Avian antimicrobial peptides in hen reproductive tract and egg

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Summary

Recently, many avian antimicrobial peptides have been predicted by bioinformatics and identified by proteomics. These compounds are generally small cationic molecules that possess activities against protozoans, fungi and viruses, as well as gram-positive and gram-negative bacteria. They are components of innate immunity and are capable of evading pathogen resistance mechanisms. Hens export protective molecules into the egg in order to anticipate defence of the embryo. In this review, we focus on avian antimicrobial peptides (AMPs) derived from the oviduct and found within the egg compartments. Indeed, the egg whose formation takes place in the oviduct is not only a source of nutrients for the embryonic development of the bird; it is also a reserve of protective molecules against physical and microbial aggression. Furthermore, enzymatic hydrolysis of egg proteins can release additional peptides that possess antimicrobial activities. Here, we describe the biochemical characteristics and the mechanisms of action of avian AMP families existing in the oviduct and in the egg. Moreover, we introduce novel applications of these peptides: they are potential candidates for marker assisted selection of increased antimicrobial egg defences and for development as neutraceuticals of major interest for human health or/and nutrition.

Keywords: antimicrobial, peptide, avian, defensins, egg, oviduct.

Introduction

Due to the growing emergence of bacteria strains and fungi that are resistant to antibiotics, much research concerning antimicrobial peptides (AMPs) is underway. These natural molecules of innate immunity ensure the defence of a multitude of multicellular organisms (bacteria, mushrooms, plants, insects and animals) against the majority of the terrestrial pathogenic organisms. Most of antimicrobial peptides even have a wide spectrum of activity which covers the main bacterial and fungi species, of the encapsulated viruses and the protozoa (Zasloff, 2002). It is noteworthy that a number of antimicrobial peptides have been described in reproductive tract tissues of mammals, in particular the defensins.

This review focuses on antimicrobial peptides that are present in the hen oviduct and the egg. Indeed, the hen egg is a unique and original model which is formed sequentially, spatially and temporally along the reproductive tract of hen. Each segment of the oviduct expresses and secretes specific molecules that become incorporated into the vitelline membrane (infundibulum), egg white (magnum), eggshell membranes (isthmus) and eggshell (uterus). Thus, the hen deposits into an enclosed medium, the egg, all the nutrients and protective systems that are necessary to support the development of an embryo during 21 days. The egg white has a natural antimicrobial activity which can be reinforce by the selection of hens for this criterion, as shown for anti-salmonella activity, of which heritability (proportion of variability due to genetic factors) is 0,16 (Sellier et al., 2007). Ovotransferrin and lysozyme are major antimicrobial molecules of the egg white, but they cannot solely explain the existing genetic variability (Sellier et al., 2007). Other antimicrobial molecules, such as peptides, are likely also involved in this variability. Recently, thanks to bioinformatics and proteomic tools and to sequencing of the chicken genome, close to 1000 proteins and peptides were defined in the various compartments of egg (Gautron et al., 2009). Moreover, expression of mRNA coding for several antimicrobial peptides has been reported in different segments of the hen oviduct. In this review, we propose to give a progress report on antimicrobial peptides (AMPs), expressed or identified in the hen oviduct and/or the egg. We will describe these AMPs, according to their families, while focusing on their structure, expression, purification, antimicrobial activity and mechanism of action.

Avian β-defensins (AvBDs) in the hen oviduct and the egg

Structure and nomenclature of AvBDs

The avian β-defensins (AvBDs) are small cationic non-glycosylated peptides (1-10 kDa), with a three-stranded β-sheet structure connected with a loop of β-hairpin turn (Evans et al., 1994; Sugiarto and Yu, 2004). Their structures are very similar to those of mammalian β-defensins (Landon et al., 2004; Pazgier et al., 2006). These peptides possess 6-12 cysteine residues that form three-six pairs of disulfide bridges, with the consensus sequence motif: x_n-C-x_{2-4}-G-x_{1-2}-C-x_{3-5}-C-x_{9-10}-C-x_{5-6}-CC-x_n where C is a cysteine, G a glycine and X an amino-acid.

Most AvBDs were simultaneously identified by two research groups which generated some confusion by naming them using two different nomenclatures (Lynn et al., 2004; Xiao et al., 2004). . Moreover, chicken beta-defensins were first identified by Lehrer's group who coined the term 'gallinacin' to describe them (Harwig et al., 1994; Higgs et al., 2005). In a collaborative venture, we proposed a novel nomenclature that adopted the numbering system used by Xiao *et al*. (2004) and replaced the term "gallinacin" by "avian beta-defensin" (Lynn et al., 2007). A total of 14 AvBD genes were identified through *in silico* studies.

Expression, identification and purification of AvBDs

Differential mRNA expression for avian β-defensins has been reported in the hen reproductive tract during the last decade. For AvBD 1, 2 and 3, the levels of mRNA expression found in the oviduct were not identical, but rather depended on the hen's physiological stage and/or environment. The onset of egg-laying activity takes place at approximately 4 months of age. The absence of AvBD 1, 2 and 3 mRNA expression was shown in the oviduct of 3 month-old hens (Zhao et al., 2001). But these 3 AvBDs were expressed in the mature oviduct of laying hens (Ohashi et al., 2005). All segments of oviduct expressed AvBD 1, 2 and 3 mRNA, with greater expression in infundibulum and in vagina (except for vaginal AvBD2). Yoshimura *et al.* (2006) reported higher levels of mRNA expression for these 3 AvBDs in the vaginal mucosa of 6-month-old hens, compared to 24-month-old hens. Decreased AvBD mRNA level in the regressed oviduct of non-laying hens suggests a modulation of AvBD expression, as a function of egg-laying activity. Furthermore, AvBDs 1, 2 and 3 mRNA expression increased when cultured vaginal cells of hens were stimulated with *Salmonella enteritidis* serovar Enteritidis or lipopolysaccharide (LPS) (Yoshimura et al., 2006). This result implies an environmental regulation of AvBD mRNA expression. Xiao *et al.* (2004) reported variable levels of

mRNA expression for AvBDs 10, 11 and 12 in two-month-old hen oviduct: i) mRNA expression of AvBD10 in the infundibulum and in a segment identified as 'oviduct' (between infundibulum and uterus, but segments are poorly characterized at this stage). ii) mRNA expression of AvBD 11 and 12 in the 'oviduct' and in the uterus. Strong expression of mRNA of AvBDs 10, 11 and 12 was also shown in the magnum and the uterus of mature laying hen (personal communication, VHG).

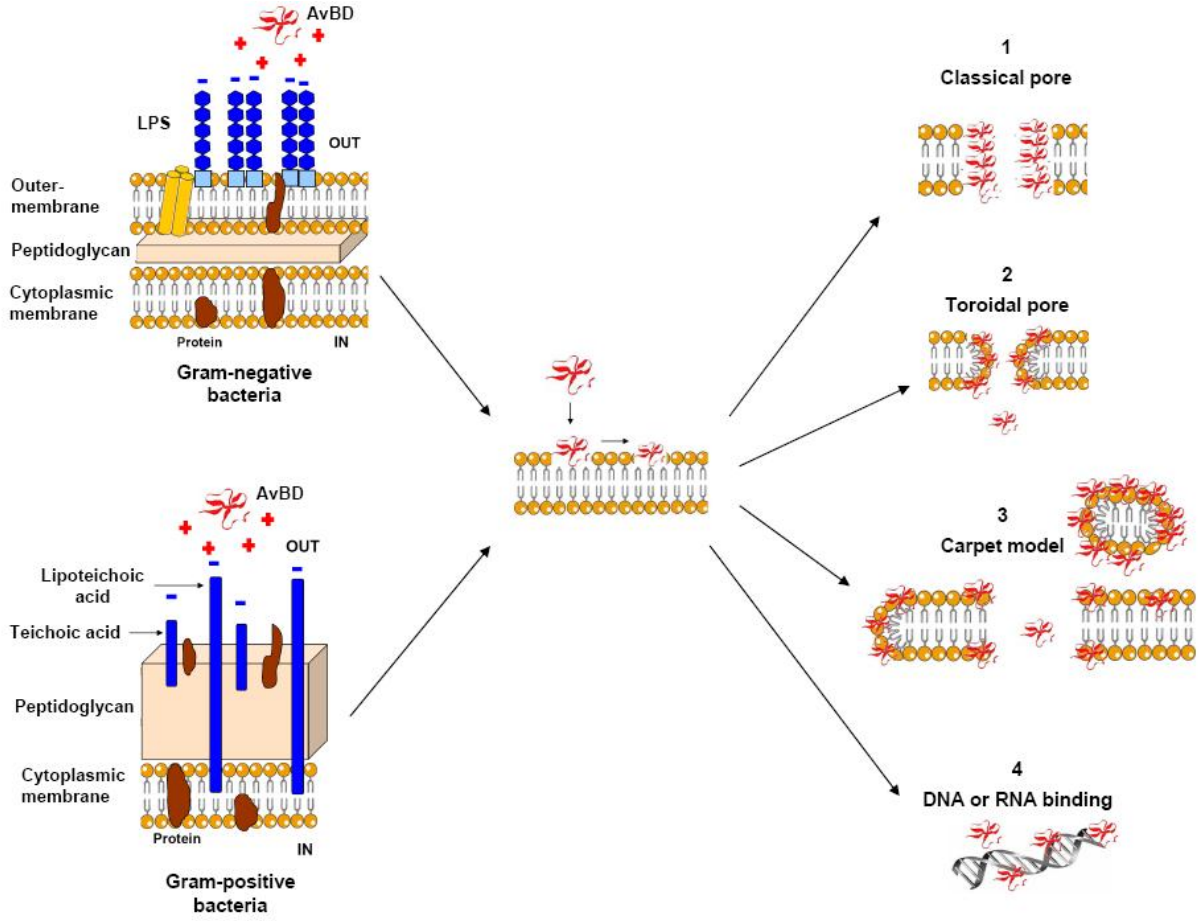
Mann *et al*. (2006, 2007, 2008a and b) used sensitive proteomic analysis to detect close to 1000 polypeptides in the different compartments of egg. Among them, some AvBDs were identified: AvBD11 was shown to be a constituent of the vitelline membrane (secreted by infundibulum), egg white (magnum) and eggshell (uterus); AvBD10 was found in the eggshell and Gallin in the egg white. These results are consistent with the strong expression of these AvBDs in the magnum and uterus, as mentioned above. Gallin is a cationic peptide of 41 residues, with 3 disulfide bonds and a similar consensus motif of β-defensin and is probably a new AvBD (personal communication, VHG).

Seven AvBDs (1, 2, 3, 10, 11, 12 and Gallin) were shown to be expressed in the hen oviduct and were identified in the egg. Moreover, AvBD 1, 2 and 3 have been purified from chicken peritoneal heterophil or neutrophil (Evans et al., 1994; Harwig et al., 1994). They were isolated using a size exclusion chromatography, followed by C18 RP-HPLC. Recently, AvBD11 was purified from egg white by affinity chromatography, followed by C18 RP-HPLC and Gallin was obtained after chemical synthesis for further characterization (personal communication, VHG).

Antimicrobial activities of AvBDs

Among the 7 AvBDs present in the Gallus gallus egg, only AvBD1 and AvBD2 have been investigated for their antimicrobial activities. AvBD1, at concentrations of 16 µg/mL or less, possesses bactericidal activity against Gram-negative bacteria (*Escherichia coli*, *Salmonella enteritidis* serovar Enteritidis, *Salmonella enteritidis* serovar Typhimurium, *Campylobacter jejuni*, *Pasteurella multocida* and *Bordetella avium*), Gram-positive bacteria (*Listeria monocytogens* and *Staphylococcus aureus*). Fungicidal activity of AvBD1 was demonstrated against *Candida albicans*, but it was not able to neutralize *Infectious Bronchitis Virus* (Evans et al., 1995; Evans et al., 1994; Harwig et al., 1994). A bactericidal activity was found for AvBD2 against *Escherichia coli* and *Listeria monocytogens* (Harwig et al., 1994). AvBD11 also possesses bactericidal activity against *Salmonella enteritidis* serovar Enteritidis, *Salmonella enteritidis* serovar Typhimurium (personal communication, VHG).

Fig. 1. Hypothetical mechanism of action of AvBDs. AvBDs could interact electrostatically with negatively charged membrane components of Gram-negative or -positive bacteria. After dimers and multimers creation, 3 models have been proposed: (1) the "barrel-stave pore", (2) the "toroïdal pore" or (3) the "carpet model" with disruption of the membrane in a detergent-like manner, resulting in the formation of micelles. (4) AvBDs could also interact with DNA, RNA, resulting in an alteration of protein synthesis.



Mechanism of action of AvBDs

The mechanism of action of AvBDs is not completely known. Several models have been proposed. AvBDs have a specific amphipatic tridimensional structure, with three intramolecular disulfide bridges, and possess opposite domains of clustered hydrophobic and cationic amino-acid chains. Exposed cationic sites are thought to interact electrostatically with negatively charged membrane components, such as LPS of Gram-negative bacteria, or acidic polysaccharides in Gram-positive bacteria (Hancock, 1997). Then, after peptide accumulation, parallel to the membrane surface, dimers and multimers could be formed, resulting in the creation of a pore. Three pore models

have been proposed (figure 1): the “barrel-stave pore” where multimers form a channel, the “toroidal pore” where multimers form a transient pore or the “carpet model” with disruption of the membrane in a detergent-like manner, resulting in the formation of micelles (Sugiarto and Yu, 2004; van Dijk et al., 2008; Wellman-Labadie et al., 2007). Subsequently, inside the bacteria, the defensins could interact with DNA or RNA, altering DNA and RNA functions including protein synthesis (Lehrer et al., 1989).

Antimicrobial peptides derived from egg proteins

Lysozyme

Lysozyme is a protein of 129 residues (14.307 kDa) which belongs to a class of hydrolases that lyses the cell wall of certain Gram-positive bacteria. It acts as a mucopeptide N-acetylmuramyl hydrolase (Mine et al., 2004). This ubiquitous protein represents around 3.4 % of the egg white proteins. Lysozyme is present in the vitelline membrane, the egg white and in the acid-soluble organic matrix of the eggshell Mann *et al*. (2006, 2007, 2008a and b; Hincke et al. 2000). Lysozyme C precursor was identified in the chicken egg yolk cytoplasmic proteome (Farinazzo et al., 2009). Antimicrobial activity of lysozyme is well-known with high activity against only some specific Gram-positive bacteria. But the sequence of lysozyme contains internal domains that are independent of the enzymatic site and capable of antimicrobial activity. Indeed, proteolysis of chicken lysozyme by pepsin and trypsin generated 20 peptides, lacking enzymatic activity. Amongst them, two peptides (amino acids 15-21 and 98-108 of the lysozyme sequence) exhibited inhibitory effect on growth of *Staphylococcus aureus* and *Escherichia coli* K-12, respectively. The former bacterium is associated with serious cutaneous infections, endocarditis and various toxinogen pathologies (Mine et al., 2004; Réhault et al., 2007). Peptide 98-108 is located in the middle part of helix-loop-helix motif of lysozyme and possesses two helices; it exhibits antimicrobial activity against Gram-negative and Gram-positive bacteria. The second peptide 15-21 possesses the residues 15 and 16 which are in the amino-terminal helix. Proteolysis of lysozyme by clostripaïn generates the peptide 98-112, which is slightly longer than peptide 98-108 Mine *et al.* (2004). Peptide 98-112 was efficient against Gram-positive, Gram-negative bacteria and *Candida albicans*, a yeast causing infections of the digestive and genital tracts (Ibrahim et al., 2001). The mechanism of action for these lysozyme-derived peptides seems to be very similar to AvBDs’one, in which structures of the helix-loop-helix type are fixed at the external membrane of

the bacterium leading to channel formation, and ultimately bacteria lyses (Ibrahim et al., 2001). In addition, polypeptides derived from peptide 98-112 inhibited DNA and RNA synthesis of *Escherichia coli* and caused bacteria lysis, by a mechanism quite different from that of native lysozyme (Pellegrini et al., 2000). These results showed that bacteriostatic peptides can be released by proteolytic digestion of native lysozyme.

Ovalbumin

Ovalbumin is a monomeric phosphoglycoprotein representing over half of the total egg white proteins (Mine and Kovacs-Nolan, 2004). In addition, small amounts of ovalbumin were identified in egg yolk, vitelline membrane, egg white and eggshell (Mann, 2007; Mann, 2008; Mann et al., 2006; Mann and Mann, 2008). It belongs to the serpin family (serine protease inhibitors), but is devoided of any antiprotease activity. To date, its biological role in the egg remains unknown. However, peptidic fragments, obtained by tryptic or chymotryptic digestion, possess antibacterial activity against many bacteria involved in various infections (*Bacillus subtillis*, *Eschericha coli, Serratia marcescens, Bordetella bronchiseptica* and *Pseudomonas aeruginosa)*, as well as the fungus *Candida albicans* (Pellegrini et al., 2004). These egg albumin fragments were able to limit the proliferation of bacteria used in food fermentation (*bifidobacterium*, *lactobacillus)* and possess immunostimulatory activities (Biziulevicius et al., 2006; Mine and D'Silva, 2008; Pellegrini et al., 2004; Réhault et al., 2007). Few peptides derived from ovalbumin exhibited an antimicrobial activity and they didn't possess a bactericidal potency as reported for the avian beta-defensins. Only one peptide was positively charged, and consequently should act as an AvBD. The others peptides which weren't positively charged should have another mode of action (Pellegrini et al., 2004).

Ovotransferrin

Ovotransferrin (OTf) is a monomeric glycoprotein, belonging to the iron-binding 'transferrin' protein family. OTf has the capacity to reversibly bind two iron ions per molecule (Ibrahim et al., 1998). It inhibits Gram-negative bacteria by depriving bacteria of iron that is essential for their growth. OTf has been identified in egg yolk, vitelline membrane, egg white and in the acid-soluble organic matrix of the eggshell (Gautron et al. 2001; Mann et al., 2006, 2007, 2008a and b). A peptide named OTAP-92, corresponding to a cationic bactericidal domain of OTf, was isolated after acid proteolysis technique and cleavage at Asp-X sequence (Ibrahim et al., 1998). OTAP-92 possesses a helix-sheet motif linked by disulfide bridges consisting of two-stranded parallel β-sheet and a helix, and has similarity to insect

defensins. Thus the mechanism of action is likely to be similar to that described for the AvBDs. OTAP-92 was able to cross the outer membrane of *Escherichia coli* by self-promoted uptake, and kill bacteria by damaging the biological function of cytoplasmic membrane (Ibrahim et al., 2000). Its strong bactericidal activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria allowed the authors to envisage therapeutic applications for this peptide, derived from a natural protein of the egg white.

Ovomucin

Ovomucin is a macromolecular and heavily glycosylated glycoprotein, consisting of a peptide-rich α-subunit and a carbohydrate-rich β-subunit (Itoh et al., 1987). It confers a gelatinous and viscous character to the egg white and thus, limits diffusion of micro-organisms. Ovomucin has been identified in the vitelline membrane and in the egg white (Mann et al., 2007, 2008a). Ovomucin glycopeptide (OGP), prepared by size exclusion chromatography after pronase digestion of hen egg ovomucin, is able to specifically bind to *Escherichia coli* O157:H7, a property which could protect against *Escherichia coli* infections. It could also be used in diagnostic applications for pathogen detection (Kobayashi et al., 2004).

Histones

Histones are principal structural proteins of eukaryotic chromosomes. They are polypeptides rich in lysine and/or arginine. Histones H2A, H2B, H3, and H4 are core proteins responsible for the basic structure of chromatin, the nucleosome core. The linker histone H1 interacts with linker DNA between nucleosomes and functions in the compaction of chromatin into higher-order structures (Kasinsky et al., 2001). In addition, there is increasing evidence that histones, in addition to their role in nucleosome formation, might play an important role in innate host defence against intracellular or extracellular microbe invasion. The structure–activity relation of a histone H2A-derived antimicrobial peptide, buforin II (from the stomach tissue of an Asian toad) was studied to understand the structural requirements for its antimicrobial activity and the mechanism of bacterial killing action. The carboxy-terminal α-helical region of buforin II seemed to be critical for the antimicrobial activity, by providing an amphipathic stable α-helical structure. Furthermore, the proline hinge was found to be a key structural factor for the cell-penetrating property (Park et al., 2000). In addition, the presence of a basic residue at the amino-terminus has been revealed to be essential for the membrane-binding activity of parasin I, a histone H2A-derived antimicrobial peptide (Koo et al.,

2008). Indeed, a single lysine residue in a random coil region has a profound effect on the mechanism of action. One hypothesis was that the lysine residue has a critical role in the formation of "barrel-stave" formation. These findings provided important information for designing potent new antimicrobial peptides acting through different mechanisms.

In *Gallus gallus*, histones H2A and H2B.V, and histone H2B carboxy-terminal fragment have been isolated from liver extract by cation-exchange and gel filtration chromatographies, followed by two-step RP-HPLC (Li et al., 2007). Histone H2A and histone H2B carboxy-terminal fragment exhibited antimicrobial activity against both Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli* D31) bacteria. Moreover, these activities were thermostable and salt-resistant. Histones H1 and H2B have been characterized in the hen reproductive system as antimicrobial proteins and their role as effector molecules for the innate host defence was demonstrated in the ovary of healthy laying hens (Silphaduang et al., 2006). Histones are found in all compartments of egg (Mann et al., 2006, 2007, 2008a and b), suggesting that they could reinforce the innate antimicrobial activity due to defensins and other molecules

Conclusions and perspectives

Even with new bioinformatic tools and the chicken genome sequence, only few antimicrobial peptides, including 7 AvBDs are clearly expressed in the hen oviduct and have been identified in or isolated from the egg. These AMPs are candidates of interest with properties beneficial to human and animal health. Concerning the AvBDs, their single nucleotide polymorphisms (SNP's) could be putative biological markers for marker assisted genetic selection (MAS) to improve egg defences and reduce the risk of food-borne disease outbreaks for consumers. Quantitative enzyme-linked immunosorbent assays (ELISA) are in progress for AvBD11 and Gallin, with the aim of evaluating the variability of these AvBDs in egg, as function of hen physiological stage and environment. Regarding the peptides which derived from egg proteins and exhibited antimicrobial activity, we suggest that the original egg proteins are not only present for a nutritive function, but they also provide a chemical defence against infectious agents. Others egg proteins have to be studied in the aim to find more derived-peptides. These peptides are not directly synthesized in the organism

itself, but produced indirectly by enzymatic cleavage of the original proteins and some of them are able to fight pathogens.

Compared to mammalian AMPs, these are at a very early stage of scientific research on avian AMPs, especially for those that are localized in the hen oviduct and deposited in the egg. Their mechanisms of action and antimicrobial selectivity have to be explored to improve strategies directed against bacteria and fungi that are increasingly more resistant to antibiotics. Moreover, the presence of these antimicrobial peptides in eggs highlights its value and putative benefits for human and animal health.

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