Antimicrobial Peptides from agro-industrial waste – a key to new antibiotics

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Antimicrobial Peptides

The latest report form the World Health Organization (WHO), 2016, describes the alarming increase of antimicrobial resistance. This fact is a huge threat to all society, principally to immunocompromised patients, such chemotherapy patients (1). Without the development of new and effective antimicrobial agents the number of deaths may increase exponential. Antimicrobial peptides (AMPs) are an essential part of the innate immunity (2) and can act as immunomodulators, making them excellent candidates for a new generation of antibiotics (3).

Pharmaceutical and cosmetic companies and food industries are particularly interested in AMPs from proteins (4). For example in the case of food industry the antimicrobial peptides are used as preservatives, instead of the traditional chemical preservations agents. Most of the AMPs described in the literature are extracted from expensive protein matrixes (e.g., food), which in most cases make their application unfeasible (5). An alternative to this process is gaining attention, agro-industries waste. The use of agro-industries has several advantages, it is protein-rich, it has a positive environmental impact which can be hydrolysed to obtain the AMPs.

The waste of the agro-industries is enormous, causing a significant environmental impact and cost of disposal (5,6). The major waste and by-products from the agro-industrial activity are microalgae (7,8), soybean meal (9), residues of olive production (10), rapeseed meal (11), chicken feathers (12), fish waste (13) and egg protein (14).

AMPs have unique characteristics that make them ideal substitutes for conventional antibiotics. The AMPs should not induce pathogen resistance, it has the ability to distinguish host from invading cells, and it has activity against a large range of microorganism. Cell membranes are negatively charged and in general the AMPs are positively charged which facilitates their association. The AMPs can easily penetrate the hydrophobic core of the cell membrane due to its hydrophobic nature. The insertion of the AMPs into the cell membrane results in membrane rupture and lysis. Bacterial membranes cannot be redesign form non-lipid molecules; therefore it is unlikely that the microorganism could develop resistance to AMPs (15,16).

In the next section several AMPs "recovered" from waste material are described, so as their antimicrobial activity and mechanism are unravelled. AMPs may hold the key to future antibiotics.

Egg Proteins

Since ancestral times eggs are part of human consumption, and is still a highly valued culinary delight. During 2012, 54 million tonnes of eggs were produced within the 9 countries with the highest yield (17). The most commonly commercialized egg belongs to *Gallus gallus domesticus* (18). Eggs are composed by the following main constituents: shell, albumen (also known as white) and yolk (19). At oviposition the egg is absent of microorganism contamination (20). Subsequently to the first microorganism contact, that occurs at the cloaca, the egg is exposed to non-antiseptic conditions during its incubation period of 21 days (20,21). For a successful egg hatching, their interior must remain free of harmful microorganisms. Besides containing all the required nutrients for the embryo development, the egg must own several physical and biochemical barriers against microbial infection, proliferation and action (20,22). The avian egg shell presents the first line of defense, being its surface composed mainly of calcite, which consists in proteinaceous matrix embedded in calcium carbonate (23,24). The shell is populated with several pores with an approximate size of 120 μm^2 that are essential for the gaseous transfer during the embryogenic process, however, are too wide to completely impede the entrance of bacteria (24,25).

Albumen consists in second barrier against microorganism contamination, comprising a bioactive physical hurdle. The albumen high viscosity hinders the motility of bacteria, obstructing their progression inside the albumen and preventing the microorganisms to reach the yolk (20). In addition to its average high pH of 9.5, albumen is composed of several proteins, some of them with renowned antimicrobial activity (20,26). The fibrous proteins of the shell are not reported to have antimicrobial activity. The same happens to the yolk proteins that are mainly constituted by low density lipoproteins. Therefore this section will only focus on the main albumen proteins that were referred in the literature as effective antimicrobial proteins, or with the potential to be explored.

Avidin, the protein with one of the lowest relative concentration in the albumen (0.05 %), is able to bind to biotin (19,27). This binding capacity was proposed to be associated with the bio-unavailability of this essential vitamin to the microorganisms, thus preventing their growth (28). Its antibacterial role is supported by the production of highly similar avidin by macrophages, and its ability to bind to the surface of several bacteria, namely to the gram-positive: bacteria *Streptococcus pyogenes*, *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* and to the gram-negative bacteria: *Serratia marcescens*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Escherichia coli* (29–31). Avidin is composed of 512 amino acid residues, has a molecular height of 68.3 kDa and a isoelectric point of 10 (23). Its motif is composed of a homotetramer, with each of the subunits being constituted by barrel shaped β-sheets (27). The biotin binding occurs in the chain D, where some polar residues present in the interior of the barrel interact with the uredo rings of biotin (27).

Ovomucin is consensually regarded as the glycoprotein responsible for the albumen viscosity (32). It is composed of two subunits that mainly differ in their carbohydrate content, α has approximately 12.5 % and β contains approximately 58 % of carbohydrates (33). The high concentration of carbohydrates, particularly in the β subunit, was reported as the responsible for the Newcastle disease virus entrapment (33). Bovine rotavirus and swine influenza virus were also reported to be inhibited by ovomucin (34,35). In addition to its anti-viral activity, the minimal inhibitory concentration (MIC) of ovomucin was determined for *Salmonella* (400 μg mL^{-1}) and for *Escherichia coli* (50 μg mL^{-1}) (36). Nevertheless its bactericidal activity should be further enlightened. Moreover, ovomucin crystal structure is not yet available limiting the amount of information available.

Ovomucoid is a major protein in albumen, comprising 11 % of the protein content of albumen (23). It is regarded as the main responsible for allergic reactions triggered by egg ingestion (37). Its potential bactericidal activity was confirmed against *Streptomyces erythraeus*, and it proved a small inhibition when in contact with *Bacillus subtilis* (38,39). Pellegrini and co-workers tested ovomucoid against several bacteria, however they were unable to obtain a clear result due to the ovomucoid contamination with lysozyme (40). Thus its mode of action remains unknown, as its crystal structure.

The second major protein is ovotransferrin (firstly named as conalbumin), which belongs to the transferrin family that comprises the principal glycoproteins responsible for the iron regulation (41,42). Hen ovotransferrin (OTf) is architecturally arranged in two elliptic globular domains, each lobe is further divided into two domains, N-terminal lobe is divided into N1 domain (1 to 91 and 247 to 332 amino acid residues) and N2 region (92 to 246 amino acid residues) whereas the C-terminal halve is divided into the C1 region (343 to 429 and 589 to 672 amino acid residues) and C2 domain (430 and 588 amino acid residues) (43). The lobes are linked through two anti-parallel β-sheets, each lobe possess one cleft with high affinity towards Fe^{3+} composed of two tyrosines, one histidine and one aspartic acid residues (43). The Fe^{3+} chelating occurs with the synergistic binding of carbonate ion (CO_3^{2-}) and is completely reversible (41,44). The antimicrobial effectiveness of OTf was assessed on numerous microorganisms, including gram-negative bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and gram-positive bacteria: *Streptococcus mutans*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (31,45). Valenti and collegues studies pointed out the bacteriostatic action of OTf, and indicated that OTf activity was not limited to its iron chelating properties (46–49). Ibrahim and co-workers obtained a cationic antibacterial peptide through acid proteolysis of OTf isolated and characterized as OTAP-92 (26). It comprised 92 residues (between leucine residue 109 and aspartic acid residue 200), and its effectiveness was evaluated (26,50). This peptide has two disulphide bridges and a sequence similar to the insect cationic peptide, defensins, which characteristic cysteine motif is analogous to the kringle region of OTAP-92. thus when in contact with the bacteria surface, several events may unfold, namely one or more of the Shai-Matsuzaki-Huang mechanisms (51–54). The peptide amphiphilic nature can interact with the bacterial membrane through two main possible mechanisms, carpet and “barrel-stave”. The peptide may bind to a bacteria surface, covering it like a carpet, thus causing a membrane charge shift, altering the cell homeostasis, and increasing metabolic burden; moreover membrane permeation and consequent disruption occurs at high protein content (52). On the “barrel starve” mechanism, the peptide molecules (starves) are inserted into the membrane and oligomerize to form a channel (barrel). It consists in forming a transmembrane pore due to the bundle of amphiphilic domains (53,54).

Finally, one of the most important biotechnologically important enzymes found in albumen is lysozyme. Lysozyme bactericidal activity has being used in several applications, namely as a preservative on food industry and an antimicrobial agent in pharmaceutical industry (38). The name lysozyme is intrinsically based on the result of its activity, the cell lysis (55). Thus it has two disulphide bounds that are mandatory for its activity (55). Consisting of a peptide chain comprising 129 amino acid residues, lysozyme is the protein with the highest isoelectric point (pI=10.7) among the albumen proteins. Lysozyme has a molecular weight of 14.3 kDa and its *modus operandi* is well known (23). The peptidoglycan layer in the bacterial cell wall protects the cell membrane from disruptive osmotic pressure (56), but lysozyme disrupts the β-(1,4)-glycosidic linkage between n-acetylglucosamine and muramic acid promoting cell aggregation and loss of bacterial viability. (23,31). Once permeable the bacteria membrane is disrupted and its constituents released. The bactericidal albumen lysozyme activity has proved to be more effective against gram-positive bacteria, with much lesser effect on gram-negative species, featured by the outer membrane preventing protein diffusion to the target site (57,58). Though, EDTA bears a synergic effect through alteration of protein conformation of the outer membrane of gram-negative bacteria, enhancing the bactericidal activity of lysozyme (59).

Keratin-based peptides

Keratin belongs to the family of fibrous structural proteins being the key structural materials of the outer layer of human skin and nails and the basic building blocks of fibres such as human hair, sheep's wool or bird feathers (60,61). These complex natural composites possess a heterogeneous morphological structure, mostly proteinaceous in nature (95 % - 97 %) while structural lipids, pigment, and other materials represent the remaining fractions (62–64). Due to its high protein content, these abundantly available fibres are commonly used as a source of functional keratin peptides. By controlling the conditions of hydrolysis, which lead to disulphide bonds breakage and reformation as well as the disruption of peptide bonds, keratin peptides with different properties may be isolated for different purposes.

Despite the fact that keratin fibres are highly cross-linked with disulphide bonds, which makes them difficult substrates for protein extraction, several methods have been used to obtain these peptides. The most popular are the alkaline and acidic methods throughout oxidative and reductive reactions. Peptides with different characteristics and properties are obtained for different applications, depending on the capacity of the process to induce the breakage of disulphide and/or peptide bonds. Structurally, keratin fibres are composed of two main elements: an external cuticle, which is a protective layer covering the core of the fibres; and a fibrous cortex comprising intermediate filament proteins (IFPs) or microfibrils and the sulphur-rich intermediate filament associated proteins (IFAPs) or matrix (61,65). The methods used for obtaining keratin peptides usually convert the IFPs and IAFPs proteins into their non-crosslinked forms by oxidation (66–69) or reduction (68,70–72), and the aminoacid cystine is converted to either cysteic acid or cysteine, respectively. The free proteins extracted with denaturing solvents produce a solution that can be purified by filtration and dialysis. More details on the methods for extracting keratins are summarized elsewhere (68,73).

In order to obtain keratin functional peptides, the enzymatic biological approach has been widely suggested. This technique allows for controlling the hydrolysis conditions, preserving the aminoacids structure due to the mildness of the process and the fact that it is an eco-friendly technique (74). In regards to obtaining antimicrobial peptides, this factor is very important since the molecular weight of the peptides and the presence of intact aminoacids such as glycine, arginine and tryptophan are critical for its antimicrobial activity. Hydrolysed keratin have been successfully obtained from human hair and wool (75), feather (76,77) and feather meal (78) via enzymatic hydrolysis using *Bacillus* spp, among others. The biological activity and biocompatibility of these peptides have been explored in the development of keratin-based materials with applications in wound healing, drug delivery, tissue engineering, trauma and medical devices where the antimicrobial activity plays a central role (79,80). For example, the presence of high content of cysteine residues leads to a high rate of cross-linking through disulphide bonds (81), explaining the high stability of the macrostructure on keratin-based products, and imparting good mechanical, thermal and chemical properties (79,82). For this reason, keratin peptides are continuously being reported as novel biomaterials in the area of tissue engineering. Other important focus of keratin-based peptides is on cosmetics, particularly in hair and skin care. Keratin peptides obtained from wool, with a molecular weight below 1 kDa were found to increase the skin hydration and elasticity (83). Moreover, a keratin peptide based on a fragment of hair keratin type II cuticular protein has proved to act as a strengthening agent for weakened relaxed and over-bleached hair (84,85).

The antimicrobial properties of keratin extracts have been scarcely reported. Yet, this paradigm is changing and the use of keratin extracts for obtaining antimicrobial peptides is increasingly growing, following the first reports on the antimicrobial properties of human corneal epithelium. It is known that human cornea possess innate defense properties against pathogens due to the production of cytoprotective antimicrobial peptides. They have been identified as peptide fragments derived from the carboxyl-terminal region of cytokeratin 6A (K6A) also known as KAMPs (86). These glycine-rich keratin 6A peptide is expressed in many types of stratified epithelial cells (87) and is upregulated in inflammation processes and tissue injury as a defense system against microorganisms (88). Based on this knowledge several works have reported the synthesis of epithelial K6A-mimicking peptides. Lee *et al*. synthesized different peptides based on the sequence of K6A peptide and demonstrated that these keratin-based antimicrobial peptides were effective against *Staphylococcus aureus* and *Pseudomonas aeruginosa* by deforming the bacterial cell envelope and inducing pore formation on the membrane (89).

In other study, keratin nanoparticles were synthesized using keratin extracts derived from an alkaline extraction from chicken feathers. These nanoparticles were found to have antibacterial activity against *Staphylococcus aureus* isolated from milk and *Escherichia coli* (90). The economical interest of antimicrobial peptides based on keratin is reflected on the increasing number of patents related with this issue (91–93).

Besides biomedical field, keratin extracts have numerous other applications. The use of keratin from feather meal as a slow nitrogen release fertilizer, due to its slow decomposition rate, is well studied (94). Also, the possibility of using keratin as a foaming agent for fire extinguishers, taking advantage on the protein biomasses available as waste from textile industry and butchery, has been reported (95).

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