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Review Article

Antimicrobial Peptides of Silk Worm: A Review

Shibani Kalita [1] and Palki Hazarika [2*]

[1]Department of Zoology, Gauhati University, Assam

[2]Department of Zoology, B. Borooah College, Guwahati-07, Assam

*Corresponding Author : shibanik203@gmail.com

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Abstract: Insect immunology and the antimicrobial peptides (AMPs), contributing to their innate immunity, serves as a very important field of research to conduct numerous studies. Among the insects, silkworms are one of the best-known groups that are used for scientific research since they have a highly developed and extremely responsive immune system. This study aims to understand the different classes of AMPs identified in silkworms by reviewing the works of various related studies and researches conducted previously. For performing various experiments and induction of AMPs, in most cases artificial immunization of microorganisms is carried out in the body of the silkworm, which in turn leads to the activation of the immune system producing specific AMPs in them. As per the study, commonly, about six different classes of AMPs have been identified that have different size, amino acid sequence and mode of action. They are Cecropin, Moricin, Defensin, Attacin, Lebocin and Gloverin. The structure and biochemistry of the AMPs has also been derived. Such researches and studies might not only open up opportunities leading to the discovery of newer AMPs but it also indicates a wide potential in leading to the discovery of natural antibiotics in the future that will not only be easily available, but also less toxic and cost effective.

Key words: amino acid, antibiotics, antimicrobial peptides, silkworm

Introduction

The silk industry and rearing of silkworms for the production of silk, known as sericulture, has been a very prominent agro-based industry since ancient times and still continues to expand continuously due to the high-end demand of raw silk and silk products all over the world. However, there is still a huge difference in the demand and production rates as *Bombyx mori*, commonly known as silkworms being a highly domesticated insect tends to largely come in contact with humans and their interferences, which in turn increases their susceptibility to infections. This increases the death rate of silkworms at various stages of its life cycle which in turn causes a reduction in production of silk. So as to develop silkworm with disease resistance capability and high quality silk, better understanding and knowledge of the various aspects of body defense mechanisms and immunity are necessary, so that infection and diseases of silkworm can be minimized while increasing its survival rates and silk production rates. Silkworm immunity can mostly be attributed to its innate immune system and its various components since it is the first in the line of defense against various microbial infections. The most important aspect of the innate immune system in case of lower organisms is the antimicrobial peptides (AMPs). They have low molecular weight and synthesized by the fat body, which is then released into the hemolymph upon infection by microorganisms (Bulet *et al.*, 1999). The AMPs break down the cell membrane of the microbes or produce by responses intracellularly (Nesa *et al.*, 2020). Numerous identified AMPs in silkworm that are responsible for carrying out a wide array

of antimicrobial functions. The AMPs of insects have become a major area of interest for the discovery of new antibiotics which is biologically active, less toxic and can combat with the present multi-drug resistance difficulty that exists currently (Buhroo *et al*., 2018). The present review work emphasizes to give a detail account of various AMPs of silkworm as well as the mode of action of those AMPs.

Antimicrobial Peptides (AMP)

Antimicrobial peptides are low molecular weight peptides or proteins with a potentially wide range of activity against bacteria and viruses. They are highly reactive chemically and have the potential to pave a new path in the field of drug discovery which in turn, might be crucial in drug designing and discovery of natural antibiotics. Along with low toxicity, low resistivity and high action potential, factors such as size, charge, hydrophobicity, amphipathic stereo geometry and self-association with the biological membrane are critically essential for the broad-spectrum antimicrobial activities of these molecules (Pushpanathan *et al*., 2013). Different studies conducted, have led to the belief that such molecules might not only be useful in bacterial and fungal infections but can also be path breaking in the treatment of cancer and viral infections. AMP expression and synthesis is initiated mostly by microbial infection and its production occurs mostly in the fat body, although some synthesis is also carried out by the hemocytes (Lavine *et al*., 2005). The most commonly seen structures of the peptides forming AMP are alpha-helix forms and beta sheets which might or might not include alpha-helical domains (Bulet and Stocklin, 2005). AMPs are positively charged peptides that are made of less than 100 amino acid residues (Buhroo *et al*., 2018). The amino acids lysine and arginine are most commonly found in them along with 30% or more hydrophobic residues (Andrea *et al*., 2007). AMPs are thus cationic in nature although some anionic varieties might also be present. Numerous AMPs have been reported in insects which are found in four groups: the á-helical peptides (e.g., cecropin and moricin), cysteine rich peptides (e.g., insect defensin and drosomycin), proline rich peptides (e.g., apidaecin, drosocin and lebocin) and glycine-rich proteins (e.g., attacin and gloverin) (Bulet and Stocklin 2005; Otvos 2000; Yi *et al.,* 2014). In Silkworm species mainly six different families of AMPs have been identified: Cecropin (Morishima *et al*., 1990; Taniai *et al*., 1992; Kato *et al*., 1993; Yamano *et al*., 1994; Kim *et al*., 1998; Yang *et al*., 1999; Cheng, *et al*., 2006; Hong *et al*., 2008), Attacin (Sugiyama *et al*., 1995), Lebocin (Hara and Yamakawa, 1995; Chowdhury *et al*., 1995; Furukawa *et al*., 1997), Moricin (Hara and Yamakawa, 1995; Furukawa *et al*., 1999), Gloverin (Cheng *et al*., 2006; Kaneko *et al*., 2007; Kawaoka *et al*., 2008) and Defensin (Kaneko *et al*., 2008; Wen *et al*., 2009).

Antimicrobial peptide of silkworm

Cecropin

Cecropins are á-helical in structure and one of the most common AMPs found in insects. There are mostly three major types of cecropins *viz.* cecropin A, cecropin B and cecropin D and they have 37, 35 and 37 amino acids respectively. Along with these three types, some other members of Cecropin and Cecropin-like peptides have been reported like Sarcotoxins, Stomoxins, Papiliocin, Enbocins and Spodopsins (Brady., 2019). Subtypes of this AMP family include Bmcec A1 (2 genes), B6 (6 genes), C (1 gene), D (1 gene) and E (1 gene) (Nesa *et al*., 2020). Cecropins have the ability to act on bacteria, both Gram-positive and Gram-negative along with fungi (Islam *et al*., 2016). Cecropins are highly potent and show action against malignant cells of some types of cancer including leukemia cell lines can also reduce infections by protozoans species like *Trypanosoma* and *Plasmodium* (Suttmann., 2008; Parachin and Fronco., 2014; Jiggins and Kim., 2005; Boisbouvier., 1998).

The first isolated cecropin was positively charged, lacking cysteine residues and isolated from the immunized hemolymph of a giant silkworm, *Hyalophora cecropia* (Steiner, 1981; Hultmark, 1982). Studies later confirmed its presence in *Bombyx mori* and *Antherea peryni* as well. Rupture of the cells of bacteria by cecropins is initiated by their binding to the lipids in the membrane that are negatively charged. This causes pores to form resulting in permeable cell membranes causing cell death (Gregory *et al*., 2008). Generally cecropins are have coiled forms but when placed in hydrophobic

conditions, it forms an alpha helix form. Antimicrobial activity of cecropin is influenced by the addition of amino groups at the C-terminus which is essential for the its interactions with liposomes (Nakajima *et al*., 1987)

Moricin

Moricin (encoded by multiple gene family) includes a group of cationic polypeptide chain (42 residues long) formed by amphipathic a-helix which consist of charged amino acids in the N terminus repeated after every interval of three to four amino acid residues (Chang *et al*., 2006; Tanka *et al*., 2012). The basisity of moricin is crucial for the resulting electrostatic interactions between negative bacterial surfaces to the positively charged peptides (Christensen., 1988). The antibacterial property of the peptide can be attributed to the charged amino acids which are repeated after every 3-4 residues in the N-terminus (Cociancich, 1993; Islam *et al*., 2016). 2-D Nuclear Magnetic Resonance (NMR) spectroscopy revealed a unique structure consisting of the á-helix with eight turns along its whole length with the exception of four residues and six residues at the N-terminus and C-terminus respectively (Axen *et al*., 1997).

Hara and Yamakawa in the year 1995 reported that the AMP, Moricin was first isolated from *Bombyx mori* that was immunized with *Staphylococcus aureus*. It revealed a 12 moricin-encoding gene forming three subtypes namely Bmmor (1 gene), moricin-like A (3 genes) and moricin-like B (8 genes) (Yang *et al.,* 2011) all of which synthesized the AMP moricin with C-termini containing positive charges and N-termini with both positive as well as negative charges. Moricin basically shows strong reactions against gram positive although in a few cases, activity against certain fungal groups like yeast has also been observed. Moricin acts in the same way as cecropin by changing membrane permeability resulting in lysis of the cell. In moricin, both the ends are extremely important as the C–terminus part is responsible for attaching to the cell membrane while the N-termini is the part performing the action.

Defensin

Defensing is also a low molecular weight peptide having a weight of about 4kDa and is positively charged. It has some conserved cysteine residues, about 6 of them, having disulphide bonds between them, which are common in most species (Xiao *et al*., 2004). It was first isolated from the flesh fly, *Saecophoga peregrine* which was bacteria immunized (Matsuyama and Natori., 1988). The consist of numerous types like phormicins, royalcins, sapecins and spodoptericis that have been identified from various insects belonging to the order Lepidoptera (Fujiwara *et al.,* 1990; Yamada and Natori., 1993). They are classified on the basis of the different types of spacing between the cysteine residues into five subdivisions - invertebrate, plant, α, β and θ subfamilies [Xiao., 2004] and their action is mostly seen on gram positive bacteria.

Defensing is encoded by a gene called Bm Defensin A which was first seen in *Bombyx mori* (Wen *et al*., 2009). This particular gene undergoes translation to produce a large peptide having an amino acid signal sequence at the N-termini of about 22 residues along with a pro-peptide of 34 amino acids as well as a mature peptide of 36 amino acids having a mass of 4 kDa consisting six conserved cysteine residues, having resemblance to defensin (Islam *et al*., 2016). It is synthesised in the hemocytes, head, silk gland, fat body as well as ovary and is associated with defence and metamorphosis (Wen *et al*., 2008). A similar AMP, BmDefB, was identified, which had almost similar amino acids like that of the cysteine residues in defensin it had about 27% homogenicity to Bm Defensin A, synthesized in the fat body and associated with activity against bacteria only (Yoichi *et al*., 2008). Bm Defens in changes the membrane permeability as well as potential affecting the respiration of cells, causing death of bacterial cells. The Bm Defensin perforates the membrane of bacterial cell causing entry of cytosolic K^+ ions, leading to change in membrane potential, reduced lysis of ATP and restriction of respiration, causing cell death (Stephen *et al*., 1993).

Attacin

Attacin is amphoteric antimicrobial peptide i.e both acidic and basic forms are present having a mass of 20-23kDa. The difference occurs in the types of amino acids in the acidic and basic amino acids, the acid form has more aspartic acid, arginine and isoleucine, the basic form has more of lysine,

tryptophan and glutamic acid (Islam *et al.,* 2016). Attacins are formed in an inactive state called pro-attacin that are converted to active attacins by an furin-like enzymes (Devi L., 1991).

The first attacin was isolated from the bacteria injected hemolymph of *Hyalaphora cecropia* (Hedengren *et al*., 2000). Studies have found that when cDNA of *Bombyx mori* was hybridized with *Hyalophoa cecropia* attacin, it coded attacin precursor protein and the resulting peptide had similarity in the amino acid residue of about 70.4, 68.3 and 18.8% with *Hyalaphora cecropia* attacins. Two subdomains present in the G domains in *Bombyx mori* as well as *H. cecropia* attacins showed that some amino acid residues are conserved during evolution that to has a role in antimicrobial activity (Sugiyama *et al*., 1995; Islam *et al.,* 2016). AMP synthesis increases on injecting or immunizing with *Escherichia coli* cells in *B. mori* larvae, occurring in the fat body and hemocytes (Carlsson *et al*., 1991). It breaks down the membranes of the bacterial cell by changing its permeability, by affecting formation of *E. coli* membrane proteins - OmpA, OmpC, OmpF and LamB (Nesa *et al*., 2020).

Lebocin

Lebocin is a proline-rich peptide with 32 amino acids residues having O-glycosylated threonine (15-Thr) and this is a characteristic feature for its antimicrobial activity (Hara and Yamakawa., 1995). Four types of lebocin has been identified and characterized - lebocin 1, 2, 3 and 4, which have homogenous peptide sequence, but dissimilar sugar moieties. Lebocin breaks down membranes of bacteria by affecting the lipid part of the plasma membrane, though it has not been completely proven. Attacin requires low ionic environment for its activity. Lebocin 3 in coordination with cecropin D is seen to highly effective against bacteria in *B. mori* (Nesa *et al*., 2020).

Gloverins

Gloverins are glycine rich AMPs, lacking cysteine and present only in the order Lepidoptera like *B. mori* and *Antheraea mylitta*. It was first found in the hemolymph of *Hyalaphora cecropia* (Axen *et al*., 1997). A gloverin like AMP has also been identified from the hemolymph of muga silkworm, *Antheraea assamensis*. Whole genome analysis of the genome of *B. mori* proved the presence of four different genes Bmglv (1-4) which are homogenous to gloverin of *Hyalaphora* (Yang *et al*., 2011). This gene has an NF-kB like motif that contains a binding site in the upstream region of the gene and studies have shown that these genes have embryonic expression and novel function (Yi *et al*., 2013; Nesa *et al*., 2020). Studies conducted by Axen *et al* and Xaio *et al* have reported that gloverin shows activity against gram negative bacteria (Axén*etal*. 1997; Xiao *et al*., 2012). Gloverins are seen to be active against *E.coli*, mutant strains of Df21f2, D21 and D22 that has Lipopolysaccharide (LPS); while it is does not act against *E. coli* strains having smooth LPS (Buhroo *et al*., 2018).

Discussion

Microorganisms are seen to become more and more resistant to various antibiotics used commonly leading to less efficiency in disease control and propagation. On the other hand, use of chemically synthesized drugs and antibiotics not only produce

Table 1. Brief overview of the various classes of AMP (Tanaka *et al* 2012; Xiao *et al,* 2004; Hara and Yamakawa,1995; Axen *et al*, 1997; Nesa *et al*, 2020).

Antimicrobial peptide	Active against	Mode of action
Cecropin (A1, B6, C, D, E)	Bacterial and human leukemic cell	Formation of pores on the plasma membrane
Moricin (A, B, Bmmor)	Gram positive bacteria	Distorts cell wall formation in fungi. In others membrane permeability is affected.
Defensin	Bacteria, fungi	Damages bacterial cell membrane
Attacin	Broad spectrum bacteria	Affects plasma membrane formation in growing cells.
Lebocin (1,2,3,4)	Bacteria	Forms leaky channels in the bilipid layer of plasma membrane.
Gloverin (1,2,3,4)	Broad spectrum bacteria, viruses but not much effective against yeasts.	Changes membrane permeability in pathogens and alters cellular function resulting in lysis and death.

Table 2. Primary structure of the AMP families (Islam *et al,* 2016; Yang *et al*, 2011; Tanaka *et al* 2012; Xiao *et al,* 2004; Hara and Yamakawa, 1995; Axen *et al*, 1997; Hedengren *et al*, 2000)

Name of peptide	Primary structure of peptide
Cecropin A	MNFVRILSFVFALVLALGAVSAAPEPRWKLFKKIEKVGRNVRDGLIKAGPAIAVIGQAKSLGK
Cecropin B	RWKIFKKEKMGRNIRDGIVKAGPAIEVLGSAKAI
Cecropin C	RWKLFKKIEKVGRNVRDGLIKAGPAIAVIGQAKSL
Cecropin D	GNFFKDLEKMGQRVRDAVISAAPAVDTLAKAKALGQ
Defensin	ATATATTTAGTTTGAGCCGTGTAACGAGTGAACATGAAGGGGGTTTATTAATTTT
	CACCCTAGTTCTAGTATACGTTGCTTCGACCTGGGCTTCACTAGATGCAGCTGA
	TGAAGTTCGAGTTATGAACGTGGAATCCCAAAGGCTGTTTCGATCCAGGAGGG
	CCTTACCATGTGCGAAGAAGAGCTGTGACAGCTGGTGCCGGAGATTGGATATT
	CCAGGCGGAGAATGTGTAACAAAGTGGAAATGCTCCTGTAATTGGATGCAGATT
	GACAAATAATAATATTTCTCTATCTCTCATCAGAACAATACTGTTGGTTATTACT
	TAAAATGTTTATCTTTTTAAAAAAAAAAAAAAAAA
Moricin	AKIPIKAIKTVGKAVGKGLRAINIASTANDVFNFLKPKKRKH
Lebocin 1	DLRFLYPRGKLPVPTPPPFNPKPIYIDMGNRY
Lebocin 2	DLRFLYPRGKLPVPTPPPFNPKPIYIDMGNRY
Lebocin 3	DLRFLYPRGKLPVPTPPPFNPKPIYIDMGNRY
Lebocin 4	DLRFLYPRGKLPVPTPPPFNPKPIYIDMGNRY
Attacin	QAGSFTVNSDGTSGAALKVPLTGNDKNVLSAIGSADFNDR
	HKLSAASAGLALDNVNGHGLSLTGTTRIPGFGEQLGVAGKV
	NLFHNNNHDLSAKAFAIRNSPSAIPNAPNFNTLGGGVDYM
	FKQKVGASLSAAHSDVINRNDYSAGGKLNLFRSPSSSLD
	FNAGFKKFDTPFYRSSWENNVGFSFSKFF
Gloverin 1	MYSKVLLSAALLVCVNAQVSMPPGYAEKYPITSQFSRSV
	RHPRDIHDFVTWDREMGGGKVFGTLGESDQGLFGKG
	GYNREFFNDDRGKLTGQAYGTRVLGPGGDST
	SYGGRLDWANENAKAAIDLNRQIGGSAGIEASAS
	GVWDLGKNTHLSAGGVVSKEFGHRRPDVGLQAQITHEW
Gloverin 2	MNTNLFYIFATTLVCVNAEVYGPSDYAEDYSISGQSS
	RRHPRDVTWDKQMGGGKVFGTLGQNDDGLFGKAGY
	NREIFNDDRGKLTGQAYGTRVLGPGGDSTNYGGRLDWANKNA
	QATIDLNRQIGGRSGMTASGSGVWDL
	DKNTHFSAGGMVSKEFGHKRPDVGLQAEIRHDW
Gloverin 3	MNSKLLFFIATVLVCV
	NAEVYRSPDYEEEYPIRG
	LFSKRHPRDVTWDTKMGGGKV
	FGTLGQNDDGLFGKAGYNREIFNDDRGQLTGQAYGTR
	VLGPGGDSTNYGGRLDWANKNAQAAIDINRQIGGRSGMTASGSGVWDL
	DKNTHISAGGMVSKEFGHRRPDVGLQAEIRHEW
Gloverin 4	MNSKLLYFFATVLVCVNAEVYWEDEEGYPVSGQFSKRHPRDVT
	WDKQVGGGKVFGTLGQNDDGLFGKAGYNREI
	FNDDRGKLTGQAYGTRVLGPAGDSTNYGGRLDWAN
	KNAEAAIDINRQIGGRSGMTATGSGVWDLDK
	NTRLSAGGMVSKEFGHRRPDVGVQAEFRHDW

numerous health hazards, side effects and complications but also are extremely expensive and take up a lot of time to be manufactured. As a result, the need for novel, natural and effective antibiotics are in high demand. AMPs seem to hold a great promise in this regard and numerous AMPs isolated from various insect families particularly silkworm, has already

been proved to have great therapeutic value. Nonetheless, much of this is yet to be explored as studies can lead to the discovery of more potent AMPs which might be effective against numerous diseases. Numerous research companies are conducting researches so as to develop AMPs at both preclinical and clinical stages. However, the commercial use and production of the peptides depend on their broad-spectrum activity; less probability for resistance development, easy production, less expense and more stability. Steps are being taken to enhance the quality of AMPs by using them in combination with other already existing medicines or slight modifications at gene level. However, the full potential of these peptides in insects including silkworm remains yet to be explored. Also, the knowledge of different types of AMPs and their mechanism of action will help us understand the workings of the immune system of such beneficial and economic insects leading to their better production.

Conclusion

Diversity of antimicrobial peptides in insects like silkworm which is economically important and easily domesticated has opened up paths that pave the probability of not only producing and discovering potent antibiotics but will also help in production of better and disease resistant breeds of silkworm species. This in turn will contribute to more silk production that will ultimately lead to economic benefits. With the advent in scientific studies and research technology, more importance can be given to the field of natural antibiotics in the future. Natural antibiotics will be a better alternative to those synthetic ones in use right now, as they are much less toxic with fewer side effects and also more cost effective in comparison to others. Antimicrobial peptides are yet to be explored fully in organisms and as such more and more studies in this field will hopefully be of immense benefit.

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