CHAPTER 14. Bioactive Minor Egg Components

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CHAPTER 14

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14.1 Introduction

A limited number of egg white proteins (less than 10) represent more than 85% of the whole amount of egg proteins. Until 2006, only 40 to 50 proteins, representing the most abundant, were identified in egg. This number has dramatically increased in the last decade, thanks to the results of functional genomic studies, which have dramatically transformed biology and biotechnology, including egg science. The recent development of high-throughput methods used in combination with the newly available chicken genome sequence[1] and bioinformatics tools to predict functions has generated new insights for the characterization of about 1000 egg components.[2,3] This large number of proteins constitutes a small quantity in the egg and they are designated as minor egg proteins. These proteins are involved in the protection and development of the chicken embryo and participate in the nutritive values of this basic food ingredient. Furthermore, the egg compartments

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contain molecules with a broad range of biological activities with potential interest for several areas, including pharmaceutical, cosmetic, food and materials industries.[4–7]

In this chapter, we will review how high-throughput and data mining technologies were used to identify and to integrate the huge amount of proteins within the whole egg and in each individual compartment. We will then report on the functionality of the numerous newly identified minor proteins, constituting a small amount in egg, but about 95% of the total number of egg proteins. In particular, we will describe how the identification of shell matrix proteins is important for shell quality improvement or biomimetic interpretation for material sciences. We will also highlight the egg as a major source of bioactive molecules including antimicrobial, immunomodulating, antioxidant and anticancer activities.

14.2 Integrative Analysis of Egg Proteomes

Since the beginning of this century, chicken (*Gallus gallus*) gene transcripts have been characterized. Sequencing of cDNAs yielded short nucleotide sequences (200–500 bp), known as expressed sequence tags (ESTs), representing a comprehensive catalogue of global or tissue-specific mRNA sequences expressed in the chicken. The combination of joint international projects allowed the characterization of 600 434 ESTs (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). These sequences were assembled to yield a genome-wide non-redundant catalogue of mRNAs (UniGene; http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=9031). The publication of the chicken genome sequence was another major advance in identifying and characterizing chicken genes and proteins.[1] With the development of next-generation sequencing technologies (NGS), several tens of millions of chicken transcript fragments are now available (NCBI Sequence Read Archive, http://www.ncbi.nlm.nih.gov/sra/?term=chicken). In March 2018, the International Chicken Genome Consortium released the Gallus_gallus-6a assembly of the chicken genome (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Gallus_gallus/104/). It consists of 34 chromosomes, and predicted 24 012 genes and 49 661 transcripts. The existence of genes and their corresponding proteins in a database is fundamental for the use of high-throughput technologies (proteomics and transcriptomics) to identify proteins in the various milieus composing the egg. Indeed, these sequences in databases are required in proteomics studies to ascribe experimental mass spectra to existing proteins.

These recent advances make possible the investigation of the egg proteome, or egg compartment sub-proteomes, using mass spectrometry-based high-throughput methods, as shown for the organic matrix of the chicken calcified eggshell layer, the egg white, the egg yolk and the vitelline membrane.

The first major proteomic analysis of egg came in 2006, with the eggshell proteome. Mann *et al.*[8] identified the acid-soluble eggshell organic matrix

and completed this approach with a phosphoproteome of the soluble eggshell proteins.[9] Both studies identified 528 different proteins as constituents of the soluble eggshell matrix. The insoluble fraction of the eggshell matrix was also investigated.[10–13] Rose-Martel *et al.*[14] performed a proteomic analysis of the outermost layer of the shell (cuticle), suspected to play a major role in preventing microbial penetration. A more recent proteomic survey allowed the identification of novel components[15,16] and the quantification of about 300 eggshell proteins at the key steps of shell calcification.[17] Finally, a recent proteomic survey on shell membranes characterized about 300 eggshell membrane proteins.[18]

Guerin-Dubiard *et al.*[19] firstly explored the egg white proteome, but the largest number of proteins identified in the egg white came from the studies of Mann and Righetti's laboratories.[20–22] Mann analyzed egg white proteins using LC-MS/MS and MS^3 of peptide mixtures prepared by in-solution cleavage of egg white proteins, which allowed the identification of 78 proteins, 54 of which were identified in egg white for the first time.[21] D'Ambrosio *et al.*[20] explored the chicken egg white proteome using combinatorial peptide ligand libraries. This method enabled the identification of 70 additional egg white proteins. By using a novel dual pressure linear ion trap instrument, the LTQ Orbitrap Velos, which increased sensitivity, Mann and Mann[22] identified 44 additional proteins. Additionally, the egg white composition was explored in different egg varieties[23] and under various storage temperatures.[24]

The chicken egg yolk is separated from the white by the proteinaceous extracellular vitelline membrane. 137 proteins were identified in the chicken egg vitelline membrane proteome.[25] Only 13 were previously known to be components of the vitelline membrane. Most of the identified components were already identified in eggshell, yolk and egg white, questioning those that are specifically constitutive of the membranes. Additionally, Mann described supplementary sequences, which improved the number of identified proteins.[25]

The egg-yolk proteome was investigated using 1D electrophoresis and LC-MS/MS; Mann and Mann[26] identified 119 egg-yolk proteins, 86 of which were not previously identified in this egg compartment. The chicken egg-yolk cytoplasmic proteome was also investigated using combinatorial peptide ligand libraries,[27] as previously reported for egg white.[20] This approach enabled the identification of 255 new yolk proteins with 54 in common with the previously determined yolk proteome.[26]

The proteomic studies allowed the identification of hundreds of egg proteins, with some of them being present in the various egg compartments. One gap in this methodology is the presence of redundancy between proteins because the proteomic studies used identifiers originated from different databases. Mann described proteins using IPI (International Protein Index database, http://www.ebi.ac.uk/IPI), which is now closed and was a merged database of ENSEMBL and GeneBank, with numerous changes to protein identifiers between 2006 (first eggshell proteomic study) and 2011 (database closing date). The other egg proteomic studies reported the proteins using identifiers originated from

GeneBank (http://www.ncbi.nlm.nih.gov/genbank/), ENSEMBL (http://www.ensembl.org) or UniProt (Universal Protein Resource, http://www.uniprot.org/). Altogether, egg proteomic studies described thousands of different protein identifiers. However, one unique protein often possesses various identifiers. To fix this problem, we have loaded the entire protein sequences originated from these different identifiers, which were aligned using a BLAST algorithm to eliminate all redundancies.

A total of 1261 non-redundant protein sequences (gene products) were finally determined using the egg proteomic studies. The different proteins were classified in the different compartments of the egg (Figure 14.1). Egg yolk, vitelline membranes and egg white constituted at least of 230, 144 and 273 different proteins, respectively. Finally, the eggshell is the compartment with the lowest amount of proteins, but it exhibits the largest variety of proteins as 904 were reported in this single compartment.

Figure 14.1 gives a representation of the specific, common and shared proteins. The egg proteins only specific to one compartment represent 82.6% (1042) of the total number, with 143 proteins specific to the yolk, 41 only found in the vitelline membranes, 149 unique proteins of the white and 709 proteins only observed in the eggshell. Surprisingly, the number of shared proteins between two or more egg compartments is limited to 219 proteins, representing less than 20% of the total number of egg proteins. Only 13 proteins are common to all egg compartments. They are riboflavin-binding protein, ovotransferrin, apolipoprotein B, glutathione peroxidase 3, immunoglobulin lambda-like polypeptide 1, vitellogenin 1, keratin 6A and 7, hemopexin, transmembrane protease serine 9, ovalbumin, ovalbumin-related X and glyceraldehyde-3-phosphate dehydrogenase. The other 206 are found within two or three egg compartments.

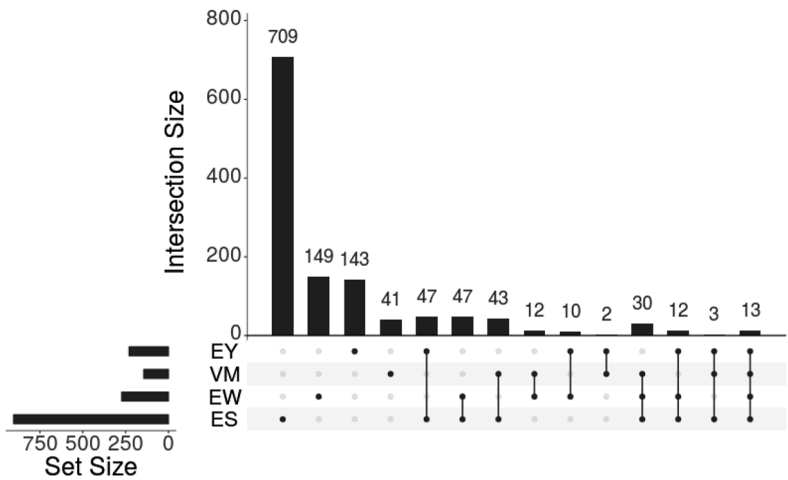

Figure 14.1 Number of proteins in the egg yolk (EY), vitelline membrane (VM), egg white (EW) and eggshell (ES). Line connectors represent the proteins shared between egg compartments.

14.3 Functional Activities of Minor Egg Proteins

14.3.1 Biomineralization of Egg

The main function of the eggshell is to protect the embryo from external aggression during its development. Consequently, the eggshell as a physical barrier must have remarkable mechanical properties. It has to be solid but also easy breakable from inside to allow hatching. Mineralized eggshell deposition occurs in the distal part of the oviduct and this process has been widely described.[28–30] Calcification is initiated in the red isthmus/uterus region (5 hours post ovulation) by deposition of spherulitic microcrystals of calcite (calcium carbonate polymorph of the chicken eggshell) on specific sites (mammillary knobs) on the surface of shell membranes. Metastable amorphous calcium carbonate (ACC) is present as an early and transient precursor phase, which rapidly evolves to supply ions to form the calcite in the shell.[31] Calcite crystals then develop into columnar crystal units with their faster growth direction (*c*-axis) nearly perpendicular to the surface developing a preferential orientation of crystals. We described four major events in the shell formation:[31] (1) Metastable ACC is first deposited and accumulated over the entire eggshell membrane, then (2) there is a rapid evolution and redistribution of ACC on specific nucleation sites (mammillary knobs) on eggshell membranes to form calcite aggregates. (3) Calcite aggregates are then enlarged into larger calcite crystals to form the mammillary layer, followed by (4) the development of columns of crystals with a preferred orientation perpendicular to the surface to form the shell's compact palisade layer. This linear deposition of mineral continues until the process is inhibited (22 hours post ovulation), and the organic cuticle is deposited on the surface of the calcified shell (22–24 hours post ovulation). Finally, the egg is laid about 24 hours after the ovulation of the yolk. Shell therefore results from the assembly of aggregated ACC particles in calcite crystals to allow a very fast controlled process during the various steps of shell calcification. These distinct phases are associated with the formation of different layers in the shell: the inner mammillary cones, the palisade layer and the cuticle.

The shell constitutes about 10% of the egg content, representing 5 to 6 g per egg. The shell is mainly composed of 95% calcium carbonate in calcitic form. Only 3.5% (175 mg) constitutes the organic shell matrix made of proteoglycans and proteins. Consequently, the 900 proteins identified in the shell represent less than 150 mg in the complete egg. In comparison, the 273 egg white proteins represent a 20–30 times greater amount. Although these shell proteins are at very low concentrations, they play a key role during the shell calcification events. Matrix proteins stabilize the ACC mineral form of calcium carbonate and select the calcite polymorph into which it is converted. Organic matrix proteins also specifically adsorb on crystal faces to control the growth and morphology of calcite, which determines the orientation of crystals in the shell.[17,29–32] The matrix–mineral interactions result in a complex ultrastructure of the eggshell, which determines its mechanical properties. A

number of experimental observations support the role of the eggshell matrix proteins in the fabric of the eggshell and its resulting mechanical properties. The first is relevant to the nature of the chicken eggshell matrix in its content of specific components (ovocleidins and ovocalyxins), which are synthesized and expressed only in tissues where eggshell calcification takes place, namely, the uterus and red isthmus. Putative functions related to the mineralization process were predicted for matrix proteins using bioinformatics. They were classified into various groups (proteins associated with shell mineralization, proteins involved in the regulation of proteins driving the mineralization, antimicrobial proteins and other functions).[17] Changes in the organic composition of the uterine fluid and in the shell during the fabrication of the eggshell allowed determination of proteins potentially associated with the shell fabric. Each phase of shell mineralization is associated with a specific electrophoretic profile in the uterine fluid, suggesting specific roles for the organic contents during the calcification process.[33] Marie *et al.*[17] used quantitative proteomics on 200 shell matrix proteins and determined patterns of abundance at the different key steps of shell formation. This study highlights 21 matrix proteins suspected to have predominant roles in the control of the different stages of shell calcification.

The role of shell matrix proteins was also evidenced by laboratory experiments. Investigations have demonstrated that eggshell organic fractions exhibit calcium binding properties owing to proteins[34–36] or keratan and dermatan sulfate proteoglycans.[37] Similarly, in the uterine fluid, protein bands corresponding to ovalbumin, ovotransferrin and the 36 kDa band during the active phase of calcification, display affinity for calcium.[33] This last band was further characterized as corresponding to clusterin[38] and ovocalyxin-36.[39] Calcite crystals grown *in vitro* with soluble eggshell protein extracts and uterine fluid delay the precipitation of calcium carbonate in a dose-dependent manner.[33,37,40,41] The uterine fluid dramatically affected the precipitation kinetics, the size and the morphology of crystals grown *in vitro*.[41–44] Similar results were observed in the presence of purified lysozyme, ovotransferrin and ovocleidin-17, which showed large modifications of the calcite morphology.[44–47] Metadynamics simulations reveal that ovocleidin-17 induces the formation of calcite crystals from amorphous calcium carbonate nanoparticles.[48–50] The strong stabilization of the amorphous calcium carbonate emulsion is attributed to ovalbumin complexes acting as nucleation centers for the amorphous phase because of their enrichment by Ca^{2+} ions.[51,52] Additionally, the dermatan sulfate glycosaminoglycan chain containing ovocleidin-116 as the protein core, ovoglycan, is polyanionic and acidic with high calcium affinity and is likely to modulate crystal growth during palisade formation.[53]

The involvement of eggshell matrix proteins was also reinforced by *in vivo* relationships between matrix proteins and eggshell quality parameters. If the eggshell matrix participates in establishing the morphology of calcite crystals, it would affect the texture (crystal size and orientation) of the eggshell and influence its mechanical properties. This hypothesis was confirmed by quantifying components of matrix proteins in parallel with variations

in eggshell mechanical properties.[54,55] Both studies showed variation of the abundance of matrix proteins (ovalbumin, ovotransferrin, and ovocleidin-116 and-17) related to shell mechanical properties and the size and orientation of crystals. These observations suggest that changes in organic matrix protein levels affect eggshell crystal size and provide mechanisms for improving the shell solidity. A complementary avenue to establish the role of matrix proteins in the variability of the eggshell physical and mechanical properties has been taken using genetic and genomic approaches.[56] These were aimed at determining the association between alleles of some eggshell matrix proteins (ovocleidins and ovocalyxins, osteopontin and ovalbumin) and measurements of eggshell solidity. This study revealed a number of significant associations between genotype (the marker) and phenotype (the trait, *e.g.*, acoustic resonance data, quasi-static compression test data and the thickness of specific components of the shell).[56] When expressed as allele substitution/standard deviation of the trait, the effect of a coding region polymorphism in ovocalyxin-32 was over 12% for breaking strength and 17% for deformation. Ovocalyxin-32 polymorphism also affects the size and the crystal orientation of the mineralized structure.[57] Takahashi *et al.*[58,59] identified the ovocalyxine-32 gene in the quantitative trait locus (QTL) region associated with shell quality in divergent lines. The effect of a coding region polymorphism in ovocleidin-116 was 17% for shell stiffness and polymorphism in the promoter of this gene accounted for around 10% of the thickness of the mammillary layer and its proportion in the shell.[56] Recently, a genome-wide association study (GWAS) associated different egg quality parameters with QTL in egg layers containing many proteins identified in the shell.[60]

The huge number of proteins identified in the shell will allow genomic improvement and will give insights for material sciences. Genes coding matrix proteins will be used as biological markers for genomic selection to reinforce eggshell breaking strength. The corresponding transcripts will be associated with published and private single nucleotide polymorphisms and mapped in QTLs related to shell quality. They will constitute candidate genes to gain precision for genomic selection to reinforce shell mechanical properties. Industrial ceramics are made at high temperature and pressure. Material science explores the biomineralization to investigate how living organisms build their shells under physiological conditions. Among various biominerals, the chicken shell is the most widely documented. Information on shell matrix proteins and how they contribute to the mechanical properties gives a chance to establish a list of natural organic compounds of added value usable in the fabrication of calcium carbonate materials/ceramics.

14.3.2 Antimicrobial Proteins

Besides the major antimicrobial proteins (lysozyme and ovotransferrin), many minor egg proteins also exhibit antimicrobial properties and contribute to the efficiency and complexity of egg defenses. These minor compounds can be classified in different groups according to their structural or functional properties.

14.3.2.1 Protease Inhibitors

The egg contains large amounts of active protease inhibitors (antiproteases). Among the most representative egg antiproteases are ovomucoid, ovoinhibitor, ovostatin and cystatin. They are ubiquitously distributed within all egg compartments but more specifically concentrated in egg white. They target proteases with more or less enzymatic specificity. Proteases are proteolytic enzymes catalyzing the cleavage/degradation of peptidic chains and are involved in many biological processes in eukaryotes and prokaryotes. Some bacteria are able to produce extracellular proteases involved in their survival, growth or invasive capacity and which play critical roles in host–pathogen interactions. Therefore, the neutralization of these virulent factors by antiproteases may lead to the inhibition of bacteria. Although the physiological function of egg antiproteases has not been well established to date, it is likely that they could potentially inhibit protease-secreting bacteria and/or have a direct bactericidal activity.

Ovomucoid is a 28 kDa protein that is highly glycosylated and possesses nine disulfide bonds. This glycoprotein contains three functional homologous Kazal-like inhibitory domains and exhibits antitrypsin and antichymotrypsin activities. The antibacterial role of ovomucoid and its participation in the protection of the egg against bacterial contamination have not been clearly demonstrated. However, some bacterial proteases, like subtilisins and *Streptomyces griseus* proteases A and B, are inhibited by turkey ovomucoid.[61]

Ovoinhibitor is a 48 kDa glycoprotein possessing seven Kazal-like inhibitory domains and 21 disulfide bonds. Ovoinhibitor can inhibit several serine proteases, such as trypsin, chymotrypsin and elastase, as well as subtilisin, a serine protease produced by *Bacillus* spp. Antibacterial activities have been reported *in vitro* against *Bacillus thuringiensis*.[62]

Ovostatin (or ovomacroglobulin) is a large homotetrameric protein belonging to the alpha-2-macroglobulin family, consisting of four disulfide-linked subunits (4 × 165 kDa). Ovostatin is known to bind and inhibit various proteases from different classes (serine-, cysteinyl-, metallo- and aspartyl-proteases) but with better efficiency towards metalloproteases (collagenase, thermolysin and stromelysin). Because of its ability to inhibit proteases produced by several virulent bacteria inducing corneal tissue damage (*Serratia marcescens, Pseudomonas aeruginosa*),[63] ovostatin can be considered as an antimicrobial.

Cystatin is a non-glycosylated protein (12.7 kDa, pI 5.1) containing two disulfide bonds and targeting mostly cysteine proteases, including ficin, papain, and cathepsins B, H and L. Cystatin exhibits antibacterial activity by preventing the growth of group A *Streptococcus*,[64] *Salmonella* Typhimurium[65] and *Porphyromonas gingivalis*.[66] Bactericidal activity has been observed for cystatin at low dose against *Acinetobacter lwoffii*, *E. coli*, *Oligella* spp. and *Pseudomonas aeruginosa*.[67] Cystatin is active not only against bacteria but also against viruses and fungi. Antiviral activities have been demonstrated *in vitro* and *in vivo*. In human cultured cells infected with poliovirus, the addition of

chicken cystatin can alter the intracellular proteolytic processing of poliovirus proteins and reduce the production of viruses.[68] Another study demonstrated that chicken cystatin was able to protect mice infected by human rotavirus.[69] The antiviral effect of cystatin observed in these studies is likely to be mediated by the inhibition of proteases involved in viral replication. Cystatin exhibits antifungal activities against azole-sensitive *Candida albicans* isolates, as well as *Candida parapsilosis* and *Candida tropicalis*. *Candida glabrata* is also inhibited but it seems to be comparatively more resistant to the action of cystatin. The inhibition of azole-sensitive *Candida albicans* isolates is achieved with minimal inhibitory concentration values comparable to those obtained with fluconazole and histatin 5.[70] Cystatin may also be antiparasitic as it is a potent inhibitor of cysteine proteases expressed by *Trypanosoma cruzi*, a parasite responsible for trypanosomiasis.[71,72]

14.3.2.2 Vitamin-binding Proteins

Among the main egg vitamin-binding proteins are avidin and riboflavin-binding protein, which are present within all egg compartments. Their physiological function is likely related to the supply of the egg and the developing embryo with vitamins: biotin and riboflavin, respectively. However, it is hypothesized that they could also be indirectly involved in antimicrobial defenses by inhibiting microorganisms' growth that requires these vitamins.

Avidin is a cationic homotetrameric glycoprotein (68.3 kDa, pI 10) exhibiting a very high affinity for biotin (vitamin B8/H). The dissociation constant is around 10^{-15} M,[73] which is one of the strongest non-covalent interactions found in nature. This protein can potentially inhibit the growth of biotin-requiring microorganisms.[74] Interestingly, avidin is able to bind to various Gram-negative and Gram-positive bacteria, including *E. coli*, *Klebsiella pneumonia*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermis*.[75]

Riboflavin-binding protein (RfBP) is a phosphoglycoprotein of ~32 kDa with a pI of about 4. The apoprotein has a high affinity for riboflavin (vitamin B2) with a dissociation constant of 1.3×10^{-9} M.[76] RfBP has a critical role in embryonic development.[77] Present in the egg white of many avian species, the concentrations and the proportion of the apoform of RfBP can vary over a ten-fold range, which may reflect the possibility that RfBP in egg white possesses antimicrobial functions.[77]

14.3.2.3 Defensins

Defensins are cysteine-rich cationic peptides of about 2–6 kDa that are involved in the host innate defense. They are found in many living species, including vertebrates, invertebrates and plants. These antimicrobial peptides contain six conserved cysteines involved in three disulfide bonds, making them extremely compact and stable. Most of these molecules

possess a broad-spectrum activity directed against Gram-positive and Gram-negative bacteria, but also against fungi and viruses. The antibacterial effect of defensins mainly results from direct interaction with the bacterial cell wall and disruption of the structure of cell membranes. Mammalian defensins are categorized into three families – alpha, beta and theta – according to the position and pairing of conserved cysteine residues; however, in birds, only beta-defensins have been identified. They are organized into two groups: the avian beta-defensins (AvBDs) and the ovodefensins (OvoDs). Proteomic studies conducted on chicken egg compartments revealed the presence of several defensins, including AvBD11 (vitelline membrane, egg white and eggshell), AvBD10 (eggshell), AvBD9 (yolk), and the ovodefensins gallin/OvoDA1 (egg white, vitelline membrane) and OvoDB1 (egg white).

AvBD11 (previously named vitelline membrane outer layer protein 2 or VMO-2) is a long size beta-defensin (9.2 kDa) composed of two beta-defensin motifs. Consequently, it contains 12 cysteines involved in six disulfide bonds. Such a structure is quite unique since no double defensins have been found in mammals to date. In chicken egg, AvBD11 is present within all compartments with the exception of yolk, but it is noteworthy that the vitelline membrane is likely the egg part where AvBD11 is the most abundant. Indeed, it is one of the major proteins of the outer layer of the vitelline membrane, together with ovomucin, lysozyme and VMO-1.[78] This antimicrobial polypeptide is active against *Salmonella* serovars Enteritidis and Typhimurium, *E. coli*, *Listeria monocytogenes* and *Staphylococcus aureus*.[79] AvBD11 is able to bind to heparin, a negatively charged glycosaminoglycan, which is used to purify this defensin from egg vitelline membrane by affinity chromatography. In turn, heparin can also inhibit the antibacterial activity of AvBD11, which suggests that the heparin-binding site on the protein may be involved in the mechanism of action.[80] AvBD10 (gallinacin-8) and AvBD9 (gallinacin-6) were identified in eggshell (matrix and membrane)[8,18] and egg-yolk plasma,[26] respectively. The physiological roles and antibacterial activities of these two defensins need to be further investigated.

Gallin or OvoDA1 (4.7 kDa) is an ovodefensin, a sub-family of beta-defensins initially found in the egg white from different avian species (chicken, turkey, swan and duck).[81] This cationic antimicrobial peptide is detected not only in chicken egg white but also in the vitelline membrane and eggshell membrane. In the egg white, this ovodefensin is gradually altered during egg storage,[82] which may affect its antimicrobial potential. Gallin/OvoDA1 is active against pathogenic and non-pathogenic *E. coli* strains, but not against *Salmonella* serovars Enteritidis and Typhimurium.[81,83,84] Avian pathogenic *E. coli* (APEC) seems, however, to be more resistant. Of note, a discrepancy regarding the susceptibility/resistance of *S. aureus* to gallin/OvoDA1 could be observed depending on the technique used to assess antibacterial activities (gelose or liquid phase).[81,84] Although the antibacterial spectrum of gallin/OvoDA1 needs to be further defined, it seems however that its antibacterial activity is limited compared to that of AvBD11. The three-dimensional

structure of synthetic gallin contains a β-defensin fold but with significant variations, which might be related to its antibacterial specificity or other yet unknown functions.[84]

Recently, another ovodefensin, OvoDB1, has been identified in chicken egg white.[85] OvoDB1 is able to inhibit *E. coli* DH5α but with lower efficiency than OvoDA1, and faint/no activity was observed with APEC, *Salmonella* and *S. aureus*.[81]

14.3.2.4 Proteins of the LBP-BPI-Plunc Family

The LBP (LPS-binding protein)/BPI (bactericidal permeability increasing protein)/PLUNC (palate, lung and nasal epithelium clone protein) family consists of proteins involved in host defense against bacteria. Members of this family are able to bind bacterial lipopolysaccharide (LPS), a surface component of Gram-negative bacteria, and mediate alterations of the outer membrane and damage to the inner membrane of bacteria. These events lead to the inhibition of bacterial growth.[86] The chicken egg contains several members of this family, including OCX-36 and TENP (transiently expressed in neural precursor).

OCX-36 (Ovocalyxin-36) is a 36 kDa protein present in the eggshell and vitelline membrane,[25,39] and exhibits growth inhibitory activity against *Staphylococcus aureus* ATCC 6538.[87] OCX-36 was shown to possess lipopolysaccharide and lipoteichoic binding activities. Interestingly, a significant difference in *S. aureus* lipoteichoic acid binding activity was observed for two natural forms of OCX-36, Pro-71 and Ser-71, with a higher binding activity detected for Pro-71.[87]

TENP or ovoglobulin G2 is a protein of about 49 kDa. By analogy with other members of this family, TENP is assumed to be antibacterial but further investigations on this protein need to be performed to characterize and confirm chicken TENP's predicted function. TENP isolated from emu egg white, however, exhibits antibacterial activities against the Gram-positive bacteria *Micrococcus luteus* and *B. subtilis*, but not against the Gram-negative bacteria *E. coli* and *Salmonella* Typhimurium.[88]

14.3.2.5 Heparin-binding Proteins

A number of proteins present at low abundance in the egg are able to interact with heparin and possess antibacterial properties. Owing to its structure and negative charges, heparin can be seen as a compound with similarities to the negatively charged molecules present at the surface of bacteria. A previous study carried out on peptides with affinity for heparin demonstrated that these peptides have antibacterial properties.[89] This observation stimulated the development of a strategy based on heparin-affinity chromatography aiming at identifying novel antimicrobial egg molecules. The approach conducted on egg white allowed the identification of 20

proteins, including known antimicrobials and new potential candidates.[80] Five proteins contained in the egg white heparin fraction were isolated and characterized: AvBD11 (see Section 14.3.2.3 Defensins), ovalbumin-related protein X, beta-microseminoprotein-like, pleiotrophin and vitelline membrane outer layer protein 1. Antibacterial activities were confirmed *in vitro* for all these candidates and, interestingly, the heparin-binding site(s) of these molecules are likely to be involved, at least in part, in the antibacterial mechanism.

Ovalbumin-related protein X (OVAX) is a glycosylated heparin-binding protein of about 45–50 kDa, present in all egg compartments. It belongs to the ov-serpin family, which includes the major egg white protein ovalbumin. The ov-serpins are structurally related to serpins, a class of serine protein inhibitors, but functionally they are devoid of any antiprotease activities. In egg white, OVAX is estimated to be 100 times less concentrated than ovalbumin. Although OVAX and ovalbumin share high sequence identity, OVAX seems to be antimicrobial, as demonstrated for two foodborne disease pathogens, *Listeria monocytogenes* and *Salmonella* Enteritidis, whereas ovalbumin has no antibacterial activity.[90] Its activity may depend on the ability of OVAX, but not ovalbumin, to bind to the anionic surfaces/molecules (*e.g.*, heparin) since heparin (negatively charged glycosaminoglycan) can inhibit the anti-*Salmonella* properties of OVAX, presumably by blocking the heparin-binding site and therefore competing with bacteria at the antibacterial site of OVAX. A cluster of positively charged amino acid residues present at the surface of OVAX is thought to interact with heparin and participate in the antibacterial mechanism.[90] Interestingly, it has been shown that an alkaline treatment of OVAX can alter the presumed heparin-binding site,[91] suggesting that egg storage and the progressive pH increase observed in egg white may alter the antibacterial activities of OVAX.

VMO-1 (vitelline membrane outer layer protein 1) is a cationic protein of about 18 kDa containing four disulfide bonds and exhibiting antibacterial activities against *Listeria monocytogenes* but not against *Salmonella enterica* Enteritidis.[80] However, it is likely that this protein possesses other physiological roles in the egg, especially within the vitelline membrane where VMO-1 is abundant.[25] The tridimensional structure of VMO-1 is organized into a singular structural motif called a beta-prism, also found in domain II of the insecticidal delta-endotoxin (a pore-forming toxin produced by *Bacillus thuringiensis*) and in the plant jacalin-like lectin domain.[92,93] Beta-prism motifs are thought to interact with carbohydrates. The heparin-binding property of VMO-1 is partly involved in the anti-*Listeria* activity of VMO-1 but other mechanisms might act complementarily to this carbohydrate-binding property to destroy or inhibit bacteria.

Pleiotrophin (15.2 kDa, five disulfide bonds) is a cationic growth factor known for its high affinity for heparin. This protein is found in the egg white,[20] vitelline membrane[80] and eggshell.[8] The pleiotrophin sequence is extremely conserved across different species, with a sequence identity of more than 90% between chicken and mammalian sequences.[94] The human homolog is antibacterial against Gram-positive and Gram-negative bacterial

strains and exerts its antibacterial action *via* a membrane disruption mechanism.[95] Antibacterial activities have also been reported for the chicken form, against *Listeria monocytogenes* and *Salmonella enterica* Enteritidis.[80]

Beta-microseminoprotein-like (gi:513191195/LOC101750704) is a small-size cationic cysteine-rich protein (9.9 kDa) recently identified in the egg white by heparin affinity. This protein also exhibits antibacterial activities against *Listeria monocytogenes* and *Salmonella enterica*.[80] Human beta-microseminoprotein possesses a potent fungicidal activity against *Candida albicans* whereas no antibacterial activity was observed against *E. coli*, *S. agalactiae*, *S. pyogenes*, *S. aureus* and *E. faecalis*.[96]

14.3.2.6 Other Antimicrobial Proteins

Ovomucin is a sulfated and heavily glycosylated protein belonging to the family of mucins, a group of proteins found in the numerous secretions of epithelial tissues and involved in the gel-like properties of secretions in which they are present. This large protein is composed of two subunits: an alpha subunit (MUC5B) low in carbohydrates and a carbohydrate-rich beta subunit (MUC6). The gel structure of egg white is attributed to the presence of ovomucin and more particularly to the complex it forms with lysozyme. The egg white's viscosity inhibits the migration of bacteria towards the yolk. Ovomucin also exhibits antibacterial and antiviral properties. The protein demonstrated inhibitory activity against colonization of *Helicobacter pylori* (a bacterium associated with peptic ulcer disease) in the stomach.[97] This effect might result from direct binding to *H. pylori* urease. In addition, hemagglutination inhibition activity was reported for ovomucin against bovine rotavirus and hen Newcastle Disease Virus.[98] While both subunits were active against the rotavirus, hemagglutination inhibition activity against Newcastle Disease Virus only requires the beta subunit moiety.

Ovocleidin-17 (OC-17) is a 17 kDa eggshell-specific protein. It belongs to the C-type lectin superfamily, a group of calcium-dependent carbohydrate-binding proteins with functions associated with cell–cell adhesion, immune response to pathogens and apoptosis. Purified OC-17 was shown to be bactericidal against Gram-positive bacteria *B. subtilis* and *S. aureus*, and exhibited enhanced activity in the presence of calcium.[99]

Histones are positively charged proteins that are able to bind to DNA. They are usually located in the eukaryotic cell nuclei nucleus, where they play critical roles in DNA compaction and chromatin regulation. However, these molecules can also be detected in the cytoplasm and in extracellular fluids. There is compelling evidence showing that extracellular histones may exert functions related to host defense and inflammatory responses. A number of studies have demonstrated the antibacterial potential of these proteins. Several histones have been identified in egg, including H1 and H2A. These two chicken histones, isolated from the hen's reproductive system, were shown to inhibit Gram-negative and Gram-positive bacteria.[100]

14.3.3 Other Activities

Many varied activities have been suggested for major proteins like ovalbumin, ovotransferrin and lysozyme. In contrast, besides antimicrobial activities, there is only limited (but promising) data illustrating the bioactive potential of egg components of lower abundance (Figure 14.2). Considering the numerous proteins and peptides, including bird-specific proteins, that have no associated functions in gene ontology databases but that are all assumed to support embryonic development, it is believed that the egg proteins still bear myriad biological surprises that deserve higher considerations.

14.3.3.1 Egg Proteins and Cancer

Cancer is associated with abnormal growth/proliferation of cells and their potential to invade or spread to other parts of the body (invasive activities). The following sub-sections present an overview of the various applications of egg proteins in cancer: (1) proteins with anti-cancerous potential (either anti-proliferative or anti-invasive), (2) proteins exhibiting specific physicochemical properties that may be useful for targeted therapy, and finally, (3) how immunoglobulins Y that are secreted into the egg yolk can also constitute a relevant approach for cancer diagnosis.

14.3.3.1.1 Anti-cancerous Activity. There is increasing evidence that food-derived proteins and peptides can be beneficial for preventing and curing cancer diseases.[101] Several studies have confirmed the tumor-inhibitory activity of lysozyme using experimental tumors, activity which essentially relies on immunopotentiation.[102] Here we will focus on emerging data on other less known egg-derived proteins such as the protease inhibitors cystatin and Kazal-like inhibitors (ovoinhibitor and ovomucoid), and phosvitin.

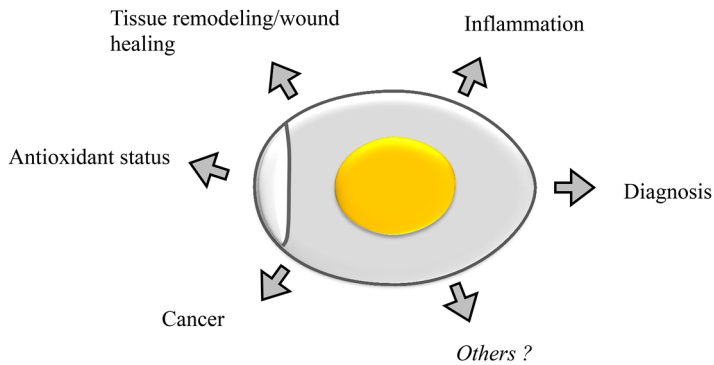

Figure 14.2 Potential therapeutic uses of minor egg proteins besides the treatment of infectious diseases. This scheme summarizes some potential applications for low abundance proteins that include avidin, glutathione peroxidases, immunoglobulin Y, OCX-36, ovomacroglobulin/ovostatin, ovomucin, ovomucoid, phosvitin and pleiotrophin.

Cancer progression is usually associated with proteolytic activities (cathepsins, serine proteases and metalloproteases) that degrade extracellular matrix proteins allowing for the proliferation and dissemination of cancerous cells. There are now compelling data that certain protease inhibitors, essentially extracted from plants, have strong anticarcinogenic activity.[103] Interestingly, egg white is characterized by several abundant protease inhibitors,[104] whose biological roles are still unclear. At least one of them, cystatin, a cysteine protease inhibitor, has revealed some anticancer properties.

Cystatins or cystatin-like proteins are present in mammals, birds, fish, insects, plants and some protozoa and regulate numerous physiological processes by inhibiting cysteine proteases (cathepsin L and B) that have a pivotal role in extracellular matrix degradation and tissue remodeling and whose activity is upregulated in metastatic progression.[105] Cystatins, either native or modified, have been proposed as anti-metastatic or cytotoxic molecules in many published studies.[106–114] In addition, ovomucoid and ovoinhibitor, which contain three and seven Kazal domains, respectively, might also have promising effects considering the antitumor activity reported for serine protease inhibitor Kazal-type 6.[115] Conversely, some Kazal inhibitors might also act as growth factors and are associated with the aggressiveness of some tumors.[116] It would be very interesting to assess such activities for both egg-specific inhibitors, which are omnipresent in egg white, whose expressions are indeed under hormonal control and which therefore may have a major role during embryonic development to drive yolk sac development/progression onto the vitelline membrane matrix.

More recently, phosvitin has revealed some cytotoxic activity against various cancer cell lines while having protective effects against the oxidative stress-induced DNA damage in leucocytes.[117]

Further studies will be needed to better appreciate the potential of all these egg molecules for cancer therapy and it might also be relevant to evaluate the synergistic activity of several of these egg candidates (or partly purified fractions) on cancer cell progression.

14.3.3.1.2 Egg Proteins in Targeting Cancer Cells. Another interesting feature of some of these egg proteins is their high affinity for some vitamins or other molecules, suggesting that they could constitute some promising carriers to deliver drugs and bioactive compounds to the tumor site and for the development of targeted therapy. The goal of such an approach is to increase the concentration of the drug in the vicinity of the cancerous cells without affecting healthy cells and while limiting side-effects. The egg archetype for such use is probably egg white avidin, *via* its very high affinity for biotin[118] and its ability to bind lectins, which are highly expressed at the surface of tumor cells.[119] A very recent article reported that ovomucin might also be a potential candidate as a mucoadhesive carrier to efficiently encapsulate and deliver drugs in various mucosal tissues.[120] Another approach is to inject peptide or protein biomarkers for cancer cells into hens, which will further

produce specific antibodies (immunoglobulin Y) that will be transferred into the yolk, which thereby serves as a noninvasive source of bioactive antibodies. This approach using chicken IgY has shown promising results in killing breast cancer cells.[121,122]

14.3.3.1.3 Egg Proteins and Diagnosis of Cancer. In parallel, the immunoglobulin Y strategy to produce antibodies against cancerous biomarkers has been extensively used to develop tools (ELISA/immunohistochemistry) that contribute to the diagnosis/prognosis of the disease.[123–130]

14.3.3.2 Egg Proteins and Tissue Remodeling/Wound Healing

Although cell proliferation and migration play a major role in pathological processes including cancer, they also have a major role in wound healing and tissue remodeling. Many proteins constituting the vitelline membrane, which supports the growth of the embryo and that of the vascularized yolk sac, are assumed to promote/regulate embryonic cell proliferation/migration onto the vitelline membrane and embryonic development. Such activities have been described for some proteins, such as ovomacroglobulin, ovomucin and pleiotrophin, which are components of the vitelline membrane and the egg white, as well as egg-yolk phosvitin, which may also act in interaction with the embryo.

Ovomacroglobulin is a broad-spectrum protease inhibitor that belongs the superfamily of alpha2-macroglobulins. Alpha-2-macroglobulins also act as carrier proteins for cytokines and growth factors. The exact role of ovomacroglobulin in eggs is not clear but some studies on mouse embryonic and primary human skin fibroblasts have shown that ovomacroglobulin was triggering cell migration by enhancing cell adhesion to the extracellular matrix, reducing inter-cellular aggregation and strengthening the cytoskeleton.[131] Ovomucin, a glycoprotein composed of alpha-ovomucin/MUC5B and beta-ovomucin/MUC6 is responsible for the gel-like structure of egg white and has also been identified in the vitelline membrane. Mucins participate in lubrication of mucous-type epithelia and modulate the cell and substratum adhesion. An ovomucin-like protein was demonstrated to be expressed by chicken primordial germ cells during early embryonic development. Ovomucin was suggested to favor migration of primordial germ cells by facilitating their aggregation and by preventing their adhesion to fibroblasts until primordial germ cells reach the gonadal ridges.[132] Better knowledge of the biological activities of these candidates on cell migration and invasion in interaction with the chicken embryo and at various stages of development may help to identify egg proteins involved in organogenesis and morphogenesis that could further constitute interesting candidates for wound healing and tissue regeneration. In this respect, various publications have also highlighted the potential role of phosvitin and pleiotrophin in bone organogenesis.

Phosvitin is an egg-yolk protein naturally generated after limited proteolysis of vitellogenins. It has unique properties suspected to be critical during egg embryo development as it stimulates differentiation of osteoblasts,

collagen synthesis, hydroxyproline formation and biomineralization, similarly to ascorbate (vitamin C), which is the only vitamin that is actually absent in egg[133,134]. In mammals, pleiotrophin is a secreted heparin-binding peptide expressed in mesodermal and neuroectodermal cells during development, but rarely in mature cartilage. However, it may be re-expressed in chondrocytes to participate in cartilage repair in the early stages of osteoarthritis.[135–137] Pleiotrophin has been identified in egg white, the vitelline membrane and in the eggshell and is highly conserved between species. To conclude, both phosvitin and chicken pleiotrophin may have interesting applications in tissue engineering and regenerative medicine.

14.3.3.3 Bioactive Proteins and Inflammation

Inflammation is a natural biological response to infections, damaged cells or irritants to maintain homeostasis, initiate repair and eliminate aggressors. Inflammation is a complex process that involves immune cells, blood vessels and molecular mediators. Chicken eggs contain several different pro- and anti-inflammatory components, such as egg phospholipids, cholesterol, the carotenoids lutein and zeaxanthin, and bioactive proteins, mainly ovalbumin and ovotransferrin or derived peptides.[138] In addition to these major proteins, recent studies have reported that the livetin fraction of yolk (and its resulting hydrolysates) may have anti-inflammatory activity by enhancing the phagocytic activity of macrophages[139] and that eggshell membrane extract also had a positive impact by reducing the secretion of pro-inflammatory molecules while stimulating those of anti-inflammatory cytokines.[140] The identity of the proteins that are responsible for such activities is still not known, but some publications have described similar effects for some proteins that were identified in the eggshell (ovocalyxin-36) or the egg white/vitelline membranes (pleiotrophin). Ovocalyxin-36 is an eggshell-specific protein initially described as an antimicrobial molecule that exhibits immunostimulatory activities after stimulation with lipopolysaccharide and that inhibits the production of pro-inflammatory cytokines *in vivo*.[141] Pleiotrophin is a universal multifunctional heparin-binding protein that possesses cytokine properties, among many other activities. Chicken pleiotrophin purified from egg white has recently been described as an antimicrobial protein, but it might also have interesting immunomodulatory activities in promoting lymphocyte survival and chemotaxis, like its human homolog with which it shares 93% sequence identity.[142,143]

14.3.3.4 Antioxidant Activities

Most organisms possess many cellular and molecular mechanisms to overcome environmental stresses, which trigger the accumulation of toxic oxygen radicals, which are associated with numerous functional dysregulations. In normal situations, potent antioxidant molecules, including dietary polyphenols and vitamins, but also multiple endogenous

enzymes, limit the toxic effects of these radicals. Chicken egg contains many antioxidant compounds that encompass vitamins, carotenoids, minerals and trace elements but also major proteins, such as ovotransferrin or egg-yolk phosvitin.[144–146] There are also increasing data related to the antioxidant capacity of hydrolytic peptides[147–150] but the antioxidant potential of minor proteins has not yet been investigated. However, proteomic approaches revealed the presence of some enzymes catalyzing the reduction of peroxides in egg white and yolk: glutathione peroxidase 3, P22352, egg white; similar to glutathione peroxidase, NP_001156704, egg yolk.[20,21,25,27]

14.3.3.5 Unexplored Potential of Enzyme Inhibitors

Egg contains several enzymes inhibitors, such as antiproteases and a lipase inhibitor of high abundance, that are bird/oviparous specific and that are distributed between the egg white and egg-yolk compartments. The fact that they accumulate in the egg suggests that they have an important role during embryonic development. For most of these molecules, the biological function is not known, likely owing to the fact that the targeted proteases have not yet been identified. Antiproteases are involved in many biological processes by regulating proteolytic/degrading activities that can have adverse effects when uncontrolled. It is quite intriguing to realize that egg is a major source of protease inhibitors with still unknown functions. In many invertebrates, Kazal-type proteinase inhibitors (a family encompassing egg white ovomucoid and ovoinhibitor) are described as defensive molecules since they alter the digestion of predators by inhibiting their digestive enzymes.[151] These mechanisms of defense are also widely encountered in plants that contain potent protease inhibitors and there is compelling evidence that they may have powerful chemopreventive applications.[103,152,153] Whether such egg inhibitors have similar physiological activities would be interesting to investigate and the increasing data published on plant antiproteases suggest that egg antiproteases may have similar positive effects on egg eaters/predators, including humans, especially when eating raw eggs. The broad-spectrum potent inhibitory activities of these molecules may have some interest for treating pathological situations associated with protease overactivity. Similarly, in the search for innovative molecules to treat obesity, there is increasing consideration of developing lipase inhibitors to counteract the excessive accumulation of fat observed in patients.[154,155] To our knowledge, there are no available studies that have investigated the potential of egg-yolk lipase inhibitor (apovitellin) in inhibiting human lipases. Apovitellenin is indeed a very potent molecule, whose physiological function is to prevent the loss of lipids from chicken very-low-density lipoproteins during their transport from the liver to the growing yolk follicles.[156]

14.4 Conclusions

Chicken egg contains many abundant molecules likely to bear original functions. Many of these proteins would be of great interest for non-food uses in human health (antimicrobials, anticancer or diagnosis molecules) and material sciences (proteins involved in biomineralization as biomimetics for ceramics). Nevertheless, a better appreciation of the functional activities of egg proteins will need further studies to consider their intimate functions that are assumed to be related to embryonic development. So far, the biological activity of egg components has been investigated using proteins or peptides that were purified from freshly unfertilized laid eggs. However, there is some evidence that these molecules may undergo some changes during incubation/embryonic development, which occurs at higher temperature (37.8 °C), that are likely to activate some latent activities. Additionally, usually, the bioactivities of eggs are screened based on the presence of domains or homologies with annotated molecules. Knowing that several egg proteins are bird-specific and do not have annotated functions in databases, the list of egg bioactive molecules is likely to be underestimated. The key is probably to analyze the kinetics of the assimilation/use of these various molecules by the embryo and to develop some specific models allowing for the depletion of one or the other candidate[157,158] to explore its impact on embryonic development. A better connection between egg protein biochemists and fundamental development biologists would be helpful to decipher the potential of egg proteins. Moreover, some differences exist in egg protein abundance or composition, and in protein sequences between avian species, which have adapted their egg contents during evolution to face various environmental challenges.[85,88,159] Thus, we believe that avian egg proteins still harbor many secrets and hidden bioactivities that may be of high value for human health.

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