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- Review -

Mode of Action of Antimicrobial Peptides Identified from Insects

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Insects represent the largest class within the animal kingdom in terms of species number. Humans had been utilized insect in the broad area, including food, agriculture, industry, pharmaceuticals and so on. At present, insects are emerging as a leading group for identifying and extracting novel bioactive substances due to enormous number and a high nutritional value. Insects rely on a suite of systemic response to resist infection such as immune cells, hemocytes, activation of enzymes cascades, and antimicrobial peptide/protein. Among the substances, antimicrobial peptides (AMPs) are main components of potent antimircrobial innate defense system into the insect hemolymph. AMPs raise influential candidate as avenue to resolve the development of antibiotic-resistant microbial organism. Insect AMPs are classified into four main classes: cecropins, insect defensins, glycine/proline-rich peptides. Insect AMPs have been purified, over 150. In this review, AMPs derived from several insects were summarized including honey bee, dung beetle, butterfly and longicorn beetle. These peptides almost exhibited potent antimicrobial activities against human microbial pathogens without causing remarkable hemolysis to erythrocytes excluding melittin, and their mode of action(s) are based on disruption of the plasma membrane or fungal apoptosis. Therefore, study of insect AMPs is expected to be useful for designing novel therapeutic antimicrobial applications.

Key words : Antimicrobial peptide, antimicrobial activity, cecropins, defensin, insect

Introduction

All multicellular organisms possess some kind of inherent molecular or cellular defense system, to fight against pathogen invasion. The innate immune system present in all plants and animals primarily functions as the first line of defense to fight against infection caused by disease producing organisms [4]. Antimicrobial peptides (AMPs) are important components of the innate defense system and are ubiquitously found in a wide variety of organisms, including plants, insects, invertebrates and mammals [13, 14]. Among them, insects represent the largest class within the animal kingdom in terms of species numbers, including more than one million described species and an equivalent number of species unidentified [13]. Insects depend on a systemic response to combat infection that classified in to two main types. Constitutive defenses always exist and ready to act. The response was relied on insect immune cells, haemocytes, and rapidly activated enzyme cascades to defend against pathogen. Another defense is the induced response which consist mainly antimicrobial peptides. The simultaneous presence of several antimicrobial peptides acting in synergy can provide insects with a more powerful defense against harmful invader such as bacteria, fungi and protozoa [6]. The first insect AMP was isolated from the pupae of *Hyalophora cecropia,* and to date, 150 AMPs have been identified [56]. A single insect is known to produce about 10-15 peptides, with each peptide exhibiting a different activity spectrum. This review provides an overview on the classification and representation of insect AMPs.

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Classification of insect AMPs

In order to fight against the constant threat of microbial infection, higher organisms produce small, cationic AMPs, which are important components of mammalian innate immune response. Insect AMPs are broadly classified into four major groups: insect defensins, cecropins, and proline-rich/glycine-rich peptides (Table 1).

Insect defensins

Defensins, which form a part of the host's innate immune

Table 1. The classification of insect AMPs by origins

Origins	AMPs	Categories	Mechanism(s)	References
Drosophila melanogaster	Drosomycin	Insect defensins	Not determined	24
	*A*ndropin	Cecropins	Not determined	45
	*C*ecropin	Cecropins	Damage in the bacteria's outer and inner membrane	35
	*D*rosocin	Proline-rich peptides	Bacterial protein deactivation	5, 34
Apis mellifera	Royalisin	Defensin	Not determined	12
	*A*pidaecin	Proline-rich peptides	Bacterial protein deactivation	7
	*A*baecin	Proline-rich peptides	Bacterial protein deactivation	8, 43
Hyalophora cecropia	Cecropin A	Cecropins	Pore formation in membrane	48
	*A*ttacin	Glycine-rich peptides	Inhibition of biosynthesis of the outer membrane proteins	11
Sarcophaga peregrina	Sapecin	Insect defensins	Membrane permeabilization	49
Copris tripartitus	Coprisin	Defensin	Apoptosis in yeast	30
papilio xuthus	Papiliocin	Cecropins	Pore formation and apoptosis in yeast	17, 29
Hyalophora gloveri	Gloverin	Glycine-rich peptides	Inhibition synthesis of bacterial outer membrane proteins and increasing permeability of the membrane	3

system, is an ancient defense strategy used by multicellular organisms, including plants, animals, and even fungi, to control their natural microbial flora and to fight against various microbial pathogens [52, 53, 56]. These defensins consist of more than 100 members that display a wide variety of antibacterial, antifungal, insecticidal activities as well as anti-HIV-1 and/or inhibitory activity against tumor cells [9, 13, 24, 25, 52, 53]. Insect defensins are mainly active against Gram-positive bacteria, including *Micrococcus luteus, Aerococcus viridians, Bacillus megaterium, B. subtilis, B. thuringiensis*, and *Staphylococcus aureus* [56]. Insect defensins kill bacteria by forming channels in the bacterial cytoplasmic membrane. This has been demonstrated using sapecin [49], which induces microheterogeneity in the bacterial lipid membrane by interacting with cardiolipin, a major phospholipid, thus forming channels responsible for the biological activity [56]. In addition to bactericidal activity, in vitro fungicidal or fungistatic activity against *Candida* species, including *C. glabrata, as well as* antifungal activity against filamentous fungi, *A. flavus* and *F. solani*, have also been assessed [51]. A previous study on the yeast, *C. albicans*, suggests that the conserved helical structure of defensins is primarily responsible for its functioning, while other regions may contribute to binding to microorganisms [56]. A recent study has suggested that intracellular signaling pathways play a vital role in tolerance mechanisms of fungal pathogens against insect defensins [44].

Cecropins

Cecropins was first identified from the hemolymph of the giant silk moth *Hyalophora cecropia* as 'immune proteins'. Cecropin is a well-studied AMP that is synthesized in fat body cells and hemocytes of insects in response to bacterial infection [1, 35, 48]. Cecropins are synthesized as secreted proteins and become active only after the removal of signal peptides [56]. All cecropins exhibit significant sequence similarity and particularly comparable number of cationic residues which are considered crucial for its selective binding to the negatively charged bacterial membranes. Cecropins have been predicted to fold into a three dimensional structure consisting of two α-helices connected by a flexible hinge region. The N-terminal region of the helix is amphipathic, whereas the C-terminal region is largely hydrophobic [1]. This helix-forming ability of the cecropins upon membrane contact is responsible for the formation of bacterial membrane pores. Cecropins are potential targets for the production of enormous quantity of synthetic peptides due to their high selectivity, broad spectrum antibacterial activity combined with low cytotoxicity towards mammalian cells [1]. Furthermore, they have been shown to display varying degree of activity against both Gram-positive and Gram-negative bacteria as well as certain fungi, metazoan, and protozoan parasites, including *Plasmodium* [54, 56].

Proline-rich/Glycine-rich peptide

Proline-rich AMPs are found in various organisms, including insects, mammals, amphibians, crustaceans, and molluscs. They were first reported in honeybees and cattle [46]. Majority of the known proline-rich AMPs have been

isolated from various insect species belonging to orders Hymenoptera, Diptera, Hemiptera, and Lepidoptera, and are classified into two major types, namely short-chain and long-chain peptides [46]. The proline-rich peptides and the glycine-rich peptides are predominantly active against Gram-negative bacterial strains [46]. One of the most striking features of insect proline-rich AMPs is their conserved selective activity spectrum directed against Gram-negative bacteria, particularly Enterobacteriaceae, at very low micromolar concentration range. However, at low concentrations, most of the Gram-positive microorganisms remain unaffected [46]. The overall mechanism of action of proline-rich AMPs in insects has been elucidated using peptides such as pyrrhocoricin from the European sap-sucking bug [38], apidaecin from the honey bee [9], and drosocin from the fruit fly [24]. In insects, proline-rich AMPs inhibit bacterial protein folding by binding to DnaK, which ultimately leads to bacterial inactivation [5, 34, 46]. Unlike active peptides such as insect defensins, and cecropins, which kill microorganisms within minutes through non stereospecific, lytic/ionophoric mechanisms, apiadaecin, and drosocin take several hours to kill bacteria [13]. In addition to proline-rich AMPs, several antibacterial glycine-rich polypeptides have also been isolated from various insect species including Diptera, Lepidoptera, Hymenoptera, Coleoptera and Hemiptera [13]. The antibacterial activity of the glycine-rich peptides including attacins, sarcotoxins, coleoptericin and holotricin-2 like proline-rich AMPs is also restricted to a limited array of Gram-negative bacterial strains [39]. Attacins from *H. cecropia* has bacteriostatic activity and inhibits directly bacterial outer membrane to increase permeability [3, 10, 11, 56]. Another glycine-rich peptide sarcotoxin II, coleoptericin, diptericin have bactericidal activity [10].

AMPs from the insects and its mechanism

Honey bee

Honey bee venom is a mixture of over 20 compounds, including active peptides such as melittin, apamine [3], apidaecin [8], and abaecin [8, 43]. Among them, melittin is the principal active component (40 to 50%) of bee (*Apis mellifera)* venom and is a powerful lytic peptide [50]. It is shown to exhibit higher efficacy against Gram-negative than Gram-positive bacterial strains. Additionally, it has been shown that melittin also exerts marked antiviral as well as anti-fungal activity [33, 47]. Characteristically, melittin at low concentration, is shown to bind to membrane lipids of erythrocytes, resulting in hemolysis within some seconds [15]. Therefore, at high concentrations, it is considered as the principal active component for strong hemolytic activity [33]. Melittin forms a pore in the cell membrane by perpendicularly inserting itself into lipid by layers in an α-helical confirmation (Fig. 1) [16, 33, 39, 55]. Therefore, analogues such as leucine zipper motif with lower cytotoxicity towards mammalian cells and simultaneous non-selective activity is useful in melittin studies [40, 58]. In general, leucine zipper motif is used as a control peptide since it exhibits definite disruption of lipid membrane [33]. Using Annexin V, DAPI, and TUNEL staining, Park *et al.* [41] confirmed diagnostic markers of apoptosis in *C. albicans* when exposed to melittin. Consecutively, Lee *et al.* [33] further characterized the intracellular mechanism of melittin-induced apoptosis in *C. albicans* [33] further studied in details the intracellular mechanism underlying melittin-induced apoptosis in *C. albicans*. They suggested that melittin exerted its antifungal activity via apoptosis by increasing reactive oxygen species (ROS) production particularly hydroxyl (OH). They further reported that upon melittin exposure mitochondrial Ca^{2+} levels increases to a large extent, suggesting the mitochondrial perturbation or rupture by the decreased mitochondrial membrane potential (Fig. 1) [33]. In addition, melittin is also shown to suppress human immunodeficiency virus-1 (HIV-1) replication by interfering with host-cell directed viral gene expression at different dose concentrations. This suggests that the innate immune system includes an antiviral pathway for rapid defense against virus spread and replication [47].

Dung beetle

Coprisin is a 43-mer defensin-like peptide, which was isolated from the dung beetle, *Copris tripartitus* in 2009. It has three disulfide bonds at positions 3-34, 20-39 and 24-41 (Fig. 2A) [31, 56]. Since *C. tipartitus* spends most of its life in fecal material, it is considered that the antibacterial properties of coprisin protect this insect from continuous pathogen invasion [28]. Coprisin exhibits broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria such as *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, and S. epidermidis* [28] as well as antifungal activity against various fungal pathogens including several species from *Aspergillus* and *Candida* genus without any cytotoxicity towards human erythrocytes [30]. Coprisin possesses antibacterial properties and synergistic activities with antibiotics. Coprisin alone and in combi-

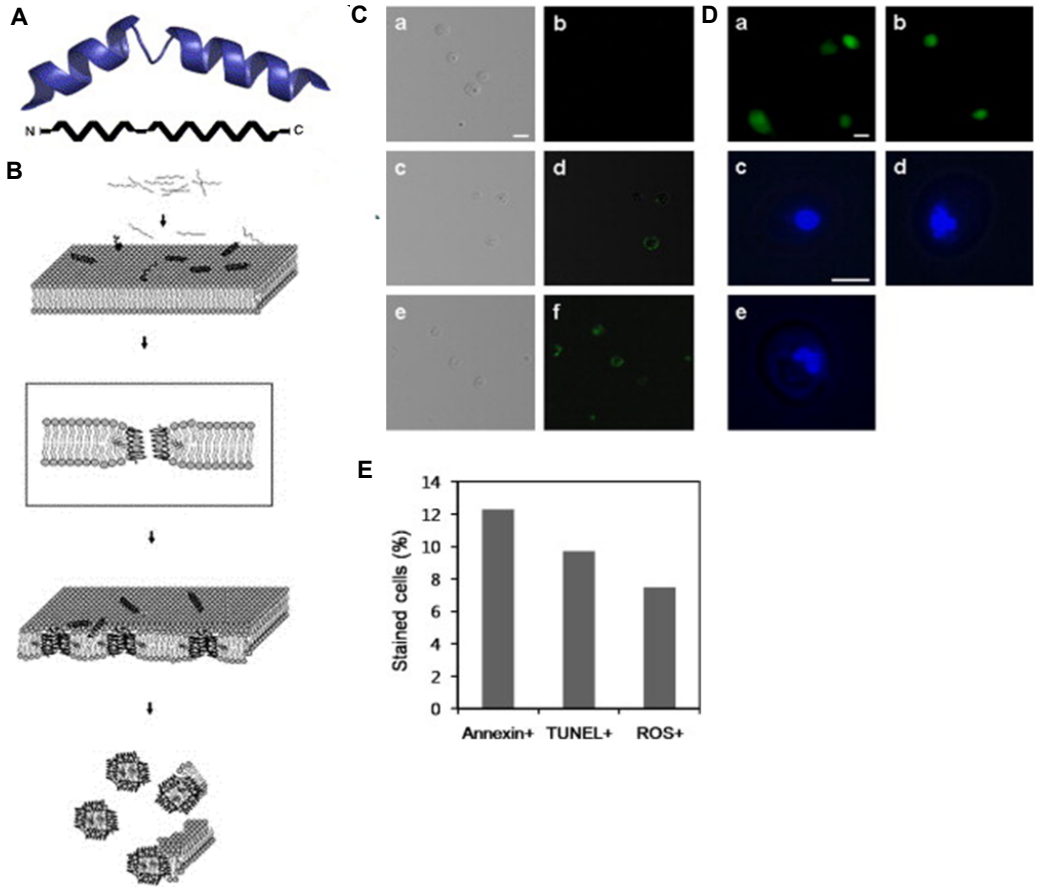

Fig. 1. (A) Melittin crystallographic structure [8]. (B) the interactions of melittin with the bacterial cytoplasmic membrane. [42]. (C) Phosphatidylserine externalization shown by FITC-annexin V staining in Melittin-treated *C. albicans* cells. Melittin (c and d), H2O2 (e and f) or not treated (a and b). Subpanels (a, c, and e) are phase-contrast micrographs. (D) DNA and nuclear fragmentation shown by TUNEL and DAPI staining in Melittin-treated cells. Melittin (a and d), H_2O_2 (b and e) or not treated. (E) Melittin-treated C. albicans showing apoptotic markers. Annexin+, TUNEL+, and ROS+ refer to the percentage of stained cells [41].

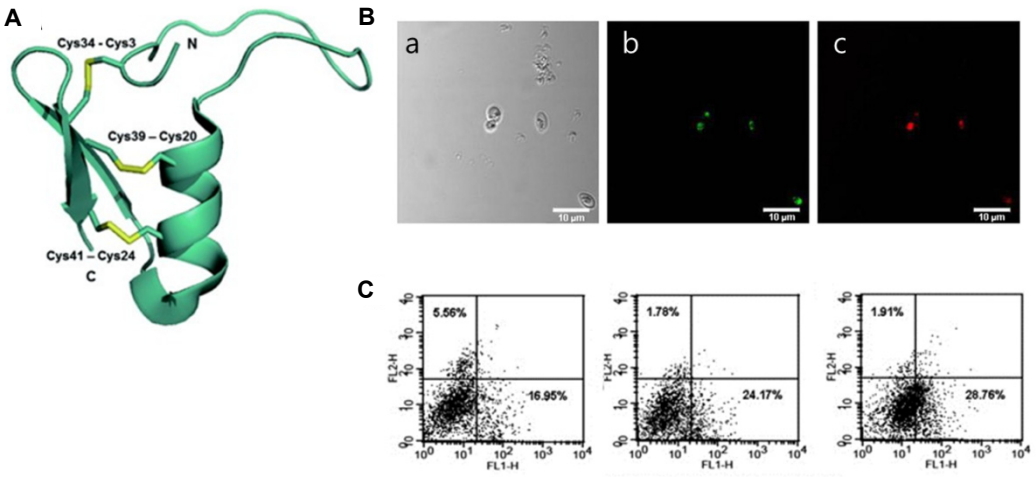

Fig. 2. (A) Solution structure of coprisin. The three disulfide bridges are shown in yellow [31]. (B) Confocal laser scanning microscopy of *C. albicans* cells treated with FITC-labeled coprisin. (a) Negative control under visible light, (b) cells treated with FITC-labeled coprisin and (c) cells treated with nuclear-staining dye. [30] (C) Phosphatidylserine externalization in *C. albicans* protoplasts induced by (a) negative (b-c) coprisin (1x, 2x, represitively) [30].

nation with antibiotics generate hydroxyl radicals, which are highly reactive oxygen forms and important regulators of bactericidal and antibiofilm activity [20]. In fungal cells, several membrane studies suggest that coprisin does not disrupt either cell plasma membrane of *C. albicans* or fungal model membranes [31]. On the other hand, some diagnostic markers of apoptosis such as phosphatidylserine externalization during early apoptosis and consecutively DNA fragmentation in late apoptosis were examined. The results confirmed that corpisin significantly induce apoptosis in *C. albicans* [31]. Coprisin additionally caused mitochondrial dysfunction and cytochrome *c* release/caspase activation as downstream events (Fig. 2) [31]. Additionally, coprisin also exhibits anti-inflammatory activity by suppressing binding of lipolysaccharides (LPS) to toll-like receptor 4, and subsequently inhibiting the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and nuclear translocation of Nuclear factor-kB (NF-kB) [27]. Coprisin exerts antibacterial effect when topically applied in both liquid and ointment bases to *S. aureus*-infected wounds in rats resulting in accelerated wound healing [30].

Swallowtail butterfly

Papiliocin is a novel 37-residue cecropin-like AMP, isolated by Kim *et al*. from the swallowtail butterfly, *Papilio xuthus* [23, 26]. It exhibits both potent anti-inflammatory activity [26] and antimicrobial activities against both Gram-positive and Gram-negative bacteria as well as fungi without cytotoxicity against human erythrocytes [23]. Two residues (Trp^2 and Phe^5) at the end terminal helix of papiliocin play a vital role in attracting it to the cell membrane of Gram-negative bacteria [56]. Papiliocin is a potent peptide antibiotic suitable for treating endotoxin shock and sepsis caused by Gram-negative bacterial infections [22]. A study on anti-fungal mechanism of paliliocin against *C. albicans* showed that papiliocin effectively perturbed the fungal plasma membrane by forming pores on the model membrane mimicking the outer leaflets of *C. albicans* within minutes [29]. Papiliocin formed pores on the model membrane mimicking the outer leaflets of the *C. albicans* within minutes [29]. Hwang *et al.* suggested that ROS accumulation and mitochondrial membrane damage are responsible for papiliocin-induced fungal apoptosis (Fig. 3C) [17]. They also examined novel antimicrobial mechanism of papiliocin in *C. albicans* and noted several apoptotic events, such as phosphatidylserine flip-flop, chromatin condensation and DNA fragmentation (Fig. 3D) [17]. Although the exact mechanism is still unclear, it will enable effective clinical approaches in treating human fungal disease.

Longicorn beetle

Longicorn beetile belonging to the insect family Cerambycidae of the insect order Coleoptera is a pest of mulverry and fig tree in East Asia [37]. Psacotheasin have been derived

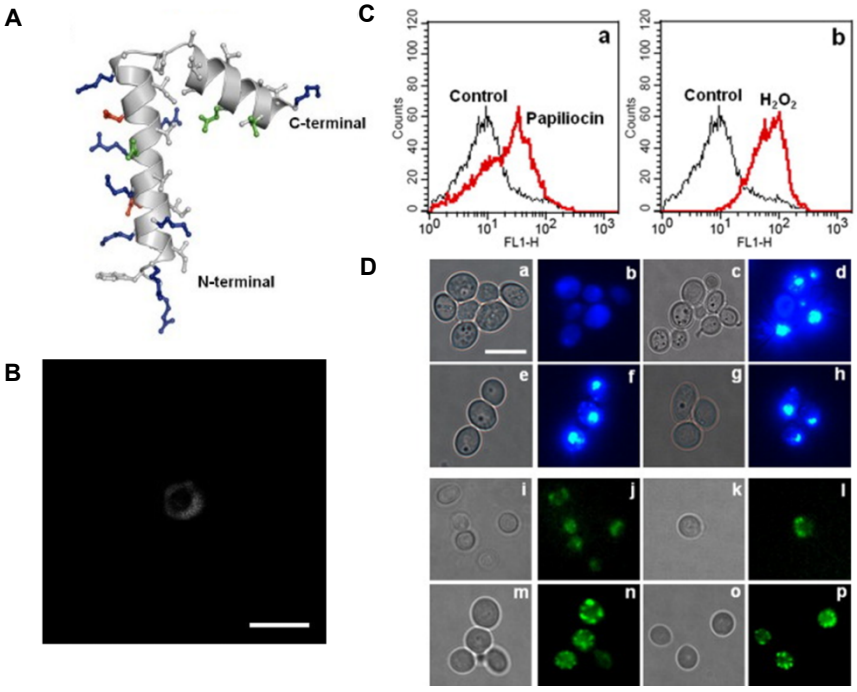

Fig. 3. (A) Ribbon diagram of the lowest structures of (C) papiliocin [26]. (B) Confocal microscopy of *C. albicans* treated with FITC-labeled papiliocin [29]. (C) ROS generation by papiliocin and H_2O_2 [29]. (D) DNA and nuclear fragmentation shown by DAPI and TUNEL staining [17].

from larvae of the yellow-spotted longicorned beetle, *Pscothea hilaris* using cDNA encoding. The primary structure is characterized by a knottin-like cysteine motif and exerted potent activities against both Gram-positive and Gram-negative bacterial strains [21]. The peptide exerts an antifungal activity without inducing hemolysis [18]. Although mode of action(s) against bacteria strains was remained unknown, the mechanisms in *C. albicans* were reported. Psacotheasin which exerts a potent antifungal activity by pore formation in the membrane eventually lead to fungal cell death [18]. Moreover, ROS accumulation, specifically hydroxyl radicals, triggers mitochondrial depolarization. Co-staining of annexin V-fluorescein isothiocyanate (FITC) and propidium iodide, and TUNEL and 4',6-diamidino-2-phenylindole (DAPI) assays exhibited that ROS-induced fungal cell death is associated with apoptosis. Finally, intracellular metacaspase, evidence of hallmark apoptosis, was activated. This corroborated that the antifungal activity against *C. albicans* is exerted by dual mechanisms which apoptotic mechanism and membrane active mechanism [19]. Overall, the above studies suggest that AMPs from centipede could emerge as a model molecule, which targets the cytoplasmic membrane or apoptotic pathway and provides a novel remedy.

Conclusion

In this review, we provide a complete overview of various kinds of insect AMPs and their underlying molecular mechanism to fight against bacterial and fungal infection (Fig. 4, 5). While insect AMPs are recognized to have novel therapeutic applications as alternatives of conventional antibiotics, more in-depth studies are required to develop and optimize these peptides as potential anti-infective drugs.

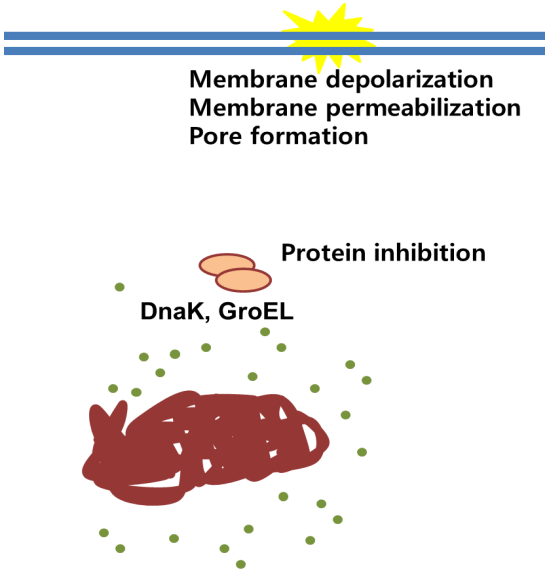

Fig. 4. The mode of actions of insect AMPs in bacteria.

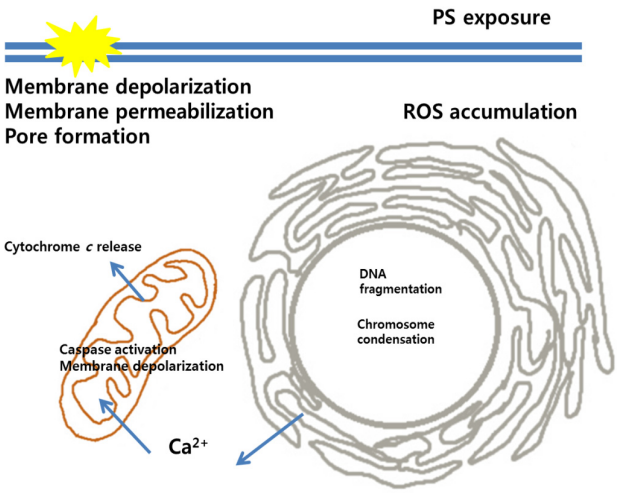

Fig. 5. The mode of actions of insect AMPs in yeast.

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초록 : 곤충 유래 항균 펩타이드의 작용 기작

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지구상에 존재하는 전체 동물 중 가장 큰 부분을 차지 하고 있는 곤충은 예로부터 인간의 식품, 농업, 산업, 의약 등의 일상 생활에 이용되어 왔다. 많은 수와 높은 영양학적 가치로 곤충의 생리활성물질이 미개발 생물자원으로 재평가 되고 있다. 곤충은 면역세포, 곤충 혈구세포, 효소들의 연쇄반응 혹은 항균 단백질/펩타이드 같은 방법으로 외부의 감염에 저항성을 가지게 된다. 항균 펩타이드는 곤충의 혈림프의 선천성 면역 시스템 중 주요한 성분중의 하나로 항생제 내성 균주의 출몰이 빈번하게 일어나 해결책으로 새로운 항생제 개발이 시급한 시점에서 유력한 후보물질로 주목 받고 있다. 곤충 유래 항균 펩타이드는 150개가 넘게 분리되었으며 크게 세크로핀, 디펜신, 글라이신/프롤린이 풍부한 펩타이드로 이루어진다. 이 논문에서, 향균 펩타이드를 생산하는 여러 곤충 중에서 벌, 소똥구리, 울도하늘소, 나비 그리고 울도하늘소에서 얻을 수 있는 펩타이드의 종류 그리고 작용 기작에 대해 알아보았다. 이 펩타이드들은 항균효과를 가지고 있으며 멜리틴을 제외하고 적혈구의 용혈 현상이 나타나지 않고 주로 세포막을 붕괴시키거나 세포자살기작을 유도하여 병원성 미생물의 성장을 억제한다. 곤충 유래 펩타이드와 같은 생리활성물질이 그 활용 가능성의 면에서 엄청난 가능성을 가지고 있어 이에 대한 연구는 앞으로 더 주목을 받을 것이다.