Comprehensive REVIEWS in Food Science and Food Safety

# Bioactive Peptides: Synthesis, Properties, and Applications in the Packaging and Preservation of Food

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**Abstract:** Bioactive peptides are protein fragments which have a positive impact on the functions and conditions of living beings. Peptides have shown several useful properties for human health, including antimicrobial, antifungal, antiviral, and antitumor activities. These compounds are produced by almost all species of life. However, they are produced in limited quantities in nature. As a result, researchers have tried to synthesize bioactive peptides to study their properties and applications in various areas. Among their applications in food preservation, peptides have been incorporated into packaging materials. This review begins with a brief description of the methods used for the synthesis, purification, and characterization of peptides. Also, the main bioproperties and mechanisms of action of peptides are discussed. Finally, some applications of peptides are presented, especially their use in active packaging, their effects on the polymeric matrix, and peptide migration.

## Introduction

Food safety is a growing concern of great importance worldwide. Recently, the estimated costs of diseases caused by foodborne pathogens was about $152 billion in the United States (Scharff 2010), and it is estimated that in the United States alone about 47.8 million illness cases, 128000 hospitalizations and 3000 deaths will be caused by foodborne pathogens in 2011.

The consumption of processed foods with chemical preservatives has led to increased consumer concern and the demand for more natural and minimally processed foods. As a result, researchers have shown a growing interest in natural antimicrobial agents such as certain peptides.

Bioactive peptides are defined as specific protein fragments that have a positive impact on the functioning or conditions of living beings, thereby improving their health (Korhonen and Pihlanto 2006). The beneficial effects are attributed to different properties found in peptides such as antimicrobial (Reddy and others 2004; Rajanbabu and Chen 2011), antioxidant (Sarmadi and Ismail 2010), antithrombotic (Wang and Ng 1999), anti-hypertensive (Erdmann and others 2008), and immunomodulatory activities (St Georgiev 1990; Gauthier and others 2006), among others.

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Peptides with antimicrobial properties are used as the first chemical barrier against microbial attack, being synthesized in response to bacterial infections. They are produced by almost all species of life, from microorganisms, plants and animals, to humans (St Georgiev 1990; Hancock and Diamond 2000). In animals, antimicrobial peptides are produced mainly in those tissues exposed to adverse conditions such as skin, eyes, and lungs, which are more likely to be in contact with microorganisms (Zasloff 2002; Papo and Shai 2003).

More than 700 antimicrobial peptides have been reported, showing significant variations with respect to their sequence, length, and structure (Papo and Shai 2003).

Antimicrobial peptides have found many applications, including those in biomedical devices, food processing equipment, and food preservation.

In food preservation, peptides can be incorporated into materials to create antimicrobial packaging (Appendini and Hotchkiss 2002). In this way, antimicrobial packaging plays an important role in maintaining the safety and quality of food, since the aim is to prolong food shelf life and to reduce bacterial growth on the product surface (Soares and others 2009a). This type of active packaging interacts with the product and/or the headspace inside to reduce, inhibit, or retard the growth of microorganisms that may be present (Soares and others 2009b).

This review highlights the main methods of peptide synthesis and noteworthy peptide bioproperties. Also, specific peptide applications in food preservation are reviewed, focusing on their incorporation in polymeric matrices. Finally, the effects of peptide incorporation on packaging characteristics as well as their migration into food are discussed.


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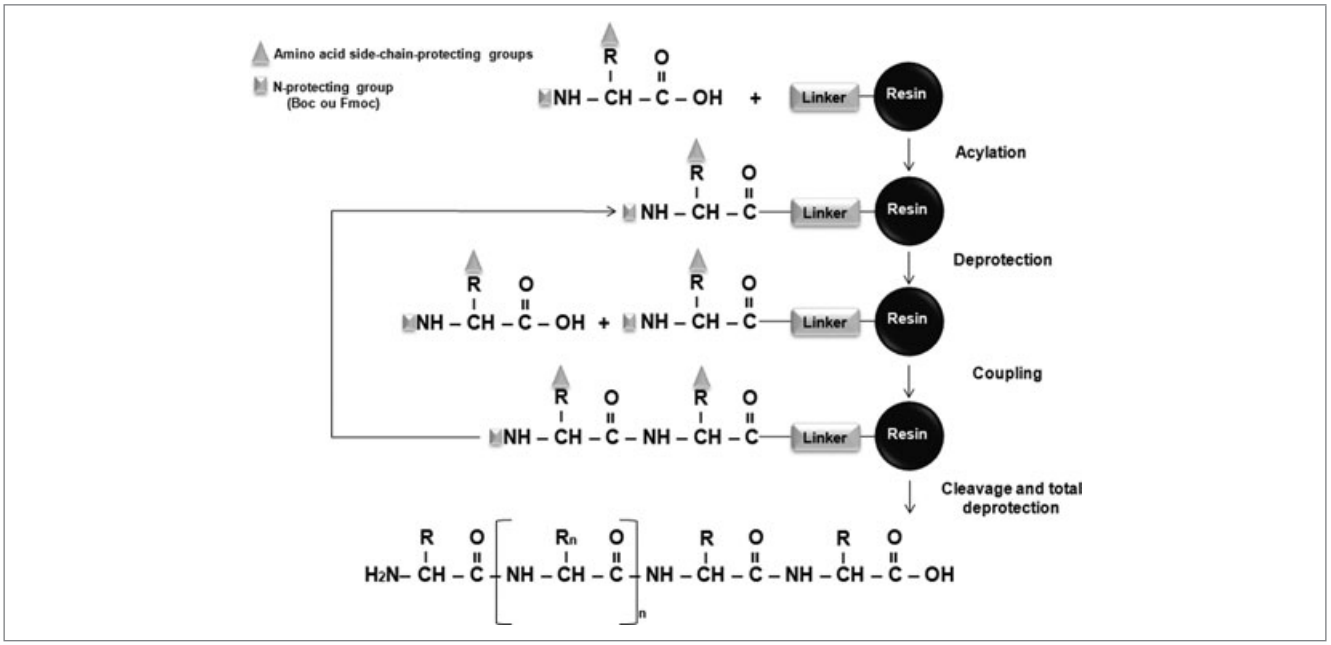


Figure 1–Peptide synthesis in solid phase Adapted with permission from Borgia and Fields (2000). Copyright (2000), Elsevier.

## Peptide Synthesis

Peptides are biomolecules that contain between 1 to several dozen of amino acid residues joined by peptide bonds.

The discovery of the different peptide activities has generated enormous interest in this class of compounds and in the methods of isolation, analysis, purification, identification, and quantification. These methods have been systematically studied and improved. However, most sources of natural peptides are poor in these compounds, thus preventing their isolation in sufficient quantities for research.

As a result, there was a growing need to synthesize peptides for application in physiological, chemical, physical, pharmacological, biochemical, and clinical studies.

Total of 3 methods of peptide synthesis have been developed and improved: Chemical synthesis, which uses chemical reagents to mediate peptide bond formation (Andreu and Rivas 2002), enzymatic synthesis, in which the peptide bond formation is catalyzed by enzymes (Bongers and Heimer 1994; Boeriu and others 2010), and the DNA recombinant technology synthesis, based on the use of cloning and ribosomal techniques from biological systems for peptide formation (Sewald and Jakubke 2002).

### Chemical synthesis

Research on this synthesis method was first initiated more than 30 y ago. However, the construction of peptides has recently become more accessible due to advances in process efficiency, including the development and use of fast coupling reagents, as well as the minimization of side reactions (Borgia and Fields 2000).

The main aspects of chemical synthesis are protection and activation. Protection strategies are intended to provide chemical selectivity necessary for the construction of a particular peptide sequence. Activation refers to the chemical coupling necessary to ensure quantitative formation of each peptide bond in the sequence (Andreu and Rivas 2002).

In chemical synthesis, chemical reagents are used to activate the carboxylic acid (RCOOH) of the amino acid, which will donate the acyl group (R-CO-) to form the peptide bond. The peptide bond presents a nucleophilic attack of the $\alpha$-amino group by another amino acid ($H_2N$-R). In this synthesis, the reactive functional groups that are not directly involved in peptide bond formation receive prior protection (Machado and others 2004). There are 2 types of chemical peptide synthesis, synthesis in solution (classical synthesis) and solid-phase synthesis.

Chemical synthesis in solution is performed with all reagents and reaction products dissolved in the medium (Kent 1988). In comparison, solid-phase synthesis (SPS) is a simple procedure to produce peptides in large quantities on a solid support which remains insoluble in the reaction medium (Shigeri and others 2001). The solid support is a polymeric resin that has a functional group on its surface (linker) that allows it to form stable bonds in the peptide sequence to the reagent used for the de-protection of the N-amino group.

Peptide synthesis in the solid phase generally consists on the acylation of an amino acid to be linked to an insoluble support (resin) via a linker (Figure 1). After that, the protecting group of the N-terminal is removed (the unprotecting step) to allow the next amino acid of the sequence to be attached to the complex "peptide-linker-resin." The unprotecting-coupling cycle is repeated until the desired sequence is complete. Finally, the cleavage reagent is used to separate the complex "peptide-linker-resin." This reagent should also remove the protecting groups of side chains that are stable to unprotecting conditions of the N-terminal group (Borgia and Fields 2000).

Peptide chemical synthesis can use 2 protocols, Boc (tert-butyloxycarbonyl) and Fmoc (9-fluorenylmethyloxycarbonyl), named according to the type of protector of the reactive group of the amino acids (N-terminal) involved in the synthesis.

The first protocol employs the tert-butyloxycarbonyl (Boc) group for N-amino protection. This protocol is based on gradual differences in their sensitivity to acids. Thus, the Boc group is typically removed with trifluoroacetic acid (TFA), while the protecting groups of the lateral chains (ester, ether, and urethane derivatives based on benzyl alcohol) are specifically designed to be stable to repeated cycles of Boc removal and are removed only with

 

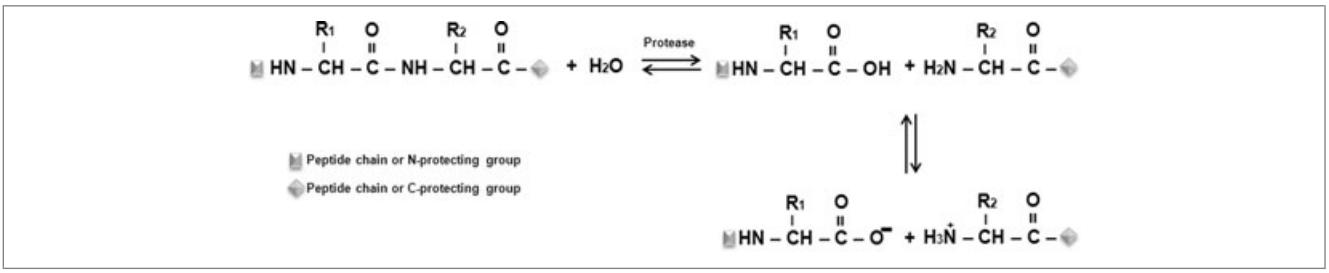


Figure 2–Enzymatic peptides synthesis by the reverse hydrolysis reaction.

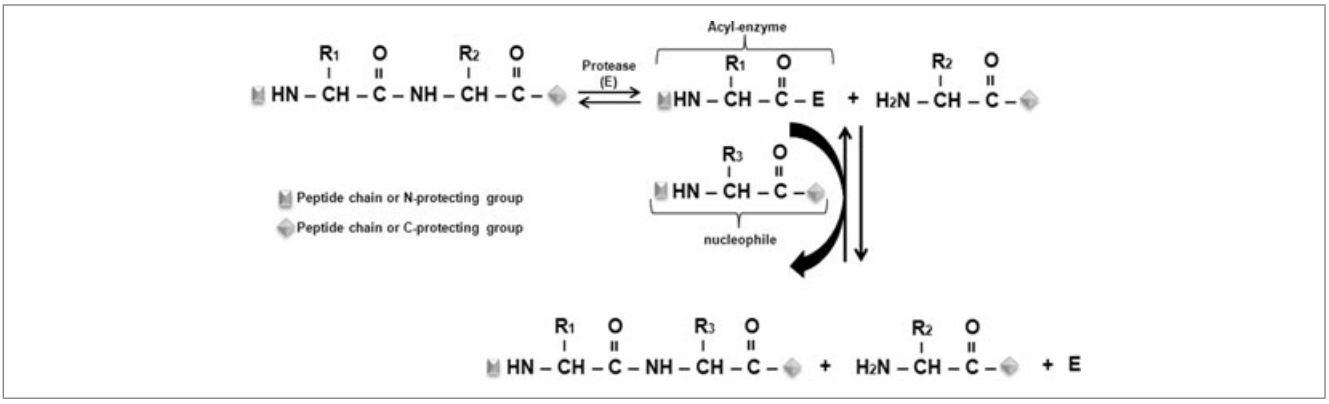


Figure 3–Enzymatic peptides synthesis by transpeptidation mechanism.

a specific reagent, a relatively stronger acid, usually hydrofluoric acid (Borgia and Fields 2000).

The second protocol uses a 9-fluorenylmethyloxycarbonyl (Fmoc) as the N-amino protecting group. This protocol provides a greater degree of chemoselectivity than the Boc protocol, since the Fmoc group is removed under basic conditions (piperidine in N, N-methylpyrrolidone or dimethylformamide), without alteration of the acid-sensitive lateral chains (Andreu and Rivas 2002).

Protection groups of lateral chains are compatible with the Fmoc protection group; these are mainly ether, ester, and urethane derivatives based on t-butanol. Protection groups of lateral chains are removed by the end of the synthesis using TFA (Borgia and Fields 2000).

## Enzymatic synthesis

In this method, the peptide bond formation is mediated by an enzyme (protease) in free or immobilized form. The enzymatic method is especially useful in the synthesis of very short peptides (2–5 oligomers) and in the condensation of large peptide fragments (So and others 1998). Proteolytic enzymes such as chymotrypsin, papain, pepsin, subtilisin, termolisin, trypsin, among others, have been used in the presence of organic solvents as catalysts for the synthesis of peptide bonds (Ogino and others 1999).

The enzymatic synthesis of peptides has several advantages over chemical methods, including good stereoselectivity and regioselectivity. However, it has certain shortcomings, such as peptide synthesis being thermodynamically unfavorable in water, as well as the secondary hydrolysis of synthesized peptide chains, which hinders their use in peptide synthesis with long sequences (So and others 1998). Thus, the main practical obstacle to employment of a protease for peptide bond formation is finding suitable conditions to allow bond formation without mediating secondary hydrolysis of the peptide or peptide fragments used as reagents (Bongers and Heimer 1994).

The formation of a peptide bond by enzyme catalysis can occur through several mechanisms, including the reverse hydrolysis reaction of amides and transpeptidation (Machado and others 2004; Boeriu and others 2010).

The mechanism of the reverse hydrolysis reaction is based on the microscopic reversibility principle. This indicates that the peptide bond formation and hydrolysis reaction come from the same intermediate (Figure 2). Thus, the reaction conditions are manipulated to shift the equilibrium towards peptide bond formation.

The transpeptidation mechanism occurs as a result of the break of a peptide bond, with the formation of an active acyl-enzyme intermediate (Figure 3). This intermediate is attacked in the presence of a nucleophile (peptide or amino acid blocked in the $\alpha$-carboxyl group) and consequently causes the formation of a new peptide bond.

For both mechanisms, the equilibrium should shift to the synthesis reaction direction, requiring the use of protective groups of $\alpha$-amino and carboxyl substrates, the addition of organic solvents to the media reaction, excess substrates, and the removal of products from the reaction medium (Machado and others 2004).

## Synthesis by recombinant DNA technology

This synthesis uses modern methods of cloning and gene expression in microorganisms, allowing the production of a recombinant peptide or several peptides simultaneously. Bacteria are the expression system generally used, with *E. coli* being the most widely used host. Since antimicrobial peptides present a natural destructive activity against the host and relative sensitivity to proteolytic degradation, peptides are often expressed as fusion proteins to

Table 1–Peptide purity levels according to their uses.

| Purity level | Uses |
|---|---|
| >70% | Immunization and polyclonal antibodies in animals |
| | Standard ELISA antibody test |
| >80% | Quantitative study of enzyme-substrate interaction |
| | Phosphorylation reactions |
| | Quantitative studies by immunocytochemistry and *in vitro* assays |
| | Electrophoresis applications |
| >95% | ELISA protocols |
| | Quantitative studies of receptor-ligand interaction |
| | Inhibition and competition assays |
| | Bioassays *in vitro* and *in vivo* |
| | Quantitative studies with enzymes (kinetics and thermodynamics) |
| | Chromatography standards |
| | NMR studies |
| | Production of monoclonal antibodies |

neutralize their innate toxic activity and increase their expression levels (Wang and others 2011).

Compared with isolation from natural sources and the other synthesis methods, the recombinant approach offers the most cost-effective alternative for large-scale peptide production (Li 2011).

## Peptide Purification and Characterization

Peptides are increasingly being produced for various purposes, and these may contain closely related impurities resulting from incomplete reactions or from several side reactions. Peptides synthesized for therapeutic and clinical research, as well as for biological and structural studies to explore the structure–activity relationships must have 95% purity or greater (Ridge and Hettiarachchi 1998). However, there are other applications where low values of purity, between 70% and 95%, are tolerable (Table 1).

Peptide purification depends on a series of separation techniques. Peptides made on a preparative scale (in gram amounts) can be obtained from a separation process, to isolate one or more individual components from a peptide mixture for future research, or on an analytical scale (about 1 mg of peptide) to identify and determine the relative amounts of some or all components in the mixture. Studies on an analytical scale are the first steps for improving separation conditions, which are developed prior to the execution of any preparative separation process (Sewald and Jakubke 2002).

After the synthesis process, peptides are submitted to a separation procedure consisting of centrifugation and washing to remove residues of the reagents used, as well as products of side reactions. Subsequently, peptides are cleaved and subjected to filtration, as well as lyophilization (Dagan and others 2002).

The most widely used methods used for the purification of peptides are reverse-phase high-performance liquid chromatography (RP-HPLC), ion-exchange chromatography, size exclusion chromatography, affinity chromatography, and capillary electrophoresis (Table 2).

The purity of a peptide must be verified by a method different from that used for purification, since the results of homogeneity derived from such a system can lead to misinformation and be misleading (Ridge and Hettiarachchi 1998).

Thus, the characterization should be analyzed by different methods of mass spectrometry. Mass spectrometry has different ionization methods, such as electrospray ionization mass spectrometry (ESI-MS), fast atom bombardment mass spectrometry (FAB-MS) or matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) that can be used for peptide characterization (Table 3).

The different mass spectrometry techniques are based on the accurate determination of the molecular mass-charge ratio of the peptide, as well as on a chemical structure determination, with high sensitivity and resolution (Sewald and Jakubke 2002).

## Peptide Bioproperties

The growing resistance of pathogens against many commonly used antibiotics has led to research of new compounds with the same functions. An interesting approach is the study of molecules of natural origin to replace antibiotics (Bechinger and Lohner 2006).

Several studies in recent decades have shown that peptides have certain bioactive properties (Agyei and Danquah 2011). Short peptides (1–50 amino acids) with cationic and hydrophobic properties are known to be potent defenses of the host organism, providing activity against a wide variety of pathogenic microorganisms such as Gram-negative and Gram-positive bacteria, fungi, viruses, and parasites (Hancock and Sahl 2006). Studies have shown remarkable results of peptide antitumor activity, observed mainly in cancer therapy (Korhonen and Pihlanto 2006).

Although several peptides have biological activity, antimicrobial activity is one of the most studied. One of the most-used analytical techniques to determine peptide antimicrobial activity is the broth microdilution test.

In this test, the microorganisms are cultured in titration microplates and the peptide to be tested is added to each well at different concentrations. The microorganism growth causes turbidity in the wells. However, when a certain concentration of the peptide tested inhibits bacterial growth no turbidity is observed. Turbidity is usually read by spectrophotometry, with the greatest frequency at 600 nm, but it can also be seen through visual inspection of the wells (Otvos and Cudic 2007).

The standard methods developed by the Clinical and Laboratory Standards Inst. (CLSI) have been used to test the activity of antimicrobial peptides. Among them are the standards for antimicrobial disk susceptibility tests (CLSI 2003), the method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically M7-A6 (CLSI 2006), and the method for broth dilution antifungal susceptibility testing of yeast M27-A2 (CLSI 2002), all of which have been widely used (Jang and others 2006; Rubinchik and others 2009; Hwang and others 2010).

### Antibacterial activity

The most studied peptides are those with antimicrobial activity, characterized by their interaction with the cytoplasmic membrane of the microorganism regardless of the final target (Powers and Hancock 2003). Factors influencing the antibacterial activity are the electrostatic interactions between the peptide and positively charged and anionic lipids on the surface of the target microorganism. Also, the hydrophobicity of the peptide (factor required for insertion into the membrane) and peptide flexibility allow peptide interaction with the microbial membrane (Jenssen and others 2006). Although these characteristics are variable according to each peptide, all of them are essential to the function of peptides as antimicrobials.

The exact mechanism of action of antibacterial peptides is not yet fully understood. However, there is a consensus among researchers regarding the first step in the initial interaction between peptide and the target cell (Reddy and others 2004).

Table 2–Peptide purification methods.

| Method | Principle | Uses |
|---|---|---|
| Reversed-phase chromatography | Based on hydrophobicity. Consisting of a stationary phase of lower polarity and a mobile phase of higher polarity | Enables rapid detection and purification of a peptide sequence from a mixture |
| Ion exchange chromatography | The distribution and surface charge of the peptide determines the interaction of charged groups with the surface of the stationary phase | Used for purification of peptides and proteins |
| Exclusion liquid chromatography | Based on separation process according to the size of the peptide relative to pore sizes in the stationary phase. Used primarily in the early stages of purification of the peptide, when performed in multiple steps | Used to separate low-molecular-weight impurities from a mixture of peptides. However, the separation of the peptide of interest with other closely related peptides is virtually impossible |
| Affinity chromatography | Based on the biological specificity of the peptide. Consists of a ligand (small specific biomolecule such as an antibody) that is immobilized in the column. The separation occurs because of highly specific biochemical interactions between the peptide and the ligand | Used when a high degree of specificity is required, for example, isolation of a target protein present in low concentration in a biological fluid or a cell extract |
| Capillary electrophoresis | Based on the migration of the peptide according to its charge in solution, depending on the application of an electric field. Complementary technique to reversed-phase chromatography | Used for peptides and proteins |

Table 3–Ionization methods used in mass spectrometry.

| Method | Fundamental principle |
|---|---|
| ESI-MS | The ions are produced from a peptide contained in a solvent (for example, an organic compound such as methanol or acetonitrile) that is scattered in a fine aerosol |
| FAB-MS | The peptide analyzed is mixed with a matrix, which is a non volatile reagent of protection (glycerol, diethanolamine, and triethanolamine, among others), and is bombarded with a beam of high-energy atoms (4000 to 10000 eV) in a vacuum. Atoms are of an inert gas such as argon or xenon |
| MALDI-MS | The peptide analyzed is bombarded by a laser beam (nitrogen), while a matrix (sinapinic acid) is used to protect the peptide. The matrix allows avoiding direct contact of the peptide with the beam, facilitating its vaporization, and ionization |

The initial attraction between the peptide and the target cell occurs via electrostatic binding between the cationic peptide and the components of the negatively-charged outer cell membrane, such as lipopolysaccharides in Gram-negative bacteria or lipoteichoic acid on the surface of Gram-positive bacteria (Jenssen and others 2006). This electrostatic interaction removes the native divalent cations ($Mg^{2+}$, $Ca^{2+}$) from the cell surface, thus destabilizing the outer membrane and facilitating the entry of the peptide and subsequent peptide contact with the cytoplasmic membrane, a process known as autopromoted uptake (Powers and Hancock 2003).

After the peptide is bound to the target cell, an arrangement of the peptide occurs on the surface of the cytoplasmic membrane. This fact is of considerable debate, since several arrangement models have been proposed, such as the barrel-stave or the carpet model among others.

Depending on the model, the peptide can permeabilize the cytoplasmic membrane and/or translocate through it. Thus, antimicrobial peptides can be classified into 2 major groups, the first consisting of those peptides which act on the cytoplasmic membrane, and the second consisting of those which have no action on the cytoplasmic membrane of the target microorganism. This means that the peptide just moves into the cell without causing major disturbances in the membrane (Powers and Hancock 2003; Jenssen and others 2006).

**Peptides acting on the bacterial membrane.** Several models have been proposed to explain how, after initial attachment, antibacterial peptides are distributed on the surface of the bacterial cytoplasmic membrane to form pores. Pore formation results in membrane permeabilization, thereby affecting cellular respiration. It also deprives the microorganisms of their source of energy by interrupting the electrochemical gradient and causing an increase in the flow of water and ions across the membrane, thus leading to cell swelling followed by cellular lysis (Bechinger and Lohner 2006).

To explain the formation of pores, the aggregate toroidal pore, barrel-stave, and the carpet models have been proposed. The last 2 models, the barrel-stave and carpet model, have been the most widely studied.

**Barrel-stave model.** This model describes the formation of a transmembrane channel (pore) through the binding of amphipathic $\alpha$-helices. The hydrophobic surface of the peptide interacts with the lipid core of the membrane, while the hydrophilic surface of the peptide is oriented inside, producing an aqueous pore (Figure 4). The progressive recruitment of additional peptides to the membrane surface increases the size of the pores, causing the loss of cell content and thus cell death (Reddy and others 2004). This model has been proposed to explain the activity of antimicrobial peptides, such as magainins (Matsuzaki and others 1998).

**Carpet model.** In this model, the peptide in high concentration is in contact with phospholipids located on the outer surface of the bacterial membrane, a fact that allows the peptide to permeate the membrane (Figure 5). The peptides bind to the surface of the target membrane and cover it like a carpet. According to this model, the peptides exhibit a preferential binding for the phospholipid groups. The binding step is followed by the alignment of the peptide on the membrane surface so the hydrophilic surface is in contact with phospholipid or water molecules, causing a reorientation of hydrophilic residues and creating a hydrophobic core. Finally, the peptide disintegrates the membrane by deformation of the membrane curvature (Reddy and others 2004). This model has been proposed to explain the action mechanism of dermaseptins (Dagan and others 2002).

**Toroidal pore model.** This model is considered a variant of the barrel-stave model. It is suggested that a perpendicular inclusion of the peptides to the membrane with their hydrophilic regions is associated with phospholipids, whereas their hydrophobic regions are associated with the lipid core. In this process, the membrane is bent inward so the pores are formed (Jenssen and others 2006). The

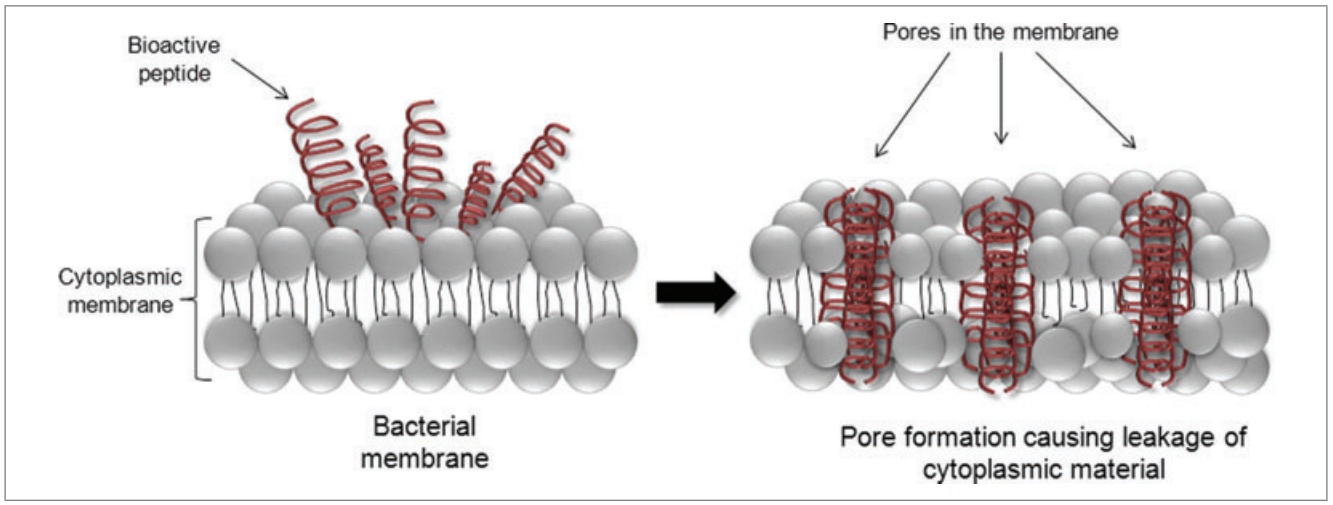


Figure 4–Barrel-stave model.

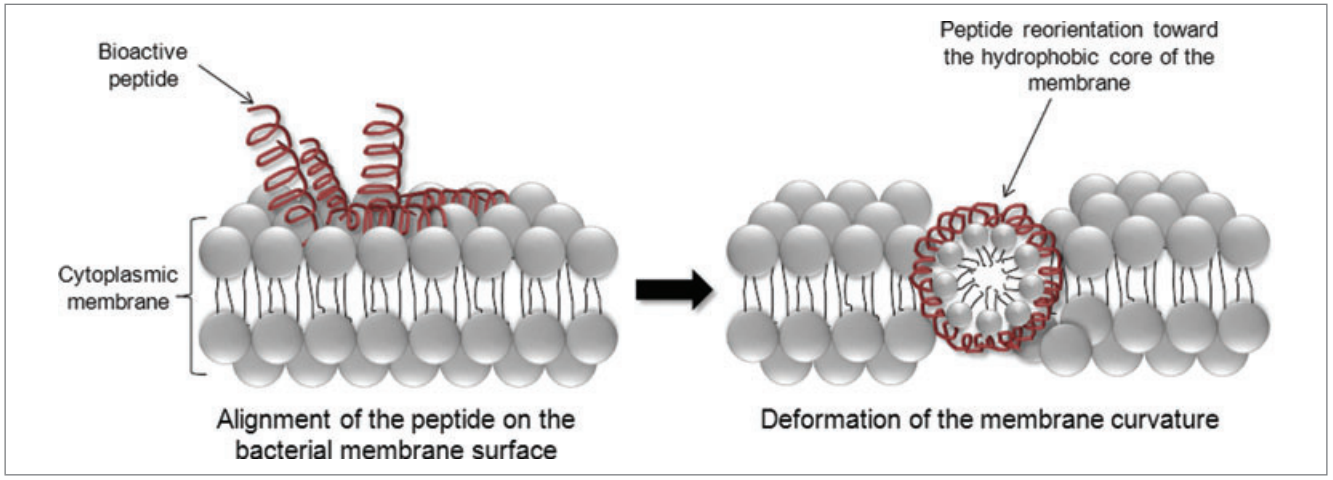


Figure 5–Carpet model.

main difference between this model and the barrel-stave model is the intercalation of the peptide with phospholipids to form the pore (Figure 6). This model has been used to explain the mechanism of action of the peptide melittin (Yang and others 2001).

**Aggregate model.** This model, proposed by Wu and others (1999), has some similarity to the toroidal pore model. This model consists mainly of the arrangement of the peptide in the membrane forming an extension and developing micelle-like aggregate of peptides and lipids, but without adopting a particular orientation (Jenssen and others 2006).

**Peptides with no activity on the bacterial membrane.** These antimicrobial peptides have the ability to translocate into bacterial cells without causing membrane permeabilization. The peptide is accumulated within the cell where it reaches a variety of essential cellular processes that result in bacterial cell death (Jenssen and others 2006). The target process includes inhibitions of nucleic acid synthesis, protein synthesis, enzyme activity, and cell wall synthesis. Peptides such as buforin II and pleurocidin have shown this mechanism of action (Park and others 1998; Patrzykat and others 2002).

## Antifungal activity

There is a growing need for new antifungal agents due to the increased resistance of molds to therapies with regularly used compounds (De Lucca and Walsh 1999). Peptides have emerged as alternative antifungal agents.

Initially, the antifungal mechanism of action was described as a result of fungal cell lysis or as a result of interferences in fungal cell wall synthesis. However, the discovery of new antifungal peptides in the last decade has led to the identification of new mechanisms of action, including membrane permeabilization, binding to ergosterol/cholesterol in the fungal membrane, the attack of mitochondria or other intracellular organelles, and the deformation of cell membrane structure (Jenssen and others 2006).

According to De Lucca and Walsh (1999), fungal peptides can be classified with respect to their mode of action into 3 groups: peptides that act through cellular lysis; peptides that cross the fungal membrane and interact with the intracellular target; and peptides that act by forming pores.

**Peptides acting by cellular lysis.** These peptides are characterized by their amphipathic nature, being molecules with 2 faces, one positively charged and the other neutral and hydrophobic.

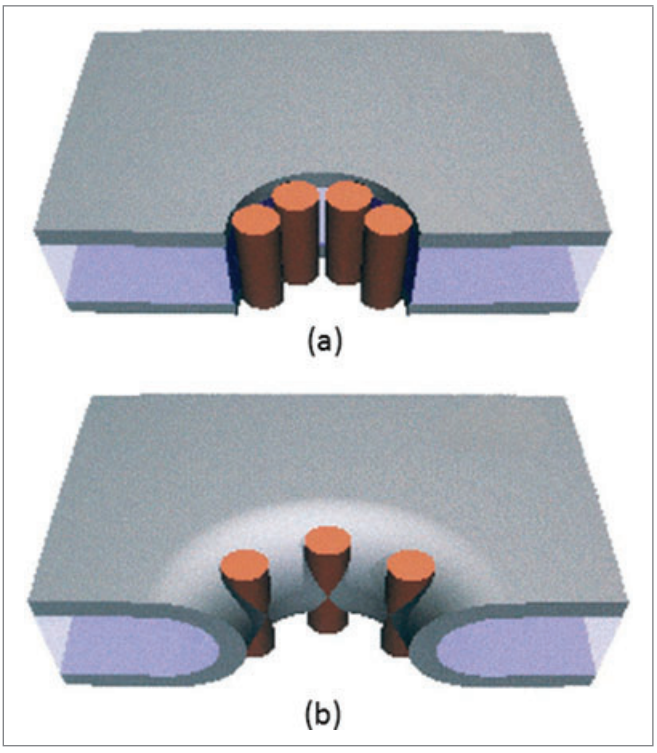


**Figure 6–Differences between barrel-stave model (a) and Toroidal model (b). Peptide monomers are represented by the cylinders. (With permission from Yang and others 2001).**

Some of these peptides bind only to the membrane surface, damaging the membrane structure, and they may or may not pass through it.

Peptide SMAP-29 (a synthetic peptide derived from the sequence of cathelicidins) has shown antimicrobial activity against the fungus *Trichosporon beigelii* by interaction, penetration, and subsequent damage to the cell membrane (Lee and others 2002). This result suggests that the main target of SMAP-29 peptide is the fungal plasmatic membrane. A similar mechanism was observed for the synthetic peptide Ib-AMPs (an analogue sequence to peptides isolated from seeds of *Impatiens balsamina*), showing antifungal activity by bonding the peptide to the fungal cell membrane and subsequent penetration (Thevissen and others 2005).

**Peptides that pass into the membrane and interact with intracellular targets.** These peptides interfere with cell wall synthesis or the synthesis of essential cellular components such as chitin or glucan. As such, the synthetic peptide omiganan (an indolicin analogue peptide, isolated from bovine neutrophils) has shown antifungal activity against *Candida albicans*, and the main mechanism of action of this peptide is related to its activity in the cytoplasmic membrane, resulting in macromolecules synthesis inhibition of macromolecules and finally cell death (Rubinchik and others 2009).

**Pore-forming peptides.** These peptides are aggregated in a selective way to form pores of varying sizes, which then allow the passage of ions and other solutes. The synthetic peptide di-K19Hc (a halocidin analogue peptide, isolated from the invertebrate marine animal *Halocynthia aurantium* known as sea peach), has shown antifungal activity against several strains of *Aspergillus* and *Candida* (Jang and others 2006). The activity of di-K19Hc results in the formation of pores on the surface of fungal membranes. Moreover, these researchers pointed out the specific binding of di-K19Hc with b-1,3-glucan, a component of the cell wall of fungi. This mechanism has also been observed with the antifungal peptide psacotheasin, isolated from the yellow-spotted long-horned beetle (*Psacothea hilaris*), which has shown activity against *C. albicans* (Hwang and others 2010). The researchers indicated that there was damage to the cell wall, membrane depolarization with the formation of pores (2.3–3.3 nm), as well as an increase in membrane permeability, all being responsible for the antifungal activity of this peptide.

## Antiviral activity

Several studies have shown the ability of cationic peptides to inhibit viral infections. The peptide cecropin A has shown antiviral activity against Junin virus (JV—which causes Argentine hemorrhagic fever). The peptide melittin inhibited JV and herpes simplex virus 1 (HSV-1) multiplication, as well as magainin I and II, and has shown inhibitory activity against HSV-1 and HSV-2 (Albiol-Matanic and Castilla 2004). Antimicrobial peptides isolated from fish, such as tilapia hepcidin 1–5, have shown activity against the nervous necrosis virus (NN virus), an infectious agent that causes mass mortality of several species of marine fish in the larval stage (Chia and others 2010). In addition, synthetic peptides consisting of arginine and tryptophan repetitions have shown activity against vaccinia virus (the cause of cowpox) (Mohan and others 2010).

The antiviral activity of peptides is often related to virus adsorption and its entry into the host cell or, in other cases, is the result of a direct effect on the viral envelope. Thus, the antiviral activity of peptides may result from multiple mechanisms of action, the most important being blocking virus entry through interaction with the host cell and blocking viral entry through interaction with the virus.

**Blocking viral entry through interaction with the host cell.** Peptides can interact directly with specific viral receptors on the host cell, thus preventing the virus from binding to the cell membrane or binding intracellularly (Jenssen and others 2006).

Proteoglycans are proteins found in all types of tissue, in intracellular granule secretions as well as in the extracellular matrix and cell surface. Proteoglycans are covalently linked to one or more chains of glycosaminoglycans (GAG), long polysaccharide unbranched structures, which have a sugar that contains nitrogen and are usually sulfated. GAG chains are present on the surface of mammalian cells and their degree of sulfation makes these compounds more anionic. This network of strong negative charges allows GAG to attract and bind to small cations, such as enzymes and proteins, and also pathogens such as viruses (Spillmann 2001).

Heparan sulfate, one type of GAG chain, is one of the most important molecules related to viral binding (Spillmann 2001). Thus, by blocking heparan sulfate molecules can be inhibited viral infection.

Jenssen and others (2006) have suggested that antimicrobial peptides which interact with heparan sulfate have the ability to block a number of viral infections. Due to the large number of amino acid residues positively charged peptides can interact electrostatically with negatively charged heparan sulfate molecules on the cell surface.

Studies on lactoferrin (LF) have shown that this peptide prevents infection of the host cell rather than inhibiting virus replication after infection of the target cell. The interaction of LF with heparan

sulfate molecules has been proposed as the mechanism responsible for LF antiviral activity (van der Strate and others 2001).

Similarly, Jenssen and others (2006) showed the antiviral activity of synthetic peptides (consisting of arginine and lysine residues) against herpes simplex virus 1 and 2 (HSV-1 and HSV-2). The peptides presented higher affinity in binding to heparan sulfate with an increasing number of cationic residues, thereby blocking the entry of HSV (-1 or -2). In addition, Luganini and others (2010) reported the inhibition of cytomegalovirus by binding synthetic peptide dendrimers with molecules of heparan sulfate on the surface of fibroblasts and endothelial cells. Thus, cytomegalovirus infection was blocked by the interaction of synthetic peptide binding sites with heparan sulfate.

**Blocking viral entry through interaction with the virus.** The interactions of peptides with the glycoproteins (gp) in the viral envelope have been proposed as another mechanism that influences the process of viral entry and virus inactivation. In this way, peptides generated from chemical modification of milk proteins, such as $\alpha$-lactalbumin, $\beta$-lactoglobulin, and lysozyme with 3-hydroxyphthalic anhydride (3-HP) inhibited infection of vero cells with HSV-1 (Oevermann and others 2003). According to those researchers, the antiviral activity of these peptides is based on their direct interaction with viral glycoproteins (gB, gC, gD), which are responsible for adsorption and penetration of the virus into the host cell. Similarly, LF has shown the ability to bind to the gp120 glycoprotein (a protein present in the outermost layer of the HIV virus) with antiviral effects, since the gp120 glycoprotein plays an important role in the adsorption and entry of HIV into target cells (van der Strate and others 2001; Pan and others 2006).

On the other hand, other peptides, such as magainins, have shown antiviral effects through direct interaction with virus cells. Egal and others (1999) have indicated that the effect of magainins is the result of the peptide acting on the viral envelope.

A similar mechanism was suggested for the activity of mucroporin-M1, a defense cationic peptide present in scorpion venom, which has shown activity against the measles virus, the coronavirus that causes severe accurate respiratory syndrome (SARS), and flu virus H5N1 (better known as the bird flu virus) (Li and others 2011). The researchers have suggested that the antiviral activity of the peptide is the result of direct interaction with the virus envelope, thereby reducing viral activity in the host cell.

## Antitumor activity

Cancer, also known as malignant neoplasm, is a general term that refers to more than 100 different diseases affecting various tissues and different types of cells. All forms of cancer are characterized by abnormal cell growth, that is, they lack the mechanisms that control normal cell division. This lack of regulatory mechanisms is the result of a multistep process involving genetic mutations induced by inheritance or environmental changes (Hütter and Sinha 2001).

Despite major advances in cancer therapy, there is considerable interest in the development of antitumor agents with a novel mode of action, since the cells have shown carcinogenic development of resistance to current chemotherapy (Hoskin and Ramamoorthy 2008).

Carcinogenic cells often become resistant to chemotherapy. This mainly occurs as a result of increased expression of intracellular enzymes for the detoxification of antitumor agents, the correction of DNA damage, generation of intracellular organelles with the ability to eliminate and/or transport the drugs out of the tumor, and irreversible defects in the cellular machinery that mediates apoptosis (Hütter and Sinha 2001).

Thus, recent studies have shown peptides as an alternative to conventional cancer treatments. However, not all peptides have selective activity against carcinogenic cells.

According to Hoskin and Ramamoorthy (2008) peptides that have antitumor activity can be classified into 2 major groups: peptides with selective activity, and peptides with non selective activity, that is, those that have activity against bacteria, carcinogenic cells, and healthy cells.

**Peptides with selective activity toward carcinogenic cells.** These peptides have activity against bacteria and carcinogenic cells, but not against normal cells. Several peptides, such as the cecropins, buforins, and magainins have shown antitumor activity without affecting normal eukaryotic cells (Cruciani and others 1991; Cho and others 2009).

Studies with magainin II have shown to inhibit the proliferation of carcinogenic cells (in bladder cancer) without any effect on normal cells (Lehmann and others 2006).

Similar results were observed by Chen and others (2009) in the study of the synthetic peptide TH2–3 (isolated from tilapia and analogous to the peptide hepcidin), with antitumor activity shown primarily by direct interaction and lysis of target carcinogenic cells (human fibrosarcoma cells). These researchers indicated that the lytic activity of the peptide and proliferative cells were restricted mainly to carcinogenic cells, since normal cells showed no significant effects.

Likewise, the synthetic peptide TH1–5 (isolated from tilapia and an analogue to the peptide hepcidin) has shown antitumor activity against carcinogenic cells, due to interaction with and penetration of the membrane. This peptide has less toxicity toward normal cells supposedly because it can discriminate between healthy cells and carcinogenic ones (Chang and others 2011). Researchers have also indicated that the interaction with the cell membrane and its subsequent damage is caused by the formation of pores on its surface. It has been suggested that the internalization of the peptide and the subsequent damage to the mitochondrial membrane activates apoptotic pathways (Chang and others 2011).

According to Hoskin and Ramamoorthy (2008) there are fundamental differences between the membranes of malignant cells and normal cells which allow the selectivity of certain peptides to attack carcinogenic cells without affecting healthy cells.

Electrostatic interactions between cationic peptides and anionic components of the cell membrane have also been considered an important factor. Carcinogenic cells typically have a negative charge due to a higher expression than normal of anionic molecules such as phosphatidylserine (PS) and mucin (glycoprotein) (Oren and Shai 1997). However, normal cells are not affected, since these cells have a neutral surface charge, conferred by the zwitterionic nature of most membrane components such as phosphatidylethanolamine (also known as cephalin), phosphatidylcholine, and sphingomyelin (Sok and others 1999).

Membrane fluidity and the surface area of the cell are also considered factors that contribute to the selectivity of peptides for carcinogenic cells. The fluidity of carcinogenic cells is greater than that of normal cells, which may increase the activity of lytic peptides through the easy destabilization of the membrane. In addition, the carcinogenic cells have a higher surface area than healthy cells due to the presence of greater numbers of microvilli, which are small projections of the cell membrane, irregular in size

 

and shape. The microvilli may allow the bonding between peptide and carcinogenic cells (Hoskin and Ramamoorthy 2008).

**Peptides with nonselective activity.** This group is comprised of peptides with activity against bacteria, carcinogenic cells, and against normal eukaryotic cells (Hoskin and Ramamoorthy 2008).

According to Papo and Shai (2003) non selective activity of these antimicrobial peptides results from their ability to interact with and cause damage to negatively charged membranes and those of a zwitterionic nature. Dathe and others (1997) have indicated that the hydrophobic moment of antimicrobial peptides exerts a substantial influence on the neutral lipidic membranes, although it has a small role in the permeabilization of highly charged lipid membranes.

Peptides of this group include melittin, isolated from bee venom; taquiplesin II, isolated from the horseshoe crab; defensins, isolated from insects; and plantaricin, a bacteriocin isolated from *Lactobacillus plantarum* (Schweizer 2009).

Plantaricin has shown activity against carcinogenic cells and against normal lymphocytes and neuronal cells (Sand and others 2010).

The mechanism of action of antitumor peptides consists of permeabilization of the cell membrane mediated by electrostatic interaction. The electrostatic interaction is generated by the negatively charged phospholipids in the cell membrane and the positively charged peptide (Schweizer 2009). Unlike carcinogenic cells, eukaryotic cells have most of their negatively charged phospholipids, particularly PS, in the inner membrane, while neutral lipids are positioned on the outside (Zhao and others 2006). However, the result obtained by Sand and others (2010) suggests that in addition to the mechanism of action related to the electrostatic interaction, there is another mechanism which explains the sensitivity of normal eukaryotic cells to plactaricin. Probably another negatively charged macromolecule present on the membrane surface of healthy cells is also involved in plantaricin activity.

Similar results were observed by Nan and others (2010) in the study of synthetic peptides consisting of lysine or arginine enriched with tryptophan. The peptide with arginine residues showed higher toxicity against human erythrocytes and mammalian cells. The hydrophobicity of the peptides has been suggested as an important factor in the increase of hemolytic activity and cytotoxicity in mammalian cells, as hydrophobic regions are required for direct interaction between peptide with membrane lipid components. The peptide with arginine residues was slightly more hydrophobic than the peptide with lysine residues. Thus, these researchers suggested that small differences in hydrophobicity of these peptides may be responsible for the cytotoxic activity of this peptide in mammalian cells.

## Applications of Antimicrobial Peptides

The growing problem of microorganism resistance to conventional antibiotics, as well as the need for new agents with antibiotic properties has stimulated interest in developing antimicrobial peptides aiming for their application in the medical field (Zasloff 2002).

Most of the studies are devoted to the development of topical agents with antibacterial and antifungal activities. Also, due to their antiviral activity, antimicrobial peptides have also been proposed as chemical preservatives.

In the food industry, antimicrobial peptides, especially those produced by bacteria, have been widely researched in recent years due to their potential use as natural preservatives (Papagianni 2003; Coma 2008; Settanni and Corsetti 2008).

The direct application of antimicrobial peptides in food preservation can be achieved by 2 methods: the direct addition of peptide to the food matrix, or the inoculation of the food matrix with the bacteriocin producer strain under the conditions favorable for the *in situ* production of the antimicrobial peptide.

Bacteriocins can be obtained *ex situ* by the cultivation of the producer strain at an industrial scale in a food-grade substrate, followed by a series of separation and purification techniques. These *ex situ* bacteriocins are commercially available in concentrated form, such as ALTA$^{TM}$ 2341 or Microgard$^{TM}$, and can be added directly to the food matrix.

The production of bacteriocins in the food matrix offers several legal and cost advantages. The use of bacteriocin producer strain requires careful selection depending on the particular food intended for inoculation to ensure the producer strains will produce bacteriocins in the necessary amounts to inhibit the target microorganism.

In addition to the peptides being studied as antimicrobial agents for direct addition to foods, they also have shown potential for being incorporated into food preparation surfaces (such as cutting surfaces) and processing equipment, as well as in food packaging (Appendini and Hotchkiss 2002).

## Peptide Applications in Food Packaging

Active packaging includes the incorporation of antimicrobial agents in the packaging material to control and extend the shelf-life of food (Soares and others 2009a). These types of packaging are considered an innovative technology in food preservation, since they allow better antimicrobial efficiency on food surfaces, thus improving stability.

The development of active packaging by incorporating antimicrobial peptides in food packaging material can be done either to prolong the life of the product or to reduce the microbial load of the packing before use (Steven and Hotchkiss 2008).

The development of active packaging with antimicrobial peptides can be accomplished by 3 main methods of incorporation: direct peptide incorporation in the polymer; peptide coating on the polymeric surface; and peptide immobilization in the polymer.

### Direct peptide incorporation in the polymer

Numerous studies have reported the incorporation of antimicrobial peptides directly in the polymeric material, especially bacteriocins. The peptides are relatively resistant to heat (Appendini and Hotchkiss 2001). However, their antimicrobial activity may be greater when heat is not used in the incorporation process. Moreover, bioactive peptides incorporated in polymer films must be able to diffuse to the package surface over time to be effective. Thus, polymers such as cellulose acetate, alginate, chitosan, and soy protein, among others, have been widely used to develop films with direct incorporation of these antimicrobials (Marcos and others 2008; Pires and others 2008; Sivarooban and others 2008; Santiago-Silva and others 2009).

Researchers have studied the antimicrobial activity of bacteriocins incorporated into polymeric materials in synergy with other antimicrobial agents. Synergistic activity against *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus* has been observed for nisin with potassium sorbate and garlic oil when incorporated into chitosan films (Pranoto and others 2005). In addition, soy protein films incorporated with nisin, grape seed extract, and ethylenediaminetetraacetic acid (EDTA) have shown inhibitory synergistic

activity against pathogenic microorganisms such as *L. monocytogenes*, *E. coli* O157: H7, and *Salmonella Typhimurium* (Sivarooban and others 2008).

The activity of bacteriocins incorporated into polymeric materials in synergy with other conservation technologies has also been reported. Films incorporated with enterocins A and B (bacteriocins produced by *Enterococcus faecium*) have shown synergistic activity when used together with high-pressure processing. Thus, the use of antimicrobial packaging developed in conjunction with the high-pressure process allowed the control of *L. monocytogenes* at below detectable levels after 90 d of storage at 6 °C (Marcos and others 2008).

## Peptide coating on polymeric surfaces

This is an alternative method when the polymer requires extreme processing conditions during packaging material manufacture, such as high pressure and temperature, which can result in inactivation of the antimicrobial agent (Appendini and Hotchkiss 2002).

In some cases, the antimicrobial coating is done by contacting the film with or immersing it in the peptide solution. In this way, linear low-density polyethylene (LLDPE) has been coated with lactocin 705 and lactocin AL705 (both bacteriocins produced by *Lactobacillus curvatus* CRL705), by direct contact of the film with a bacteriocin solution, showing antimicrobial activity *in vitro* against *Lactobacillus plantarum* CRL691 and *Listeria innocua* 7 (Massani and others 2008).

Similarly, Scannell and others (2000) used alternatively lacticin 3147 and nisin adsorbed on the surface of plastic bags (polyethylene/polyamide) through direct contact of the polymeric material with bacteriocin solution. The film coated with nisin showed inhibitory activity against *L. innocua* and *S. aureus*, maintaining its activity for 3 mo either at room temperature or under refrigeration. However, the film coated with lacticin 3147 did not show antimicrobial activity. The researchers suggested that lacticin 3147 was not retained by the polymer (Scannell and others 2000).

Proper handling of solvents and polymeric structures has been suggested to increase the adsorption of the peptide into the polymer matrix (Appendini and Hotchkiss 2002). For example, the polymeric surface can be coated by applying a filmogenic solution that can be deposited on the film surface by the casting method. Accordingly, Chollet and others (2009) developed a laminated film of polyethylene (PE) and polyamide (PA), with the structure PE/PA/PE, coated with a filmogenic solution of hydroxypropyl methyl cellulose (HPMC) and adsorbed with nisin. The developed film presented antimicrobial activity *in vitro* against *Kocuria rhizophila* (Chollet and others 2009).

## Peptide immobilization on polymeric surfaces

Peptides can be immobilized or attached to solid supports by physical methods, such as layer-by-layer assembly, or by chemical methods, such as covalent bonding (Onaizi and Leong 2011).

**Layer-by-layer assembly.** In this process, the peptide is sandwiched between 2 polyionic polymers and the number of peptides and polymers is flexible (Figure 7). The effectiveness of the peptide depends on its relative mobility. The advantage of this method is that it allows the slow release of the peptide embedded in the surface of the polymer. However, a key drawback of this method is that the peptide immobilized in the layers closest to the solid support will not be in direct contact with the target surface, thus reducing peptide activity. This peptide must be able to diffuse through the different layers of the assembly to the interface (Onaizi and Leong 2011), to ensure efficient release and consequent bioactivity.

The diffusion process of the peptide in the different layers is more complex than its diffusion in solution, since additional factors such as tortuosity of the diffusion path, the number of layers, and the polymer–peptide interactions can affect the diffusion process (Appendini and Hotchkiss 2002; Sukhishvili 2005).

**Covalent bonding.** In this process, the antimicrobial peptide will react chemically with a given surface to form a stable bond, which results in the formation of an antimicrobial coating on the polymeric surface (Haynie and others 1995). Covalent bonding offers several advantages, including a more stable attachment between the peptide and the polymer surface (Goddard and Hotchkiss 2007). Covalent bonding reduces attached peptide ability to destabilize and improves its bioactivity, by protecting it from denaturation.

Due to the inert nature of most polymers, they must be subjected to a functionalization process on the surface before bonding with the peptide. The polymers can be functionalized with different spacers, which are reactive functional groups that allow peptide attachment on the spacer surface (Humblot and others 2009).

The quantity of reactive functional groups generated in the functionalization process results in a restricted number of covalent bondings, which limits the amount of peptide that can be attached to the packaging. According to Goddard and Hotchkiss (2007), direct bioactive compound applications require small amounts to be effective. However, for the applications of peptides in polymeric matrixes it is necessary to maximize the amount of peptides per unit area. To accomplish this, the functionalization technique must be optimized with the objective of linking the desired type and quantity of reactive functional groups.

Stiff or flexible spacers have been used as reactive groups for the functionalization of polymers (Figure 8). Stiff spacers, such as polymethyl-methacrylate (PMMA) and polyvinyl chloride (PVC), restrict the lateral mobility of the peptide bond, keeping the peptide firmly in a specific orientation. On the other hand, flexible spacers, such as polyethylene glycol (Sand and others 2010) allow lateral mobility of the peptide bound, which can result in different orientations of the peptide molecules at the interface (Onaizi and Leong 2011).

Among the different types of spacers, polyethylene glycol (PEG) is widely used in the immobilization of peptides. The use of PEG has several advantages, such as rapid and free peptide orientation, promoting peptide–bacteria interactions (Costa and others 2011).

Although the potential penetration and translocation of peptides through the microorganism cytoplasmic membrane is low due to the covalent bond that attaches the peptide to the polymer, it has been reported that peptides have sustained their bioactivity after attachment to polymeric matrixes.

The synthetic peptide 6K8L (a peptide sequence derived from magainin) was covalently bound to polystyrene (PS) resin by functionalization with PEG and showed antimicrobial activity against foodborne pathogenic microorganisms, including *E. coli* O157: H7, *L. monocytogenes*, and *Pseudomonas fluorescens* (Appendini and Hotchkiss 2001). Similarly, the synthetic peptide E14LKK was covalently immobilized in LDPE film after chromium oxidation and functionalization with PEG. This active packaging had antimicrobial activity against *E. coli* showing a reduction of 3 log cycles when compared with the control (Steven and Hotchkiss 2008).

The sustained bioactivity of attached peptides is caused by the presence of spacers which allow sufficient freedom of motion for the active portion of the peptide to contact microorganisms on the food surface (Appendini and Hotchkiss 2002). Haynie and

 

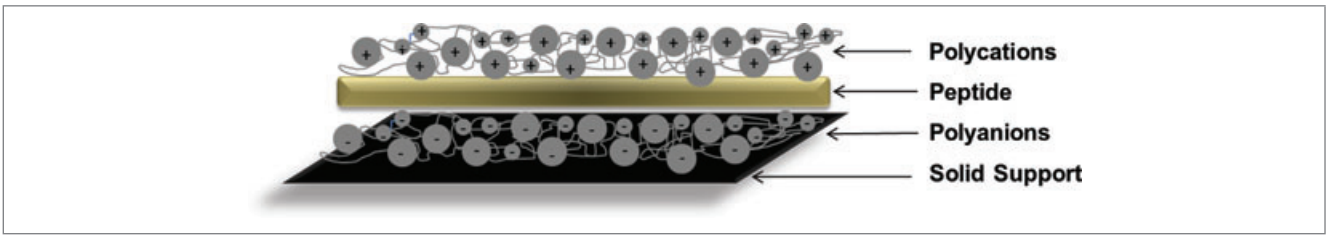


Figure 7–Peptide immobilization by layer-by-layer assembly method. (Adapted with permission from Onaizi and Leong 2011).

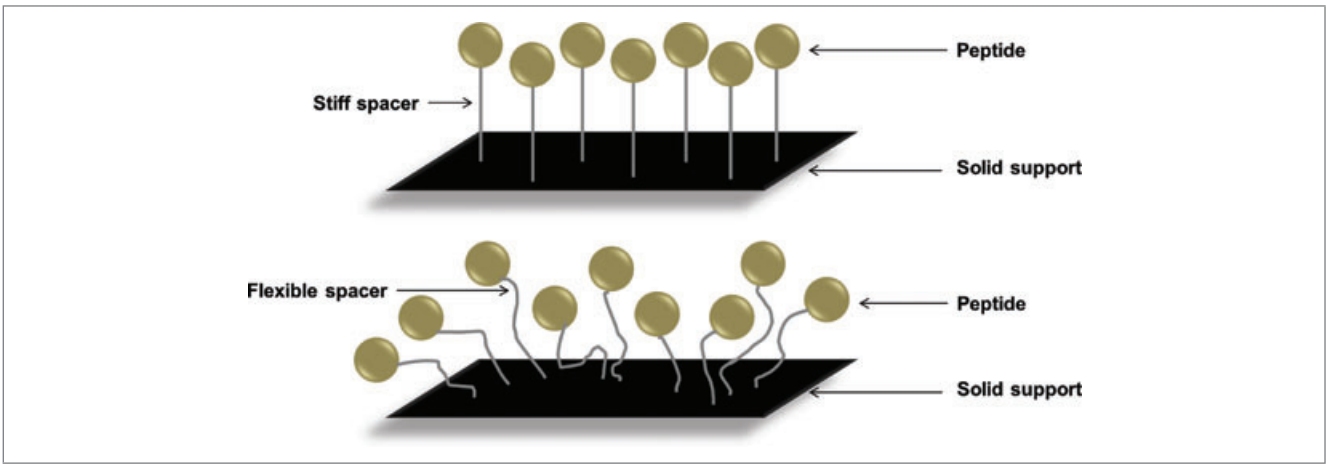


Figure 8–Types of spacers used for polymer functionalization. (Adapted with permission from Onaizi and Leong 2011).

others (1995) have previously demonstrated that peptide–bacteria interactions are sufficient for peptide bioactivity. Moreover, Costa and others (2011) have indicated that the efficacy of attached peptides could possibly result from a higher peptide-relative surface availability, contrary to the other methods of peptide applications in which peptide aggregation can occur, producing uneven distribution.

On the other hand, the diffusion of attached peptides into the food surface is restricted due to the covalent bonding. However, diffusion to the food product can occur in extreme conditions, such as high temperatures, which can promote hydrolysis reactions.

## Characterization of Food Packaging Incorporated with Peptides

The characterization of active packaging involves 2 processes: structural analysis and measurement of their properties (Table 4).

According to Goddard and Hotchkiss (2007), the type of analytical tool used in the structural characterization of polymers depends on the kind of modification, the specificity required, and available resources. Some of the techniques used in structural analysis of active packaging with antimicrobial peptides are the contact angle, X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM).

Steven and Hotchkiss (2008) used the techniques of contact angle and XPS to assess changes in the surface of LDPE films after treatment with chromic oxide and functionalization with PEG as spacer, subsequently covalently binding a synthetic peptide. The contact angle for the film before being subjected to any process of functionalization showed values of 101°. However, after chromic oxidation and PEG bonding, films presented values of 61° and 45°, respectively; which indicated that the film surface became hydrophilic. These researchers concluded that the decrease in the contact angle is the result of an increased ionization at the film surface after oxidation, due to the presence of functional groups, such as carboxylic acid (COOH); and a value even lower after functionalization was observed due to solubility of PEG. The XPS technique showed changes in chemical composition on the film surface, resulting in the detection of nitrogen (2.6%) and an increased percentage of oxygen (initially from 6.7% to 13.3% after oxidation and 19.4% after functionalization). The oxygen increase was due to the presence of carboxylic acid after chromic oxidation. Also, this increase was the result of functionalization with PEG due to the main presence of $O_2$ in the PEG chain backbone. In addition, the functionalization with PEG also introduced nitrogen originating from its amino-terminal functions ($NH_2$-PEG-$NH_2$).

The FTIR spectroscopic technique has been used by Pranoto and others (2005) to study the interactions between chitosan films and nisin. They observed an increase in the band of the amide I corresponding to the wave number 1638 $cm^{-1}$ related to the increased concentration of nisin incorporated in the film. According to the researchers, this is probably due to the interaction between the amine functional groups of chitosan and functional groups of nisin, which resulted in covalent bonds, and consequently in a larger peak.

Microscopic techniques have also been widely used to evaluate morphological changes in the surface of films that have been incorporated with antimicrobial peptides. Pires and others (2008) used SEM and observed that cellulose-based films incorporated with nisin, or a mixture of nisin and nantamicin, showed crystals deposited on the surface. These results indicated a heterogeneous distribution of the peptide in cellulosic films, while the control film presented a homogeneous structure.

Similarly, Santiago-Silva and others (2009) used SEM to observe changes in surface morphology of cellulose acetate film incorporated with pediocin. When the concentration of peptide was increased, the films incorporated with pediocin had a rough surface

Table 4–Characterization techniques of packaging incorporated with antimicrobial peptides.

| Type of characterization | Technique | Factor studied |
|---|---|---|
| Structural analysis | Contact angle | Quantifies surface hydrophobicity by measuring how far a droplet of water spreads on a surface |
| | X-ray photoelectron spectroscopy (XPS) | Determines the atomic composition of the top several nanometers of a solid. This technique can be used to quantify the percent atomic composition and stoichiometric ratios |
| | Fourier transform infrared spectroscopy (FTIR) | Detects and identifies the chemical functional groups present in the polymer |
| | Scanning electron microscopy (SEM) | Allows the characterization of the polymer surface morphology and the observation of the dispersion quality of the peptide in the polymeric matrix |
| Property measurements | Mechanical properties | Measurement of the mechanical performance of the polymer. Generally according to the standard method ASTM D882 (ASTM 2010a) |
| | Barrier properties | Measurement of water vapor permeability. Generally according to the standard method ASTM E96/E96M (ASTM 2010b); ASTM F1249 (ASTM 2006) |

due to large amounts of pediocin granules dispersed in the matrix. This resulted from the lack of peptide solubility. On the other hand, the control film showed a homogeneous and transparent surface.

In addition to the analysis of structural changes and interactions between the peptide and the polymeric matrix, the study of packaging properties is important as well. These properties show the performance of the developed material and how it will relate to the primary functions of food packaging, such as physical integrity. Thus, mechanical and barrier properties have become increasingly relevant and are more frequently studied.

The mechanical properties of films incorporated with antimicrobial peptides serve as the basis for assessing the effects on the mechanical performance resulting from the modification made to the polymer (Table 5).

Guiga and others (2009) investigated the effect of Nisaplin® (2.5% purity of nisin) coating on the mechanical properties of laminated films (PE/PA/PE), studying the mechanical properties of elongation at break and Young's modulus. Their results showed a significant difference in mechanical properties between the films incorporated with the peptide and the control treatment; peptide-incorporated films showed an increase of Young's modulus and a decrease in elongation at break. The polymer-based coating (HPMC) applied in laminated film, as well as the interaction between proteins and salts present in Nisaplin, may have modified the mechanical behavior of the manufactured packaging, thereby increasing the rigidity of the film with the consequent decrease in its elongation.

A similar result was observed by Santiago-Silva and others (2009) in their study of cellulosic films incorporated with pediocin. The researchers indicated that the addition of 25% of the peptide increased the maximum load required for film rupture when compared to the control. The researchers pointed out that a possible interaction between the pediocin and the polymeric matrix allowed the development of a more resistant film. However, a significant drop in the maximum load value was observed at a 50% concentration. According to the researchers, there was an excessive amount of the peptide incorporated, which weakened the cellulose chains of the film and resulted in a reduction of film resistance.

A decrease in the mechanical strength of films incorporated with antimicrobial peptides has also been observed. Sivarooban and others (2008) reported a decrease in puncture resistance and tensile strength values of soy protein films incorporated with nisin. Similarly, Pires and others (2008) indicated that nisin incorporated into cellulose-based films affected the film structure, reducing maximum load and elongation values. These resulted from the heterogeneous distribution of the peptide in the polymeric matrix, which consequently lead to the formation of stress points and reduced film resistance.

Although several studies have indicated changes in film properties, in some cases peptide incorporation into polymeric matrices had no significant effects. Massani and others (2008) reported no significant difference in tensile strength, elongation, and water vapor permeability of LDPE films coated with lactocin705 and lactocin AL705. Similar results were observed by Chollet and others (2009) who indicated that the incorporation of nisin into PE/PA/PE laminated films, by coating with HPMC, showed neither changes in tensile strength at break nor in water vapor permeability. Similarly, Guiga and others (2010) reported that the direct incorporation of nisin into multilayer films of ethyl cellulose (EC) and HPMC (EC/HPMC/EC) did not alter the properties of tensile strength, Young's modulus, or elongation at break.

Barrier properties of packaging include resistance to water vapor or gases ($O_2$ and $CO_2$). The water vapor barrier property of the packaging can be determined by calculating the water vapor permeability (WVP) or the water vapor transmission rate (WVTR). Both parameters cover the determination of the passage of water vapor through a polymeric material. However, the WVP considers vapor pressure difference between 2 specific surfaces (internal and external) of the analyzed packaging (ASTM 2010b). Both parameters, WVP as well as WVTR, have been used to indicate that the addition of nisin in different polymeric matrixes, such as LDPE film coated with a cellulose-based solution (Grower and others 2004; Massani and others 2008), sodium caseinate films (Kristo and others 2008) and PE-film coated with HPMC (Chollet and others 2009), causes no significant changes in the water vapor barrier property.

On the other hand, studies regarding gas permeability in active packaging have been limited due to their applications as films or coatings for food products.

## Migration of Peptides Incorporated into Food Packaging Materials

During the evaluation and characterization of antimicrobial packaging it is important to research the transference of antimicrobial substances from the packaging material into the food, since this information allows the determination of how the antimicrobial agent is released from the active packaging.

Table 5–Mechanical properties of active packaging incorporated with antimicrobial peptides.

| Mechanical property | Measurement unit | Significance |
|---|---|---|
| Maximum load | N | Measurement of the maximum load on the film registered in the tensile strength test |
| Tensile strength | MPa | Measurement of the ability of the film to withstand a defined load when submitted to traction |
| Load at break | N | Measurement of the load registered on the film at break point when performing the tensile strength test |
| Tensile strength at break | MPa | Measurement of film resistance to a load at break |
| Elongation at break | % | Measurement of the maximum deformation of the film presented before its rupture |
| Elastic modulus (Young's modulus) | MPa | Measurement of the film stiffness |
| Penetration resistance | N | Measurement of the ability of a flexible sheet material to withstand elongation and/or puncture by a driven probe |

The mass transfer can occur by the diffusion mass transfer mechanism or by the convective mass transfer mechanism. The convective mass transfer mechanism occurs in a moving fluid, known as natural convection, if the movement is caused by differences in the density, or as forced convection, if the movement is caused by external agents or when a fluid is flowing on the solid surface by forced movement.

On the other hand, diffusion mass transfer consists of a random motion of individual molecules as a result of a concentration gradient (Crank 1975; Geankoplis 1993).

The migration of substances from packaging materials takes place through the diffusion mass transfer mechanism, since the active packaging and the food contain a concentration gradient for the antimicrobial agent incorporated in the packaging.

Unlike the research on the release of active substances from drugs or the release of solvents from polymers, the study of antimicrobial release from active packaging is still limited (Buonocore and others 2003; Bastarrachea and others 2010).

The knowledge of diffusion parameters allows an efficient design of active packaging. Several factors must be considered when studying the migration from antimicrobial packaging, including the release rate of the antimicrobial molecules from the packaging. If this rate is high, the active packaging would release the antimicrobial rapidly, resulting in a large concentration at a determined time. However, the large concentration would not be maintained over time, depending on the solubility of the antimicrobial in the selected food. If the solubility is very high, the antimicrobial will migrate rapidly to the food matrix, and therefore result in a decreased concentration of the antimicrobial on the food's surface along time. On the other hand, if the release rate is low, the antimicrobial agent will be slowly released in a desired concentration and if it presents a low solubility in the selected food, the antimicrobial can accumulate on the food surface and slowly migrate into the food matrix. In this situation the release rate should not be slower than the microbial growth (Bastarrachea and others 2011).

In either case, the release of the antimicrobial agent from the packing material is indicated by the diffusion coefficient ($D$). Thus, the diffusion characteristics of the antimicrobial agent can be used to determine the amount needed to maintain the proper concentration on the food surface (Buonocore and others 2003).

The literature reports a few migration studies of antimicrobial peptides incorporated in active packaging. Most evaluate the migration of nisin, probably due to the fact that this is the only antimicrobial peptide substance indicated as *generally recognized as safe* (GRAS) for direct contact with food in the United States (FDA 2011). Nisin is also widely accepted as a food preservative in the European Community where it is classified as a safe preservative for food contact, coded as E 234 (FSA 2010), as well as in Brazil where the use of nisin is permitted by Brazilian law as a natural preservative for biological products (ANVISA 1996).

Diffusion of several antimicrobial agents, such as potassium sorbate or lysozyme incorporated in active packaging, has been explained by Fick's second law (Han and Floros 1998; Gemili and others 2009):

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \quad (1)$$

where, $C$ is the diffusing substance concentration; $D$ is the apparent diffusion coefficient; $t$ is the diffusion time; and $x$ the distance.

Depending on the conditions of the migration test, different analytical solutions have been applied to solve Fick's second law and to calculate the $D$-value in the migration of the antimicrobial peptide nisin incorporated in different types of packaging materials (Table 6).

Different analytical solutions of Fick's second law have been used in previous studies to calculate the $D$-value of nisin at a specific temperature (Table 7).

Some of these studies were conducted at different temperatures to characterize the $D$-value as a function of this parameter. Protein films (corn zein or wheat gluten) and poly(butylene adipate-co-terephthalate) (PBTA) films incorporated with nisin showed an increase in $D$-value with increasing temperature, indicating that the peptide concentration was higher in the simulant at equilibrium state with increasing temperature (Teerakarn and others 2002; Bastarrachea and others 2010). Similarly, at low temperatures, lower $D$-values of nisin diffusivity indicate that the film retains larger amounts of the peptide in the polymer matrix while in contact with the simulant.

The Arrhenius activation energy model (Eq. 2) has been shown to confirm the dependence of the diffusivity with respect to temperature.

$$\mathrm{D} = \mathrm{D}_0 \exp\left(-\frac{\mathrm{E_a}}{\mathrm{RT}}\right) \quad (2)$$

where, $D_0$ is a constant; $E_a$ is the activation energy for the diffusion process (J/mol); $R$ is the universal gas constant (8.314 J/mol K); and $T_{abs}$ is the absolute temperature (K). Thus, $E_a$ can be calculated by Eq. 3.

$$\mathrm{Ln\,D} = -\frac{\mathrm{E_a}}{\mathrm{R}}\left(\frac{1}{\mathrm{T_{abs}}}\right) + \mathrm{Ln\,D_0} \quad (3)$$

**Table 6–Analytical solutions of Fick's second law in migration studies of nisin incorporated in active food packaging.**

| Polymeric matrix | Analytical solution | Description of variables | Reference |
|---|---|---|---|
| Protein films (corn zein and wheat gluten) | $\frac{M_t}{M_o} = 1 - 4\left[\frac{Dt}{\pi h^2}\right]^{\frac{1}{2}}\left\{\pi^{-\frac{1}{2}} + 2\sum_{\pi=1}^{\infty}(-1)^n ierfc\frac{nh}{2\sqrt{Dt}}\right\}$ | $M_0$, is the initial amount of nisin in the film; $M_t$, released amount of nisin at time t; h, film thickness; D, diffusion coefficient; ierfc, associated function of the mathematical error function | Teerakarn and others (2002) |
| Paper coated with acrylic polymer and ethylene-vinyl acetate co-polymer (EVA) | $\frac{M_t}{M_\infty} = \frac{2}{L_p}\left[\frac{Dt}{\pi}\right]^{0.5}$ | $M_t$, is the amount of nisin released at time t; $M_\infty$, is the migration in a state of equilibrium; $L_p$ coating layer thickness, D, diffusion coefficient | Kim and others (2002) |
| Hydroxypropyl methyl cellulose (HPMC) films | $C = \frac{M_0}{\sqrt{\pi \cdot D \cdot t}} e^{\left(\frac{-x^2}{4 \cdot D \cdot t}\right)}$ | C, is nisin concentration in the simulant; $M_0$, amount of nisin in the film; D, diffusion coefficient; x, length of the gel (simulant); t, diffusion time | Sebti and others (2003) |
| Paper coated with ethylene-vinyl acetate co-polymer (EVA) | $\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty}\frac{8}{(2n+1)^2\pi^2}\exp\left(\frac{-(2n+1)^2\pi^2}{4L_p^2}Dt\right)$ | $M_t$, is nisin amount in the simulant at time t; $M_\infty$, nisin amount in the simulant at equilibrium; D, diffusion coefficient; $L_p$, coating thickness | Ho Lee and others (2004) |
| Polybutylene adipate co-terephthalate (PBTA) films | $\frac{M_{s,t}}{M_{F,0}} = \frac{\alpha}{1-\alpha} - \sum_{n=1}^{\infty}\frac{2\alpha}{1+\alpha+\alpha^2 q_n^2}e\left[-\frac{dq^2 nt}{l^2}\right]$ | $M_{s,t}$, is the amount of nisin in the simulant at time t; $M_{F,0}$, amount of nisin in the film when t = 0; $\alpha$, mass ratio between the amount of nisin in the simulant and in the film at equilibrium; $q_n$, is the "n" root of $\tan q_n = -\alpha q_n$; l, is a half of the film's thickness | Bastarrachea and others (2010) |

**Table 7–Diffusion coefficient (*D*) of nisin incorporated in different active food packagings.**

| Polymeric matrix | Food simulant | T (°c) | $D$ ($\times 10^{-12}$ m$^2 \cdot$ s$^{-1}$) | Reference |
|---|---|---|---|---|
| Paper coated with EVA | Emulsion (distilled water and paraffin oil) | 10 | 11.3 | Ho Lee and others (2004) |
| HPMC films | Agarose gel | 10 | 12.4 | Sebti and others (2003) |
| Paper coated with acrylic polymer and EVA | Distilled water | 10 | 9.3 | Kim and others (2002) |
| | 2% NaCl | | 6.0 | |
| | 2% Sucrose | | 11.3 | |
| | 2% Citric acid | | 12.2 | |
| Corn zein films | Distilled water | 5 | 0.00065 | Teerakarn and others (2002) |
| | | 25 | 0.00770 | |
| | | 35 | 0.03100 | |
| | | 45 | 0.06400 | |
| Wheat gluten films | Distilled water | 5 | 0.00510 | Teerakarn and others (2002) |
| | | 25 | 0.03500 | |
| | | 35 | 0.07500 | |
| | | 45 | 0.13000 | |
| PE/PA/PE films coated with HPMC | Agarose gel (5% fat) | 25 | 65 | Chollet and others (2009) |
| | Agarose gel (30% fat) | | 181 | |
| EC/HPMC/EC films | 0.8% NaCl | 28 | 0.0254 | Guiga and others (2010) |
| | | 5.6 | 93 | Bastarrachea and others (2010) |
| PBTA films | Distilled water | 22 | 472 | |
| | | 40 | 578 | |

Teerakarn and others (2002) indicated $E_a$ values of 85.8 and 53.1 kJ/mol for corn zein and wheat gluten films, respectively, and Bastarrachea and others (2010) obtained an $E_a$ value of 38.3 kJ/mole for PBTA film incorporated with nisin.

The value of $E_a$ represents the degree of molecular interactions between the antimicrobial substance incorporated and the polymeric matrix. Thus, higher $E_a$ values represent stronger antimicrobial–polymer interactions, which is reflected in a lower *D*-value due to the greater energy level required for antimicrobial release (Bastarrachea and others 2010).

The relationship between temperature and diffusivity of the antimicrobial agent is the result of structural changes in the polymer matrix, since above the glass transition temperature ($T_g$) the molecular mobility in the system increases along with temperature, which leads to an increase in the ability of the packaging material to transport substances through its polymeric matrix (Teerakarn and others 2002).

In addition to the interactions between polymer and antimicrobial agent, the *D*-value is also influenced by interactions between the antimicrobial and the food matrix. Thus, the food

 

Table 8–Mentioned peptides and their amino acid sequence.

| Peptide | Amino acid sequence | Reference |
|---|---|---|
| Buforin II | TRSSRAGLQFPVGAVHRLLRK | Park and others (1996) |
| Cecropin A | KWKLFKKI | Andreu and others (1992) |
| $\alpha$-Defensin | CYCRIPACIAGERRYGTCIYQGRLWAFCC | Ganz (2003) |
| Dermaseptin S4 derivative | ALWKTLLKKVLKA-$CONH_2$ | Dagan and others (2002) |
| Di-K19Hc | KWLNALLHHGLNCAKGVLA | Jang and others (2006) |
| Enterocin A | TTHSGKYYGNGVYCTKNKCTVQWAKATTCIAGMSIGGFKGGAIPGKC | Aymerich and others (1996) |
| Enterocin B | ENDHRMPNELNRPNxLSKGGAKGAAIAGGLFGIPKGxLAW* | Casaus and others (1997) |
| Ib-AMPs | EWGRRCCGWGPGRRYCVRWC | Thevissen and others (2005) |
| Lacticin 3147 | AADhbNDhbFALADYWGNNGAWAAbuLAbuHEAMAWAK**,*** | Willey and van der Donk (2007) |
| Lactocin 705 | GMSGYIQGIPDFLKGYLHGISAANKHKKGRL | Palacios and others (1999) |
| Magainin II | GIGKFLHSAKKFGKAFVGEIMNS | Matsuzaki and others (1998) |
| Melittin | GIGAVLKVLTTGLPALISWIKRKRQQ-$NH_2$ | Yang and others (2001) |
| Mucroporin-M1 | LFRLIKSLIKRLVSAFK | Li and others (2011) |
| Nisin | MSTKDFNLDLVSVSKKDSGASPRI | van der Meer and others (1994) |
| Omiganan | ILRWPWWPWRRK-$NH_2$ | Rubinchik and others (2009) |
| Pediocin | KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC | Fimland and others (1998) |
| Peptide SMAP-29 | RGLRRLGRKIAHTVKKYG-$NH_2$ | Lee and others (2002) |
| Peptide TH2–3 | QSHLSLCRWCCNCCRSNKGC-$NH_2$ | Chen and others (2009) |
| Plantaricin | | |
| $\alpha$ peptide | NWSLQMGATAIKQVKKLFKKWG | Nissen-Meyer and others (1993) |
| $\beta$ peptide | NAYSLQMGATAIKQVKKLFKKWG | |
| Pleurocidin | GWGSFFKKAAHVGKHVGKAALTHYL | Patrzykat and others (2002) |
| Psacotheasin | CIAKGNGCQPSGVQGNCCSGHCHKEPGWVAGYCK-$NH_2$ | Hwang and others (2010) |
| Synthetic peptide 6K8L | HOOC-LKLLKKLLKLLKKL-$NH_3$ | Appendini and Hotchkiss (2001) |
| Synthetic peptide E14LKK | LKKLLKLLKKLLKL | Steven and Hotchkiss (2008) |
| Synthetic peptide TH1–5 | GIKCRFCCGCCTPGICGVCCRF-$NH_2$ | Chang and others (2011) |
| Tachyplesin II | NH2-R-W-C-F-R-V-C-Y-R-G-I- C-Y-R-K-C-R-CONH2 | Miyata and others (1989) |

*x = Means that the specific identity of an amino acid cannot be determined unambiguously.
**Dhb = (Z)-2,3-didehydrobutyrine.
***Abu = 2-Aminobutyric acid.

composition, as well as the solubility of the antimicrobial in these components also affects the *D* coefficient. In the study of paper coated with EVA incorporated with nisin, the *D*-values varied according to the composition of the food in contact with the active packaging (Kim and others 2002). The highest *D*-value was observed when the film was in contact with a 2% citric acid solution, and the lowest value was observed when in contact with a 2% NaCl solution. Characteristic parameters of each solution, such as pH and ionic strength, have been shown to influence nisin solubility. Nisin has a high solubility (up to 40 $mg{\cdot}mL^{-1}$ at pH 2) at low pH, but at high concentrations of NaCl (above 1 M) nisin solubility dependence on pH almost disappears and the solubility decreases to values below 1 $mg{\cdot}mL^{-1}$ at any pH (Rollema and others 1995).

Chollet and others (2009) also investigated the influence of food composition on the migration of nisin incorporated in PE/PA/PE films coated with HPMC by changing the fat percentage. They found that increasing the fat content in the food resulted in an increased *D*-value and, therefore, in a greater diffusion of incorporated nisin. In their experiment, nisin diffusion mechanism was governed by the fat content. The increase in fat content resulted in microstructural changes, such as enlargement of pore size in the food matrix, which favored nisin diffusion into it.

## Conclusions and Future Prospects

Consumer demand for minimally processed foods and additive-free products has led to the development of antimicrobial packaging. Peptides have shown various bioproperties, among them antimicrobial activity, leading to the application of these compounds in the food preservation area by either direct addition or incorporation into packaging materials (Table 8).

Active packaging materials incorporated with antimicrobial peptides have shown effectiveness in inhibiting pathogenic microorganisms, an improvement in food safety. Moreover, antimicrobial peptides incorporated into the polymeric matrix may affect the engineering characteristics of the packaging material, and lead to differentiated diffusion performance. This review highlights the characteristics of pure peptides, as well as their incorporation into polymeric matrices. Several studies have indicated significant changes in mechanical properties and surface morphology of the films incorporated with antimicrobial peptides. However, research related to the study of barrier properties to gases and water vapor is still limited.

More studies on the release of other peptides, different from nisin, from food packaging materials are needed to better understand the mechanism of dissemination of antimicrobial agents.

Finally, in the years ahead, the advent of nanotechnology will lead to research on the synergistic effects of antimicrobial peptides and nanoparticles, such as metals, metal oxides, and nanoclays, with the objective being to improve the mechanical and barrier properties of antimicrobial packaging.

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