https://doi.org/10.46344/JBINO.2022.v11i06.06

FUNCTIONALITY AND METHODOLOGIES FOR OBTAINING BIOACTIVE PEPTIDES: A REVIEW

Xochilt Yoseline Marcos-Méndez, Laura Acosta-Domínguez, Frixia Galán-Méndez*

Universidad Veracruzana, Facultad de Ciencias Químicas, Circuito Gonzalo Aguirre Beltrán esq. con Calle La Pérgola, Zona Universitaria, Xalapa, Veracruz, México, C.P. 91000

Email ID: fgalan@uv.mx

ABSTRACT

Bioactive peptides are short-chain peptides of 3 to 20 amino acids, which can exert biological activities after their release from the precursor protein. Foods of plant or animal origin are the main source of these compounds, so there is a range of studies focused on obtaining peptides from these sources, using these conventional methods and new emerging technologies. The purpose of these studies is to obtain a biological benefit for human health and some of them, a use in the food industry. The aim of this work was to present updated scientific evidence of the different bioactivities that have been identified in food varieties, as well as applied production methodologies and the possible implementation of peptides in food products. In conclusion, there is currently a notable interest in finding food sources that provide bioactive peptides, however, there are challenges related to their methodology and scalable production.

Keywords: bioactive peptides, biological activity, food source, food products.

INTRODUCTION

In the daily diet, human obtain biomolecules such as carbohydrates, lipids, proteins, among others; which through the digestive system are absorbed in the intestine and then, metabolized at the cellular level (Gutierrez and Gonzales, 2016). Proteins play an important role as a nutrient and perform physicochemical functions for the well-being of the human body (Rizzello *et al*., 2016). These are made up of a sequence of amino acids linked by peptide bonds (Espósito *et al*., 2018), and during gastrointestinal digestion they can be released as short-chain peptides (3 to 20 amino acids), which are considered bioactive peptides when they have biological activity. Bioactive peptides are obtained for different methods., for example, gastrointestinal digestion can be simulated outside an organism by reproducing the process in vitro through enzymatic hydrolysis, or industrial methods such as chemical hydrolysis and fermentation can be used, too (Pepe *et al*., 2016; Toldrá *et al*., 2018).

Biopeptides have beneficial biological effects on human health when they are released from the protein that contains them, and their amino acid sequence will determine the biological activity they develop; some are considered multifunctional, that is, they can perform more than one potential function in the body (Chakrabarti *et al*., 2018; Daroit and Brandelli, 2021). The bioactivities reported in the literature are antimicrobial, anticancer, antidiabetic, antithrombotic, antihypertensive, antioxidant and anti-inflammatory activity (Siow and Gan, 2016; Daliri *et al*., 2017; Sánchez and Vázquez, 2017; Siregar *et al*., 2020). These bioactivities allow biopeptides have application in the food and pharmaceutical industry, through their use as food additives or nutraceutical ingredients (Wang and Selomulya, 2020).

The objective of this work was to present updated scientific evidence of the different bioactivities that have been identified in food varieties, as well as the applied production methodologies and the possible implementation of peptides in food products.

BIOLOGICAL ACTIVITIES OF BIOACTIVE PEPTIDES

Antihypertensive

Arterial hypertension is characterized by high blood pressure levels, whose control is given by the regulation of the renin-angiotensin-aldosterone system. This condition is regulated by fast-acting pharmaceutical products; however, their continuous use can generate side effects such as dry cough, irritating cough, headache, loss of taste, hypokalaemia, hyponatremia and angioedema (Ciau-Solís *et al*., 2018). In this way, antihypertensive peptides, named for their inhibitory effect on the excess of activities carried out by the enzyme renin and ACE (angiotensin I converting enzyme), are highly relevant as they are an alternative that allows control of blood pressure without negative impacts on health (Aluko, 2018; Wagner-Grau, 2018). Therefore, in recent years, the inhibitory effect of ACE has been demonstrated in vitro, from various sources of dietary protein, such as milk, fish, yeast, rice, corn, eggs, and bovine blood plasma (Ciau -Solis *et al*., 2018).

Anticancer

The World Health Organization (WHO) defines cancer as "a large group of diseases that can begin in almost any organ or tissue of the body when abnormal cells grow uncontrollably" (WHO, 2021: paragraph 1). This growth spreads into the surrounding tissues and the most promising treatment to slow the transformation of normal cells into cancer cells is chemoprevention, which turns out to be expensive and carries unwanted side effects such as damaging normal cells. In this way, bioactive peptides with anticancer effect have been shown to be a natural alternative for cancer control (Chalamaiah *et al*., 2018). So far, in vitro, and in vivo studies of peptides with potential anticancer effect from different food sources such as insects, larvae, fungi, peptides of marine origin and amphibians have been found (Chalamaiah *et al*., 2018; Eghtedari *et al*., 2021).

Antimicrobial

Antimicrobial agents are highly used food additives in the food industry; however, a large amount of those that are chemically synthesized are used, generating concern in the consumer (Habinshuti *et al*., 2019). However, there are foods that are part of the diet and have important components such as antimicrobial peptides that provide a bacterial inhibitory effect. These peptides are capable of inhibiting bacteria, protozoa, fungi and viruses. Within the studies carried out, peptides from seeds have been isolated and identified, as is the case with cumin (Mijiti *et al*., 2018). On the other hand, other lines of research focus on the antimicrobial activity of Maillard reaction products (MRP) from vegetable protein hydrolysates (Habinshuti *et al*., 2019).

Antidiabetic

Diabetes mellitus is a metabolic disease characterized by high blood glucose levels (Sanzana and Durruty, 2016) and a large part of its diagnoses can be divided into two types: type I diabetes mellitus and type II diabetes mellitus (Kinariwala and col., 2019; Naya and Álvarez, 2016). These conditions are commonly treated through medications; however, their consumption can cause adverse effects such as nausea, vomiting, weight gain, pancreas infection, among others; therefore, there are various treatment alternatives. One of them is the implementation of antidiabetic peptides, whose activity consists of inhibiting the action of the enzymes a-amylase, a-glucosidase, and dipeptidyl peptidase 4 (DPP-IV), since these enzymes are related to the increase in blood glucose (Kehinde and Sharma, 2020; Kinariwala *et al*., 2019; Akan, 2020; Han *et al*., 2021). According to a study by Khan *et al*. (2018), some sources that provide antidiabetic peptides are: sheep's milk, whey, beans, barley flour, pumpkin seeds, and oats.

Antithrombotic

Antithrombotic activity is responsible for preventing the formation or increase of blood clots in the body. Within this process, coagulation depends largely on the interaction of the enzyme thrombin with fibrinogen. The antithrombotic action is focused on the inhibition of thrombin, and therefore, this interaction is the basis of various studies carried out for the formulation of drugs (Bhandari *et al*., 2020), as well as for the identification of bioactive peptides, which they inhibit the formation of blood clots in the intrinsic pathways (Cian *et al*., 2018). Antithrombotic peptides interact with thrombin exosite 1, resulting in inhibition of the enzyme thrombin. Food sources of antithrombotic agents can be of animal or vegetable origin, such as peanut protein, amaranth, egg white, rapeseed, among others (Chen *et al*., 2019).

Antiinflammatory

Inflammation is a necessary immune response when aggressions or causative agents of different natures occur (Caballero-Gutiérrez and Gonzáles, 2016). It has important relevance in the causes and development of various diseases, therefore, there is an interest in the discovery of compounds from food, such as bioactive peptides with anti-inflammatory effect, whose mechanism is the inhibition of enzymes that participate in the pathology , for example, LOX, COX-1 and COX-2. Although another mechanism is through the inhibition of the secretion of inflammatory markers or the reduction of proinflammatory cytokines, NF-κB, RAS and MAPK; on the contrary, it helps in the increase of TGF-β or IL-10 (Gutierrez and Gonzales, 2016; Jakubczyk *et al*. 2019). Whey, the marine sponge Theonella swinhoei, and the microalgae Spirulina are examples of sources of these biopeptides (da-Silva *et al*., 2021; Dullius *et al*., 2018; Festa *et al*., 2011).

Antioxidant

The composition, structure and hydrophobicity are key factors in the antioxidant properties of the fragments with biological activity. The antioxidant capacity minimizes oxidative stress, thus avoiding alterations in the functionality of the organism; said stress is a consequence of an increase in free radicals (Viada-Pupo *et al*., 2017). Antioxidant peptides inhibit lipid peroxidation, scavenge free radicals, induce ferritin, and enhance the activity of GSH-Px and SOD, enzymes that protect cells against oxidative damage (Gutierrez and Gonzales, 2016; Li *et al*., 2019). In this way, antioxidant peptides are a natural alternative that converts the food source that provides them into a dietary supplement option that promotes health (Yu *et al*., 2021); as well as, they can be obtained through the reuse of bones, as is the case of bovine bone, which in a study by Yang *et al*. (2021), showed to have peptides with antioxidant activity.

CONVENTIONAL METHODS OF PEPTIDE PRODUCTION

Bioactive peptides are traditionally produced by three methods: enzymatic hydrolysis, chemical hydrolysis, and fermentation; however, recombinant DNA technology is currently another method commonly used in the production of peptides. On the other hand, during the processing of food, some reactions may occur that generate its release. It should be noted that the production of compounds with biological activity usually begins with studies at the laboratory or pilot level and later, they are scaled up to industrial production, which leads to the generation of conditions for obtaining them. In this way, the lack of viable strategies for industrial production generates the search for new emerging technologies that satisfy this need (Dullius *et al*., 2018; Marciniak *et al*., 2018). Table 1 shows the different methods for obtaining peptides that have been used in recent years associated with a specific biological activity, as well as their source of production. Enzymatic hydrolysis

Enzymatic hydrolysis is considered a safe process that uses food-grade enzymes; these molecules have a particular importance in gastrointestinal simulation, since it is a representation of the human digestion process that allows determining the effectiveness of peptide release in the protein; however, digestive enzymes do not necessarily have to be applied in enzymatic hydrolysis, different types of proteases can also be used. For the optimization of this method, there must be a strict control of key parameters such as pH, temperature, agitation, and the amount of enzyme needed. This last aspect is a delimitation because it can generate high costs (Marciniak *et al*., 2018; Ulug *et al*., 2020). Likewise, the specificity of the enzyme influences the cut made on the original protein and enables the release of bioactive peptides; as demonstrated by Mojica and Mejía (2016), by optimizing the enzymatic production of antidiabetic peptides from black bean proteins (Phaseolus vulgaris L.). For this, they used 8 proteases (proteinase K, pepsin, trypsin, papin, alcalase, aromazyme, thermolysin and chymotrypsin), with different proportions of layers and hydrolysis times. The results showed that alcalase in a 1:20 (p/p) ratio and for 2 h released better antidiabetic peptides.

Fermentation

During fermentation, some microorganisms can express proteolytic enzymes, which cause peptide formation (Chai *et al*. 2020; Kehinde and Sharma, 2020). Of course, aspects such as the exposure time and the selected microorganism will influence the final product (Rizzello-Pérez *et al*., 2016). Lactic acid bacteria are convenient microorganisms for this process, as is the case of fermented milk from specific strains of Lactobacillus, which, according to Aguilar *et al*. (2017), allows to obtain multifunctional peptides. Also, there are fungal microbial sources (Martínez-Medina *et al*., 2019), such as Aspergillus oryzae and Aspergillus flavipes, used in bovine and goat milk to produce proteases, through solid state fermentation, to obtain peptides with antimicrobial and antioxidant properties (Zanutto-Elgui *et al*., 2019). Microbial fermentation is an economical method compared to enzymatic hydrolysis; however, the low yield in obtaining the compounds and the specificity of their formation are aspects that hinder their industrial use (Raveschot *et al*., 2018).

Chemical hydrolysis

Chemical hydrolysis is a simple and less expensive process, which involves the breaking of peptide bonds by means of acids or alkalines, with the aim of generating peptides and free amino acids (Wang *et al*., 2017); however, with this method molecules that damage health can be generated (Martínez *et al*., 2019). Wisuthiphaet *et al*. (2016) analyzed fish protein hydrolysates by means of acid (hydrochloric acid) and enzymatic (papain and alcalase) hydrolysis; these authors concluded that enzymatic hydrolysis provided better nutritional activities, with respect to hydrolysis in acid medium. Therefore, chemical hydrolysis is a process that has limitations by reducing nutritional qualities (Ulug *et al*., 2020).

Recombinant DNA technology synthesis

Recombinant DNA technology synthesis by recombinant DNA technology is generally carried out in microorganisms, that is, in prokaryotic cells, mainly bacteria, leading to the production of one or many recombinant peptides simultaneously. Escherichia coli (E. coli) is the most widely used host as a prokaryotic cell; however, they are also produced in eukaryotic cells from plants, seeds, tubers, or roots, where rocombinant proteins are stored in organs such as vacuoles, endoplasmic reticulum and plastids, protected from the activity of proteases (Satei *et al*., 2021; Varasteh-Shams *et al*., 2020). The recombinant technology method is relatively more cost-effective and is mainly used in large-scale peptide synthesis using affordable starting materials, but like chemical and enzymatic methods of production, bioactive peptides need to be purified and characterized to determine their value functional activity (Sosalagere *et al*., 2022).

NEW PRODUCTION TECHNOLOGIES

Currently, the conventional methods used in the production of bioactive peptides have some limitations or disadvantages, which prompt the search for new technologies that allow these compounds to be obtained or complement conventional methods (Ulug *et al*., 2020). High hydrostatic pressure (HHP) is an effective non-thermal technology, which is based on the application of high pressures, generally between 100 MPa and 700 MPa; this process uses water as a transmitting medium and allows the inactivation of microorganisms as well as the modification of enzymatic reactions (Bonfim *et al*., 2019; Calderón-Santoyo *et al*., 2019). This treatment performs structural and conformational modifications in proteins, which results in a benefit in the hydrolysis of those with a globular structure, for example, whey proteins, in which enzymatic hydrolysis does not generate a high hydrolytic degree (Landim *et al*., 2021). Despite this, the enzymatic hydrolysis in combination with the HHP processing allows to increase the biological activity of the peptides, resulting in a higher yield. Such is the case of casein peptides, which have been shown to have a high degree of hydrolysis and an improvement in their antioxidant properties, through the implementation of both methods (Bamdad *et al*., 2017). Ultrasound Assisted Extraction (UAE) Ultrasound-assisted extraction (UAE) is considered an ecological method that makes it possible to improve the extraction yield of bioactive compounds from foods, as well as preserve their molecular properties (Yang *et al*., 2021). The application of ultrasound to a solid-liquid medium results in an acoustic cavitation phenomenon, considered as the necessary force for the release of the components (Baite *et al*., 2021), since the implosion of the cavitation bubbles leads to a micro-jet causing the peeling of the product surface, as well as the erosion and decomposition of particles (Chemat *et al*., 2017). Therefore, because of these forces on proteins, there is evidence of its application to obtain bioactive peptides from different sources such as Indian edible macroalgae (Kumar *et al*., 2020), Porphyra haitanensis (red algae) (Wen *et al*., 2020), pineapple by-products (Mala *et al*., 2021) and aburon viscera (Wu *et al*., 2021).

Ohmic heating

Ohmic heating is an emerging technology that consists of heating food through the passage of electrical energy to thermal energy, with the help of two inserted electrodes, which promotes a rapid and uniform increase in temperature (Cappato *et al*., 2017); in addition, it is convenient due to minimal thermal degradation and nutrient retention (Ulug *et al*., 2020). Therefore, it is an alternative with less destructive impact of bioactive compounds compared to conventional heating, such is the case of sweet whey, for which, Costa *et al*. (2018) compared both heatings and the sweet whey showed a higher number of bioactive peptides when ohmic heating was used.

Pulsed Electric Fields (PEF)

Pulsed Electric Fields (PEF) are a non-thermal processing method. Its operation involves the application of electric fields through short pulses with intensity from 10 kV/cm to 80 kV/cm in certain microseconds (Pal, 2017). It has a potential effect for the inactivation of microorganisms and can be used for the extraction of components, since they have low energy demand and selectivity for their release (Frey *et al*., 2017). In recent years, studies have shown the effectiveness of PEFs in enhancing peptide activity, especially antioxidant activity. Such is the case of a study conducted by Franco *et al*. (2020), in which they obtained extracts with potential antioxidant activity from the application of PEF in fish residues. On the other hand, Liu *et al*. (2019) demonstrated that the application of PEF and heating improved the antioxidant and anti-inflammatory activity of ovomucin-depleted egg white.

Microwave assisted extraction

Microwave-assisted extraction, a non-conventional technology, refers to the extraction of bioactive compounds through the application of non-ionizing electromagnetic waves, through frequencies ranging between 300 MHz and 300 GHz (Nabet *et al*., 2019); which results in a reduction of the surface and solvent tension, as well as an increase in the solubility of the analytes in the extraction medium (Rodríguez-Pérez *et al*., 2016). Two mechanisms responsible for the transfer of energy and the generation of heating of the sample or food are needed, ionic conduction and dipole rotation (Feki *et al*., 2021). Likewise, its use is susceptible due to the reported high yields, the lower amounts of solvent and the short extraction times in the production of biologically active compounds (Muñiz-Márquez *et al*., 2020).

In addition, microwave-assisted extraction in combination with enzymatic hydrolysis optimizes the obtaining of bioactive peptides derived from sources such as rice bran protein (Hayta *et al*., 2021), sea cucumber collagen (Jin *et al*., 2019) and chia (Urbizo-Reyes *et al*., 2019). Another example is the study reported by Habinshuti *et al*. (2020), where they obtained antioxidant peptides from sweet potato protein by applying ultrasonic microwave-assisted hydrolysis.

ISOLATION, PURIFICATION AND CHARACTERIZATION

During the process of obtaining bioactive peptides, these are subjected to isolation, purification, and characterization, which allows identify their biological activity. First, the selection of the food must be considered, to later extract and purify them. In plant matter, these last steps use techniques such as ion exchange chromatography, gel electrophoresis (SDS-PAGE), reverse phase high resolution liquid chromatography, circular dichroism spectral analysis (CDS), two-dimensional gel electrophoretic analysis and assisted laser desorption-ionization (MALDI-TOF MS/MS) (Tang *et al*., 2018). Agrawal *et al*. (2016) isolated, purified and characterized peptides from pearl millet (Pennisetum glaucum). First, they isolated the protein from this food and applied enzymatic hydrolysis with trypsin; second, they purified by gel filtration liquid chromatography and successively, reverse phase chromatography. Finally, the characterization consisted of the sequential analysis and molecular mass determination, using a MALDI-TOF-TOF-MS/MS mass spectrometer; the obtained sequence was SDRDLLGPNNQYLPK and it turned out to have antioxidant activity. Mijiti *et al*. (2018), obtained antimicrobial peptides from *Cuminum cyminun* L. seeds. They isolated proteins and extracted peptides that were purified by reverse phase C18 column chromatography, as well as ion exchange chromatography, which allowed the separation of the peptide fractions. Subsequently, they were characterized by SDS-PAGE and high-pressure liquid chromatography. Regarding molecular weights, these were defined through liquid chromatography and mass spectrometry. Finally, they confirmed the antimicrobial activity through tests with bacterial strains (E. coli and *Staphylococcus aureus*) and a fungal strain (*Candida albicans*).

APPLICATION IN FOOD PRODUCTS

Wang and Selomulya (2020), to use peptides as food additives or nutraceutical ingredients, carried out a study on drying them directly, with technological adjuvants or through microencapsulation; the latter, aims to hide the odors or flavors of the compound. Wang and Selomulya presented some examples of applied drying in peptides from chicken, dairy, fish, soybean, and egg white protein. Among them is the encapsulation of casein hydrolysates and antioxidant peptides, from a coating of maltodextrin and gum arabic (Rao *et al*., 2016). Other investigations are given in antioxidant peptides for extruded snacks (da-Silva *et al*., 2021), marine collagen peptides added in biscuit flours (Kumar *et al*., 2019) and peptides as constituents of active packaging and food preservatives (Santos *et al*., 2018; Tkaczewska, 2020). In the latter, camel milk protein hydrolysates have proven to be an effective additive for the preservation of minced fish (Al-Shamsi *et al*., 2018).

On the other hand, bioactive hydrolysates are of interest for those foods with special uses, such as infant formula with enzymatically hydrolyzed proteins (Chakrabarti *et al*., 2018) and a fruit punch with liquid protein ProMod (Pro-Stat), manufactured by Abbott (United States), whose product is based on a collagen hydrolyzate and has an effect in the treatment of ulcers caused by constant pressure in an area of the skin (Chalamaiah *et al*., 2019). In recent years, the ideology of taking advantage of food by-products has increased; therefore, new research focused on these by-products, considered waste, is expected for their possible use in the preparation of antimicrobial edible films through peptides, such as those derived from cheese whey (Dinika *et al*., 2020). However, even though there are studies aimed at the encapsulation of these bioactive compounds, there is little evidence of their respective addition to food products under this technique, therefore, future research on their possible implementation is expected. Meanwhile, the industry will have to face some challenges related to scalable production, as well as the impact they have on the applicable food matrix (Chakrabarti *et al*., 2018).

CONCLUSIONS

Bioactive peptides present diverse biological activities that are important and beneficial for human health, which have prompted the interest of scientists in the development and improvement of the methodologies for obtaining them. As well as, the study of food sources containing these compounds has evolved through in vitro and in vivo models. Research in food sciences has a substantial role in the implementation of new technologies and methodologies that facilitate the obtaining of bioactive peptides with a higher yield, to generate alternatives for their use and application as an ingredient in food products that have a direct impact on human health.

Table 1. Sources and methods for obtaining bioactive peptides with biological activity.

Source/ host of expression	Obtaining method	Biological activity |	Application/Evaluation of the activity	Reference
Lima bean (*Phaseolus lunatus*)	Two systems of enzymatic hydrolysis	Antihypertensive	Angiotensin converting enzyme and renin enzyme	Ciau-Solis *et al.*, 2018
Wasp venom *Oreumenes decoratus*	Extraction of venom sacs with acetonitrile-water containing 0.1 % TFA	Anticancer	Breast cancer MCF-7	Torres *et al.*, 2018
Hemoglobin from raw bovine blood	Enzymatic hydrolysis with pepsin	Antimicrobial	Ground beef	Przybylski *et al*., 2016
Sunflower flour	Enzymatic hydrolysis with xylose and cysteine	Antimicrobial	MRP in strains: *Staphylococcus aureus* and *E.coli*	Habinshuti *et al.*, 2019
Clones of transgenic of tobacco	Recombinant DNA technology	Antimicrobial	*Enterococcus faecium* (ATCC 8459), *Bacillus cereus* (ATCC 11778), and *Escherichia coli* (ATCC 8739)	Varasteh-Shams *et al.*, 2020
E. coli	Recombinant DNA technology	Antimicrobial	Methicillin resistant *Staphylococcus aureus*	Satei *et al.*, 2021
Bovine milk	Fermentation with cultures of *Lactobacillus*	Antidiabetic	Enzymes α-amylase, la-glucosidase and pancreatic lipase	Kinariwala *et al.*, 2019
Camel milk casein	Gastrointestinal digestion *in vitro* with pepsin and pancreatin	Antidiabetic	a-glucosidase enzyme	Akan, 2020
Spent grain of brewers	Simulated gastrointestinal digestion	Antithrombotic	Thrombin	Cian *et al.*, 2018
Tenebrio molitor proteins	Enzymatic hydrolysis with pepsin and trypsin	Antithrombotic	Yang method	Chen *et al.*, 2019
Cutaneous secretions of *Bombina maxima*	Lipopolysaccharide (LPS) solution to obtain secretion and application in Superdex 10/300 peptide column for separation.	Anti-inflammatory	Inflammatory cytokines	Guo *et al.*, 2021
Sprouted soybeans	Hydrolysis with pepsin/pancreatin	Anti-inflammatory	Human colon cancer cells	González-Montoya *et al.*, 2018
Stracchino white cheese	Gastrointestinal digestion *in vitro* with pepsin	Antioxidant	Intestinal epithelial cells (IEC-6)	Pepe *et al.*, 2016
Lotus seeds (*Nelumbo nucifera* Gaertn.)	Enzymatic hydrolysis with Flavourzyme	Antioxidant	Peroxidase/DPPH free radical based system	Yu *et al.*, 2021

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