

# Functional genomics reveals numerous novel egg proteins

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## Summary

The avian egg is a nutritious food and also a major source of biologically active compounds that are beneficial for human health. These biologically active molecules are widely used by pharmaceutical, cosmetic and food industries. Egg proteins were previously studied using classical biochemical techniques. The development of molecular biology in the late 80's, the recent publication of the chicken genome sequence, and the development of new bioinformatics tools were major scientific advances leading to the identification and characterization of a number of minor egg components, which were not previously identified. Using recent data on the characterisation of egg proteins, we illustrate in this review the most recent developments in egg biochemistry (proteomics), molecular biology (transcriptomics) and in the identification of proteins constituting the eggshell gland secretome. The newly identified molecules may be a source of useful active compounds beneficial for human and animal health. They may be putative biological markers, the single polymorphisms of which could be used in marker-assisted genetic selection (MAS) to improve egg defences and to reduce the risk of food-borne disease outbreaks for humans. Experimental studies to explore the biological activities of these novel egg proteins and to investigate their potential will constitute the next challenge of egg science.

Keywords: Egg, transcriptome, proteome, secretome, proteins, biological activities

## Introduction

The biological role of the chicken egg is to be a natural container of nutrients and bioactive molecules for the extra-uterine development of the embryo (Anton et al., 2006; Réhault et al., 2007). This implies that the egg must contain the entire components that are essential for the development of a reproductive cell into a mature chick. The egg contains the vitamins and proteins (egg white and yolk), the lipids (yolk), and the mineral (eggshell) necessary for embryonic development. The egg is also a basic food for humans all around the world. The egg is of high nutritive value because it is a well-balanced source of amino acids, which are easily assimilated (Nys, 2001; Nys and Sauveur, 2004).

To face physical and microbial aggressions, the egg has two major protective systems. The first natural defence of the egg is the eggshell, which acts as a physical barrier against bacteria as long as it remains intact. The eggshell is a highly ordered structure made of calcium carbonate and of an organic matrix. The matrix plays a crucial role in the control of mineralization and in determining the mechanical properties of the shell (Gautron and Nys, 2007). The second natural defence of the egg is a chemical protection system consisting of yolk, egg white and eggshell proteins with antibacterial and antiviral properties (Gautron and Nys, 2007; Réhault et al., 2007). In addition, the egg compartments contain molecules with a broad range of biological activities of major interest for several industrial areas, including pharmaceutical, cosmetic and food industries. Thus, the chicken egg is a major source of active molecules such as anti-hypertensive, anticancer, antioxidant, cryoprotective, immunomodulating, and anti-adhesive components (Anton et al., 2006; Mine and Kovacs-Nolan, 2006; Réhault et al., 2007). These remarkable properties mainly rely on proteins present in egg yolk, egg white and the eggshell. Consequently, the characterisation of egg proteins and the study of their functions is an important challenge that stimulates the egg science.

The fractionation of the proteins of white and yolk was initiated more than 50 years ago. The major proteins of albumen and yolk were separated and purified using ammonium sulphate precipitation, chromatographic methods and electrophoretic techniques (Li-Chan et al, 1995). This catalogue of classical biochemical techniques was extended by molecular biology tools in the 80's. Despite these efforts, the composition of the egg was still not completely understood. Only the major proteins were identified and many minor egg proteins were not recognized at all.

During the last ten years, the results of genomic studies have dramatically transformed biology and biotechnology, including egg science. The recent development of high-throughput methods used in combination with the newly available chicken genomic sequence (International Chicken Genome Sequencing Consortium, 2004) and the development of bioinformatics tools to predict functions generated new insights for the characterization of new and minor components (Gautron et al., 2007a). Some of these molecules may be a source of active compounds with specific properties beneficial to human and animal health. In this review, we describe how these high-throughput technologies were used in recent contributions to enable major advances in the characterization of egg proteins.

## **The egg proteome**

Proteins are the functional units of biological processes and consequently their study is of great interest. The term proteome refers to the complete set of proteins present in a given cell or organism under defined conditions. Proteomics is the study of the proteome, with the goal of identifying and quantify all proteins, to elucidate their interactions, and to determine their post-translational modifications. The study of proteomes usually involves mass spectrometry-based high-throughput methods for protein identification (Mann and Steen, 2004). Typically the proteins of a given cell or tissue are extracted and degraded by specific proteases. This highly complex mixture is then introduced into a mass spectrometer for determination of peptide masses as precisely as possible. For the identification of proteins, the masses of these peptides, and of fragments produced from peptides by collision-induced dissociation (CID) in the mass spectrometer, are compared to the theoretical masses predicted by dedicated computer programs from *in silico* digestion and fragmentation of the entire sequences stored in databases. Such an approach depends of course on the availability of databases containing the sequences of the proteins to be analyzed. The recent publication of 90-95% of the chicken genome (International Chicken Genome Sequencing Consortium, 2004), makes possible the investigation of the egg proteome, or egg compartment sub-proteomes, using mass spectrometry-based high-throughput methods, as shown recently for the organic matrix of the chicken calcified eggshell layer, the egg white, the egg yolk and the vitelline membrane (Table 1).

Table 1: Number of egg proteins identified using proteomic facilities

Egg compartment	Number of identified proteins	References
Eggshell	528	Mann et al., 2006 ; 2007 ; Miksik et al., 2003; 2007
Egg white	148	Raikos et al., 2006; Guérin-Dubiard et al., 2006; Mann 2007; D'Ambrosio et al., 2008
Vitelline membrane	137	Mann, 2008
Egg yolk	316	Mann and Mann, 2008; Farinazzo et al., 2009

## The eggshell proteome

LC-MS/MS (mass spectrometric sequence analysis after separation of the peptides by liquid chromatography) of peptides obtained from enzymatic cleavages of the acid-soluble eggshell organic matrix identified a total of 528 different proteins as constituents of the eggshell matrix (Mann et al., 2006; Mann et al., 2007; Mann et al., 2008). All the previously identified matrix proteins were identified in this eggshell proteome, as were proteins encoded by mRNAs with increased expression triggered by uterus dilation upon egg entrance and the start of shell calcification. This study also identified some proteins of unknown function, which were not present in other egg compartments, and were thus predicted to be new specific eggshell matrix proteins. In addition to specific proteins, the eggshell matrix contained many egg white proteins, extracellular growth factors and other signal transduction chain components, lipid-binding proteins, immune system-related and antimicrobial proteins. It also contained many proteins previously identified in body fluids, such as serum albumin, hemopexin and vitamin D-binding protein. Many of these proteins may be synthesized by cells lining the lumen of the shell gland, and may play a role in shell matrix assembly or eggshell mineralization control. However, the matrix protein mixture also contained numerous components which may originate from decaying cells and basement membranes lining the oviduct, or may be remnants of secretory processes along the oviduct. These proteins may have been incorporated into the mineralized shell merely due to their presence in the uterine fluid during mineralization.

The insoluble fraction of the eggshell matrix was also investigated (Miksik et al., 2003; 2007). Surprisingly, only proteins already identified in the acid-soluble shell matrix were identified. These were the four eggshell specific matrix proteins [ovocleidin-116 (Hincke et al., 1999), ovocleidin-17 (Hincke et al., 1995), ovocalyxin-36 (Gautron et al., 2007b), and ovocalyxin-32 (Gautron et al., 2001a)], the ubiquitous extracellular chaperone clusterin, which was also present in egg white (Mann et al., 2003), and the egg white protein ovalbumin (Hincke 1995).

## **The egg white proteome**

For hen egg white proteomic analysis, different strategies and methods have been applied to bypass some technical problems due to egg white properties. Until 1989 only 13 proteins had been identified in the egg white (Li-Chan and Nakai, 1989). This was mainly due to the unfavorable composition of the albumen with 6 major proteins constituting about 86% of the total protein content, and to its high viscosity (D'Ambrosio et al., 2008). Using 2D electrophoresis and MALDI-TOF mass spectrometry, Raikos et al (2006) identified five proteins. Three of them (ovalbumin, ovotransferrin, clusterin) had already been identified previously, and the other two were activin receptor IIA and the hypothetical protein FLJ10305. Guérin-Dubiard et al. (2006), separated egg white proteins by chromatography combined with 2D electrophoresis. The 69 excised protein spots from 2-D gels were cleaved in-gel with specific proteases and the masses of the peptides were measured using MALDI-TOF and LC-MS/MS. Altogether 16 proteins were identified, two of which, Tenp and VMO-I, had not been previously identified as egg white proteins. Both were also detected in the shell matrix (Mann et al., 2006). Many of these 16 proteins were present in more than one spot in 2D gels, possibly due to differences in post-translational modifications.

Major advances came recently from the studies of Mann (2007) and D'Ambrosio et al. (2008) who were able to identify a total of 148 proteins in the egg white. Using the methods described above, Mann (2007) demonstrated the presence of 78 proteins in egg white. Among these, 54 were described for the first time in albumen. Several new egg white proteins potentially of interest were related to antibacterial defense. A protein that was annotated as "similar to acyloxyacyl hydrolase" (IPI00589382) shared 62 % identity with mammalian acyloxyacyl hydrolases, which are known to cleave acyl chains from bacterial lipopolysaccharides (LPS). A similar antibacterial function was also suggested for a 74 kDa protein (IPI0058627.1), predicted to contain several bactericidal/permeability-increasing (BPI) domains. This domain binds to bacterial LPS and thereby kills the bacteria. Such domains were already reported in Tenp (Guérin-Dubiard et al., 2006) and in ovocalyxin-36, a new specific eggshell matrix protein (Gautron et al., 2007b).

Lymphocyte antigen 86 (MD-1) (IPI00590040), a protein involved in cellular response to LPS, was also present in egg white and eggshell. Additionally, avian beta-defensin 11 (IPI00574804), histones H2A (IPI00585292.2), H3 and H4 domains of histone protein (IPI00576977.1), and gallin (IPI00681274.1) were identified as novel egg white components with possible antimicrobial activity. D'Ambrosio et al. (2008) explored the chicken egg white proteome using combinatorial peptide ligand libraries. This method reduced the concentration of high-abundance proteins while simultaneously accumulating the low-abundance species. These findings made possible the identification of 70 additional egg white proteins not identified in the previous studies, including a novel protein similar to BPI (NCBI accession number 118082796).

## **The egg yolk and vitelline membrane proteomes**

Chicken egg yolk is separated from the egg white by the vitelline membrane. Yolk constituents are synthesized in the liver, and then transported in the blood to the ovary with circulating blood. The vitelline membrane is a proteinaceous extracellular membrane surrounding the yolk. The components of the inner layer, the perivitelline membrane, are mainly secreted by the granulosa cells surrounding the follicle. The outer layer components are deposited in the infundibulum after ovulation, before the forming egg starts its migration through the oviduct.

Proteomic analysis of the chicken egg yolk identified 119 proteins. Eighty six were not previously identified in yolk (Mann and Mann, 2008). The identified proteins can be classified in different groups. The first group is constituted by the vitellogenin-derived yolk proteins and apovitellin. Vitellogenins are constituents of blood high density lipoproteins, which are cleaved in yolk to yield the mature proteins. The yolk proteome also contained other lipid-binding proteins, vitamin-binding proteins, proteases and protease inhibitors, serum proteins, proteins previously identified in egg white and miscellaneous proteins. The chicken egg yolk cytoplasmic proteome was also investigated using combinatorial peptide ligand libraries (Farinazzo et al., 2009). This approach enabled the identification of 255 new yolk proteins with 54 in common with the previously determined yolk proteome (Mann and Mann, 2008). Altogether, a total of 316 proteins were identified in egg yolk. More work will be necessary to determine the function of these proteins.

Proteomic analysis of the chicken egg vitelline membrane identified 137 proteins. Only 13 were previously known to be components of the vitelline membrane. Most of the identified components were already identified in yolk, egg

white and eggshell (Mann, 2008). This study identified the vitelline membrane outer layer protein II (VMO-II) as the avian beta defensin-11. The vitelline membrane proteome survey also showed the presence of altogether 8 zona pellucida (ZP) proteins, five of which were not previously identified in egg compartments. ATPases were also detected, confirming previous studies indicating the presence of ATPase activity in the vitelline membrane (Etheredge et al., 1971). Unexpectedly, the vitelline membrane proteome demonstrated the presence of specific eggshell matrix proteins (ovocleidins-116 and -17, ovocalyxins-32 and -36), which were thought to be specifically expressed in the eggshell gland (uterus). However, ovocleidin-116 was recently detected in skeletal tissues (Horvat-Gordon et al., 2008) indicating a more widespread distribution of these proteins.

## **Egg transcriptome**

Egg proteomics is a very efficient way to identify egg proteins. However, as reported before, some of the proteins might derive from decaying cells and basement membranes lining the oviduct and or may have been left over from previous oviduct sections secreting the corresponding egg layer. Some of them may therefore not be secreted by the tissue which secretes the respective egg compartment and may not have a function in all egg compartments in which they were identified. Therefore, the study of the expression of genes coding egg proteins in the various and specialized part of the oviduct is an alternative and complementary approach.

Analysis of the transcriptome is a way to study the genes specifically expressed in the tissues responsible for the deposition of specific egg compartments. The transcriptome quantifies all mRNA molecules (or transcripts) in one cell type or a population of cells for a given set of physiological circumstances. Transcriptomics depict the expression level of genes, often using techniques capable of screening thousands of different mRNA molecules at a time. This technique uses DNA arrays, which are a collection of microscopic DNA spots attached to a solid surface, such as glass or nylon membrane, forming an array. DNA arrays provide a medium for matching known and unknown DNA samples based on base-pairing rules. RNAs extracted from two conditions that have to be compared (different tissues or physiological stages) are reverse transcribed into cDNAs, which are then labelled with fluorescent probes. cDNAs of the first physiological stage or tissue can then be labelled with, for instance, a green fluorescent dye, while cDNAs sampled from the same source but at a different stage, or from another tissue, can be labelled with a red fluorescent dye. The labelled cDNAs (probes) are then mixed and put on slides for hybridization. After washing, the fluorescence present on the surface of the glass is measured. Genes occurring only in the first sample will be revealed by a green

fluorescence, while those expressed in the second sample only will fluoresce red. When a gene is expressed in both selected conditions, green and red colours are mixed to appear more or less yellow. The next step is to use statistical and bioinformatic tools to link the expressed genes to the identified clones present on the arrays, and to analyze the differential expression of genes. The result is a list of genes largely expressed under only one of the two conditions chosen and of those proteins produced under both conditions. Differentially expressed genes are then classified according to their known functions, and consequently yield information on the specificity of tissue expression and potential biological role of the translated proteins.

Transcriptomic analyses of the hen oviduct are currently being developed as part of two European programs (SABRE for Cut Edge for Sustainable Animal BREeding, 2006-2010, and RESCAPE for Reducing Egg Susceptibility to Contaminations in Avian Production in Europe, 2006-2009). Microarrays were used to identify novel proteins expressed in relation with the deposition and synthesis of egg components (Gautron et al., 2008; Jonchère et al., 2009). Egg formation in the oviduct occurs daily by sequential secretion onto the yolk of the various compartments of the egg. Each part of the oviduct, magnum, isthmus and uterus, has a very specific role in the synthesis of egg components and large change in expression of genes coding proteins of the corresponding egg components (egg white, eggshell membranes and eggshell matrix, respectively), are therefore to be expected. Consequently, genes specifically expressed in the different segments of the oviduct (magnum, egg white deposition; white isthmus, eggshell membrane deposition; uterus, eggshell calcification), were analysed. (Table 2). Using this method, 828 genes were found to be specifically over-expressed in the magnum where egg white proteins are deposited, 135 in the white isthmus (eggshell membranes synthesis) and 627 in the uterus (eggshell calcification).

Table 2: Number of identified genes in various regions of oviduct using microarrays

Oviduct Regions	Egg compartment deposited	Number of specific genes over-expressed
Magnum	Egg white	828
White isthmus	Eggshell membranes	135
Uterus	Calcified eggshell	605

This approach allows a screening of proteins that might be molecules of the egg or involved in the cellular mechanisms producing the egg components. It is therefore of interest to explore the putative functions of these proteins by analogy to their role in other organs of various species. Functional descriptions of the list of genes were performed using SIGENAE facilities ([www.sigenae.org](http://www.sigenae.org)). Nineteen percent of the specifically expressed genes have no correspondence in databases (218/828 genes for magnum, 28/135 for white isthmus and 55/605 for uterus). These genes observed only in the hen oviduct might code for novel proteins specific to the hen egg and not expressed in mammalian species.

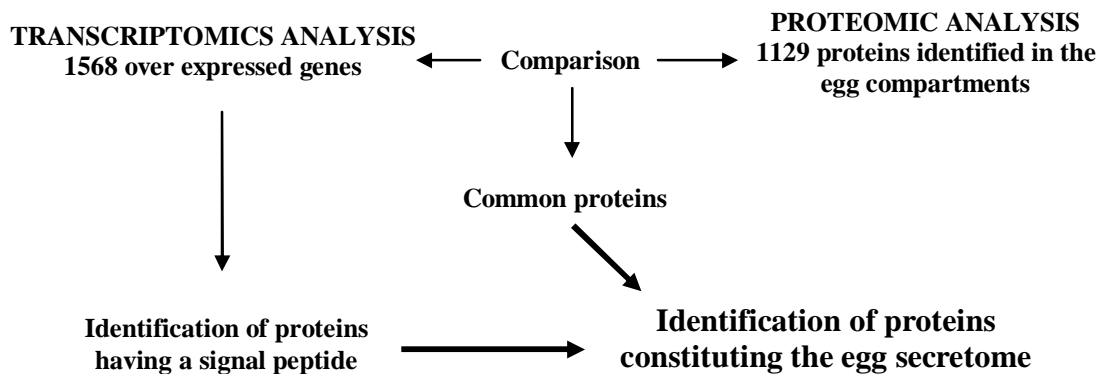
Finally, genes were classified according to their potential antimicrobial activities. This classification is still in progress and particular attention is paid to the following groups: *i) Proteins with potential antimicrobial properties.* This group includes peptides and proteins containing bactericidal permeability increasing (BPI) domains. The TENP protein which has high similarity to BPI, was over-expressed in magnum compared to uterus (10 fold) and compared to white isthmus (54 fold). Similarly, beclin-1 which is also related to BPI proteins, was over-expressed in magnum. These two proteins may be specifically expressed in the magnum to be deposited in the white, where they will play an antimicrobial role. *ii) Proteins involved in antioxidant and inflammatory process, which could be related to host defence.* As an example, a superoxide dismutase, which destroys radicals known to be toxic to biological systems, is over-expressed in uterus compared to magnum (1.57 fold) and white isthmus (1.19 fold), and is predicted to be a specific component of the shell. *iii) Genes coding for proteases and protease inhibitors.* Proteases may exert an antimicrobial activity either by degrading bacterial components or by generating antimicrobial peptides by limited proteolysis of other egg proteins. The expression of some proteases was shown to be specific of some oviduct regions. Thus, matrix metalloprotease 23B seemed to be highly specific of magnum (84 fold compared to uterus and about 30 fold compared to white isthmus). Protease inhibitors (antiproteases) could act by inhibiting bacterial proteases that are necessary for proliferation and invasive capacity of pathogens (Réhault et al., 2007; Mine, 2007).

## **Oviduct Secretome**

Transcriptomics and proteomics are very efficient methods allowing the identification of hundreds of egg components in a short time. The challenge will be to determine which proteins are specifically deposited in a specific egg compartment to exert a biological function, and how to sort out the most important ones in a huge amount of data. The genes coding for oviduct proteins can be divided into two groups: *i) The intracellular proteins with a role in*

oviduct metabolism related to the deposition of egg components, but which are not secreted into the lumen of the oviduct, ii) the extracellular proteins, which are secreted to be deposited in the egg and consequently should have a function in the egg. One way to tackle this problem is to determine the oviduct secretome by determination of proteins present in the egg with an over-expression in particular oviduct tissue involved in the deposition of the single egg compartments. In a first approach the over expressed genes which were identified using a transcriptomic approach in the various region of the oviduct will be translated into proteins. The protein sequences will then be compared to the proteins already identified in the corresponding egg compartment by other methods. In a second step, the protein sequences have to be analysed to determine the presence of a signal peptide sequences necessary for proteins to be secreted (Fig 1).

Figure 1: Schematic representation of the method used to determine the egg secretome.



This approach has been already used for the eggshell and allowed the identification of 54 eggshell proteins secreted to be deposited in the eggshell (Jonchere et al., 2009). These proteins were classified in various groups (involvement in the mineralization process, antimicrobial activity, proteases and inhibitors etc.). This approach will also be applied in the near future to the other egg compartments.

## Conclusion

The recent development of high-throughput technologies boosted by the availability of the chicken genomic sequence and the development of *in silico* functional annotations, allowed the identification and characterization of many new egg components. These newly identified molecules are currently characterized functionally to clarify their biological role, but also to determine the biological activities of major interest for industrial applications. Many of the new proteins may be a source of active compounds with properties useful for industries. Some of them are involved in the natural defences of the egg and we currently explore the variability in the biological activities of these components. The eggshell acts as a physical barrier against bacterial penetration and the chemical system consisting of antimicrobial proteins present in all compartments of the egg. These natural defences of the egg can be reinforced by selection or by stimulating their activity through environmental factors. On one hand, these proteins are candidates to be used as biological markers for a marker-assisted selection to reinforce the protective systems of the egg. On the other hand, the control of factors which might affect their activity levels can be used to optimize the egg defence and, consequently, contribute to reduce the risk of food-borne disease outbreaks for egg consumers.

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