Diversity of Antimicrobial Peptides in Silkworm

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Antimicrobial resistance is a phenomenon that the world is witnessing that poses a serious threat to global health. The decline in the development of novel therapeutics over the past has exacerbated the situation further. In this scenario, the pursuit of new alternative therapeutics to commonly used antibiotics has gained predominance amongst researchers across the world. Antimicrobial peptides (AMPs) from natural sources have drawn significant interest as promising pharmacological substitutes over the conventional antibiotics. The most notable advantage of AMPs is that microorganisms cannot develop resistance to them. Insects represent one of the potential sources of AMPs, which are synthesized as part of an innate immune defence against invading pathogens. AMPs from different insects have been extensively studied, and silkworm is one of them. Diverse classes of AMPs (including attacins, cecropins, defensins, enbocins, gloverins, lebocins and moricins) were identified from silkworm that exhibit antimicrobial property against bacteria, fungi and viruses, indicating their potential therapeutic benefits.

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1. Isolation of Antimicrobial Peptides (AMPs) from Silkworms

The larval stage of silkworm, *B. mori*, has five instars during which the larvae go through four moults. Silkworms are challenged with pathogens to isolate AMPs during their fifth instar as the duration is longer (6–8 days), which allows for enough time to develop infection. Furthermore, silkworm's fat body content is at its peak during this instar, which is the primary source of AMPs [1][2]. Upon infection, the immunocompetent tissues are lysed in a suitable buffer to extract the proteins and subjected to various chromatographic techniques such as ion-exchange chromatography, gel filtration chromatography and RP (reverse phase)-HPLC (High performance liquid chromatography) for purification. Finally, the purified peptides are identified via mass spectrometry and de novo sequencing [3]. The proteomic data are analysed using the following tools: Mascot Distiller coupled with Mascot Server [4], Thermo proteome discoverer [5], PEAKS [6], Maxquant and a companion software, Perseus [7][8]. The antibacterial activity of the isolated AMPs against test cultures can be determined by employing any of the following techniques viz., agar disc diffusion, agar well diffusion, agar plug diffusion, antimicrobial gradient method, broth microdilution, broth macrodilution and agar dilution method [9].

2. Different AMPs in Silkworms

2.1. AMPs Reported from Mulberry Silkworm B. mori

2.1.1. Cecropins

Cecropin, α-helical linear AMP (37 amino acids) lacking cysteine residues was first isolated from *Hyalophora cecropia* moth infected with bacteria [10]. In *B. mori*, five types of cecropins are found including cecropin A, cecropin B, cecropin C, cecropin D and cecropin E. A total of eleven *Bmcec* genes (*BmcecA1*, *BmcecA2*, *BmcecB1*, *BmcecB2*, *BmcecB3*, *BmcecB4*, *BmcecB5*, *BmcecC*, *BmcecD1*, *BmcecD2* and *BmcecE*) encoding cecropins are reported in silkworms [11]. Another AMP, enbocin, whose amino acid composition indicated that it belonged to the cecropin family, was also reported in *B. mori* [12][13]. Cecropins are primarily produced in the host mainly in response to Gram positive or Gram negative bacterial infections. They possess random coil structures in aqueous solution, but when they interact with cell membrane of microorganisms, they adopt α-helical conformations [14][15]. Although certain aspects of cecropins' mode of action are still unknown, it is presently believed that they bind to the bacterial cell membrane along the axes of α-helical domains parallel to lipid bilayer. The polar residues of cecropins attach to the lipid phosphates, whereas the non-polar side chains burrow into the membranes hydrophobic core. The continuous accumulation of cecropin molecules forms a carpet structure on lipid bilayer surface, which has a detergent-like property and disintegrates the bacterial membrane [15][16]. However, *H. cecropia* cecropins at lower concentrations interact with membranes to form channels or pores, affecting cellular electrolyte balance, thereby causing cell death [15][17].

Cecropins at very low concentrations exhibit antibacterial activity against a wide range of Gram positive and Gram negative bacteria. Two modified *B. mori* cecropins, CecXJ-37C and CecXJ-37N with an amino acid addition on C-terminal, are also reported to be active against diverse bacterial strains [18]. Cecropins and cecropin-type peptides are also known to inhibit growth of *Aspergillus* spp., *Fusarium* spp. and yeasts indicating antifungal properties of this AMP [19]. These peptides are shown to have low cytotoxicity and negligible haemolytic activity to the host cells at concentrations exhibiting antimicrobial activity. The ability of cecropins like any other AMP to preferentially target microbes without interfering with host cells is due to differences in the makeup and composition of the respective cell membranes [20]. Reports suggest that *B. mori* cecropins did not exhibit any cytotoxic or haemolytic effects at concentrations up to 200 μM, but they inhibited growth of microbial pathogens at much lower concentrations [14].

Apart from antimicrobial properties, cecropins are reported to selectively induce apoptosis in cancer cells. CecropinXJ, a newly isolated cationic AMP from *B. mori* inhibited growth of hepatocellular carcinoma (HCC) cell line Huh-7 cells and induced apoptosis in HCC cells [21]. CecropinA is also reported to induce apoptosis in human leukaemia (HL-60) cells [22]. Cecropins, like most AMPs, are known to specifically target tumour cells by binding to the phospholipid phosphatidylserines found on the outer surface of tumour cell plasma membranes. This sort of membrane architecture differs in normal cells, where phospholipid phosphatidylserines are found in the inner surface of plasma membrane and phosphatidylcholines and sphingomyelins are located on the outer surface [23].

2.1.2. Defensins

Defensins are cysteine containing peptides that were first reported from *Sarcophaga peregrina*, the flesh fly [24]. Defensins are cationic in nature containing conserved cysteine residues (6 no's) and have molecular weight of 4 kDa. Defensins have a complex structural topology with arrangement of α-helixes and β-sheets stabilized by three

disulfide-bonds and therefore known as cysteine-stabilized αβ motif [25]. *BmDefensinA* found in *B. mori* genome is a defensin ortholog of *Spodoptericin*. A group of researchers reported expression of *BmDefensinB* gene in *B. mori* after infection with *E. coli*, *Bacillus subtilis* and *Beauveria bassiana* [26]. Defensins exhibit antibacterial activity against Gram-positive bacteria, namely *B. subtilis*, *B. thuringiensis*, *B. megaterium Micrococcus luteus*, *S. aureus* and *Aerococcus viridians* [27]. A defensin-like anionic antimicrobial peptide BmDp from *B. mori* is also reported, which is identical to BmDefensinA and is close to galiomicin and spodoptericin [28].

Defensins inhibit bacterial growth by membrane disruption and through the formation of voltage dependent anion-selective channels in cell membranes [29][30][31]. Recent findings suggest that β-defensin binds to specific phospholipids on the cell membrane forming oligomeric complex to facilitate cell lysis [32]. However, insect defensin's mode of action appears to be complex and information on the same is limited. Specific targets for insect defensins are yet to be found, and structure-activity studies may aid in unravelling the molecular process behind their bioactivity [25].

2.1.3. Moricins

Moricin, a cationic, amphipathic α-helix AMP shows the presence of charged amino acid residues after every three to four amino acids, which is responsible for its antimicrobial properties against bacteria and few strains of yeasts. Moricin consists of 42 amino acid residues and was first isolated from the *B. mori* haemolymph. It was found to be active against Gram positive bacteria *S. aureus* [3]. In *B. mori*, a total of twelve genes encoding moricin have been reported and divided into two subfamilies on the basis of sequence similarity. Out of twelve moricin genes, four belong to subfamily *BmmorA* (A1 to A4) and eight belong to subfamily *BmmorB* (B1 to B8) [11].

A very limited amount of literature is available on the mechanism of pore formation in bacterial cell membrane by moricins from *B. mori*. Moricin contains N-terminal fragment (5–22 amino acids), which is amphipathic, α-helical and is the active site for antibacterial activity. The C-terminal region of moricin initially interacts with the surface of bacterial membrane and then permeability of the membrane is altered by N-terminal amphipathic a-helix. It is reported that the voltage-dependent pores could be formed through interaction between three or more amphipathic α-helices spanning a lipid membrane [3][33]. Moricins exhibit higher antibacterial activity against Gram positive bacteria than Gram negative bacteria.

2.1.4. Gloverins

Gloverins are glycine-rich AMPs of molecular weight 8–30 kDa and were first reported from haemolymph of giant silk moth *H. gloveri* pupae [34]. Gloverins possess flexible random-coil structure in aqueous solution. Gloverins from different insects are active against bacteria, fungi and virus while inactive against *E. coli* strains possessing smooth LPS. Reports suggest that the binding of gloverins to the inner part and Lipid A region of LPS is required for its activity. A conformational change occurs in the gloverins when they penetrate the hydrophilic regions of LPS layer and interacts with negatively charged hydrophobic regions made of lipid A [34][35]. BmGlvs binds to rough LPS leading to conformational transition of this peptide from random coil to α-helix which is believed to be the main

reason for pore formation on bacterial cell membrane [35]. Binding of gloverin to microbial surface is prerequisite for its conformational change and antimicrobial activity.

In silkworm *B. mori*, four genes encoding gloverins (namely *Bmglv1*, *Bmglv2*, *Bmglv3* and *Bmglv4*) were identified. All four gloverin genes were activated by *E. coli*, *B. subtilis*, and *Salmonella abortusequi* while the expression of gloverin genes was reduced when challenged with *S. aureus* [12]. The differences in the structure and compositions of bacterial cell wall among the bacterial strains may be reason for differential expression of gloverins [12]. BmGloverin2 (BmGlv2), along with other AMPs of silkworm, is reported to have synergistic antifungal activity against *B. bassiana* [36]. It is also reported that BmGLv2 inhibited growth of two Gram negative bacteria (*E. coli* JM109 and *Pseudomonas putida*) by enhancing the cell membrane permeability [37], resulting in disruption of the ion gradient between cytoplasm and external milieu and leading to cell death.

2.1.5. Attacins

Attacins are low molecular weight (20–23 kDa) AMPs that were first isolated from the haemolymph of *H. cecropia* pupae inoculated with bacteria [38]. On the basis of isoelectric points (pI: 5.7–8.3), attacins are divided into two groups, namely acidic (E and F) and basic (A to D). Attacin-A1 is reported to possess antimicrobial activity against *E. coli* and *Trypanosoma brucei* [39], whereas attacin-B has antibacterial activity against Gram negative bacteria (*E. coli* and *Citrobacter freundii*) and also antifungal activity (*C. albicans*) [40]. Attacins inhibit the bacterial growth by hindering the synthesis of outer cell membrane proteins viz., OmpC, OmpF, OmpA and LamB in bacteria or by altering the permeability of bacterial outer membrane [41][42].

2.1.6. Lebocins

Lebocins (32 amino acids) are proline-rich AMPs with O-glycosylated residues that were isolated from *B. mori* haemolymph challenged with *E. coli*. Lebocin family consists of four protein encoding genes (*Leb1*, *Leb2*, *Leb3* and *Leb4)*. The expression of lebocin genes is induced by LPS in haemocytes and fatbody [43]. Lebocin is reported to exhibit antimicrobial activity against Gram negative (*Acinetobacter* sp. and *E. coli*), Gram positive bacteria and fungi. Lebocin-B and Lebocin-C isolated from another lepidopteran insect, *Manduca sexta*, differ from *B. mori* Lebocin and are reported to possess antibacterial activity against *Serratia marcescens*, *S. typhimurium* (Gram negative); *S. aureus*, *B. cereus* (Gram positive) and *Cryptococcus neoformans* (fungi) [44].

2.2. AMPs Reported from Non-Mulberry Silkworms

In addition to the AMPs from the domesticated mulberry silkworm, *B. mori*, AMPs have also been identified from the nonmulberry silkworms belonging to the family Saturniidae, namely *Antheraea assamensis* (muga), *Antheraea mylitta* (tropical oak tasar), *Antheraea pernyi* (temperate oak tasar), *Antheraea yamamai* (Japanese oak tasar) and *Samia cynthia ricini* (eri).

An antifungal peptide named gallerimycin is reported to be isolated from the fatbody of *S. cynthia ricini*. A cDNA clone of *Scr-gallerimycin* (AB366558) gene encodes 74 amino acids and the gallerimycin protein has 6.21 kDa of

calculated molecular mass and 7.6 pKa [45]. A lebocin-like gene induced in the fatbody of eri silkworms upon challenging with bacteria was also reported. The cDNA of the lebocin-like gene encodes for 162 amino acids, which has homology with *B. mori* and *Trichoplusia ni* lebocin precursor proteins [46]. The cDNA clones of two Attacins (A and B) were reported from the fatbody of *S. cynthia ricini* challenged with bacteria. Both the attacin genes were coded for 233 amino acids and shared 98% identity at protein level, whereas at nucleotide level, 92% identity was reported [47]. Another attacin-like gene was reported from *A. pernyi* whose expression level significantly increased in fatbody upon *E. coli* infection [48]. A gloverin-like peptide of molecular mass 9.052 kDa active against Gram negative bacteria was isolated and characterized from muga silkworm immunized with *C. albicans* [49]. In *A. mylitta*, a glycine-rich antimicrobial peptide (GGGGGGHLVA) was reported to be active against MDR *E. coli* associated with urinary tract infections [50]. A tri-peptide AMP, NH_2-Gln-Ala-Lys-COOH (QAK) was reported to be isolated and purified from haemolymph collected from immunized *A. mylitta*. Acetylated and non-acetylated QAK peptide exhibited antibacterial activity against *E. coli* and *S. aureus* [51]. A cecropin-like peptide isolated from the Japanese oak silkworm, *A. yamamai* exhibited antimicrobial activity against Gram negative bacteria (*E. coli*, *K. pneumoniae* and *P. aeruginosa*), Gram positive bacteria (*S. aureus*, *Enterococcus faecalis* and *M. luteus*) and fungi (*C. albicans*), indicating its broad spectrum potential. The authors reported that MIC values against the tested Gram negative bacteria, Gram positive bacteria and fungal strain ranged between 1–2 µg/mL, 64–128 µg/mL and 64 µg/mL, respectively [52]. In another study, AMPs were isolated from haemolymph samples of *A. mylitta* and fractionated by HPLC. The fractions were assessed for their antibacterial activity against MDR bacteria including *E. coli*, *P. aeruginosa* and *B. pumilus.* It was found that fraction II exhibited antibacterial activity against *E. coli* (zone of inhibtion-9 ± 0.35 mm) and *P. aeruginosa* (6.5 ± 0.40 mm), whereas fraction III was active against only *B. pumilus* (7.5 ± 0.30 mm) [53].

3. Factors Affecting the Activity of AMPs

The AMPs isolated from natural sources are generally unstable, and it is therefore imperative to determine their stability before going ahead with application in various fields. AMPs are affected by several factors such as metal ions, temperature, pH and proteases. Metal ions affect the self-assembly and activity of AMPs, while pH may have varied effects depending upon the charge of the peptides [54]. The majority of the AMPs show poor stability at ambient temperatures. The stability of peptide is determined at different temperatures ranging from 4 °C to 90 °C incubated for minutes to days depending upon the application of AMP [55][56]. Upon incubation, AMPs are again evaluated for antimicrobial activity via the microdilution well method to determine the minimum inhibitory concentration (MIC). In the case of some AMPs, MIC increased with incubation time, while a few reports suggested that AMPs are stable even at higher temperatures and longer incubation times [55][56]. Proteases exert a highly destructive effect on AMPs. The effect of proteases on AMPs is assessed by exposure of the AMPs to proteinase K, chymotrypsin and trypsin. All three proteases are known to reduce the antimicrobial activity of AMPs as they act by degradation of AMP or by inhibition of the AMP activity [55][57].

In order to overcome the influence of different factors mentioned above, the identified bioactive peptides could be synthesized chemically through solid-phase peptide and peptide synthesis in solution. Chemical synthesis of AMPs

is advantageous over extraction of AMP from natural sources, as synthetic peptides are easy to modify as per the specific requirement [58][59]. More efficient analogues of AMPs may be prepared with better activity and stability. The stability of AMPs against proteases is reported to be improved by different chemical modifications such as capping (acetylation or amidation of residues), residue phosphorylation, cyclization, the addition of unnatural amino acids or D-amino acids and peptidomimetics [54]. In view of these reasons, designed AMPs have attracted many researchers for obtaining the desired effects. During the designing of AMP, the length, net charge, secondary structure, hydrophobic and amphiphilic properties of the peptide have to be considered to ensure its bioactivity. Additionally, the conjugation of fatty acid to side chain of peptide helps in improvement of stability, antibacterial selectivity and antibiofilm activity of AMPs [54][60].

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