical
XP_416687.3	ADAMTS1	PREDICTED: A disintegrin and metalloproteinase with thrombospondin motifs 1 [Gallus gallus]	Classical
XP_001235093.1	TMBIM4	PREDICTED: transmembrane BAX inhibitor motif-containing protein 4	Non-classical
NP_001001609.1	GAL10	gallinacin-10 preproprotein	Classical
XP_427542.3	PSMB4	PREDICTED: proteasome subunit beta type-4	Non-classical

Table 4. Highly over-expressed genes for WI/Ma and WI/Ut (-fold≥2)

Accession No.	Description (Gene symbol)	Gene ID	Over-expression level (-fold)	
			WI/Ut	WI/Ma
XM_003641007.1[a]	PREDICTED: Gallus gallus collagen alpha-1(X) chain-like (LOC100858979)	100858979	111.3	155.8
XM_417686.2	PREDICTED: Gallus R3H domain and coiled-coil containing 1 (R3HCC1)	419534	79.5	136.1
XM_003641007.1[a]	PREDICTED: Gallus gallus collagen alpha-1(X) chain-like (LOC100858979)	100858979	50.3	94.5
XM_001236414.1	PREDICTED: Gallus similar to spore coat protein SP75 (LOC776923)	776923	70.0	57.9
XM_003643477.1[b]	PREDICTED: Gallus gallus IgGFc-binding protein-like (LOC100859738)	100859738	22.6	34.0
XM_001233273.1	PREDICTED: Gallus hypothetical protein LOC769959 (LOC769959)	769959	46.0	15.6
XM_420734.2	PREDICTED: Gallus gallus LIM and calponin homology domains 1 (LIMCH1)	422780	11.6	15.5
NM_001012911.1	Gallus MAX interactor 1 (MXI1)	423888	14.1	9.8
XM_003643477.1[b]	PREDICTED: Gallus gallus IgGFc-binding protein-like (LOC100859738)	100859738	7.6	8.8
XM_421393.2	PREDICTED: Gallus gallus tudor domain containing 9 (TDRD9)	423488	3.7	2.7
XM_413815.2	PREDICTED: Gallus fibrillin 1 (FBN1)	373992	45.9	2.5
XM_422226.2	PREDICTED: d-3-phosphoglycerate dehydrogenase	424381	1.7	2.4
XM_413719.2	PREDICTED: Gallus sorbitol dehydrogenase (SORD)	415332	1.8	2.3
XM_421286.2	PREDICTED: Gallus gallus transmembrane protein 63C (TMEM63C)	423372	2.5	2.2
NM_205453.1	Gallus thioredoxin (TXN)	396437	2.6	2.1
XM_417267.2	PREDICTED: Gallus similar to hemopexin (LOC419076),	419076	2.9	1.6
NM_001001315.1	Gallus thymosin beta 4, X-linked (TMSB4X)	408047	3.6	1.6

a. Two Clone IDs were annotated as collagen alpha-1(X) chain-like
b. Two Clone IDs were annotated as IgGFc-binding protein-like

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Chapter III Proteomic analysis provides new insight into the chicken eggshell cuticle

Abstract

The cuticle is the outermost layer of the avian eggshell, whose protein constituents remain virtually unknown. We hypothesize that cuticle components play a major role in microbial resistance, since eggs with incomplete or absent cuticle are more susceptible to bacterial contamination. In this study we extracted proteins from the outermost non-calcified layer of the cuticle of chicken eggs and subjected them to LC/MS/MS proteomic analysis. We identified 47 cuticle proteins with high confidence and reproducibility. Two proteins, similar to Kunitz-like protease inhibitor and ovocalyxin-32 (a carboxypeptidase A inhibitor), were the most abundant of the cuticle proteins. A number of proteins known to have antimicrobial activity in the egg were detected (lysozyme C, ovotransferrin, ovocalyxin-32, cystatin, ovoinhibitor) as well as possible new candidates (myeloperoxidase, ovocalyxin-36 and members of the SERPIN family). This is the first comprehensive report of cuticle proteome, a starting point to determine cuticle function and the molecular basis of its antimicrobial properties.

1. Introduction

Integrated defense strategies that operate at biomineralized barriers are a hallmark of sophisticated biological structures. One example, although not well understood, is the calcified eggshell that is essential for reproduction in birds and reptiles. The avian calcified shell is a complex structure that is deposited while the forming egg is retained in the distal oviduct (uterus / shell gland) during an extended period. Genetics controls the shell permeability for metabolic gases and water, which depends on the characteristics of its pores - number, density, branching pattern and caliber. Ultimately, a cuticle is deposited onto the eggshell surface during the final phase of egg formation [1]. The cuticle mainly consists of proteins (> 85%) and possesses two layers: the innermost is mineralized and contains hydroxyapatite-containing vesicles deposited during the final phase of eggshell calcification (termination); the outermost remains non-mineralized [2,3]. The cuticle is distributed unevenly over the surface of the egg; its thickness ranges from 5 to 10 μm [4]. The cuticle covers the calcified shell and fills the entry to its pores (up to 50 μm in depth), creating a barrier which inhibits water movement across the shell and prevents dehydration of the egg interior [5]. Moreover, the cuticle physically excludes bacterial penetration of pores and limits microbial colonization on the egg's surface [6,7]. Eggs with absent or incomplete cuticle are more susceptible to bacterial contamination [8]. Cuticle desiccation during egg storage or incubation leads to cracks that expose eggshell pores and leave the egg vulnerable to contamination by pathogens [9]. A pathogen-free egg is extremely important for avian reproduction and survival of the developing embryo, in addition to food safety of the nutritious unfertilized egg intended for human consumption. Eggs and egg-containing foods are the main vehicles for Salmonella enteritidis intoxication (poisoning) [10], in addition to potential contamination by E. coli, Pseudomonas sp.,

Micrococcus sp. and various other bacterial strains [7]. In order to understand the molecular basis for the antimicrobial function of the eggshell cuticle and gain further overall insight into cuticle function, we performed a comprehensive proteomic analysis of the outermost (non-calcified) layer of the cuticle.

2. Material and Methods

2.1 Cuticle protein extraction

Unwashed freshly laid eggs from Lohmann LSL-Lite chickens were obtained from Laviolette Poultry Farm, St-Isidore, Ontario. In two independent trials, intact eggs (n = 5) were individually extracted in sterile plastic bags containing 2 mL of extraction solution: 1% SDS or 1% SDS, 2mM DTT. The egg surface was manually massaged for 5 minutes with extraction solution to solubilize the cuticle layer. An empty plastic bag was treated with extraction solution to assess possible contamination arising from the extraction method (none was detected). The individual extraction samples were transferred to Amicon Ultra-4 Centrifugal Filter Devices (molecular weight cutoff of 3000 Da, Millipore Corporation, Billerica, MA) and centrifuged at 4000 rpm to concentrate the dilute cuticle extracts. The protein concentration of each concentrated extract was measured with the bicinchoninic acid (BCA) assay (Thermo Scientific, Hampton, NH) before pooling for electrophoresis. Pooled extracts were resolved by SDS-PAGE using a precast 4-12% BisTris gel (Invitrogen, Carlsbad, CA). The gels were sectioned and sent to the Proteomics Platform of the Eastern Quebec Genomics Centre (Laval, QC) for LC/MS/MS analysis (services include in-gel digestion, mass spectrometry and Mascot data- base searching, Sections 2.2–2.5).

2.2 Protein in-gel digestion

Gel sections were placed in 96-well microplates, washed with water and digested with trypsin on a MassPrep liquid handling robot (Waters, Milford, MA) [11,12]. Proteins were reduced with10mM DTT, alkylated with 55mM iodoacetamide and digested with 126nM of modified, sequencing grade, porcine trypsin (Promega, Madison, WI) at 58 °C for 1 h. The digestion products were extracted using 1% formic acid, 2% acetonitrile followed by 1% formic acid, 50% acetonitrile, pooled, vacuum centrifuged dried and then resuspended into 7μL of 0.1% formic acid. Only 2μL of the resuspended extracts were analyzed by mass spectrometry.

2.3 Mass spectrometry

Online reversed-phase nanoscale capillary liquid chromatography and electrospray mass spectrometry (ES MS/MS) were used to separate and analyze peptide samples. The experiments were performed with a Thermo Surveyor MS pump connected to a LTQ linear ion source (ThermoFisher, San Jose, CA). Peptide separation occurred on a Self-Pack PicoFrit column (New Objective, Woburn, MA) containing Jupiter packing material (Phenomenex, Torrence, CA) 5u, 300A, C18, 10cm × 0.075mm internal diameter. Peptides were eluted by a 2–50% solvent B (acetonitrile, 0.1% formic acid) linear gradient in 30 minutes, at 200nL/min (obtained by flow-splitting) while the mass spectra were obtained using Xcalibur software version 2.0 and a data dependent acquisition mode. Collision-induced dissociation of the seven most intense ions followed each full scan mass spectrum (400 to 2000 m/z). Other parameters include an enabled dynamic exclusion (30 second exclusion duration) function and relative collisional fragmentation energy set to 35%.

2.4 Database searching

The MS/MS results were analyzed with Mascot (Matrix Science, London, UK; version 2.2.0), searching the uniref-100.2010.06.Gallus.gallus.9031database (37461 entries), with trypsin digestion. Search parameters included a fragment ion mass tolerance of 0.50Da and a parent ion tolerance of 2.0Da. The iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification, while oxidation of methionine was specified as a variable modification. Two missed cleavages were allowed. The same procedure was repeated with Mascot set up to search uniref-100_2010_06_Bacteria_2 database (5093156 entries) to assess possible bacterial contamination of the unwashed egg surface by bacteria from the farm environment or chicken feces. No peptides of bacterial origin were detected.

2.5 Criteria for protein identification

Validation of MS/MS based peptide and protein identifications were performed using Scaffold (version Scaffold-3_00_08, Proteome Software Inc., Portland, OR) and were accepted if they were identified at $p \leq 0.05$ probability, specified by the Protein Prophet algorithm [13,14]. Protein identifications also required at least 2 unique peptides to be accepted. The principles of parsimony were utilized to group proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone.

2.6 Bioinformatic analysis

The relative quantification of the identified proteins were calculated using the exponentially modified protein abundance index (emPAI) = 10^(Nobserved /Nobservable) − 1, where Nobserved is the amount of unique parent ions obtained and Nobservable corresponds to the amount of peptides expected after digestion with trypsin [15]. The

protein Basic Local Alignment Search Tool (pBLAST) was used to align proteins identified by Scaffold against the non-redundant protein sequence database for the species Gallus gallus (9031). Potential signal peptides in the predicted full-length protein were assessed using the SignalP4.0 Server (www.cbs.dtu.dk/services/SignalP) and were only accepted as valid if both the neural network and hidden Markov models identified a signal peptide ($p \leq 0.05$). Gene Ontology (GO) term enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Functional Annotation Tool (DAVID Bioinformatics Resources 6.7, NIAID/ NIH) was used to highlight the more relevant GO terms associated to biological processes and molecular functions [16].

3 Results

3.1 Cuticle extraction

The amount of cuticle extracted from each egg was variable; an average of 123 (± 59) µg of protein/egg was extracted with 1% SDS, 2mM DTT [range 74–215 µg/egg]. However, significantly less cuticle protein was extracted with SDS alone, revealing an average of 62 (± 14) µg/egg [range 42 –78 µg]. The same 1% SDS, 2mM DTT protocol repeated with grocery store eggs revealed only 39 (± 4) µg /egg of extracted cuticle. The lower cuticle protein of the grocery store eggs is likely a consequence of the commercial washing process (cleaning with brushes and alkaline detergents) before eggs are sold for consumption [17]. Individual extraction samples were pooled together for electrophoresis in order to obtain proteomic results representative of multiple eggs. SDS-PAGE revealed intense Coomassie Blue-stained bands at 32kDa and 10kDa as well as less intensely stained bands at 27, 20, 14 and 8kDa (Fig. 1). The entire extraction process and proteomic analysis was repeated twice to confirm reproducibility.

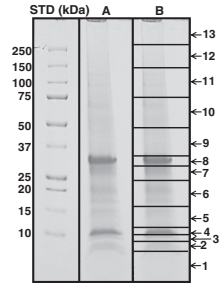

Figure 1: SDS-PAGE analysis of 30 µg of cuticle proteins from 5 pooled fractions, all extracted with 1% SDS, 2 mM DTT. Bars and numbers indicate where the gel was sectioned and the fragments that were sent for LC/MS/MS sequencing analysis (lane B).

3.2. Cuticle proteome

Each gel was sectioned as depicted in Fig. 1 and subjected to LC/MS/MS analysis. Two independent proteomic analyses were merged by combining the entire dataset of spectra, in order to increase the threshold for significance to detect the less abundant proteins. Table 1 lists the 47 proteins that were confirmed in the cuticle extract by at least 2 unique peptides ($p \leq 0.05$). The most abundant cuticle proteins were predicted: similar to Kunitz-like protease inhibitor and ovocalyxin-32 (OCX-32) with emPAI values of 14.08 and 112.62, respectively. Ovalbumin also had an elevated emPAI of 35.79, however this value was quite variable between individual extracts (2.44 and 35.79). We hypothesize that this is caused by the inconsistent contamination of the egg surface by egg white. The protein sequence of predicted: similar to Kunitz-like protease inhibitor in the NCBI protein database, (REFSEQ ID: XP_001235178.2), has recently been updated and confirmed by the EST database. However, 3 unique peptides identified during proteomic analysis are only present in the predicted C-terminal sequence of the original protein sequence (REFSEQ ID: XP_001235178.1), suggesting that it also is a valid sequence (Table 2). Alternative splicing to produce both versions of the protein is a possible explanation for this discrepancy. Each gene product has a different predicted molecular weight: XP_001235178.1 : 36kDa;

XP_001235178.2 : 23kDa (Fig. 2). Protein migration in the gel was quite disperse, possibly due to breakdown products; but the majority of the assigned spectra were present in gel slice 6 (15–23kDa) (Fig. 1).

The other most abundant protein, OCX-32, was found to reflect 4 different isoforms of the same protein (Table 3, Fig. S1). We were able to obtain the emPAI value of three isoforms: 112.62 for UNIPROT ID: C7G541, 71.78 for D3KYT5 and 52.92 for C7G542.

3.3. Classification of cuticle proteins

Using the DAVID Functional Annotation Tool, we were able to identify the more relevant GO terms corresponding to the molecular functions and biological processes of our 47 proteins (Table 4). The software was able to identify two clusters. The first group consists of 10 proteins involved in enzyme regulator activity, 7 of which are predicted to possess serine-type endopeptidase inhibitor activity, also known as serine protease inhibitors (SERPINs). These include predicted: similar to α-2- plamin inhibitor, ovalbumin, ovoinhibitor, ovalbumin-related protein Y, predicted: similar to Kunitz-like protease inhibitor, neuroserpin and predicted: ovalbumin-related protein Y. The second cluster consists of proteins involved in lipid metabolism and transport such as apolipoprotein A-IV, vitellogenin-1 and vitellogenin-2. Although not detected by the DAVID software, another cluster can be made with proteins possessing known antimicrobial activity such as lysozyme C, ovotransferrin, OCX-32, cystatin and ovoinhibitor.

4. Discussion

The chicken eggshell cuticle is in direct contact with the outside environment and therefore represents the first line of defense against a harsh external environment. Eggs

with absent or incomplete cuticle layers are more susceptible to microbial contamination [8]. The cuticle provides a physical barrier to restrict bacterial entry through pores [6]; however, this does not explain the observation that the cuticle limits microbial colonization of the egg surface [7]. In order to gain more insight into cuticle function, a comprehensive proteomic study was undertaken.

A novel and successful feature of our study was the minimalistic processing approach to concentrate the dilute cuticle extract, which allowed us to identify for the first time a large number of cuticle constituents. Extended dialysis of dilute solutions, which can lead to protein losses and/or degradation, was avoided. Cuticle protein extraction with 1% SDS, 2mM DTT, followed by LC/MS/MS proteomic analysis, produced a comprehensive catalog of the most abundant proteins situated in the outermost (non-calcified) layer of the avian egg. A previous proteomic study of the calcified eggshell started with shell that had been first treated to remove the cuticle; over 520 protein constituents of the mineralized eggshell matrix were identified [18]. Another proteomic study identified only seven cuticle proteins after scraping the EDTA insoluble proteins off the surface of the intact egg [19]. Our data shows limited overlap with their results. We confirmed the cuticle presence of ovocleidin-116 (OC-116), OCX-32, similar to Kunitz-like protease inhibitor, OCX-36 and clusterin, but not OC-17 or collagen I chain, all of which were previously identified in both the cuticle (mixture of mineralized and non-mineralized layers) and egg- shell matrix [18,19]. Compared to other proteomic studies of the various egg compartments (yolk, vitelline membrane, egg white, mineralized shell [18–26]), several novel identifications were made in our study: predicted: selenium-binding protein 1-like, similar to myeloperoxidase precursor, hemoglobin subunit α-A, epithelial cell adhesion molecule and

predicted: thrombospondin type-1 containing protein 4.

A previous study of the egg white proteome identified a selenium binding protein-1-A-like (REFSEQ ID: XP_003642729.1) [23], which is not the same protein detected in this study. The function of the selenium-binding protein in cuticle is not clear. Selenium and its binding proteins are being investigated in cancer cell growth inhibition; these are reported to interact with glutathione peroxidase [27], a protein important in oxidative stress which we also identify in the cuticle. A few studies also observed the inhibitory effects of selenium on E. coli and S. aureus growth [28,29]. Another previously unidentified constituent, myeloperoxidase, exerts an antibacterial effect by generating reactive oxidizing products. It is a resident antimicrobial in mammalian neutrophil leukocytes and a key player in host defense mechanisms, showing inhibitory activity towards a variety of bacteria including S. aureus, E. coli and P. aeruginosa [30]. Little information is available for predicted: thrombospondin type-1 containing protein 4. This protein is known to be part of the protease and lacunin superfamily and possesses metal loendopeptidase activity according to GO term annotation. The thrombospondin type-1 domain is present in many extra- cellular proteins involved in cell-matrix binding and signaling [31].

Eggshell matrix proteins have previously been organized into 3 broad groups: eggshell matrix-specific proteins, egg white proteins and ubiquitous / miscellaneous proteins [1,18]. This is also a convenient manner to catalog the cuticle proteins identified in this study.

4.1. Eggshell-specific proteins

One of the most abundant cuticle protein, similar to Kunitz- like protease

inhibitor (also annotated as ovocalyxin-25, [Gautron, Hincke et al., manuscript in preparation, 32]), possesses a motif placing it in the Bovine Pancreatic Trypsin Inhibitor (BPTI)/kunitz family of SERPINs, which is categorized as an enzyme regulator (Table 4). This family of protease inhibitors is basic in nature and characterized by a disulfide rich α-β fold structure with three highly conserved disulfide bonds and small molecular mass [33]. A highly studied member of this family, BPTI, possesses antimicrobial activity and inhibits the growth of a variety of both Gram-positive and Gram–negative strains of bacteria [34]. A wide range of opportunistic pathogens possess membrane-bound proteases, or release extracellular proteases, which increases their pathogenicity; these proteases are an excellent target for antimicrobials [35]. Our study documents two forms of this protein, which may represent alternative splicing, and reflect distinct biological activities (Fig. 2). Since extracts from multiple eggs were pooled before analysis, it is not clear whether different versions of this protein co-exist in the same animal. The other abundant cuticle constituent was found to be OCX-32, previously described as an eggshell-specific protein that is highly expressed in the isthmus and uterine regions of the hen reproductive tract [1,36]. Immunofluorescence revealed that it is enriched in the cuticle layer, but also found in the outer calcified layer [36]. It is enriched in the uterine fluid associated with the terminal phase of calcification, the components of which modify calcite crystal growth in vitro: the calcite crystals are decreased in size and the lag time for their nucleation is much shorter [37]. OCX-32 is categorized by the biomineral tissue development GO term due to the hypothesis that this protein regulates the arrest of eggshell calcification. However, the OCX-32 protein sequence possesses 30% identity to a carboxypeptidase A inhibitor, leading to the alternative hypothesis that it is an antimicrobial protein that targets microbial proteases [1]. In support of this prediction, purified recombinant OCX-32 inhibits

carboxypeptidase A activity and inhibits the growth of Gram-positive bacteria B. subtilis [38]. The impact of non-synonymous single nucleotide polymorphisms (SNPs) on the activity of different version of the protein, as verified in this study, is not yet clear.

OCX-36 is an eggshell-specific matrix protein that previous proteomic analyses have detected in vitelline membrane, egg white and eggshell, in addition to the eggshell membranes where it is particularly abundant. It is a member of the Bactericidal/Permeability-Increasing (BPI) family of innate immune proteins that bind bacterial lipopolysaccharides (LPS) [39]. Thus GO terms associated with OCX-36 involve lipid binding functions.

OC-116 is a major eggshell-specific matrix protein which is classified with biomineral tissue development due to its pro- posed role in shell calcification [1]. OCX-21 (gastrokine-2), the most recently identified OCX protein, possesses a BRICHOS domain and is proposed to function as a chaperone to promote correct protein folding and stability during eggshell mineralization [32].

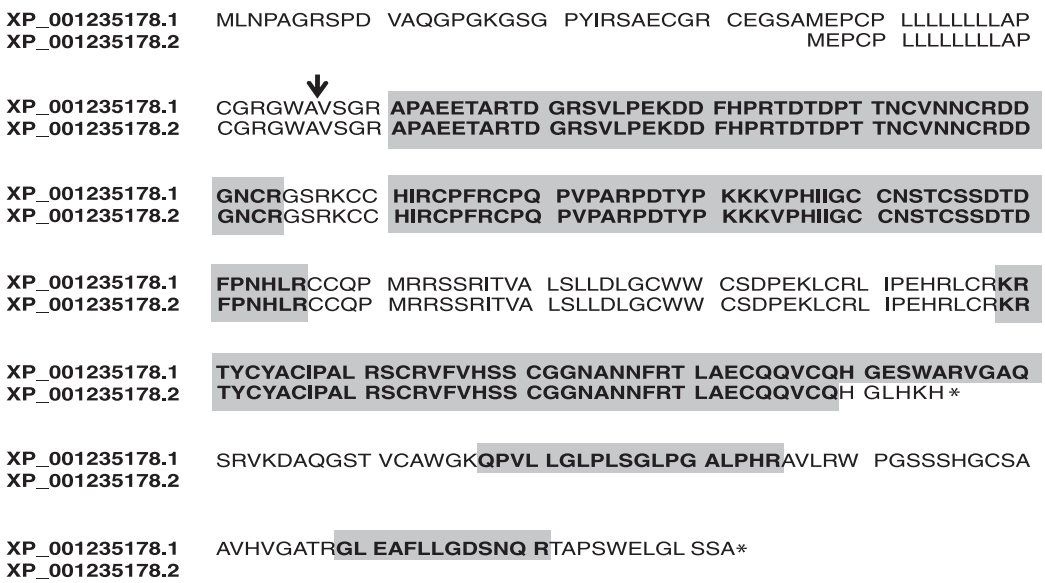

Figure 2: Protein sequence alignment of the two versions of predicted: similar to Kunitz-like protease inhibitor. REFSEQ ID: XP_001235178.1 represents a previous version of the protein while REFSEQ ID: XP_001235178.2 corresponds to the newly updated sequence. The identified peptides corresponding to each sequence are highlighted in grey. Asterisks (*) indicate the location of the STOP codons predicted in conceptual translation product, and an arrow designates the cleavage site of the signal peptide, predicted by SignalP 4.0.

4.2. Egg white proteins

Ovalbumin, ovotransferrin, lysozyme C, ovoinhibitor and cystatin are well-known egg white proteins that this study identified in the cuticle layer. This is consistent with previous reports of lysozyme and ovotransferrin on the shell surface [40–42]. It is possible that internal egg constituents derived from broken / cracked eggs during automated collection and processing may contaminate the shell surface. The cuticle presence of chain A, structure of an avian Igy-Fc 3–4 fragment, vitellogenin-1 and − 2, which are abundant yolk proteins, also supports this hypothesis. GO term associations identify lysozyme C and ovotransferrin as antimicrobial proteins, which are grouped with proteins involved in the response to stress and biotic stimuli. Lysozyme C is expressed in all segments of the hen oviduct and possesses hydrolytic activity against cell wall polysaccharides of Gram- positive bacteria. Ovotransferrin stunts bacterial growth by

sequestering iron, a necessary growth factor for pathogens, and is classified in the iron binding and iron transport GO term categories. Ovalbumin, the most abundant egg white protein, is a non-functional member of the SERPIN family and possesses no antimicrobial activity; however, digestion with trypsin generates several peptides which inhibit B. subtilis growth [43]. Ovoinhibitor is also a SERPIN proteinase inhibitor and possesses trypsin, chymotrypsin and elastase inhibitory activity as well as inhibitory activity against bacterial and fungal proteases [44]. Cystatin is an inhibitor of cysteine proteinases which are present in certain viruses; it also inhibits growth of Gram-positive and -negative bacteria [45]. GO terms place ovalbumin, ovoinhibitor and cystatin in the enzyme regulator activity category (Table 4).

4.3. Ubiquitous/miscellaneous proteins

A variety of low abundance proteins were significantly identified in our extracts (Table 1). Proteins such as serum albumin, clusterin, nucleobindin-2 and apolipoproteins are detected in many different tissues and cellular compartments. A previous study identified clusterin in the chicken eggshell matrix and egg white, and suggested a chaperone role during egg formation [46]. A number of proteins are typically described as cellular constituents and lack a predicted signal peptide for secretion: predicted: mucin-5B, hemoglobin subunits α-A and β, actin, saposin, tubulin and annexin. These are all described as cytoplasmic proteins by the cellular component GO terms. This is similar to the vast majority of the proteins detected in the mineralized eggshell proteome [1]. It has been proposed that abrasion of the luminal wall by the eggshell during calcification (a process lasting over 17 hours), in addition to normal turnover of the uterine epithelial cells, leads to release of cellular contents into the lumen and their incorporation into the calcifying shell

and cuticle as a non-specific background phenomenon [18].

4.4. Function of the cuticle constituents

Previous studies examining the antimicrobial activity of the cuticle noted an inhibitory effect on the growth of Gram- positive (B. subtilis, S. aureus) and Gram-negative (P. aeruginosa) bacteria [47,48]. The results of this study reinforce the notion of an antimicrobial role; proteins with known antimicrobial activity were identified that could reduce microbial colonization of the egg surface. In addition to OCX-32, lysozyme and ovotransferrin, our analysis revealed other proteins with known antimicrobial activity, as well as some promising new candidates. Cystatin has bactericidal activity against both Gram- positive (S. aureus, S. gallinarum) and Gram-negative (P. aeruginosa, P. gingivalis, E. coli) bacteria [45] while ovoinhibitor inhibits fungal and bacterial proteases [44]. One of the most abundant proteins in our cuticle extract is a protein predicted to be similar to Kunitz- like protease inhibitor; we hypothesize that this protein, along with the other members of the SERPIN family of proteins (Table 4), also inhibit bacterial and fungal proteases. Other potential antimicrobial proteins are OCX-36 based on its similarities to well-known innate immune proteins, and myeloperoxidase by generating reactive oxidizing products.

The benefits of a protective barrier surrounding the egg to promote food safety are evident. Gram-positive bacteria frequently contaminate the eggshell surface since they are capable of withstanding desiccating conditions, while Gram- negative bacteria more frequently contaminate the interior of the egg [49]. Our results demonstrate that washed grocery eggs have less cuticle protein compared to the farm eggs. One industrial strategy is to coat eggs with edible material such as mineral oil, chitosan-lysozyme, soy proteins or

whey proteins [50,51]. These coatings act as a physical barrier only or do not possess the wide range of antimicrobials that the natural cuticle possesses. Additional manipulations during egg processing result in an increased incidence of microcracks [52] and elevated opportunities for bacterial contamination.

In summary, we have currently identified at least 47 proteins localized in the outer cuticle layer of the chicken eggshell. Only two of these proteins (similar to Kunitz-like protease inhibitor and OCX-32) comprise the vast majority of the cuticle proteins. This study is the first step towards understanding the role of the cuticle and its individual constituents. Since eggs with in- complete or absent cuticles are more susceptible to bacterial contamination, identification of cuticle-resident antimicrobials could provide new targets for selective breeding programs (i.e. marker assisted selection) to enhance the innate immune defenses of the egg for enhanced food safety.

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2012.03.019.

5. Conflict of interest statement

The authors have no financial or commercial conflicts of interests to declare.

6. Acknowledgments

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Table 1: Merged results for cuticle proteins extracted with 1% SDS, 2mM DTT identified in two independent analyses.

Identified proteins	MW (kDa)	UniProt ID	No. of unique peptides	Coverage (%)	emPAI [a]	Signal peptide [b]	Also present in [c]	Gel section [d]
Ovocalyxin-32 [e]	31	C7G541	34	85	112.62 (54.46,16.61)	Yes	vm, w, s, c	1–13
Ovalbumin	43	P01012	18	56	35.79 (2.44,35.79)	No	y, vm, w, s	1–13
Similar to Kunitz-like protease inhibitor	23	N/A (REFSEQ ID: XP_001235178)	23	50	14.08 (8.41,14.78)	Yes	w, s, c	1–13
Lysozyme C	16	P00698	9	50	9.53 (6.96,6.84)	Yes	y, vm, w, s	2, 4
Glutathione peroxidase 3	25	F1NPJ8	7	36	4.89 (1.43,1.90)	Yes	y, vm, w, s	5–6
Ovoinhibitor	52	P10184	18	48	4.15 (4.15,0.64)	Yes	y, vm, w, s	4–8, 10
Ovotransferrin	78	P02789	24	41	2.56 (0.48,2.17)	No	y, vm, w, s	8, 10
Similar to prostate stem cell antigen	13	F1NXM7	2	24	1.61 (0.90,0.90)	Yes	y, s	5
Serum albumin	70	P19121	16	28	1.54 (0.75, 0.86)	Yes	y, vm, w, s	10
gastrokine-2 (OCX-21)	21	E1C2G7	4	33	1.26 (0.5, 0.84)	Yes	s	2, 4
Hemoglobin subunit β	16	P02112	3	30	1.19 (1.19, 1.19)	No	s	4, 13
Ovocalyxin-36	49	Q53HW8	6	23	0.88 (0.88, 0.72)	Yes	vm, s, c	5, 8–9
predicted: selenium-binding protein 1-like	78	N/A (REFSEQ ID: XP_003642728)	14	23	0.75 (0.65, 0.25)	No		8–13
Cystatin	15	P01038	3	27	0.75 (0.75, 0.75)	Yes	y, vm, w, s	4
Hemoglobin subunit α-A	15	P01994	2	15	0.75 (0,0.75)	No		7
β-hexosaminidase subunit α precursor	59	F1NEX5	11	18	0.69 (0.69, 0.16)	Yes	s	10
Chain A, structure of an avian Igy-Fc 3–4 fragment	25	N/A (PDB ID: 2W59_A)	5	35	0.67 (0.67, 0.67)	No	y	4, 10
predicted: similar to SDF3	47	E1C7H6	7	20	0.61 (0.47, 0.10)	Yes	y, vm, s	9
Vitellogenin-2	203	E1BYN6	17	11	0.47 (0, 0.47)	Yes	y, vm, w, s	2, 6–13
Epithelial cell adhesion molecule	34	Q5F381	4	10	0.46 (0.46, 0)	Yes		8
Nucleobindin-2	54	F1NGB1	4	11	0.44 (0.44, 0)	Yes	w, s	10
Similar to FKSG18	39	F1NWW1	3	10	0.41 (0.26, 0.12)	No	s	8
Clusterin	49	F1NGP2	4	10	0.31 (0.20, 0.09)	Yes	y, vm, w, s, c	8
Leucine-rich repeat-containing protein 19	43	F1P096	4	12	0.28 (0.13, 0.13)	No	vm, w, s	8
predicted: annexin A8	37	E1C8K3	2	5	0.27 (0, 0.27)	No	s	9
Similar to myeloperoxidase precursor	81	F1P3V5	5	9	0.25 (0.18, 0.12)	No		10–11
Tubulin α-1 chain	46	P02552	2	7	0.25 (0, 0.25)	No	vm, s	
Actin, cytoplasmic 1/2/5	42	P53478, P60706, Q5ZMQ2	4	20	0.24 (0.24, 0.24)	No	y, vm, w, s	8
Apolipoprotein A-IV	41	O93601	4	14	0.24 (0.24, 0)	Yes	s	8
Ovalbumin-related protein Y	44	P01014	3	10	0.22 (0.11, 0.22)	No	y, vm, w, s	10
Tubulin β-3 chain	50	P09206	3	11	0.22 (0, 0.22)	No	vm, s	6
Similar to neuronal pentraxin-2	48	E1C7S1	2	6	0.18 (0.18, 0)	No	s	10
Similar to α-2-plasmin inhibitor	51	F1NAR5	3	4	0.17 (0.17, 0.08)	Yes	y, s	10
Proactivator polypeptide (saposin A-D precursor)	58	O13035	2	5	0.16 (0.16, 0)	Yes	y, s	10
Polymeric immunoglobulin receptor precursor	69	N/A (REFSEQ ID: NP_001038109)	2	4	0.13 (0.13, 0.05)	Yes	vm, w, s	10
Aminopeptidase N	109	O57579	3	4	0.13 (0.09, 0.15)	No	y, w, s	11
Ovocleidin-116	77	F1NSM7	3	6	0.12 (0.12, 0)	Yes	y, vm, w, s, c	8, 10
predicted: mucin-5B	322	N/A (REFSEQ ID: XP_421033)	5	2	0.11	No	s	12–13
Neuroserpin	47	Q90935	2	6	0.10 (0, 0.10)	Yes	s	4–5
predicted: hyaluronidase-1	49	N/A (REFSEQ ID: XP_424356)	2	6	0.09 (0.09, 0)	Yes	s	9
Similar to ovomacroglobulin, ovostatin, partial	166	F1NEW8	3	2	0.05 (0.05, 0.06)	No	s	10–11
predicted: similar to Mesothelin	88	N/A (REFSEQ ID: XP_414835)	2	4	0.05 (0.05, 0.05)	Yes	s	11
Apolipoprotein B	484	F1NV02	11	3	0.04 (0, 0.04)	Yes	y, vm, w	10–13
predicted: thrombospondin type-1 domain-containing protein 4	114	N/A (REFSEQ ID: XP_413780)	2	2	0.04 (0.04, 0)	No		11
Similar to basement membrane-specific heparan sulfate proteoglycan core protein, partial	129	F1NAT6	2	2	0.03 (0.03, 0)	No	s	9, 11
Vitellogenin-1	211	P87498	4	2	0.02 (0, 0.02)	Yes	y, vm, w, s	10

Table 1 *(continued)*

Identified proteins	MW (kDa)	UniProt ID	No. of unique peptides	Coverage (%)	emPAI [a]	Signal peptide [b]	Also present in [c]	Gel section [d]
predicted: ovalbumin-related protein Y	45	N/A (REFSEQ ID: XP_418984)	2	5	N/A	No	y, vm, w, s	5, 9, 11

[a] The relative quantification of the identified proteins were calculated using the exponentially modified protein abundance index (emPAI) = $10^{(N_{observed}/N_{observable})} - 1$, see Materials and Methods Section 2.6. For each identified protein, the emPAI value of the merged analyses is given, with the individual values in parentheses (Cuticle Extract: CE-1, CE-2).

[b] Signal peptides were predicted using SignalP 3.0 server.

[c] Egg compartment where previously detected: y, yolk; vm, vitelline membrane; w, egg white; s, eggshell; c, cuticle.

[d] Gel section in which peptides corresponding to the identified proteins were identified, see Fig. 1.

[e] Several isoforms of this protein were identified in the sample. This table lists the isoform with the highest emPAI value. See Table 3 for a list of the identified peptides of all isoforms.

Table 2: Identified peptides corresponding to two versions of the predicted: similar to Kunitz-like protease inhibitor sequence.

	REFSEQ ID	
Unique peptide sequences	XP_001235178.1	XP_001235178.2
APAEETAR	x	x
CPFRCPQPVPARPDTYPK	x	x
CPQPVPARPDTYPK	x	x
CPQPVPARPDTYPKK	x	x
GLEAFLLGDSNQR	**x**	
KKVPHIIGCCNSTCSSDTDFPNHLR	x	x
KRTYCYACIPALR	x	x
KVPHIIGCCNSTCSSDTDFPNHLR	x	x
QPVLLGLPLSGLPGALPHR	**x**	
RTYCYACIPALR	x	x
SCRVFVHSSCGGNANNFR	x	x
SSCGGNANNFR	x	x
SVLPEKDDFHPR	x	x
SVLPEKDDFHPRTDTDPTTNCVNNCR	x	x
TDGRSVLPEKDDFHPR	x	x
TDTDPTTNCVNNCR	x	x
TDTDPTTNCVNNCRDDGNCR	x	x
TLAECQQVCQ	x	x
TLAECQQVCQHGESWAR	**x**	
TYCYACIPALR	x	x
VFVHSSCGGNANNF	x	x
VFVHSSCGGNANNFR	x	x
VPHIIGCCNSTCSSDTDFPNHLR	x	x

[a] Peptides that do not correspond to the updated version REFSEQ ID: XP_001235178.2 are indicated by gaps.

Table 3: Identified peptides corresponding to four isoforms of ovocalyxin-32

Identified peptide sequences	UNIPROT ID			
	D3KYT5	C7G540	C7G541	C7G542
AQVSSVKQQIR	x	x	x	x
CVHAQNKK	x	x	x	x
DNAVAFK	x	x	x	x
EASPSRPLALHK	x	x	x	x
EAVWAAWTALHYINSHEASPSR	x	x	x	x
EAVWAAWTALHYINSHEASPSRPLAL	x	x	x	x
EAVWAAWTALHYINSHEASPSRPLALHK	x	x	x	x
EAVWAAWTALHYINSHEASPSRPLALHKVVK	x	x	x	x
ERLPWPQVPGVMHPLNPSHR		x	x	
ERLPWPQVPGVMRPLNPSHR	x			x
FIVLLHEIPTQQLNV	x		x	
FIVLLHEIPTQQLNVCHMYLVWTLGHPIR	x		x	
FIVLLHEIPTQQMNVCHMYLVWR				*
FIVLLHEIPTQQMNVCHMYLVWTLGHPIR		*		
FYEYLQHQK	x	x	x	x
FYEYLQHQKK	x	x	x	x
GSSHIMWKQSTEHTGYLLAQVSSVK			*	
KDNAVAFK	x	x	x	x
KDNAVAFKFIVLLHEIPTQQLNVCHMYLVWTLGHPIR	x		x	
KPITANYIPDSH	x		x	
KPITANYIPDSHGNIAHD	x		x	
KPITANYIPDSHGNIAHDHLQLWGLAIVGSSHIMWK			*	
KQIQEEDHR	x	x	x	x
KQIQEEDHRFYEYLQHQK	x	x	x	x
KSPPVVHAK	*			
KSPPVVHGK		x	x	x
LPWPQVPGVMHPLNPSHR		x	x	
PLNPSHR	x			x
PQVPGVMHPLNPSHR		x	x	
QIQEEDHR	x	x	x	x
QIQEEDHRFYEYLQHQK	x	x	x	x
QQIRKDNAVAFK	x	x	x	x
QSTEHTGYLLAQVSSVK	x	x	x	x
QSTEHTGYLLAQVSSVKQQIR	x	x	x	x
QSTEHTGYLLAQVSSVKQQIRK	x	x	x	x
SPPVVHAK	*			
SPPVVHAKCVHAQNK	*			
SPPVVHGK		x	x	x
YSCAPDNHGLEDGSGQDSGSAAGTSHETK	x	x	x	x
YYVHCTTEGYIHGENAGSCFATVLYLK	x	x	x	x
YYVHCTTEGYIHGENAGSCFATVLYLKK	x	x	x	x

[a] Peptides that are exclusive to a single isoform are indicated by asterisk and their spectra depicted in Fig. S1. More information on peptides exclusive to a single isoform can be found in Table S2.

Table 4: GO term clusters corresponding to the biological processes and molecular function of the cuticle proteins.

Clusters		GO Terms	P-Value [a]	Proteins [b]
Enzyme regulator activity				
E-score [c] 8.71	GO:0004866	Endopeptidase inhibitor activity	6.57E−11	F1NAR5; P01012; P10184; XP_418984; XP_001235178; Q90935; P01038; P01014; F1NEW8
	GO:0030414	Peptidase inhibitor activity	1.13E−10	F1NAR5; P01012; P10184; XP_418984; XP_001235178; Q90935; P01038; P01014; F1NEW8
	GO:0004857	Enzyme inhibitor activity	9.75E−10	F1NAR5; P01012; P10184; XP_418984; XP_001235178; Q90935; P01038; P01014; F1NEW8
	GO:0004867	Serine-type endopeptidase inhibitor activity	7.59E−09	F1NAR5; P01012; P10184; XP_418984; XP_001235178; Q90935; P01014
	GO:0030234	Enzyme regulator activity	5.00E−07	F1NAR5; O93601; P01012; P10184; XP_418984; XP_001235178; Q90935; P01038; P01014; F1NEW8
Lipid metabolism and transport				
E-score 2.54	GO:0010876	Lipid localization	1.67E−04	O93601; P87498; E1BYN6; F1NEX5
	GO:0005319	Lipid transporter activity	0.002178	O93601; P87498; E1BYN6
	GO:0006869	Lipid transport	0.004484	O93601; P87498; E1BYN6
	GO:0033036	Macromolecule localization	0.006045	O93601; P87498; E1BYN6; F1NAT6; F1NEX5
	GO:0051179	Localization	0.019084	P19121; O93601; P01994; P87498; E1BYN6; F1NAT6; P02112; F1NEX5

[a] P-value corresponds to the EASE score determined by the DAVID software.
[b] Corresponds to DATABASE ID # utilized in Table 1.
[c] E-score represents the enrichment score determined by the DAVID software.

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Chapter IV Preliminary results for a new candidate from the cuticle layer for innate antimicrobial protection of chicken egg, the similar to Kunitz-like protease inhibitor (OCX-25).

1. Background Introduction

1.1 Formation of the Cuticle

The cuticle layer is produced by secretory cells in the uterus and deposited on the calcified shell surface during the last 1.5 hours before oviposition (Wyburn, 1973). This layer, also known as the bloom, is moist when the egg is just laid, taking a few minutes to solidify. It is a relatively thin layer of variable thickness ranging from 5 to 10 μm(Romanoff, 1949). According to previous studies by light and electron microscopy (Baker, 1962), the dry cuticle mainly covers the pores of the eggshell calcified layer (Figure 1). The cuticle consists of two layers, the outermost non-calcified layer and an inner calcified layer. They can be seen clearly in thin sections of the shell by using an electron microscope. It is important to note that not all bird species have a cuticle (i.e. pigeons), and a small proportion of chicken eggs (~15%) also have absent or incomplete cuticle layers (Board, 1973 and 1974).

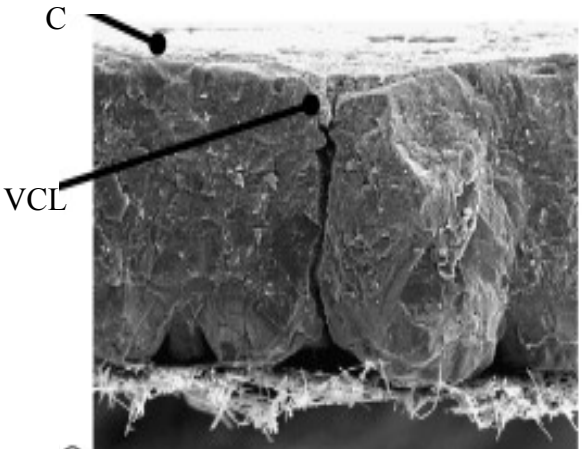


Figure 1: Scanning electron microscope cross-section of eggshell, illustrating the external cuticle layer. VCL, vertical crystal layer; C, cuticle. (Reprint from Cabeza et al., 2011, with permission from Innovative Food Science and Emerging Technologies)

1.2 Function of the Cuticle

Two principal defense mechanisms protect the egg embryo. The intact eggshell

acts as a physical barrier to protect the egg embryo from infection by bacteria, physical damage and small predators. The shell and vitelline membranes also add to the physical barrier by acting as bacterial filters, keeping bacteria away from the yolk while allowing nutrients to traverse. The last component of the physical barrier is the egg white protein ovomucin. This protein gives the albumen its viscosity that can hinder bacterial movement. In addition to physical barriers, a chemical protection system is composed of egg white and eggshell matrix proteins that function as an antimicrobial defense (Anton et al., 2006;Seuss-Baum et al., 2007; Mine, 2008; Hervé-Grépinet et al., 2009).

The cuticle is a proteinaceous layer coating the outer eggshell surface and filling the pores of the eggshell and blocking entry points for pathogens. Some of the cuticle's protein constituents have antimicrobial activity that helps in the egg's chemical protection. Antimicrobial proteins such as lysozyme (Hincke et al., 2000), ovotransferrin (Gautron et al., 2001) (against Gram-negative bacteria) and ovocalyxin-32 (inhibit *B. subtilis*), have been detected in the cuticle layer and shown to have activity against certain bacteria (Wellmann- Labadie et al., 2008). The cuticle layer also participates in controlling the exchange and loss of water in dry environments.

1.3 Components of the cuticle proteins revealed by proteomic analysis

Due to the crucial role of the cuticle in the chemical defense of the egg, we considered it very important to properly characterize the constituents of this layer. Until now there was no comprehensive list of cuticle proteins. This study aimed to identify the entire cuticle proteome and potential antibacterial proteins. In the last year of research, we extracted proteins from the non-calcified cuticle layer of fresh farm and grocery eggs in order to compare the protein constituents and to assess possible antimicrobial activity.

Proteomic analysis of SDS-soluble cuticle proteins gave us an opportunity to identify some of its constituents.

A total of 47 proteins were identified in the fresh farm eggshell cuticle by LC/MS/MS (Chapter III). The most abundant protein, Ovocalyxin-32, which protein sequence possesses 30% identity to a carboxypeptidase A inhibitor, leading to the hypothesis that it is an antimicrobial protein that targets microbial proteases. Another abundant protein is similar to kunitz-like protease inhibitor.

The predicted kunitz-like protease inhibitor found in the cuticle of the eggshell has been annotated as Ovocalyxin-25 (OCX-25). Kunitz motif is the domain responsible for the inhibitory activity, belonging to one of three families of serine protease inhibitors. OCX-25 was found to be similar to bovine pancreatic trypsin inhibitor (BPTI) corresponding to the kunitz family motif of serine protease inhibitors. There is an increasing interest in trypsin inhibitors because of its possible role in altering the nutritive value of food, and the unique pharmacological potential for clinical application. Due to this interest, scientists are devoting a lot of effort on isolating, purifying, and characterizing trypsin inhibitors found in nature. The Kunitz-like protease inhibitor is part of the serine protease inhibitor family (Pouvreau et al., 2003). This is the largest and most diverse family of protease inhibitors that control the activation and catabolism of proteins via the inhibition of serine proteases. The inhibitor acts as a competitive substrate analog and binds to the serine protease to form an inactive complex, rendering the protease inactive (Zhou, 1989). We hypothesize that this enzyme is responsible for inhibiting bacterial proteases used to degrade or digest the cuticle layer in order to penetrate the shell.

2. Material and Methods

2.1 Extractions of Cuticle Layer Proteins

The cuticle proteins were extracted from fresh farm eggs (LSL-Lite and brown Lohmann Brown domestic chicken, *Gallus gallus*) from the Laviolette Poultry Farm in St-Isidore, Ontario, Canada.

Eggs were individually and completely immersed in either 1N HCl or 0.5M EDTA, pH7.5. These methods are specific for the extraction of the cuticle proteins from the outer calcified eggshell.

(1) EDTA —Eggs (ten eggs for each time point) were individually immersed in 45ml EDTA (0.5M EDTA, 0.5M NaOH, pH7.5) at room temperature for various time periods (15, 30 and 60mins). Part of the eggshell dissolved and the cuticle was then scraped off into the extraction solution. The samples were centrifuged before dialysis using JA16.25 rotor (Beckman Avanti J-25) at 13, 000rpm for 30 min. Samples were dialyzed in water for 7 days (15 minute sample) or 10 days (30 and 60 minute samples) and were centrifuged at 13,000rpm for 30mins to remove any heavy particles before freeze-drying. The supernatant samples were divided into 30ml volumes for lyophilization.

(2) 1N HCl — each egg (80 eggs) was treated with 40ml 1N HCl in 100ml beaker at room temperature for 4mins. The sample was neutralized (the sample was neutralized to pH7.5 with NaOH and 10mM Tris-HCl pH 8.0), and centrifuged using JA10 rotor (Beckman Avanti J-25) at10,000rpm for 20 min before dialysis process. The supernatant samples were dialyzed against water for 8 days before freeze-drying. The sample after dialysis was centrifuged to remove insoluble material, and separated into

30ml volumes for lyophilization.

For the antimicrobial activity test, the sample was dissolved in PBS, and for the trypsin inhibitory assay the sample was dissolved in sterilized water. After being dissolved in PBS or water, the sample was centrifuged at 13,000rpm for 5mins to separate the supernatant and pellet. Only the supernatant sample was used in antimicrobial activity and trypsin inhibitor activity assays.

Samples from both methods were tested for protein concentration using a BCA assay and were analyzed by SDS-PAGE gel to compare protein composition. Coomassie Blue staining and/or silver staining (SilverQuest Silver Staining Kit, Invitrogen) was applied to stain the gels. Western blot was used to detect and confirm the presence of the kunitz-like protease inhibitor that was the most abundant in the proteomic analysis. The supernatant of 1N HCl sample was subjected to LC/MS/MS analysis.

2.2 Antimicrobial activity of cuticle protein extracts

Bacteria cultures were grown overnight in Luria-Bertani (LB) broth. Bacteria were diluted in LB broth and incubated for 2 h. The bacteria were concentrated through centrifugation twice at 3000rpm for 10 min in PBS. The absorbance of 0.2 at 600nm corresponds to ~$1X10^6$ CFU/ml, and bacteria were adjusted to this concentration with PBS. The sample (μ g) was dissolved in PBS, added to the bacteria in a 1:1 ratio, and incubated for 1 h at 37°C at 250rpm shaking. Dilutions of bacteria (10^{-1}-10^{-4}) were made in order to create a growth curve for analysis. LB broth was added, and bacterial growth was measured over~18 h at 600nm using the Bioscreen C instrument (OY Growth curve) measured by EZExperiment software. The growth of Gram-positive (*B. subtilis* ATCC 19659 and *S. aureus* ATCC 6538) and Gram-negative bacteria (*E. coli* O119:B4 and *S. tyhimurium* ATCC

1535) was measured. Controls of PBS for full growth, and kanamycin for inhibited growth were performed for all bacteria. Data was analyzed using Prism Version 5.0 software.

2.3 Trypsin inhibitor activity assay for cuticle extracts

The standard curve for the assay was established with soybean inhibitor (SBTI, Sigma). 60µg /ml of trypsin in 1mM HCl (bovine pancreas, Sigma) was preincubated with different concentrations of inhibitor ranging from 10µg /mL to 30µg /mL for 30min at room temperature. The inhibitor activity was determined at room temperature by adding 0.5mg/mL L-BAPNA in 50mM Tris-HCl buffer pH 8.2, 20mM CaCl2 as a substrate and monitoring the increase in absorbance at 405nm using a 96-well microplate reader (Bio-Tek instruments). The reaction rate was determined by calculating the slope of the color development over time.

3. Results and Discussion

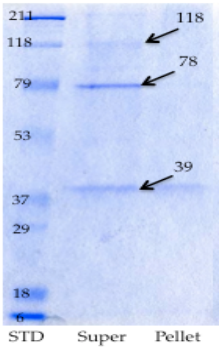
3.1 Comparison of two different extraction methods

Our objective was to determine extraction and buffer conditions that solubilized cuticle proteins, especially for Kunitz-like protease inhibitor (OCX-25). Several preparations of cuticle extracts were prepared by the HCl-method and EDTA-method. Two examples of these extraction methods are presented in the next section.

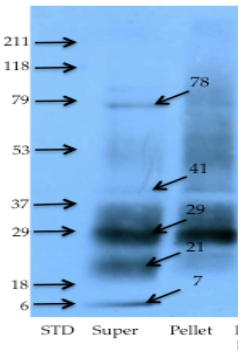
EDTA Method for 15mins (Figure 2)

SDS-PAGE gel indicated a faint band in both supernatant and pellet at the 39kDa. In addition, the supernatant showed an intense band at 78kDa, as well as a faint band at 118kDa. Western blot had an intense band at 29kDa in both samples. Lower molecular weight reactive bands were visible predominantly in the supernatant at 7 and 21kDa, as well as faint higher molecular weight bands at 41kDa and 78kDa. Total protein yield was

3.78µg of pellet and supernatant; approximately 40% of the protein was soluble in the supernatant with PBS.



A.



B.

Figure 2: Coomassie Blue gel (A) and Western Blot (B) of eggshell cuticle extraction from EDTA-method. Samples loaded onto the 12.5% polyacrylamide gel. The bands of the SDS-PAGE are visible by Coomassie Blue staining. Western Blot uses a 1:15 000 OCX-25 antibody with an exposure time of 3 sec.

HCl Method (Figure 3)

SDS-PAGE gel stained with Coomassie Blue showed no significant bands, possibly due to the low protein concentration. Therefore, silver staining was used to study protein bands in these samples. Western blot had an intense band at 10, 20 and 25kDa in the supernatant with slightly less intense bands of similar size in the pellet. Total protein yield was 8.33µg ; about 25% of the protein was dissolved with PBS.

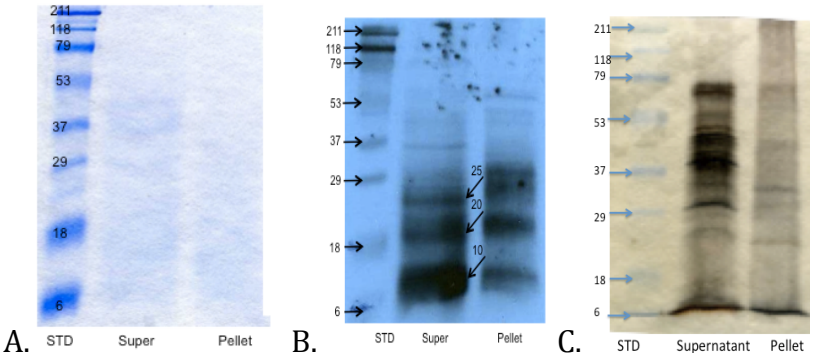


Figure 3: Coomassie Blue staining (A), Western blot (B) and Silver staining (C) of eggshell cuticle extraction prepared by the HCl-Method. The amount of protein loaded was 1.6µg for supernatant, 0.5µg for Pellet onto the 12.5% polyacrylamide gel. The amount of protein loaded for the Western Blot was 0.4µg for Super, 0.1µg for Pellet onto the 12.5% polyacrylamide gel. Western blot uses a 1:15 000 OCX-25 antibody with an exposure time of 15 sec.

A difficulty was detected with the 0.5M EDTA extraction method since EDTA interfered with the BCA assay. The BCA assay is able to accurately quantify protein in a sample when there is less than 10mM EDTA. Regardless of increasing the time of dialysis to remove the EDTA, the BCA assay again indicated EDTA contamination. Many researchers have encountered difficulties to remove EDTA through dialysis, due to its ability to form a network large enough to remain in the dialysis bag. In addition, antimicrobial assay with EDTA alone demonstrated substantial antimicrobial activity.

An alternative extraction method was to use 1N HCl to extract the cuticle proteins. Silver staining indicated that the OCX-25 immunoreactive bands were not the major components of the supernatant or pellet in this manner. Instead, the major bands in the supernatant sample were at 6kDa, 29kDa, 39kDa and 78kDa.

In summary, EDTA and HCl are both methods to remove the outer eggshell and associated cuticle, but the EDTA is difficult to remove by dialysis. HCl is a better extraction method to prepare samples for assays of antimicrobial activity and trypsin inhibitor activity.

3.2 Antimicrobial activity assay

The supernatant of the protein that was extracted by 1N HCl method was tested with different bacteria, B. *subtilis*, E. *coli* and S. *typhimurium*, and S. *aureus*. Growth of B. *subtilis* was not inhibited by the cuticle extracted. However, E. *coli* and S. *aureus* displayed reduced growth when incubated with the extracts (Figure 4). Unfortunately, the 1N HCl control buffer displayed similar inhibition of S. *aureus* growth (Figure 5).

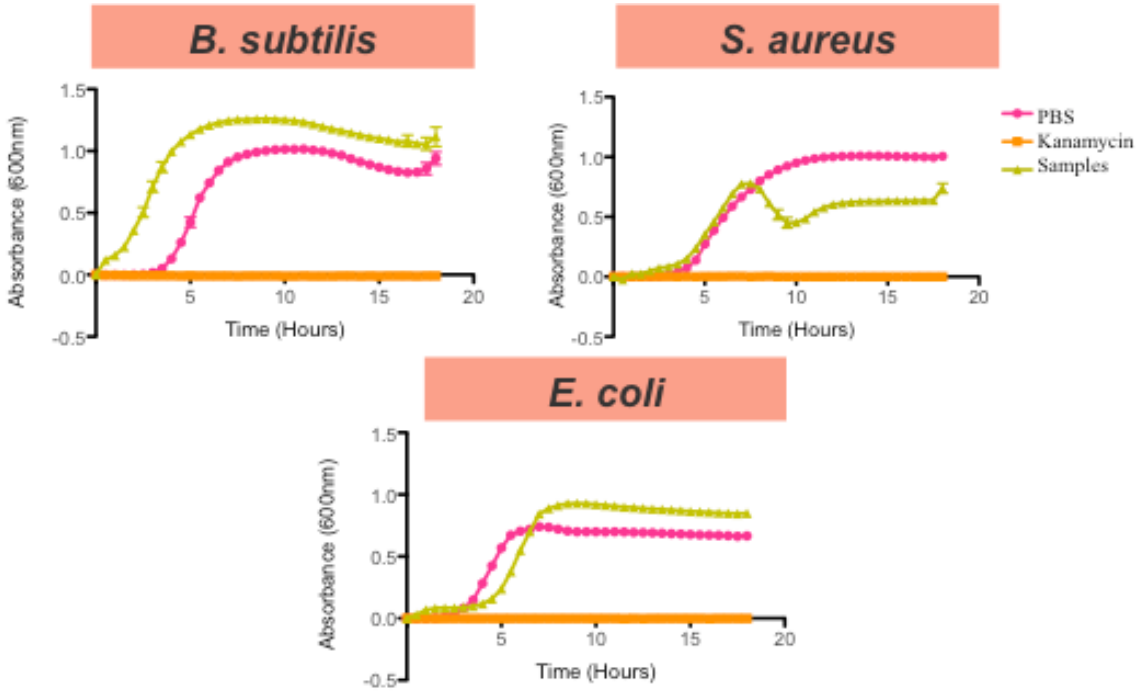


Figure 4: Graphs of triplicate absorbance readings (at 600nm) of (A) Gram-positive *B. subtilis,* (B) Gram-positive *S. aureus*, (C) Gram-negative *E. coli*, measured by the Bioscreen C instrument over 18 h. Errors bars with the standard deviation of the in triplicate absorbance are marked. Preincubation with bacteria, 0.3µg of cuticle extract was tested. Negative control for inhibition is PBS. Positive control for inhibition is kanamycin (10mg/ml preincubation).

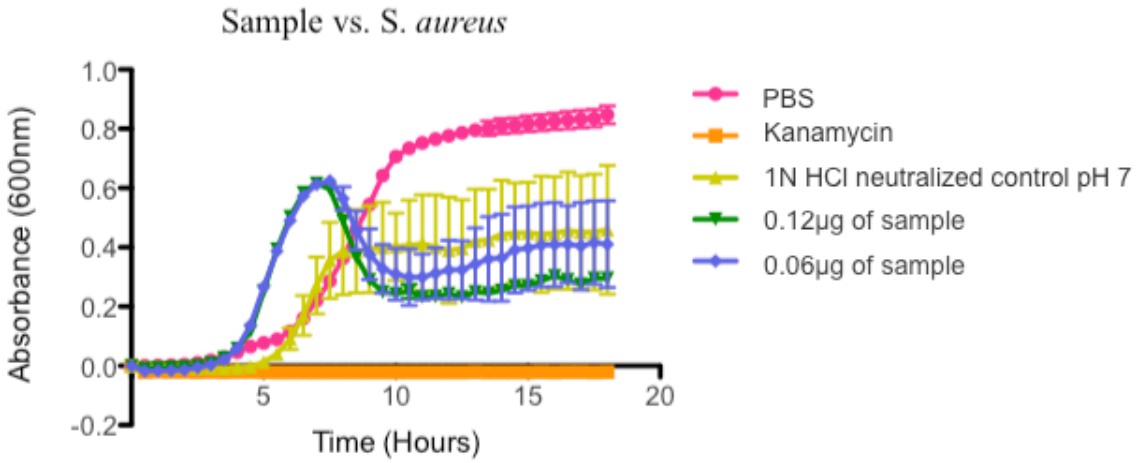


Figure 5: Graph of in triplicate absorbance readings (at 600nm) of Gram-positive *Staphylococcus aureus* turbidity, measured by the Bioscreen C instrument over 18 h. Errors bars with the standard deviation of the in triplicate absorbance are marked. Preincubation with bacteria, 0.12µg , and 0.06µg of cuticle extract was tested. Negative control for inhibition is PBS, and neutralized 1N HCl after 24 h of dialysis. Positive control for inhibition is kanamycin (10mg/mL preincubation).

In conclusion, different bacteria were tested with cuticle proteins containing OCX-25 to test the predicted antimicrobial activity. Although, the cuticle proteins showed significant antimicrobial activity, the effect was similar to that observed with the vehicle control. It was not possible to test higher concentration of cuticle extract to determine whether it was a specific effect due to the cuticle protein.

3.3 Trypsin inhibitory activity assay

Similar to Kunitz-like protease inhibitor is predicted to possess a Kunitz domain, the active domain of the protein, which inhibits the function of protein-degrading enzymes. According to the LC/MS/MS results, one of the most abundant cuticle proteins is Kunitz-like protease inhibitor of the cuticle extracts (Chapter III).

First of all, the reliability of the trypsin inhibitory assay was tested by using a known trypsin inhibitor, Soy Bean Trypsin Inhibitor (SBTI) (Figure 6). The rate of color development by cleavage of the chromogenic substrate was calculated by monitoring the

increase in absorbance for up to 1h. Linearity of this reaction during the first 10 min was verified, and the rate of color development during this period was calculated. When the concentration of trypsin inhibitor was increased, the color development rate decreased, indicating a decrease in trypsin enzymatic activity. When molar amount of SBTI was approximately equal to the molar amount of trypsin, the activity was completely inhibited, as expected. I hypothesized that the similar to Kunitz-like protease inhibitor (OCX-25) extracted from the egg cuticle will have a similar effect on trypsin enzymatic activity.

In the next step, two cuticle protein extracts (extracted by 1N HCl) were tested with the trypsin inhibitory activity assay to verify the hypothesis. The entire extraction process and trypsin inhibitory activity assay was repeated twice to confirm reproducibility. Freeze dried sample was dissolved in H_2O, and tested by trypsin inhibitor activity assay (section 2.3). Figure 7 shows the rate of color development with various concentrations of cuticle proteins using 30μg /ml of trypsin. We observed a decrease in trypsin activity, suggesting inhibition by cuticle proteins. When the concentration of cuticle proteins was increased, the color development rate decreased, indicating a decrease in trypsin enzymatic activity. The cuticle protein sample concentration is from 10 to 40 times higher than the trypsin; it could not totally inhibit the reaction since the cuticle protein is not purified Kunitz-like protease inhibitor.

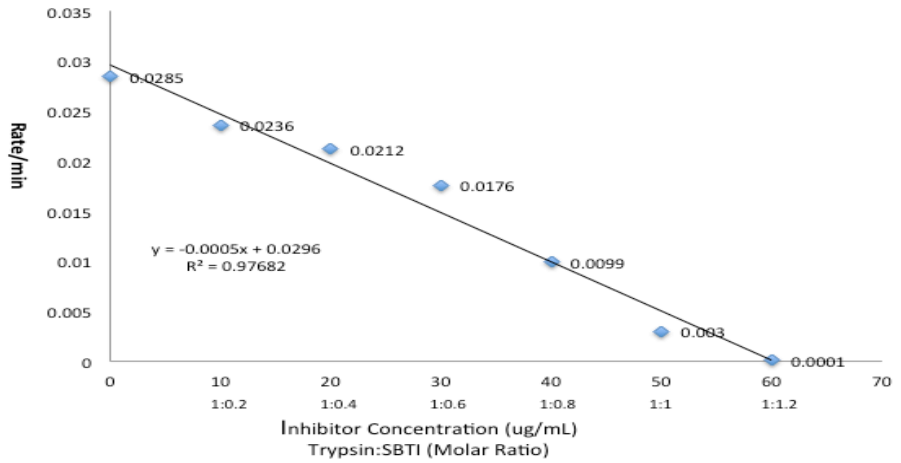


Figure 6: Rate of color development during the trypsin inhibitory assays using various amounts of SBTI.

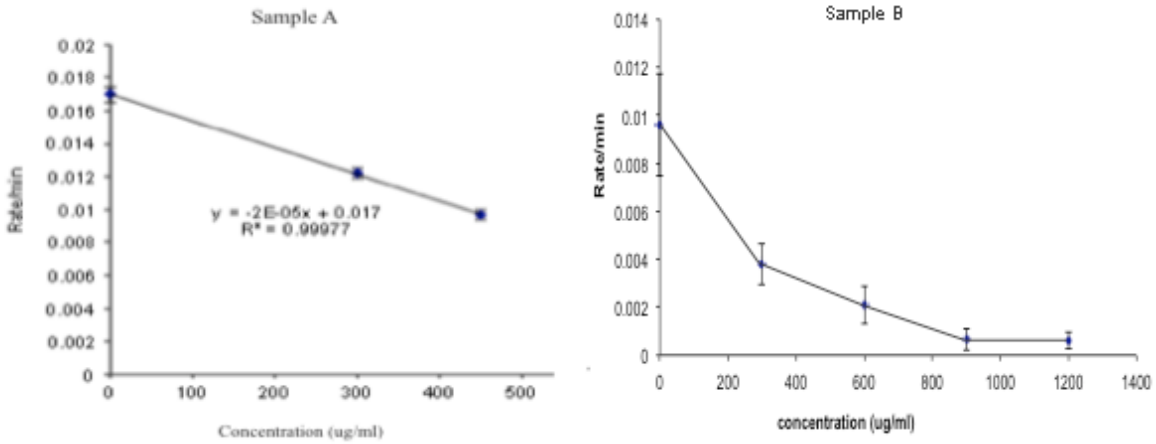


Figure 7: Rate of color development of trypsin assay during a 10mins period (Sample A and B indicates two extracts). A: 300µ**g**/mL or 450µ**g**/mL of cuticle protein (10µl) were incubated with 30µ**g**/mL of trypsin (10µl). B: 300µ**g**/mL, 600µ**g**/mL, 900µ**g**/mL and 1200µ**g**/mL of cuticle protein (10µl) were reacted with 30µ**g**/mL trypsin (10µl).

3.4 Proteomic analysis of cuticle extracts

1N HCl extracts were resolved by SDS-PAGE using a precast 4-12% Bis Tris gel (Bio-Rad). The gels were sectioned and sent for Proteomic analysis (Proteomics Platform of the Eastern Quebec Genomics Centre, QC), and the entire extraction process and proteomic analysis was repeated twice to confirm reproducibility (Figure 8).

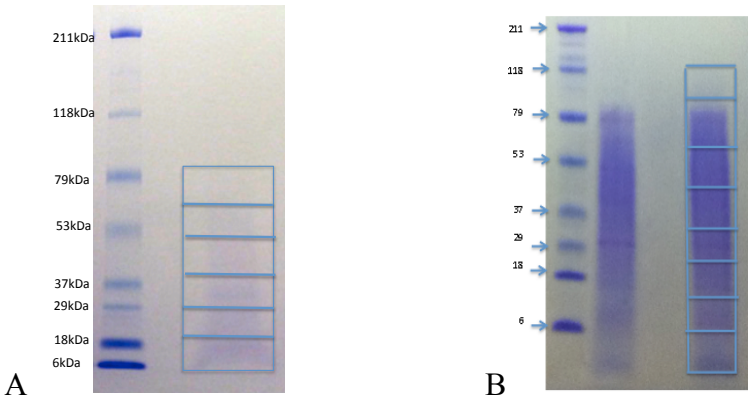


Figure 8: SDS-PAGE analysis of 30ug cuticle proteins, extracted with 1N HCl (Sample A and B correspond to two samples sent for proteomic analysis). Bars indicated where the gel was sectioned and the fragments that were sent for proteomic analysis.

The two proteomics analysis results indicated that at least 8 proteins were common to both extracts prepared by the 1N HCl extraction method (Table 1). The results indicated that similar to Kunitz-like protease inhibitor (OCX-25) was present in **sample A** extraction sample but not the **sample B** extraction (Figure 8 and Table 1). Therefore, the trypsin inhibitory activity that we observed was likely due to other serine-type protease inhibitors present in the extracts; these candidates include ovoinhibitor, neuroserpin, trypsin inhibitor C1TI-1 and predicted: ovalbumin-related protein Y. Ovoinhibitor from egg is a multidomain Kazal-type serine protease inhibitor; purified egg white ovoinhibitor was shown to inhibit trypsin and subtilisin, and it also exhibited antimicrobial activities against *Bacillus thuringiensis* (Bourin et al., 2011). Other proteins that have antimicrobial activity were detected in our protein sample, such as Gallinacin-9 (Avian beta-defensin 9 (AvBD9), Lynn et al., 2007), which was shown to have the antimicrobial activity against S. *serovars* (Milona et al., 2007). In addition, recombinant OXC-32 was demonstrated to inhibit growth of *B. subtilis* (Xing et al., 2007), and Lysozyme C is a well-known antimicrobial protein (Hughey et al., 1989;Hincke et al., 2000)

Our previous proteomic study of the SDS-soluble cuticle identified at least 47 proteins by using 1%SDS, 2mM DTT extraction solution (Chapter III, Table 1). However, in our analysis, only 8 of these proteins were also identified (Ovocalyxin-32, Vitellogenin-1, Ovoinhibitor, Ovalbumin, Serum albumin, Ovalbumin-related protein, Neurosepin and similar to Kunitz-like protease inhibitor). We hypothesized that this is due to the differences between two extraction solutions, since 1N HCl extracted the outer calcified eggshell in addition to the cuticle layer. A very important difference is that SDS is expected to solubilize all cuticle proteins, while in my current study we were only able to detect the proteins that remain soluble in 1N HCl. Therefore, our goal to identify conditions to extract and dissolve the cuticle proteins in non-denaturing solutions was not successful.

Table 1: Results for cuticle proteins extracted with 1N HCl identified in two independent analyses

Identified protein	Accesstion No	Molecular Weight	Sample A Coverage (%)	Sample A No of unique peptide	Sample A em PAI	Sample B Coverage (%)	Sample B No of unique peptide	Sample B em PAI
Ovoinhibitor *	P10184	52 kDa	51.40%	35	16.49	56%	32	10.2
Lysozyme C *	P00698	16 kDa	42.60%	7	5.11	50%	8	5.11
Serum albumin*	P02769	69 kDa	5.90%	5	0.41	17%	17	1.08
Ig lambda chain C region	P20763	11 kDa	-	-	-	28%	3	0.51
Cystatin	P01038	15 kDa	-	-	-	20%	3	0.91
Trypsin I-P1	Q90627	26 kDa	-	-	-	23%	5	0.66
PIT 54	Q98TD1	51 kDa	5.70%	2	0.22	9.60%	3	0.22
Ovalbumin*	P01012	43 kDa	-	-	-	9.10%	2	0.17
Fibronectin	F1NJT3	273 kDa	-	-	-	2.60%	7	0.07
Ovomucoid	P01005	23 kDa	36.80%	9	7.64	26%	5	1.05
Alpha 1-acid glycoprotein	Q8JIG5	22 kDa	19.00%	3		13%	2	1.19
Vitamin-D binding protein	Q9W6F5	54 kDa	-	-	-	14%	7	0.28
Ovocalyxin-32*	Q90YI1	31 kDa	-	-	-	9.50%	2	0.39
Neuroserpin*	Q90935	47 kDa	-	-	-	13%	4	0.16
Hemopexin (Fragment)	Q90WR3	29 kDa	26.00%	4	0.58	15%	4	0.26
Connective tissue growth factor	Q98TQ8	37 kDa	6.70%	2	0.41	10%	3	0.39
Golgi apparatus protein 1	Q02391	130 kDa	-	-	-	2.00%	3	0.08
Vitellogenin-1*	P87498	211 kDa	-	-	-	1.20%	2	0.03
Avidin	P02701	17 kDa	-	-	-	9.20%	2	0.83
Avidin-related protein 1	O13153	16 kDa	-	-	-	9.30%	2	0.83
Trypsinogen	A2JDL7	27 kDa	21.00%	3	1.7	-	-	-
Trypsin inhibitor ClTI-1	P85000	6 kDa	51.00%	2	0.66	-	-	-
Complement regulatory membrane protein	Q9DEG0	49 kDa	8.40%	3	N/A	-	-	-
Ovalbumin-related protein Y*	OVALY	44 kDa	12.00%	4	0.37	-	-	-
PREDICTED: similar to ribosomal protein S7 isoform 6	F1NN16	23 kDa	17.00%	2	N/A	-	-	-
Polyubiquitin-B	UBB	19 kDa	19.00%	3	N/A	-	-	-
Chondrogenesis associated lipocalin	Q8QFM7	21 kDa	17.00%	2	N/A	-	-	-
Gallinacin-9	Q6QLR1	7 kDa	33.00%	2	0.52	-	-	-
PREDICTED: similar to Kunitz-like protease inhibitor*	F1NPR2	21 kDa	22.40%	5	N/A	-	-	-

Sample A and Sample B: Two independent cuticle protein extracts (They correspond to Figure 7 A and B, and Figure 8 A and B)

N/A: emPAI is very low that cannot calculate.

- : Protein was not detected in this sample.

* : Protein was also identified in the proteomic study of the SDS-soluble cuticle extract.

4. Conclusion

The cuticle is the outermost layer of the egg. It prevents the egg from losing water in the dry environments and is an important element in antimicrobial protection of the egg (Board and Tranter, 1986). This is confirmed by studies illustrating that bacterial contamination of egg contents is significantly higher in eggs with incomplete or absent cuticles. Little was known about the protein constituents of cuticle layer, and its potential contribution to the chemical aspect of antimicrobial defense. In our project, the entire proteome of fresh farm eggs cuticle layers were analyzed by LC/MS/MS. One of the abundant proteins, similar to kunitz-like protease inhibitor (OCX-25), were studied more in depth with trypsin inhibitory assays and antimicrobial assays in order to assess its function in the cuticle and its potential role as an antimicrobial protein involved in the innate defense of the egg.

The Bioscreen was used to test the antimicrobial activity of the extracted proteins. These proteins were tested against Gram- positive bacteria *B. subtilis, S. aureus* as well as Gram-negative bacteria *E. coli, S. typhimurium.* However, inhibition was also detected in buffer and these activities cannot be verified at this time. More complete study would be necessary. The trypsin inhibitor activity assay indicated that the cuticle proteins could inhibit the reaction of trypsin and substrate; however, proteomics indicates a number of other candidates, ovoinhibitor, ovalbumin-related protein Y and Trypsin inhibitor ClTI-1 which could be importance for cuticle function.

Therefore, the current research has provided some insight into the antimicrobial and enzymatic aspects of the cuticle proteins, and its function for egg protection. This information may contribute to food safety awareness, and protection against associated risks of contamination in the poultry industry.

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Chapter V

General Discussion

Agriculture and Agri-Food Canada indicates that poultry is the most consumed animal protein in Canada. There were approximately 2,800 regulated chicken producers in Canada. Canadian consumption of chicken and its byproducts has increased approximately 50% over the previous decades. In Canada, annual egg consumption has increased in recent years, and consumption was 16.1 dozen eggs per year per person in 2010. Chicken meat and eggs are Canadian's favorite proteins. Poultry production and processing are among the most highly mechanized sectors in agriculture, and the chicken industry is a major part of the Canadian economy. At the global level, total poultry meat production increased from 69 million tons (2000) to 94 million tons in 2008.

Bacterial infection of chickens and eggs remains a major concern in the poultry industry all over the world. A variety of microbes, such as *Campylobacter jejuni, Listeria monocytogenes, Chlamydia psittaci, Staphylococcus faecalis, Staphylococcus aureus, Escherichia coli, Salmonella enterica serovar Typhimurium, Salmonella enterica serovar Enteriditis* and *Yersinia enterocolitica*, are found on eggshells and can grow in egg contents (Burley et al., 1989; Board et al., 1994; Ricke et al., 2001). Infectious diseases can be a serious problem in poultry. It is important to know these diseases, and can separate the sick bird in the early stage for treatment. In addition, poultry has been associated with more than 2000 types of Salmonella world-wide, and some of them cause outbreaks of severe diarrhea sometimes blood-stained and death. However, some types of Salmonella can be carried by chicken without causing any diseases. These types of bacteria can contaminate the chicken egg and meat, and may cause food poisoning in humans.

Factors that affect bacterial invasion of the egg are its shell quality, egg albumen pH, eggshell pores, and the environmental storage conditions (temperature, humidity and

vapor pressure) (Cox et al., 2000). Subsequently, human infection occurs through the consumption of a contaminated egg or chicken meat. The bacteria begin their infection by colonizing the human intestines. Virulence factors from both Gram-positive and Gram-negative bacteria allow for them to proliferate, and colonize the intestinal epithelial cells. Bacterial serine proteases, one type of virulence factor, can produce peptides in order to defend themselves against the acidic environment. Some bacteria can use their flagella to burrow into the mucous of the intestines. Symptoms begin as the bacteria grow in number. Severe dehydration occurs from diarrhea and vomiting, abscesses form in the intestinal wall, Reiter's syndrome, and colitis occur, requiring immediate antibiotic treatment (Slonczewski et al., 2010). Therefore, in order to increase food safety from consumption of poultry products, it is necessary to understand antimicrobial mechanisms in order to reduce the transmission of bacteria from poultry meat and from eggs. For instance, *E coli.* can cause septicaemia when chicken are 3 to 6 weeks old. The *Salmonella enterica (S.) serovars Typhimurium* and *Enteriditis* infect both poultry and humans, while S. serovar Typhi, can cause typhoid fever, and only infects humans through oral-fecal transmission.

Bacterial infection of chickens and eggs remains a major concern in the poultry industry. In poultry, it is recognized that infections occur horizontally and vertically. Horizontal infections involve bacteria passing through the shell, via either pores or cracks, and either remaining in the egg albumen or infecting the nutritious egg yolk. Vertical transmission involves a contaminated chicken passing the infection onto the egg through its reproductive tissues during the egg formation process. In order to reduce bacterial surface contamination of table eggs, eggs are washed with detergents to remove feces and feathers from the surface of the egg. However, this process can also produce microcracks in the

eggshell surface, which allows bacteria to remain associated with eggshell. In addition, the washing process also removes a proposed protective barrier on the outer egg – the eggshell cuticle. The cuticle is the outmost layer of eggshell surface which is approximately 90% protein. It is the first layer to resist bacterial infection of the egg by forming a physical barrier and contains antimicrobial chemical defenses. It plugs eggshell pores to prevent the egg from losing water in dry environments and is an important element in antimicrobial protection of the egg (Board and Tranter, 1986). This is confirmed by studies illustrating that bacterial contamination of egg contents is significantly higher in eggs with an incomplete or absent cuticle. Little is known about the protein constituents of the cuticle layer, although some studies have indicated that a few proteins were detected in it, such as ovocalyxin-32 (OCX-32) and lysozyme.

In order to understand the antimicrobial function of the eggshell cuticle and gain further insight into cuticle function, we performed a proteomic analysis of the cuticle layer. We hypothesized that cuticle components play a key role in microbial resistance, since eggs with incomplete or absent cuticle are more susceptible to bacterial contamination. In the proteomic analysis, we extracted proteins from the non-calcified layer of the cuticle of fresh chicken eggs and subjected them to LC/MS/MS proteomic analysis. At least 47 proteins were identified in the cuticle layer. **Ovocalyxin-32 (a carboxypetidase A inhibitor)** and **similar to Kunitz-like protease inhibitor** are two of the most abundant proteins. A number of egg proteins that are known to have antimicrobial properties were also detected in the cuticle, such as lysozyme C, ovotransferrin, ovocalyxin-32, cystatin and ovoinhibitor. This study is the first step to understand the function of the cuticle and its protein constituents. This is the first comprehensive report of the cuticle proteome, and is a starting

point to determine cuticle function and the molecular basis of its antimicrobial properties.

Similar to Kunitz-like protease inhibitor was found in the cuticle layer, a protein also annotated as ovocalyxin-25 (OCX-25). The sequence of this protein suggested that it possesses serine protease inhibitor activity; therefore, we attempted to extract cuticle proteins in a non-denaturing buffer in order to assess trypsin inhibitory activity. Tested two conditions to dissolve the very outer shell to remove the cuticle: 1N HCl and EDTA. They each had drawbacks and were not optimal for dissolving the cuticle proteins in order to test antimicrobial activity. An assay of trypsin inhibitory activity was developed, in order to determine whether the **similar to Kunitz-like protease inhibitor in the** cuticle could inhibit trypsin. Trypsin inhibitory activity was detected; however, the cuticle extracts also contained other proteins that potentially have this activity, such as Neuroserpin, Trypsin inhibitor C1TI-1 and ovalbumin-related protein Y. In addition, antimicrobial assays were performed in order to assess OCX-25 function in the cuticle and its potential role as an antimicrobial protein involved in the innate defense of the egg. The Bioscreen assay was used to test antimicrobial activity of the extracted cuticle proteins against Gram-positive bacteria (*B. subtilis, S. aureus)* as well as Gram-negative bacteria (*E. coli, S. typhimurium).* Although some protein constituents (including lysozyme C, ovotransferrin, OCX-32, cystatin and ovoinhibitor) are known to be antimicrobial, no such activity was detected in our preliminary investigation, possibly due to the very low quantities of cuticle proteins that could be extracted. In future studies to investigate the function of OCX-25 in the cuticle layer, it will be necessary to find more efficient methods to extract this relatively insoluble proteins and to purify it, possibly by HPLC. Our current research has provided some insight into the antimicrobial and enzymatic aspects of the cuticle proteins, and its

function for egg protection, although more work will be necessary in order to completely understand the role of specific cuticle constituents.

The cuticle layer of eggshell surface is the first barrier to protect the chicken embryo or the unfertilized table egg from bacterial contamination. If bacteria pass though the cuticle layer and eggshell calcified layer, the eggshell membranes are another barrier to resist infection of the contents. The eggshell membranes are formed in the white isthmus segment of the hen oviduct. The eggshell membrane is composed of two cross-linked fibre layers, which are connected by additional fibres. The eggshell membranes are a surface upon which calcification occurs in order to form the eggshell. Therefore the properties of the eggshell membranes have a direct influence upon eggshell quality, which is a major factor in resisting bacterial contamination of the egg. Since the eggshell membrane is very stable and insoluble, it is difficult to analyze its structural constituents by protein-based methods. Therefore, we decided to pursue a transcriptome strategy to determine the gene-encoded protein constituents of the membranes.

We found that a total of 135 transcripts were over-expressed in the white isthmus compared with the other segments of hen oviduct, magnum and uterus (over-expression levels from 1.1- to 156-fold difference). These white isthmus transcripts correspond to 103 unique genes (101 UniGene IDs), which encode 85 annotated proteins. Gene Ontology (GO) analysis was used for interpretation of protein function. It is important to note that the over-expressed genes identified in this report are hypothesized to be involved specifically in the formation of the eggshell membranes. However, proteins that are key components in its formation or its antimicrobial protection could also be highly expressed in the uterus (eggshell) or magnum (egg white) and therefore were eliminated as over-expressed white

isthmus transcripts in this comparative analysis. Fouty-six proteins were predicted to be secreted proteins by SignalP 3.0 and SecretomeP 2.0 analysis. Fourteen of these proteins are predicted to possess a signal peptide, and 32 have the potential to be secreted by an unconventional pathway.

Some interesting proteins were found in this study. One of the most overexpressed genes was **Collagen X, which** has been previously shown as a structural protein in the eggshell membrane by traditional biochemistry methods. Another overexpressed gene is **similar to spore coat protein SP75**, which was recently detected in a proteomic study of eggshell membrane (Kodali et al., 2011). We also detected overexpression of several enzymes associated with collagen formation: Prolyl 4-hydroxylase subunit alpha-2 (P4HA2) and Peptidyl-prolylcis-trans isomerase C (ppiC). P4H2A catalyzes the formation of 4-hydroxyproline that is essential for the proper three-dimensional folding of newly synthesized procollagen chains (Bassuk et al., 1989; Annunen et al., 1997). PpiC is another collagen-associated protein, which catalyzes the cis-trans isomerization of proline imidic peptide bonds. Overexpression of Lysyl oxidase genes was detected in WI; Lysyl oxidase homolog-3 and homolog -2. One of these (homolog-3) was also detected in the study by Kodali et al., 2011. In addition, two of the predicted secreted proteins are potential antimicrobial components that could contribute to the egg antimicrobial defense mechanisms. The first is putative phospholipase B-like 1; the second, gallinacin-10 (Avian beta-defensin 10 (AvBD10)) is encoded by the GAL10 gene (Lynn et al., 2007). Beta-defensins are a group of cysteine-rich antimicrobial peptides that are effective against Gram-positive and Gram-negative bacteria, fungi and yeast. Proteomic analyses of egg compartments have detected avian beta defensins in multiple egg

components (Mann et al., 2006; Mann et al., 2008).

Our study focused on the over-expressed genes in the white isthmus during eggshell membrane formation. Another approach to this problem is proteomic analysis of eggshell membranes to directly detect the protein constituents of the eggshell membrane, which is difficult because the membrane fibres are extensively crosslinked and resist tryptic digestion. Due to the difference between these two methods, we found little overlap between our results and that of Kodali et al., 2011.. Only the most abundant consitituents were identified in both approaches: Collagen X, similar to spore coat protein SP75 and Lysyl oxidase homolog-3.

Therefore, gene expression profiling of the chicken oviduct during the formation of eggshell membrane has revealed numerous differentially expressed genes. The function remains unknown for most of the proteins that are encoded by the over-expressed genes identified in this study.

The egg is produced by all bird and most reptiles. The chicken egg is a very important food for human daily life; it contains abundant vitamins, trace elements, choline, potassium, protein and omega-3 fatty acid for the human body to absorb. Egg also provides small amount of lutein that contributes to eye health and help to prevent age-related blindness. It also provides some essential amino acids that cannot be synthesized by humans, such as methionine, lysine, tryptophan, phenylalanine, threonine and histidine. In addition, the egg is one of the few foods that naturally contain vitamin D, and is an important source of calcium and sodium. For all of these reasons, the chicken egg plays a crucial role in human diet. Therefore safety from bacterial contamination of egg and egg

byproducts is necessary to ensure continuing quality of health.

In summary, there are two principal defense mechanisms to protect the egg embryo, physical defenses and chemical antimicrobial protection. The physical defense is the intact eggshell that functions as a physical barrier to protect the egg embryo from infection by bacteria, physical damage and small predators. The chemical protection system is composed of the egg white and eggshell matrix proteins that provide antimicrobial defense. Our investigations of the cuticle protein constituents and their antimicrobial properties will help to understand the mechanisms of protection of the table egg from bacterial contamination. The structural proteins of the eggshell membranes are important for formation of a strong eggshell, which resists contamination. All the information that we have gained from our research will help to contribute to food safety of this nutritious food.

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