Applications of AMPs in Packaging

Subjects: Materials Science, Biomaterials | Agriculture, Dairy & Animal Science | Agricultural Engineering
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Antimicrobial Peptides can be defined as the molecules of the innate immune system present in all life forms, ranging from bacteria to human beings. The innate immune system is a defence system working non-specifically against injury or infection in the barrier surface. AMPs are composed of a sequence of amino acid ranging from 5 to 50 chains, usually L-amino acids.

Keywords: antimicrobial peptides ; synthesis ; isolation ; harvesting ; food packaging ; active packaging ; impregnation

1. Introduction

Most of the AMPs are positively charged peptides (due to a high content of arginine and lysine residues) that are capable of destroying pathogens directly. The benefit of using peptides as antimicrobial agents is that it shows effectiveness against Gram-positive and Gram-negative bacteria, including multidrug-resistant bacteria [1][2]. Most of the AMPs function by disrupting the physical structure of the microbial membrane and/or by targeting intracellular bodies [3]. AMPs have proven to have the ability to fight against various health issues due to the presence of immunomodulatory properties [4]. AMPs are effective in preventing skin cancer, treating wound infection and limiting autoimmune diabetes [5][6][7]. Promising results of AMPs have suggested its use in novel therapeutic drugs [3].

AMPs can be synthesised from animals, plants, protozoa, fungi and bacteria. Among all the species, insects are found to be the largest living entity of AMPs because of their excellent ability to tolerate changes and their resistant property towards pathogens [8]. Insects are considered an excellent source of AMPs because of their huge biodiversity [9]. The characterisation of the innate immune system of insects depends on cellular immunity, which includes phagocytosis, and humoral immunity, which includes the excretion of proteins and peptides [10]. The way an insect responds to fight infections can be categorised in two categories: The constitutive response, which is perpetually ready to fight, and the induced response, which takes 1–3 h to generate [11]. The first insect AMP derived was Cecropin from lepidopteran Hyalophora cecropia [12][13]. More than 3000 AMPs are known till now, among which 2301 are isolated from animals. Hermetia illucens (the black soldier fly) is found to be the most propitious source due to its resisting ability against a huge range of pathogens [14]. Hermetia illucens has a strong immune system as it feeds on decaying organism and manure, which contains high amount of pathogens. **Figure 1** represents the history of antimicrobial peptides derived from insects.

Figure 1. A history of antimicrobial peptides derived from insects.

In recent years, AMPs have gained a huge interest in various fields due to their low toxicity and ability to fight a wide spectrum of pathogenic microbes. One is food packaging, involving studies based on the incorporation of AMPs into packaging material that will be in direct contact with food or interact with the headspace to resist microbial contamination

in food [15]. The advantages of using antimicrobial peptides in food packaging over antimicrobial agents [16]: Improves food safety by preventing the development of resistant strains of microorganisms; Prohibited use of some of the antimicrobial agents due to toxicological reasons; Distinctive mode of action of antimicrobial peptides with effective results.

2. Categorisation of Antimicrobial Peptides

The antimicrobial activity of AMP depends on peptide length, charge and hydrophobic nature, and this leads into grouping of AMPs based on some dissimilarities in attributes. Based on the structure, AMPs can be categorised into four types: α helical peptide, cysteine-rich peptide, proline-rich peptide and glycine-rich peptide [17]. Some studies have also discussed classification into α helical peptide and β sheet peptide. In α helical peptides, cysteine is present, which forms an intracellular disulphide bridge, and in β sheet peptides, a disulphide bond is found, which is useful in stabilizing the structure as well as crossing the cell membrane [18][19]. According to [20], AMPs can be classified based on the electrostatic charge into cationic (which have positive charge) and non-cationic (consists of negative charge) peptides. Based on the mode of action, peptides are divided into two categories: the membranolytic mechanism and non-membranolytic mechanism [21][22]. AMPs derived from insects are divided into three types: Defensins, Cecropins and peptides with an overrepresentation of Proline and/or Glycine residues [23]. In a study 50 genes encoding putative AMPs were identified from Hermetia Illucens, among which 6 attacins, 26 defensins, 7 cecropins, 10 diptericins and 4 knottin-like peptides were recorded [24]. **Table 1** includes the classification of antimicrobial peptides.

Table 1. The classification of antimicrobial peptides.

S.No.	Criteria for Classification	Peptides	Description	Examples	References
1.	Structure	α helical peptide	Intramolecular disulphide bridge is formed by the cysteine	Cecropins	[18]
		Cysteine rich peptide	Peptides with cysteine residues	Defensis	[9]
		Glycine rich peptide	Consists of 14% to 22% glycine residues	Attacins	[25]
		Proline rich peptide	Composed of 14–39 amino acids and contains proline residues	Drosocins	[9]
		β sheet peptide	Consist of a disulphide bond, which helps in stabilizing the conformation	Defensis	[26]
2.	Mode of action	Membranolytic	These peptides enter the microbial cell wall by disruption	Scolopendin 2	[27]
		Non-membranolytic	Peptide that enters the cell by endocytosis	Scolopendin 1	[27]
3.	Electrostatic charge	Cationic	Peptide with positive charge	Cecropins	[28]
		Non- cationic	Peptides with negative charge and isolated from mammalian epithelia	Enkelytin	[28]

3. Synthesis of Antimicrobial Peptide

AMPs are found to be useful in various fields of interest, which has led to the study of different methods for the efficient synthesis of peptides from the source. At present, there are three methods of synthesising peptides, which are chemical synthesis, enzymatic synthesis and synthesis using recombinant DNA technology. A brief comparison between all the three mechanism is discussed in the **Table 2** .

Table 2. A comparison between all the three synthesis techniques.

S.No.	Type of Synthesis	Advantages	Challenges	References
1.	Chemical	Easy separation from side products and impure compounds.	Toxic byproducts and low yields.	[29][30]
2.	Enzymatic	Helpful in synthesis of short chain peptides. It also has good stereo selectivity.	It becomes challenging while synthesis of long chain peptides. Low productivity and high cost of catalyst.	[30][31]

S.No.	Type of Synthesis	Advantages	Challenges	References
3.	Recombinant DNA Technology	Convenient for large scale production.	Takes more time due to lengthy process.	[32][33]

The synthesis of peptide using chemical reagents to conciliate peptide bonding was performed for the first time 50 years ago. Chemical synthesis of AMPs can be performed in two ways: synthesis in a solution and solid-phase peptide synthesis (SPPS). Synthesis in a solution is implemented by dissolving all the components in the solution [34]. While in SPPS, an insoluble solid is attached to a N-α-derivative of an amino acid through a linker. Then the protecting group (N-α group) is removed, followed by washing the complex with the solvent. Coupling of the second amino acid (containing N-α group) to the complex, either in the presence of activator or as a preactivated species, takes place. A solvent is used to wash the oligopeptide–linker–support complex, and unreacted matter is discarded. Repetition of withdrawing the protection group and the coupling cycle is performed until the required sequence of amino acids is achieved. Using the cleavage agent, peptide is generated in the form of free acid or amide (**Figure 2**) [35].

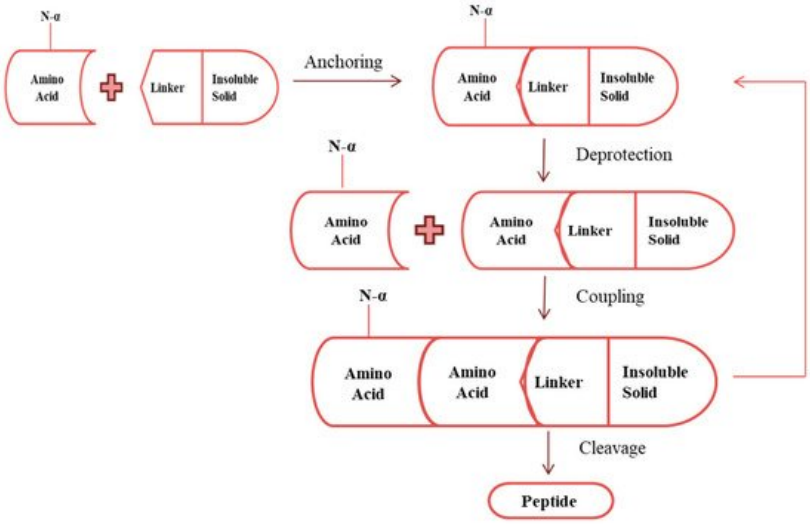

Figure 2. A representation of chemical synthesis of peptides via SPPS.

Enzymatic synthesis of peptides uses enzymes, such as pepsin, papain, trypsin and others, to conciliate a peptide bond. Enzymatic synthesis is better than chemical synthesis as it can be used to manufacture small peptides (2–5 oligomers), but it has a drawback that it is ineffective in the synthesis of long sequences [36]. It can be implemented in two ways: (i) reverse hydrolysis, which involves reversibility principle under the conditions that equilibrium is deliberately shifted towards peptide formation (**Figure 3**), and (ii) transpeptidation, which involves breaking the peptide bond and forming an active acyl-enzyme intermediate that further results in peptide formation in the presence of nucleophile (**Figure 3**) [31][37][38].

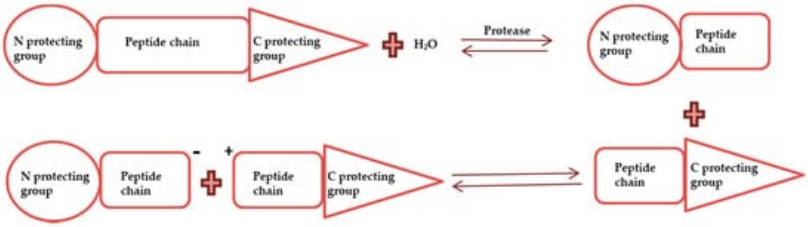

Figure 3. A representation of enzymatic synthesis of peptide using reverse hydrolysis.

This mechanism involves cloning and gene expression, which produces a recombinant peptide, and E.coli is considered the most common host [39]. Synthesis of peptides using recombinant technology is considered the most economical method for massive production [40].

4. Harvesting of Antimicrobial Peptides from Hermetia illucens

Hermetia illucens are present ubiquitously, and since it survives in highly contaminated environment, it is capable of producing a huge spectrum of antimicrobial peptides. H. illucens consume cheaper feed and also grow faster, which is a highly recommended attribute for AMP isolation [41][42]. Defensins, cecropin, attacin and lysosymes are considered as

representative antimicrobial peptides of H. illucens [9]. The mechanism of harvesting AMP from insects involves some important fundamentals, as discussed below. General steps in AMP harvesting are mentioned in the flow chart (**Figure 4**).

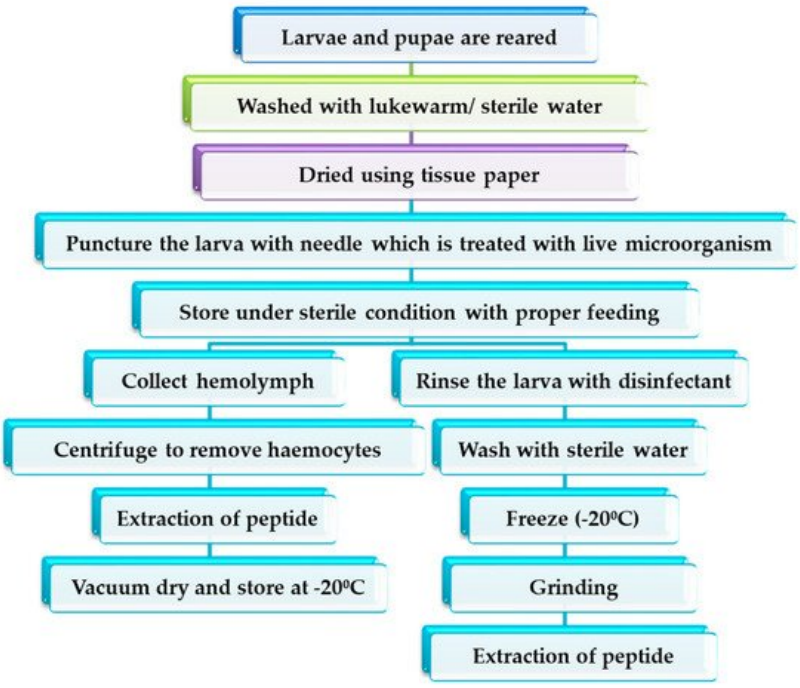

Figure 4. The general methodology for harvesting of AMP from *H. illucens*.

In the process of insect formation from larva, several growth and development stages occur, which are usually known as instar. There is no specification for considering any specific instar larva for the AMP collection. However, in a study, the lowest transcription level was observed during first-instar larval stages and the highest transcription level during the fifth-instar larval stage [43]. At the fifth stage, growth is completed, and metamorphosis is initiated [44]. Therefore, considering fifth-instar larva will be beneficial in a higher rate of synthesis of peptides. If the larva is to be used as a feed, prepupa is better than larva because of its high chitin content [45]. Rearing of larva varies from study to study, depending on the criteria of the research. In an investigation, researchers observed that aqueous extracts of black soldier fly larvae fed a cellulose diet and protein-rich diet showed antimicrobial activity against Gram-negative bacteria, while larvae fed chitin, cellulose, bacteria and plant oil had higher inhibitory activity against Gram-positive bacteria [24]. Chloroform extract of larva showed the highest inhibitory activity when fed lignin, bacteria and plant oil diets. In contrast, in a report by [46], larvae were starved after inoculating the Lactobacillus sp . to study the antimicrobial activity of H. illucens extract. At the industrial level, cottonseed press cake is a good choice as feed for H. illucens due to its sustainability and cheaper cost [47].

Microorganisms are injected into the body of a larva or haemolymph using a needle. Inoculated microorganisms should be in the stationary phase because natural microbial infection in insects occurs in the stationary phase [11]. The microorganism is injected either to isolate and analyse the characteristics of the AMP formed for that specific microorganism or to examine the influence of immunisation on the antimicrobial activity towards different microorganisms. Several reports have claimed the antimicrobial activity of AMPs harvested from H. illucens, among which a few are mentioned in **Table 3** . In a study, AMPs from H. illucens were purified and investigated. AMPs were harvested from three samples: larvae that were immunised during the last instar stage, unimmunised larvae and mutilated larvae. From all the samples, haemolymph was collected and tested against E.coli and methicillin-resistant Staphylococcus aureus. Among all the samples, larvae that were immunised during the last instar stage showed the highest antimicrobial activity, while the mutilated group showed the least [42]. Zdybicka-Barabas observed that M. luteus -immunised haemolymph of H. illucens larvae showed inhibitory activity against Gram-positive bacteria, whereas E.coli -immunised larvae showed activity against Gram-positive and Gram-negative bacteria. Additionally, it was concluded that synthesis of AMPs vary depending on the bacteria used for the immune challenge [48]. Usually, when AMPs are treated with enzymes, such as trypsin or chymotrypsin, it results in a loss of antimicrobial activity. However, when H. illucens are immunised with L. casei and given the same treatment, they remain unaffected [46].

Table 3. Antimicrobial activity of peptides extracted from *H. illucens*.

S.No.	Source	Peptide	Harvesting Technique	Microorganisms Inhibited	References
1.	Haemolymph		Solid phase extraction	Helicobacter pylori	[49]
2.	Crushed larva	stomoxynZH1	RNA extraction using Trizol	*S. aureus*, *E. coli*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*	[50]
3.	Grounded larvae		Maceration	*E. coli*, *P. fluorescens*, *M. luteus* and *B. subtilis*	[23]
4.	Larvae		Directly used as feed for piglets	*Lactobacilli*, *D-streptococci*	[51]
5.	Lyophilized larvae		Homogenised and extracted with acidic methanol	*Methicillin resistant Staphylococcus aureus*	[52]
6.	Haemolymph	cecropin-like peptide 1	Solid-phase extraction and reverse-phase chromatography	*E. coli*, *Enterobacter aerogens* and *Pseudomonas areuginosa*	[53]
7.	Grounded larva		Maceration using methanol	*Salmonella* and *E. coli*	[54]
8.	Larvae whose digestive tract was removed. Followed by treating with liquid nitrogen	Hidefensin-1, Hidiptericin-1 and HiCG13551	TRIeasy- RNA isolation kit	*Streptococcus pneumonia*, *E. coli* and *Staphylococcus aureus*	[55]

For AMP extraction from larvae, either haemolymph is collected, or the whole larva body is utilised. Hetru and Bulet collected the haemolymph in a precooled tube consisting of protease and melanisation inhibitors. Additionally, for small-sized insects, they froze the insects using liquid nitrogen, followed by grinding. Then, the powder was placed in water acidified with trifluoroacetic acid embodied with protease and melanisation inhibitor, followed by centrifugation and filtration [56]. Similarly, in most of the research, it was found that haemolymph is collected in ice-cold tubes consisting of phenylthiourea crystals to avoid coagulation. After collection, haemolymph is centrifuged to remove haemocytes [42]. Tabunoki proposed a method for collecting haemolymph from larva using a collection tube. This tube was prepared by making a hole at the bottom of a 0.5-mL centrifuge tube and then placing this tube in a 1.5 mL tube [57]. Samples were collected from grounded larvae in a solution of methanol, water and acetic acid for characterisation. Additionally, the method to isolate RNA was considered as follows: dissect larvae in ice-cold PBS, soak foregut/ midgut, hindgut and salivary glands in lysis buffer, and then freeze [23]. Another protocol was reported in which larvae were ground and suspended in 20% acetic acid solution, followed by boiling and then centrifugation [46].

5. Applications of Antimicrobial Peptides in Active Packaging

Food packaging is a phenomenon of holding food in a material that fulfils four basic functions: containment, convenience, communication and protection. Containment refers to the fact that packaging material should be compatible with the food in terms of holding/carrying the product. Convenience means that the packaging material should be easy to use by the consumers. Communication means that the packaging material should include all the mandatory information required to be known to the consumer. Additionally, protection focuses on the prevention of undesirable changes in the physical, chemical and biological attributes of the food. Packaging can be classified based on the type of material, type of use and novel packaging. Based on the material packaging, there are four types: paper and paperboard, plastic, metal and glass. Based on usage, packaging has three types: primary, secondary and tertiary. Novel packaging includes innovative packaging, such as active packaging and intelligent packaging. Active packaging is defined as a packaging system that interacts with the surroundings of food to extend its shelf life [58]. Active packaging helps in achieving the selective permeation. Methods to incorporate active components into the packaging are coating, micro perforation, lamination, co-extrusion, or polymer blending [59]. Active packaging includes oxygen scavenger, carbon dioxide scavenger and emitters, ethylene scavenger, antimicrobial packaging and antioxidant packaging. Intelligent packaging provides information about the packaged product by sensing some of the properties of food, it includes indicators, sensors and data carriers [60]. In recent years, active packaging has attained a lot of attention due its capability to fulfil consumers demand as well as its positive impact towards biodegradability and sustainability.

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