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Conventional and *in silico* approaches to select promising food-derived bioactive peptides: A review

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ABSTRACT

The interest for food-derived bioactive peptides, either from common or unconventional sources, has increased due to their potential therapeutic effect against a wide range of diseases. The study of such bioactive peptides using conventional methods is a long journey, expensive and time-consuming. Hence, bioinformatic approaches, which can not only help to predict the formation of bioactive peptides from any known protein source, but also to analyze the protein structure/function relationship, have gained a new meaning in this scientific field. Therefore, this review aims to provides an overview of conventional characterization methods and the most recent advances in the field of *in silico* approaches for predicting and screening promising food-derived bioactive peptides.

1. Introduction

The growing interest in natural alternatives to treat and prevent different diseases has turned the spotlight on foods that may contain beneficial compounds, such as bioactive peptides (Daliri, Oh, & Lee, 2017).

Besides the nutritional role of dietary proteins as the source of amino acids for the growth and maintenance of body cells and tissues, they also carry out a functional and biological role, through of specific peptides, called bioactive peptide (BP), that can modulate physiological responses, resulting in a positive effect on health (Daroit & Brandelli, 2021). According to BIOPEP-UWM™ database of bioactive peptides (formerly BIOPEP), over 4300 BP have been reported to date, which may be classified based on the bioactivity they exhibit, *i.e.*, antimicrobial, antithrombotic, antihypertensive, opioid, immunomodulator, mineral-binding and antioxidants, among others (Minkiewicz, Iwaniak & Darewicz, 2019). Such activities have been related to the prevention or treatment of different diseases, including, but no limited to cancer, immune disorders, and cardiovascular diseases (Chalamaiah, Yu & Wu, 2018; Lammi, Aiello, Boschin & Arnoldi, 2019; Skjånes, Aesoy, Herfindal, Skomedal, 2021). Both the composition and the sequence of amino acids are known to be key factors on their bioactivity (Sánchez & Vázquez, 2017). These short chains of amino acids (2–20 amino acids) are encrypted within the parent protein sequence and must be released to exert their effects.

In this sense, BP can be released from dietary proteins through hydrolysis by using digestive, microbial, plant or animal enzymes, by fermentation with starter or nonstarter cultures, or by ripening process (Daroit & Brandelli, 2021). These methods have been found to be laborious, time-consuming, and expensive on an industrial scale. Hence, new bioinformatic approaches have emerged to obtain and characterize functional peptides, that, unlike conventional methods, can be useful to select the appropriate protease(s) for protein hydrolysis by narrowing down the number of enzyme combinations, to predict possible bioactivity or interaction with specific molecules and receptors by homology-based searches in specific databases and by molecular docking and structural alignments (Agyei, Tsopmo & Udenigwe, 2018). Therefore, the use of these *in silico* tools avoids the expense of laboratory time and reagent money. Additionally, they have allowed to find new BP from alternative sources, e.g., kefir milk (Amorim et al., 2019a), collagen (Nuñez, Guzmán, Valencia, Almonacid & Cárdenas, 2020), fish skin (Elaziz, Hemdan, Hassanien, Oliva & Xiong, 2017), invasive sea grass *Halophila stipulacea* (Kandemir-Cavas, Pérez-Sánchez, Mert-Ozupek & Cavas, 2019), *Chlorella sorokiniana* (Tejano, Peralta, Yap, Panjaitan & Chang, 2019), cyanobacterium *Arthrospira platensis* (Ji et al., 2018), among many others.

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Therefore, this review presents an overview of conventional characterization methods and the most recent advances in the field of *in silico* approaches for the generation, identification, and validation of promising food-derived bioactive peptides.

2. Common and non-conventional sources of bioactive peptides

The process of obtaining BP begins with the selection of the protein source. According to Udenigwe & Aluko (2012), the protein selection is mainly based on two criteria: 1) granting added value to protein rich residues from the food industry, and 2) the use of specific proteins that contain sequences with the desired bioactivity. Nonetheless, it is important to consider that biological activities are directly related to the amino acid sequence; therefore, the selection of protein source is crucial to produce BP (Daroit & Brandelli, 2021).

Plants and animals have commonly been used as a source of BP, being legumes (soybeans, beans, and lentils), cereals (oat and wheat), and oilseeds (flaxseed) the most exploited plants; while eggs, milk, and meat are the most widely exploited sources of animal protein (Chakrabarti, Guha & Majumder, 2018). However, other less exploited sources to obtain BP have begun to attract attention such as seaweed, residues from the food industry, edible insects, cyanobacteria, and some edible fungi (Table 1), which have made possible to obtain BP with a variety of bioactivities unexplored.

Once the protein source (food matrix) has been chosen, the next step is to select the method to obtaining the BP. In this regard, the so-called conventional methods have widely been used at laboratories scale. However, interest on *in silico* studies has raised due to they are less expensive and less time-consuming.

3. Conventional approaches for generation, identification, and validation of bioactive peptides

Currently, conventional approaches for production of BP are still widely used. These approaches typically include the selection of the dietary protein source, the hydrolysis with the selected proteinase, the purification and identification of BP, as well as the evaluation of biological activities (Daroit & Brandelli, 2021). Additional pretreatments, such as high pressure, microwave, and ultrasound, are sometimes used to facilitate the release of peptides and to prevent interferences during biological activities analyzes (Munir et al., 2020).

On the other hand, new methods based on supercritical and subcritical fluids have been used as alternative to obtain new BP, either as pretreatment or to carry out hydrolysis (Olivera-Montenegro, Best, & Gil-Saldarriaga, 2021; Ulug, Jahandideh & Wu, 2020). However, for the purposes of this review, these technologies were considered within the conventional approach.

3.1. Methods for bioactive peptide generation

BP can be obtained from food proteins by different methods. Conventional methods include enzymatic (by using either digestive, plant, or microbial enzymes) and microbial digestion (fermentation). In some cases, the combination of methods has proven to be necessary to obtain peptides of small size (Chakrabarti et al., 2018). Additionally, BP may be released during seed germination, and food processing (e.g., meat curing, cheese ripening, heat- or pressure- treatments) (Sandoval-Sicairos, Milán-Noris, Luna-Vital, Milán-Carrillo, & Montoya-Rodríguez, 2021; Toldrá, Gallego, Reig, Aristoy, & Mora, 2020; Martini et al., 2020; Zielińska et al., 2018). Therefore, in the next section, the conventional methods to produce BP will be discussed.

3.1.1. Enzymatic hydrolysis by digestive enzymes

Overall, the enzymatic hydrolysis is a controlled method that, not only improve the biological activity of protein by-products, but also enhances their functional properties, such as digestibility (Cotabarren et al., 2019). The method consists of subjecting the protein to enzymatic treatment under specific pH and temperature conditions (Table 2; Chakrabarti et al., 2018). The selection of the protease and the hydrolysis time are decisive factors in the types of peptides to be generated (Daliri et al., 2017). In other words, the type of enzyme, the hydrolysis time, the temperature, and the enzyme-substrate ratio, may affect the extent of hydrolysis and, therefore the type of peptides generated. It should be highlighted that no specific enzymes to produce specific BP are known so far.

Different enzymes can participate in the hydrolysis of proteins to obtain BP, either alone or in combination (Daliri et al., 2017), just as happens during the digestive process. Indeed, this biological process has widely been emulated and used to evaluate the formation of BP produced after the consumption of food-protein. In this sense, it has been recognized that this biological process can influence the peptide profile of foods, either by degrading BP or by releasing new ones, which implies that the health benefit of dietary proteins is obtained after the hydrolysis by gastrointestinal digestion (Giromini, Cheli, Rebucci, & Baldi, 2019). Hence, the impact that digestive process has on the production and presence of BP is highly relevant considering that some of peptides are resistant to digestion, but others are released during its passage through the gastrointestinal tract. Properties of such bioactive peptides include angiotensin-converting enzyme (ACE) inhibitor activity, antioxidant, immunomodulatory (Wada & Lönnerdal, 2015).

According to information described above, the simulation of gastrointestinal digestion has been used to assesses several foods for different purposes. For instance, Liu & Pischetsrieder (2017), evaluated the formation/degradation of BP during gastrointestinal digestion of kefir, a fermented food already considered beneficial, by using a three-stage model, which simulate the oral (α-amylase), gastric (pepsin) and intestinal (pancreatin) phases. Results showed that different BP were released by simulated digestion, particularly the ACE-inhibitor β-casein$_{203\text{-}209}$, which increased its concentration 10,000-fold after digestion. Conversely, these results have not been observed in other studies (Mora, Bolumar, Heres, & Toldrá, 2017; do Nascimento et al., 2021). Such differences could be related to the digestive model selected, since it has been reported that both the concentration of digestive enzymes and the hydrolysis time used can influence the release and concentration of BP (Daliri et al., 2017).

3.1.2. Proteolysis by plant or microorganism enzymes

The *in vitro* hydrolysis using endogenous proteases of plants or microorganisms have previously been reported (Daroit & Brandelli, 2021). Proteases play an important role in plants metabolism; besides, they also have multiple biotechnological uses, including the production of BP. Papain and bromelain, obtained from papaya and pineapple, respectively, are the most used (Mazorra-Manzano, Ramírez-Suarez, & Yada, 2018; dos Santos-Aguilar & Sato, 2018).

In this sense, papain has been used to produce BP from animal proteins sources, such as camel whey and buffalo milk. The bioactive peptides obtained showed antimicrobial activity (*in vitro*), as well as hepatoprotective action in a murine model. Such bioactivities were associated to the antioxidant properties of the BP generated after the hydrolysis, even though the degree of hydrolysis (DH) achieved with papain was<30% after 4 h (Abdel-Hamid, Goda, De Gobba, Jenssen, & Osman, 2016; Abdel-Hamid, Osmand, El-Hadary, Romeih, Sitohy, & Li, 2020). It is important to note that the selection of the enzyme will depend on the protein to be hydrolyzed and the DH expected, which have been related to the size and structure of the peptides obtained, that in turn has direct influence on the biological activity.

Protein hydrolysates with low DH have shown biological activity. For instance, Cotabarren et al., (2019), obtained hydrolysates, from defatted chia expeller, with antioxidant activity by using commercial papain, the DH achieved was of 14.3% after 40 min. Similarly, Borrajo, Pateiro, Gagaoua, Franco, Zhang, & Lorenzo (2020) found that peptides with antibacterial capacity can be obtained from hydrolysis of porcine

Table 1
Conventional and unconventional sources of bioactive peptides from food-derived proteins.

Peptides Source	Protein substrate	Peptide(s)	Obtaining method	Bioactivities	References
Common sources					
Dairy products	Buffalo cheese	Water-soluble peptides	Fermentation	Antimicrobial against *Enterococcus faecalis* (12.5 mg/mL) and *Bacillus subtilis* (25 mg/mL), Antioxidant activity ABTS (from 33.39 to 63.27% of scavenging of cation radical with concentrations from 2.5 to 20 mg/mL) and DPPH (IC_{50} 5 mg/mL)and anti-hypertensive due to angiotensin-converting enzyme (ACE)-inhibitory activity (26.17 to 58.79% for concentrations between 2.5 and 20 mg/mL).	da Silva et al., 2019
	Prato cheese	β-CN (f193-209)	Fermentation by *Lactobacillus helveticus* LH-B02 and ripening time	Inhibitory activity of the ACE (90.22% after 120 days of ripening).	Baptista, Negrão, Eberlin & Gigante, 2020
	Parmigiano-Reggiano cheese	β-CN fractions α_{S1}-CN fractions α_{S2}-CN fractions	Ripening time and enzymatic digestion, and *in silico* analysis	ACE-inhibitory, anti-hypertensive, antimicrobial, immunomodulatory, antioxidant, dipeptidyl peptidase IV-inhibitory (DPP-IV), and anxiolytic peptides identified in the literature using Milk Bioactive Peptides Database (100% homology)	Martini, Conte & Tagliazucchi, 2020
	Goat milk	AFPEHK	Fermentation by *Lactobacillus casei* NK9	ACE-inhibitory of 10 kDa permeates (25.16 %)	Parmar, Hati & Sakure, 2018
	Camel milk	3 kDa and 10 kDa fractions	Fermentation by *Lactobacillus bulgaricus* NCDC and *Lactobacillus fermentum* TDS030603	ACE-inhibitory (76.75% after 48 h of fermentation with *L. bulgaricus* and 73.93% after 48 h of fermentation with *L. fermentum*).	Solanki, Hati & Sakure, 2017
	Buffalo milk	α_{S1}-CN variant AA α_{S1}-CN variant BB	Enzymatic digestion	ACE-inhibitory, anticancer, antioxidant, and anxiolytic (predicted by BIOPEP).	Li et al., 2020
Meat	Cooked beef	IVAPGKGILAADESTGSIAK	*In vivo* gastric digestion and *in silico* analysis	ACE-inhibitor, antithrombotic, antiamnestic, DPP-IV inhibitor (predicted by BIOPEP).	Sayd, Dufour, Chambon, Buffière, Redmon, & Santé-Lhoutellier, 2018
	Duck meat	< 5 kDa fractions	Post-mortem aging	Antioxidant assessed at 3 days of aging by DPPH (59.83%), FRAP (greater than 300 μM $FeSO_4{\cdot}7H_2O$ Eq/g sample), and ORAC (1000 μMTEq approximately)	Liu, Chen, Huang, Huang, & Zhou, 2017
	Pork, beef, chicken, and turkey meat	< 3 kDa fractions	*In vitro* enzymatic digestion	Antioxidant activity evaluated with ABTS (594.9, 535.2, 714.3, and 651.9 μmol Trolox/g of peptide of beef, chicken, pork, and turkey, respectively), ACE-inhibitory (IC_{50} from 81.2 μg/mL (chicken) to 238.0 μg/mL (Turkey)), and DPP- IV (IC_{50} from1.88 mg peptides/mL (pork) to 2.71 mg peptides/mL (chicken))	Martini, Conte, & Tagliazucchi, 2019.
Fish	Fish skin gelatin	Glycopeptides	Enzymatic hydrolysis with alcalase and flavourzyme, and then glycosylated	Antimicrobial activity against *E. coli* (MIC 40 mg/mL) and antioxidant activity evaluated with DPPH (EC_{50} from 2.6 to 3.4 mg/mL)	Hong, Gottardi, Ndagijimana, & Betti, 2014
	Tilapia skin collagen	GPAGPAGEK, DGPSGPKGDR, GLPGPSGEEGKR, and DGPSGPKGDRGETGL	Enzymatic hydrolysis with trypsin, pepsin, neutral protease, alkaline protease and protamex	Iron-chelating (83.47% at a hydrolysate concentration of 5 mg/mL)	Lin et al., 2021
Other animal products	Egg white	Egg white peptides	Enzymatic hydrolysis	Calcium-chelating (44.1 mg of calcium/kg).	Huang et al., 2021
Cereals	Wheat gluten proteins	YYP, IP, YVP, YP	*In silico* analysis	Opioid peptides identified using BIOPEP database	Garg, Apostolopoulos, Nurgali, & Mishra, 2018
	Maize	19ZP1, 19ZP2 and 19ZP3	*In silico* analysis	ACE-inhibitory (IC_{50} from 14.19 to 202.04 μM) and antioxidant evaluated by ORAC (from 9.47 to 1349.36 μM TE/g of peptide)	Díaz-Gómez, Neundorf, López-Castillo, Castorena-Torres, Serna-Saldívar, & García-Lara, 2020
	Brown rice	FGGSGGPGG and FGGGGAGAGG	Enzymatic hydrolysis with bromelain	ACE-inhibitory (IC_{50} value of 0.20 mg protein/mL).	Selamassakul, Laohakunjit, Kerdchoechuen, Yang, & Maier, 2020
	Quinoa	QHPHGLGALCAAPPST	Enzymatic hydrolysis by bromelain, chymotrypsin, and Pronase E	DPP-IV (*p*-value of 0.009151) and α-glucosidase inhibitory (*p*-value 0.006893), and ACE-inhibitory (*p*-value 0.000117) activities elucidated by *in silico* docking study with Pepsite2	Mudgil et al., 2020
Legumes	Soy milk	10 kDa fraction			Singh & Vij, 2017

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Table 1 (*continued*)

Peptides Source	Protein substrate	Peptide(s)	Obtaining method	Bioactivities	References
			Fermentation by *Lactobacillus plantarum C2*	Antioxidant evaluated by ABTS (1831 TEAC μm) and DPPH (50.74% of inhibition) and ACE-inhibitory (73.35% of ACE inhibition)	
	Black beans	SGNGGGGGASM, SKPGGGSPVA, VELVGPK, KPTTGKGALA	*In vitro* enzymatic digestion	DPP-IV and ACE inhibitors peptides identified using BIOPEP database.	(Moreno-Valdespino, de Mejia, Mojica, Luna-Vital, & Camacho, 2019)
	Pea	YSSPIHIW, ADLYNPR, and HYDSEAILF.	Enzymatic hydrolysis	Antioxidant evaluated by PeptideRanker (scores from 0.53 to 0.74)	Ding, Liang, Yang, Sun, & Lin, 2020
Unconventional sources					
Algae	Red seaweed	GGSK and ELS	Enzymatic proteolysis	α-amylase inhibitory (IC_{50} of 2.58 mM and 2.62 mM for GGSK and ELS, respectively)	(Admassu, Gasmalla, Yang, & Zhao, 2018)
	Spirulina platensis	EYFDALA	Enzymatic hydrolysis using pepsin	Antioxidant evaluated by ABTS and DPPH (IC_{50} 11.07 μg/mL and 4.83 μg/mL, respectively, with a molar concentration of 13.4 μmol/mL of peptide).	Zeng, Wang, Zhang, Song, Liang, & Zhang, 2020
Plant	Yam (*Dioscorea cayennensis*)	< 3.5 kDa fractions	*In vitro* enzymatic digestion	Antioxidant (value close to 80% of DPPH radical scavenging), ACE-inhibition (IC_{50} of 90 μg/mL), and antimicrobial (MIC of 0.094 mg/mL against *E. coli*)	Do Nascimento et al., 2021
	Edible rhizomes (turmeric, and ginger)	VTYM (ginger), CGVGAA, DVDP, and CACGGV (turmeric).	Enzymatic hydrolysis by pepsin and trypsin	Antioxidant evaluated by DPPH (EC_{50} = 19.9 μmol/L for VTYM), and ABTS (EC_{50} = 24.0 μmol/L for VTYM), and ACE-inhibition (IC_{50} = 16.4, 18.3, 19.0, and 25.0 μmol/L for VTYM, CGVGAA, DVDP, and CACGGV, respectively).	Sompinit, Lersiripong, Reamtong, Pattarayingsakul, Patikarnmonthon, & Panbangred, 2020
Edible insects	*Schistocerca gregaria*	FDPFPK	Baked and *in vitro* enzymatic digestion	Antioxidant evaluated by DPPH (EC_{50} = 0.35 mg/mL), ABTS (EC_{50} = 0.08 mg/mL), and anti-inflammatory by lipoxygenase inhibitory activity (LOX, IC_{50} = 2.85 mg/mL) and Cyclooxygenase 2 inhibitory activity (COX 2, IC_{50} = 7.40 mg/mL).	Zielińska, Baraniak, & Karas, 2018.
	Gryllodes sigillatus	IIAPPER	*In vitro* enzymatic digestion	Antioxidant evaluated by DPPH (EC_{50} = 1.01 mg/mL), ABTS (EC_{50} = 15.62 mg/mL), and anti-inflammatory by LOX (IC_{50} = 8.21 mg/mL) and COX 2 (IC_{50} = 8.16 mg/mL).	Zielińska et al., 2018.
	Tenebrio molitor	AGDDAPR	*In vitro* enzymatic digestion	Antioxidant evaluated by DPPH (EC_{50} = 1.83 mg/mL), ABTS (EC_{50} = 1.89 mg/mL), and anti-inflammatory by LOX (IC_{50} = 7.03 mg/mL) and COX 2 (IC_{50} = 9.01 mg/mL).	Zielińska et al., 2018.
	Gryllodes sigillatus	Cricket protein hydrolysates (60–85% of degree of hydrolysis)	Enzymatic hydrolysis with alcalase and enzymatic digestion	ACE inhibition (IC_{50} from 0.062 to 0.066 mg/mL)	Hall, Johnson, & Liceaga, 2018
Food industry waste	Meat myofibrillar proteins	Acidic peptides (fractions 6–10) Crude hydrolysates	Enzymatic proteolysis with a bacterial-derived protease	Antioxidant evaluated by ORAC (above 12 mmol/L TE).	Ryder, Bekhit, McConnell, & Carne, 2016
	Fish muscle (*Cyprinus carpio*)	5.3 kDa fraction	Enzymatic proteolysis with protease produced by *Halobacillus andaensis*	Antioxidant evaluated by DPPH (20% of radical inhibition) and ABTS (above 15 % of radical inhibition).	Delgado-García et al., 2019
	Tofu whey wastewater	Lunasin	Tofu processing	Anti-inflammatory and immunomodulatory, assessed using RAW 264.7 murine macrophages (30% reduction of TNF-α with 190 μM lunasin).	Nieto-Veloza et al., 2021
	Spent coffee grounds	YGF and GMCC	Fermentation process by *Bacillus clausii*	ACE and DPP-IV inhibitors obtained using BIOPEP database and classified by PeptideRanker (score of 0.97)	Ramírez, Pineda-Hidalgo, & Rochín-Medina, 2021
	Spent brewer yeast	SPQW, PWW and RYW	Autolysis and enzymatic hydrolysis	ACE-inhibition (IC_{50} = 84.2 μg/mL) and antioxidant evaluated by ORAC (IC_{50} from 5.5 to 7.25 μg/mL)	Amorim et al., 2019b
Microorganisms	Edible cyanobacterium *Arthrospira platensis*		*In silico* enzymatic digestion with pepsin, trypsin, and chymotrypsin.	ACE and DPP-IV inhibitors (39.1 and 47.7 % of peptides from cyanobacterium, respectively) obtained using BIOPEP database	Ji et al., 2018
	Kluyveromyces marxianus	LPESVHLDK, VLSTSFPPLK	Sonicated-enzymatic hydrolysis (trypsin and chymotrypsin)	Antioxidant (IC_{50} = 5568 μM TE/mg protein for VLSTSFPPLK) and ACE-inhibition (IC_{50} = 22.88 and 15.20 μM for LPESVHLDK and VLSTSFPPLK, respectively).	Mirzaei, Mirdamadi, Ehsani, & Aminlari, 2018.
Fungi	*Agrocybe aegerita*	25 novel antioxidant peptides			Song et al., 2020

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Table 1 (*continued*)

Peptides Source	Protein substrate	Peptide(s)	Obtaining method	Bioactivities	References
			Enzymatic hydrolysis by neutral protease	Antioxidant evaluated by the total antioxidant capacity assay (0.73 μmol TE/mg).	
	Edible mushroom (*Agaricus bisporus*)	Ultrafiltered fractions (1–3 kDa)	Enzymatic hydrolysis by alcalase and pancreatin	Antioxidant evaluated by FRAP (0.62 of absorbance at 3 mg/mL)	Kimatu et al., 2017
	Pleurotus ostreatus	Mushroom protein hydrolysates (65% of hydrolysis degree)	Enzymatic hydrolysis by proteinase K	Antioxidant evaluated by DPPH (IC_{50} = 5.24 mg/mL), ABTS (IC_{50} = 1.80 mg/mL), and hydroxy radical (IC_{50} = 1.09 mg/mL), and ACE inhibitory (87%) at 10 mg/mL.	Goswami, Majumdar, Das, Barui, & Bhowal, 2021

liver protein with bromelain for 4 h. Although it has been reported that reaction times is another key parameter, when reaction time was extended, no significant increase in the DH was observer in these two studies.

On the contrary, by prolonging the hydrolysis time (3 h) of a corn gluten meal with papain, resulted in a higher DH (16%) and peptides (5–10 kDa) with higher antioxidant activity (Hu, Chen, and Li, 2020). In this same study, authors reported an optimal hydrolysis time of 4 h for bromelain and ficin to obtain antioxidant peptides, although the DH was close to 12%. For this study, papain showed to be more effective to obtain a higher DH at shorter time; however, this does not always ensure a higher bioactivity, since in this case the hydrolysate with bromelain obtained the highest DPPH antioxidant capacity. The authors attributed the differences to the specificity of each enzyme, since papain is a monothiol cysteine endoprotease, and bromelain prefers to break out proteins to poly- and oligopeptides (Holyavka et al., 2019), having a specificity in hydrophobic and non-polar amino acid residues (Selamassakul et al., 2020). In contrast, ficin cleave tyrosine and phenylalanine bonds (Holyavka et al., 2019).

The specificity of the enzymes and their impact on bioactivity was also reported in the study carried out by Borrajo et al. (2020), who observed an increase in the antioxidant activity when papain or alcalase were used instead of bromelain, under the same conditions. Hence, these studies suggest that in order to obtain peptides with a specific bioactivity, the selection of the enzyme (specificity) and the nature of the protein source should be considered first, rather than the reaction time and degree of hydrolysis.

Antioxidant, antimicrobial, and ACE inhibitor are the main bioactivity exerted by hydrolysates obtained with plant enzymes. Furthermore, these enzymes have been used in the food industry for their flavor-enhancing properties (García, Puchalska, Esteve & Marina, 2013). Thus, studies have been conducted to stablish the relationship between biological property and flavor. In this regard, Selamassakul et al., (2020) reported that peptide fraction <1 kDa, obtained by using bromelain and brown rice as source of protein, showed improved antioxidant and ACE-inhibitory activities as well as bitter and umami taste. These results indicated that the bioactivities and flavor could be related with low-molecular weight peptides. The establishment of this relationship evidence the potential of BP to serve as functional ingredient providing both, health benefits and good taste.

Similarly, the peptides obtained from the hydrolysis with microbial enzymes may also improve the flavor. Therefore, enzymes such as alcalase, flavourzyme and protamex have been intentionally added to foods to impart flavor, taste, and enhance texture (Chew, Toh, & Ismail, 2019). Microorganisms are considered the main source of proteases due to their economic and technological advantages (dos Santos-Aguilar & Sato, 2018). Consequently, the application of microbial proteases to obtaining BP from different food proteins has not been long in coming, e.g., alcalase has been used to hydrolyze the proteins of edible insects. In their study, Hall et al., (2018) shown that an 3% enzyme level yield up to 85% DH. However, as previously mentioned, higher DH did not necessarily correlate to higher bioactivity, since antioxidant activity did not show significant differences between hydrolysates with a degree of hydrolysis (DH) ranging from 15 to 85%, whereas ACE and DPP-IV inhibition activities were significant higher for hydrolysates with 60–85% DH.

On the other hand, bioactive peptides have also been obtained from trout skin proteins by using alcalase and flavourzyme enzymes, being the latter more effective than the former, under the same conditions, to obtain peptides with significant higher ($p < 0.05$) antioxidant activity (Yaghoubzadeh, Ghadikolaii, Kaboosi, Safari, & Fattahi, 2020). Therefore, these data corroborate that the selection of specific enzymes can release bioactive peptides with desired properties. Thus, research efforts have been devoted to use combined enzymes as strategy to obtain peptides with improved bioactivities, as showed by Ayala-Niño, Rodríguez-Serrano, González-Olivares, Contreras-López, Regal-López and Cepeda-Saez (2019), who combined alcalase and flavourzyme to obtain a greater inhibition of ACE, thrombin, and antioxidant activities with bioactive peptides from amaranth.

Once hydrolysis is complete, a heat treatment is performed to stop the activity of enzyme and the hydrolysates are separated by centrifugation. Subsequently, the peptides are recovered through methods such as lyophilization (Ayala-Niño et al., 2019), desalting (Amorim et al., 2019a), membrane filtration (Selamassakul et al., 2020), or column chromatography. The gel filtration method is also used to desalt low molecular weight peptides and separate them according to size (Zielińska et al., 2018).

3.1.3. Food processing and other technologies

During processing (e.g., fermentation, heat, and high-pressure treatments), food components can suffer alterations improving texture, flavor, stability, and even generation of BP (Toldrá, Reig, Aristoy, & Mora, 2018).

Fermentation is a preservation method that has been used for a long time. Microorganisms have enzymes with the ability to hydrolyze proteins to generate BP. Fermentation can be spontaneous or controlled (direct inoculation of a known microorganism). In the case of spontaneous fermentation, it is not necessary to adapt the microorganisms, fermentation is directly started at the desired temperature (Mazorra-Manzano et al., 2020). While for controlled fermentation, the selected microorganism should be grown under optimal conditions until reaching it exponential phase, harvested, and adjust the inoculum to the desired concentration to inoculate the protein source (Chakrabarti et al., 2018; Singh & Vij, 2017).

Enzymatic hydrolysis and microbial fermentation are the two biotechnological methods more used to release BP, the latter is a more complex method compared to the former, since different factors intervene during fermentation, including reaction/fermentation time, protein source, and bacterial strain used, while *in vitro* hydrolysis is performed by enzymes with known specificity. Despite of this, microbial fermentation may present certain advantage over other peptide delivery techniques, for instance, the process is cheaper than enzymatic hydrolysis. Besides, peptides with greater biological activities may be generated due to the high diversity of microbial proteases, with high level of

Table 2
Controlled parameters in enzymatic hydrolysis for obtaining bioactive peptides.

Enzyme/ substrate ratio (% w/ w)	pH	Temperature (°C)	Hydrolysis time (min)	Identified peptides	Protein substrate —Bioactivity	Reference(s)
Pepsin						
1	2	37	120	No identified	Deer velvet — ACE-inhibitory (17.57% ACE activity)	Haines, McCann, Grosvenor, Thomas, Noble, & Clerens, (2019).
Pancreatin						
4	7.5	37	120	No identified	Deer velvet — ACE-inhibitory (17.57% ACE activity)	Haines et al., (2019)
Chymotrypsin						
1	7.8	50	360	QHPHGLGALCAAPPST and other 34 peptides identified	Quinoa — ACE-inhibitory (IC_{50} = 0.22 mg/mL) and DPP-IV inhibitory (IC_{50} = 0.72 mg/mL)	Mudgil et al., (2020)
Papain						
0.5 – 4	6.0 – 7.0	37 – 50	40 – 480	No identified in most investigations; 22 peptides containing the LPF tripeptide (Corn Gluten Meal).	Camel whey — Antibacterial activity against *Salmonella typhimurium* (MIC 0.91 mg/mL), *Escherichia coli* (MIC 1.00 mg/mL), *Bacillus cereus* (MIC 0.91 mg/mL) and *Staphylococcus aureus* (MIC 0.09 mg/mL). Porcine Liver — antioxidant activity evaluated by DPPH (from 304 to 325 μg Trolox/g), FRAP (from 36.9 to 57.0 μmol Fe^{2+}/100 g), ABTS (from 352 to 416 mg ascorbic acid/100 g), and ORAC (from 31.7 to 44.3 mg Trolox/g). Chia (*Salvia hispanica* L.) expeller — antioxidant activity evaluated by ABTS (IC_{50} from 25.1 to 31.6 μg/mL) and DPPH (IC_{50} from 316.2 to 398.1 μg/mL). Corn Gluten Meal — antioxidant activity evaluated by DPPH (greater than 70% of radical scavenging with 5 mg/mL of hydrolysates).	Abdel-Hamid, et al., (2016; 2020); Borrajo et al., (2020); Cotabarren et al., (2019); Hu et al., (2020).
Bromelain						
1 – 100	5.0 – 7.0	40 – 50	180 – 420	No identified in most investigations; 7 peptides containing the LPF tripeptide (Corn Gluten Meal); APAAIGPYSQAVLVDR and other 34 peptides (Porcine liver); FGGSGGPGG, FGGGGAGAGG and other 6 peptides (Brown rice)	Porcine Liver — antioxidant activity evaluated by DPPH (from 322 to 379 μg Trolox/g), FRAP (from 57.2 to 69.8 μmol Fe^{2+}/100 g), ABTS (from 280 to 335 mg ascorbic acid/100 g), and ORAC (from 31.5 to 36.4 mg Trolox/g), and antibacterial activity against *Brochothrix thermosphacta* and *Listeria monocytogenes* Corn Gluten Meal — antioxidant activity evaluated by DPPH (greater than 80% of radical scavenging with 5 mg/mL of hydrolysates). Porcine Liver — peptides with antioxidant activity identified by SWATH and correlated with antioxidant capacity. Quinoa — ACE-inhibitory (IC_{50} from 0.90 to 1.12 mg/mL) and DPP-IV inhibitory (IC_{50} from 0.18 to 0.24 mg/mL). Brown rice — antioxidant activity evaluated by DPPH, ABTS, and hydroxyl radical-scavenging activities (0.19, 2.28, and 24.64 mM Trolox, respectively), ACE-inhibitory activity (IC_{50} value of 0.20 mg protein/mL)	Borrajo et al., (2020); Hu et al., (2020); López-Pedrouso et al., (2020); Mudgil et al., (2020); Selamassakul et al., (2020).
Ficin						
22.5	6.0	50	240	LLPFYQ and QQILLPF	Corn Gluten Meal — antioxidant activity evaluated by DPPH (greater than 60% of radical scavenging with 5 mg/mL of hydrolysates)	Hu et al., (2020)
Flavourzyme						
1	5.5	50	420	APAAIGPYSQAVLVDR and other 34 peptides	Porcine Liver — peptides with antioxidant activity identified by SWATH and correlated with antioxidant capacity.	López-Pedrouso et al., (2020).
Alcalase						
1 – 3	8.0	50	240–480	No identified		

(*continued on next page*)

Table 2 (*continued*)

Enzyme/ substrate ratio (% w/ w)	pH	Temperature (°C)	Hydrolysis time (min)	Identified peptides	Protein substrate —Bioactivity	Reference(s)
					Porcine Liver — antioxidant activity evaluated by DPPH (from 443 to 562 μg Trolox/g), FRAP (from 43.3 to 82.9 μmol Fe^{2+}/100 g), ABTS (from 761 to 1068 mg ascorbic acid/100 g), and ORAC (from 35.8 to 53.2 mg Trolox/g), and antimicrobial activity against *B. thermosphacta* and *L. monocytogenes* Cricket — antioxidant activity evaluated by DPPH (from 872.4 to 1490.5 μmol TE/mg), FRAP (from 719.6 to 991.3 μmol TE/mg), ABTS (from 381.1 to 663.3 μmol TE/mg), and DPP-IV inhibitory (0 to 50%) and ACE-inhibitory (IC_{50} from 0.015 to 0.096 mg/mL)	Borrajo et al., (2020); Hall et al., (2018)

activity (dos Santos-Aguilar & Sato, 2018).

It should be highlighted that the type of microorganism used is crucial for optimal production and high bioactivity of peptides obtained. For example, Chi and Cho (2016), reported the formation of smaller peptides from soybean meal when fermented with *Bacillus amyloliquefaciens* U304, compared to that fermented with *Lactobacillus acidophilus*, *L. plantarum* or *Saccharomyces cerevisiae* CJ169. This could be due to the presence of a highly active protease in *B. amyloliquefaciens*. Additionally, an increase in antioxidant activity was found, attributed to the formation of low molecular weight of BP, which proves that the bioactivity also depends on the proteolytic capacity of the microorganisms.

A combination of microorganisms has also been used to promote the release of BP. In this regard, soybean protein fermented with a mixture of *B. subtilis* GD1, *B. subtilis* N4, *B. velezensis* GZ1, *L. bulgaricus,* and *Hansenula anomala* CICC 1728 showed a greater peptide content (301.3 mg/g), with a higher *in vitro* antioxidant activity (86% DPPH), compared to soybean protein fermented with each microorganism independently. Besides, authors also reported that the administration of the mixture of microorganisms was able to inhibit the intense exercise-induced metabolite accumulation, liver damage, and oxidative damage, and relieve fatigue in mice (Cui, Xia, Zhang, Hu, Xie, Xiang, 2020).

Some authors haver reported that different factors may intervene in the growth of the fermenting microorganism (e.g. temperature), thereby affecting the DH and therefore in the release of BP. Mazorra-Manzano et al. (2020) described that temperature and time influences the release of peptides with ACE-inhibitory activity during the fermentation of cheese whey with native microorganisms. However, the authors overlook the fact that repeatability is not assured, since it was performed with endogenous microbiota of cheese whey; and as mentioned by Daliri, Lee, & Oh (2018) in a previous research, certain conditions during fermentation, including temperature, time of fermentation, and microorganisms, can result in the production of non-reproducible peptide profiles.

On the other hand, treatments such as heat, high pressures, sonication, and microwaves, used during food processing, can cause changes in the food and thus in protein conformation. Hence, these treatments can be used as pretreatments to enhance BP release after proteolysis (Daroit & Brandelli, 2021). In this regard, an increase in ACE-inhibitor and antioxidant peptides during cheese ripening was achieved by ultrasound pretreatment (specific energy = 41 J/g, 20 kHz) in milk before cheese production. This same trend was observed with high pressure (400 MPa for 15 min) and microwaves (specific energy = 86.6 J/g) pretreatments but in a lesser extent (Munir et al., 2020). The authors attributed the increase in proteolysis to the conformational changes of milk proteins due to the cavitation effect of the sonication, while the effects of high pressure were related with the disruption of the casein micelles.

These treatments are also effective to improve the hydrolytic efficiency of proteases. Garcia-Mora, Peñas, Frias, Gomez, and Martinez-Villaluenga (2015) reported a greater hydrolysis of lentil protein when enzymatic hydrolysis was carried out under pressure between 100 and 300 MPa with alcalasa, protamex, savinase or corolase 7089. The high-pressure treatment also increased the ACE inhibitory and antioxidant activities with each enzyme, except for alcalase. These data are in agreement with those reported by Hall and Liceaga (2020), who performed a treatment of microwave-assisted hydrolysis treatment combined with alcalase addition to hydrolyze cricket protein. Results showed an increase in the inhibition of ACE and DPP-IV activities.

Heat treatments have also been shown to improve peptide release. Three edible insects (*Gryllodes sigillatus, Tenebrio molitor,* and *Schistocerca gregaria*), were treated with heat for 10 min (in boiling water at 100 °C or baked at 150 °C) before carrying out the *in vitro* enzymatic digestion. Data showed a positive effect on the antioxidant and anti-inflammatory properties of peptides by the heat treatment process (Zielińska et al., 2018). Similarly, it has also been shown that a heat treatment of cooking does not affect the bioactivity of antioxidant and ACE-inhibitor peptides formed during the ageing of meat (Mora et al., 2017). These results showed that pretreatments can be a strategy to improve de DH and to improve the formation of BP. Conformational change of proteins and peptide formation during heat treatment, may facilitate the participation of the enzyme. However, the importance of enzyme specificity and its accurate selection based on the nature of the protein should not be overlooked (Mazorra-Manzano et al., 2018).

In addition to the technologies described above, there is growing interest in subcritical water as an alternative method for the hydrolysis and production of peptides (Powell, Bowra, & Cooper, 2017). In this method, the water is kept in a subcritical state, that is, between the boiling point (100 °C and 0.1 MPa) and the critical point (374 °C and 22 MPa). In addition, due to its characteristics, it does not leave residues like organic solvents, it is non-toxic and is considered a green technology (Ulug et al., 2020). Koh, Lee, Ramachandraiah, & Hong, et al., (2019) investigated the impact of this approach on the formation of peptides hydrolysates from bovine serum albumin. Data evidence that optimization of processing parameters may improve the production of valuable peptides; however, research is still required to document the bioactivity of derived peptides with this technology.

Seafood waste, such as skin, bones (Ahmed & Chun, 2018), and viscera (Lee et al., 2021) have also been hydrolyzed by this method. Melgosa et al., (2020), used subcritical water extraction to obtain protein hydrolysates with bioactive peptides from sardine waste. Results showed that extraction yield as well bioactive properties, viz, antioxidant and antiproliferative activity, where improved when samples

where defatted before hydrolysis with subcritical water. Furthermore, the bioactive properties were positively affected as extraction temperature increased.

This same trend was observed in quinoa protein hydrolysates, obtained with Corolase®, when pretreated with supercritical CO_2. Authors conclude that the elimination of unwanted metabolites, such as lipids and phenolic compounds, improved the degree of hydrolysis and antioxidant activity of quinoa protein hydrolysates (Olivera-Montenegro, Best, & Gil-Saldarriaga, 2021). Similarly, Zhang et al. (2019) extracted wheat germ protein with subcritical water, which was later enzymatically hydrolyzed with alcalase, obtaining small peptides (<1 kDa) with antioxidant activity.

3.1.4. Combination of methods

A combination of conventional methods has been used to improve hydrolysis, to facilitate the release of BP, and to enhance their biological activity. The release of BP during milk fermentation with lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) has been reported to increase almost 5.6-fold, after 5 h, when assisted by the enzyme flavourzyme (Tsai, Chen, Pan, Gong, & Chung, 2008).

Proteolysis by endogenous enzymes, enzymatic hydrolysis, or microbial fermentation, followed by *in vitro* digestion is one of the most commonly combinations used. This combination, besides being used to promote the release of BP, is used to evaluate their stability once consumed. For instance, ACE-inhibitory peptides released from soymilk fermentation with *L. plantarum* C2 have been reported to be stable after *in vitro* digestion, attributable to its amino acid composition, but an increase in antioxidant activity was also observed, probably due to proteolysis by digestive enzymes (trypsin, pepsin and pancreatin) (Singh & Vij, 2018). In a related work, Martini et al. (2020) studied the effect of ripening and simulated digestion on peptide profile of Parmigiano-Reggiano cheese. Data demonstrated that resulting bioactive peptides may vary after simulated digestion and can be grouped according to the bioactivity and evolutive trend respect to the ripening time. For instance, ACE-inhibitory peptides, showed an increase trend after digestion according to the ripening, while antimicrobial peptides, showed a release increased after digestion reaching an equilibrium after 18 months of ripening. Finally, opioid peptides displayed a decreasing trend after digestion as a function of the ripening time. These data agree with those reported by Liu & Pischetsrieder, (2017), who observed an increase in the ACE-inhibitor peptide concentration (*ca.* 10,000-fold) after *in vitro* digestion of kefir.

3.2. Bioactivity screening and structure function

After selecting the protein and carrying out the proteolysis, the hydrolysates are fractionated according to their size, and purified according to their structure. Subsequently, *in vitro* tests are carried out to determine its bioactivity. Overall, those fractions with the highest bioactivity are selected and, finally, they are identified by liquid chromatography coupled to a mass spectrometer (Daliri et al., 2018). *In vitro* tests are dependent on the bioactivity. Those BP with antioxidant, antihypertensive, and antimicrobial activities, are usually the most studied.

The scavenges of radicals DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2, 29-azinobis ((3-ethylene bezothiazoline) 6-sulphonic acid)] assays are the most common methods for the assessment of antioxidant capacity of a peptide, which is determined in terms of absorbance changes of artificial, stable, and colored radicals DPPH• or $ABTS^{\bullet+}$. The reduction degree of colored radical during reaction is recorded at 515 and 734 nm, respectively (Singh & Vij, 2017).

More sophisticated methods have also been used to assess the free radical concentration. Electron paramagnetic resonance spectroscopy was used to evaluate antioxidant activity with DPPH of pea protein hydrolysates (Ding et al., 2020). Hence, the antioxidant activity of the peptides is evaluated through a chemical reaction in which the structure of the peptide intervenes by stabilizing the radical, either by the transfer of a proton or an electron. In this concern, aromatic amino acids (Tyr, His, Trp, and Phe) can donate protons, which could supply antioxidant activity to the peptides (Toldrá et al., 2018). Other methods used are the chelating capacity of Fe^{2+}, and the ferric-reducing power, based on a colorimetric reaction that are also evaluated by means of an absorbance reading at 562 and 700 nm, respectively (Zielińska et al., 2018).

It has been reported that the metal chelating activity of a peptide is related to the presence of side amino and carboxyl groups of acidic amino acids such as glycine and asparagine and basic amino acids such as Lys, His, and Arg (Saiga, Tanabe & Nishimura, 2003). Therefore, it can be assumed that the amino acid composition of the peptide, in addition to playing an important role in the biological activity of the peptide, may also be related to the mechanism by which it exhibits the antioxidant activity.

In the case of antihypertensive peptides, it is common to evaluate the inhibitory activity of ACE, which is one of the main enzymes involved in the renin-angiotensin system (Daliri et al., 2017). The method to evaluate this activity consists of determining the amount of hippuric acid formed by a reaction of Hippuryl-L-histidyl-L-leucine with the sample previously incubated with ACE. The quantification of hippuric acid can be carried out using high-resolution reversed-phase liquid chromatography or by spectrophotometry. The results are expressed as ACE-inhibitory activity (IC_{50}) (Tsai et al., 2008; Parmar et al., 2018; Auwal, Zainal-Abidin, Zarei, Tan, & Saari, 2019). Antihypertensive peptides have been shown to contain sequences between 2 and 12 amino acid residues. However, its activity is mainly related to the type of amino acid present in the sequence; for example, peptides that have Pro, Phe, Tyr, or Trp at the C terminal (Auwal, et al., 2019), as well as Val, Leu, and Ile at the *N*-terminal, are present in most reported ACE inhibitor peptides (Daskaya-Dikmen, Yucetepe, Karbancioglu-Guler, Daskaya, & Ozcelik, 2017).

On the other hand, the agar well diffusion method and disc diffusion method are widely used to evaluate the antimicrobial activity of antimicrobial peptides. For this, the peptide solution is filled into a hole/well created on the agar medium or placed in a filter paper disc on the agar surface inoculated with the microorganisms to be inhibited. After incubation under optimal conditions, the presence of antimicrobial activity is indicated by the absence of bacterial growth around the hole/disc. Indicator microorganisms generally used for this test include *Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogenes, Salmonella enterica,* and *Staphylococcus aureus.* Nevertheless, it has also been evaluated with microorganisms of food interest such as *Bacillus cereus* and *Brochothrix thermosphacta* (Abdel-Hamid et al., 2016; Borrajo et al., 2020).

The antimicrobial activity of peptides has been related to the physicochemical properties (size, charge, hydrophobicity, amphipathicity, and solubility), the number (12 to 50 amino acids), and type of amino acids (Jakubczyk, Karaś, Rybczyńska-Tkaczyk, Zielińska, & Zieliński, 2020). It has been reported that the positive charge or the presence of hydrophilic and hydrophobic amino acids are the main structural motifs with which antimicrobial peptides interact with microorganisms. In this sense, the presence of positively charged basic amino acids Lys and Arg are highly related to the antioxidant activity of the peptides (Daliri et al., 2017). Moreover, it is well known that some antimicrobial peptides show more activities than antimicrobial, such as antioxidant (Borrajo et al., 2020).

Other activities such as immunomodulatory and opioid have also received the attention of researchers. *In vitro* immunomodulatory tests are performed using cell lines such as RAW 264.7 (Chalamaiah et al., 2018). Similar to other bioactivities, immunomodulatory properties depend on the amino acid composition, sequence, length (2–10), charge, hydrophobic nature, and structure of the peptide (Ahn, Cho, & Je, 2015). Therefore, hydrophobic amino acids (Gly, Val, Leu, Pro, and Phe) negatively charged (Glu), and aromatic (Tyr) are the ones that mostly prevail in immunomodulatory peptides (Toldrá et al., 2018). While for

opioid activity, exorphins show the sequence Tyr-Gly-Gly-Phe or Tyr-Pro at their *N*-terminus, while at their c-terminus, the peptide sequence and length vary. It has been reported that Tyr is essential for the function of the peptide since its removal could result in the loss of activity (Guesdon, Pichon, & Tomé, 2006; Toldrá et al., 2018).

3.3. Validation: ex-vivo and in vivo studies

In vitro evaluation of bioactivities is the first step in the search for new BP, However, this potential must be validated by using *in vivo* or *ex vivo* assays with complex biological systems.

In vivo studies of novel peptides and hydrolyzed fractions have been evaluated using animal models, allowing to establish not only the bioactivity but also the implications that it would have under specific conditions of stress. For instance, a mixture of microorganisms (*Bacillus subtilis* GD1, *Bacillus subtilis* N4, *Bacillus velezensis* GZ1, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Hansenula anomala*) with a high proteolytic activity was used to produce a fermented soybean food. Then, male C57BL76J mice (5-month-old), stressed by intense exercise, were daily administered with fermented soybean. The administration resulted in an attenuation of fatigue indices (blood glucose, blood urea nitrogen, lactic acid, hepatic glycogen, and lactate dehydrogenase). Besides, treatments were effective to up-regulated the expression levels of oxidative stress-related signaling genes (*Nrf2, NQO1, GCLC* and *GCLM*) in liver (Cui et al., 2020).

The antihypertensive activity of peptides obtained from plant sources has also been evaluated *in vivo* and *ex vivo*. Suárez, Aphalo, Rinaldi, Añón, and Quiroga (2020) proved the administration (a single dose for 3 h) of either isolated protein and hydrolysates from amaranth (1.9 g protein/kg), as well as the synthetic peptide VIKP of the 11S amaranth protein (50 mg peptide/kg), in order to determine the mechanism by which amaranth protein isolate hydrolysate, and synthetic peptide exert their hypotensive effect.

The authors observed the reduction of systolic blood pressure (from 200 to 220 to 140–170 mmHg) of spontaneously hypertensive rats, as well as a decrease in plasma renin levels and an increase in ACE levels, which was inversely proportional to its activity. According to the authors, these results could mean that hydrolysate, peptide, and protein may act as ACE competitive inhibitors. The antihypertensive effect was also validated with an *ex vivo* study in thoracic aorta rings of rats in presence of potassium ions and norepinephrine (compounds known to cause vasoconstriction), in order to evaluate the contractile activity. In this case, the thoracic aorta rings of the animals treated with the protein isolate, the synthesized peptide, and the hydrolysates showed low contractile activity, indicating a vasorelaxant effect. In their comprehensive analysis, authors point to amaranth as an important source of antihypertensive peptides. Furthermore, they proposed two mechanisms: peptides act as a competitive inhibitors of plasma enzymes (ACE and renin), and they also have a vasorelaxant effect.

Milk is known to be an important source of BP; hence, different effects of peptides derived from milk have been evaluated *in vivo*. β-casofensin (GVSKVKEAMAPKHKEMPFPKYPVEPFTESQ), a peptide, that cannot be release from milk during digestion, but can be obtained from lactic acid bacteria fermentation, was administered daily to Wistar pup rats (0.1 μM; 10 μL / g of body weight, postnatal 10–20 d). The resulting data showed an increase in the population of goblet cells, as well as an increase in the expression of *Muc2* mRNA, which indicates that the peptide has a protective effect on the intestinal barrier. However, a decreased in intestinal permeability to FD4 was observed in an *ex vivo* study performed in jejunum segments from male rats, stimulated with the β-casofensin (A1 variant), while no effect was observed with A2 variant, with respect to the control treatment (Bruno et al., 2017). These results evidence that the changes on a single amino acid (from Gln (A1 variant) to Glu (A2 variant) at position 117) within the peptide sequence can alter the expected benefits.

Hydrolysates from buffalo milk, obtained with papain, have shown hepatoprotective effect on albino rats stressed with carbon tetrachloride. Protective effect was associated either to the possible antioxidant activity of hydrolysate, the indirect inhibitory activity of ACE, or the regulation of expression of antioxidant enzymes (SOD and CAT) (Abdel-Hamid et al., 2020). Besides, Kefir has shown antihypertensive effect on Wistar rats. Such effect was related to 35 peptides identified in kefir, which according to an *in silico* study are potential ACE inhibitors (Amorim et al., 2019). Therefore, the use of *in vivo* and *ex vivo* studies are adequate tools to define the health benefit and the mechanisms, but if they are carried out in conjunction with *in silico* approaches, they can further facilitate the search for new and promising bioactive peptides, as well as new benefits and in turn elucidate the probable mechanisms of action.

On the other hand, the number of clinical studies is less abundant than studies with animals. The clinical studies are mostly based on BP from animal proteins or their derivatives, such as milk or eggs, and some of these BP are already commercialized. For instance, the postprandial glycemic effect of milk-derived alanine-proline dipeptide, marketed under the name Pep2Dia®, has been evaluated in prediabetic subjects in a randomized cross-over trial, where the subjects received six weeks a single dose of dipeptide before a high carbohydrate meal. Results showed the regulation of postprandial hyperglycemia and a slightly, but significant, reduction of HbA1C levels. Hence, this product could potentially reduce the risk of suffering diabetes mellitus type 2, even in prediabetic subjects (Sartorius, Weidner, Dharsono, Boulier, Wilhelm, & Schön, 2019).

Similarly, Akazawa et al., (2018) have hypothesized that a lactotripeptide with ACE inhibitory activity and capacity to increase the production of a vasodilator, could reduce cognitive decline and cerebral atrophy by improving the speed of blood flow to the brain in mild-aged and older adults, which decreases with age. Therefore, a randomized, placebo-controlled, double-blind design was used to assess the healthy benefits of a daily supplementation, for 8 weeks, of a casein hydrolysate, containing VPP and IPP peptides, to healthy middle-aged and older adults. The results evidenced that supplementation increased the velocity of cerebral blood, which in turn may reduce the risk of loss of cognitive function (Akazawa et al., 2018).

The constant search for therapeutic agents to improve the health status of thousands of people with non-communicable diseases has increased the interest in the search for novel BP. *In vivo* studies in humans should be the optimal stage in which it will be defined whether the potential benefit obtained from *in vitro* tests, and animal models of a peptide, can be transferred to a human with favorable results. In most of the reported cases the results are favorable, however this is not always the case. For instance, Lucey, Heneghan, Manning, Kroon, and Kiely, (2018), reported that there was no reduction in blood pressure, or the modification of cardiovascular risk factors, in adults between 50 and 70 years of age with a systolic pressure of between 130 and 150 mmHg, after consuming egg ovalbumin protein hydrolysates (3 g/day) for a period of 6-week.

4. *In silico* tools to analyze bioactive peptides

Data from biological systems can be managed, curated, and interpreted through computational methods (*in silico*), which would allow, in the case of the discovery of new BPs, to save time and resources compared to conventional methods (FitzGerald, Cermeño, Khalesi, Kleekayai, Amigo-Benavent, 2020). In this context, *in silico* analyzes make possible to select the appropriate enzymes and protein sources, perform proteolysis, predict possible biological activity, as well as allergenicity and toxicity, and determine action mechanisms by molecular docking. These approaches will be addressed in the following sections.

4.1. Selection of suitable enzyme(s) and appropriate protein source

The protein source and the protease enzyme are the most important factors in the successful generation of BP. Therefore, *in silico* experimentations have been based on the proper selection of these factors. Such selection would be impossible under conventional methods, since performing the hydrolysis and verifying the formation of BP from several sources at the same time would be a tedious, time-consuming, and excessively expensive work. Hence, researchers have taken advantage of the speed of *in silico* analysis to obtain important information, before evaluating *in vitro* activities and further clinical trials (Ibrahim, Bester, Neitz, & Gaspar, 2019).

The selection process of both factors involves a series of successive steps. The first step consists in acquiring the sequence of the study proteins, by using specialized databases such as UniProt Knowledgebase (http://www.uniprot.org/). Then, simulate the cleavage of the peptide bonds with the enzymes of interest using databases. For instance, the sequences of 10 storage proteins from five oilseed sources and three bovine proteins were retrieved from UniProt Knownledgebase, in order to investigate the potential in the production of BP with ACE and DPP-IV inhibitory properties. *In silico* hydrolysis was performed using the Enzyme Action tool in the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), with the enzymes subtilisin and pepsin (pH 1.3 and > 2). Thus, this comparison allowed to observe that the oilseeds proteins can be better precursors of ACE inhibitory peptides with the enzyme subtilisin than bovine proteins. However, low yields are obtained in the generation of DPP-IV inhibitory peptides under these same conditions (Han, Maycock, Murray, & Boesch, 2019).

In the same way, an *in silico* study allowed the selection of thermolysin, followed by papain, as the appropriate enzymes to obtain the best ACE inhibitory peptides from yak milk proteins (α_s1-, α_s 2-, β- and κ-casein). This selection was based on the frequency of bioactive fragments occurrence in a protein sequence (A), the potential biological activity of the protein (B). Both parameters were obtained from the BIOPEP database (Lin et al., 2018). In this same context, Han et al. (2019) report that these parameters (A and B) are calculated based on the information available in the BIOPEP database. However, despite the adequate selection of both the protein and the enzyme to be hydrolyzed, the study is limited to the information contained in databases, therefore it can be modified over time due to continuous updating of databases.

In this perspective, databases play a very important role in the development of *in silico* analyzes, since they provide a collection of peptides with various bioactivities. There are specialized databases in single bioactivity, for example antihypertensive peptides (AHTPDB), while others include peptides with different functions (e.g., BIOPEP-UWM, and BioPepDB). There are also specialized databases on peptides from food proteins (FeptideDB), from specific protein sources such as milk (MBPDB, MilkAMP), or from fermented foods (FermFooDB) (Iwaniak, Darewicz, Mogut, & Minkiewicz, 2019; Panyayai et al., 2019; Chaudhary, Bhalla, Patiyal, Raghava & Sahni, 2021). Therefore, if the study is on specific bioactivity or a special source, these tools facilitate the task of generating peptides.

4.2. In silico proteolysis (GI enzymatic digestion)

The digestive process is a physiological process used to absorb the greatest amount of nutrients from food. When protein material is digested, the peptide profile is altered, which may cause the formation of new peptides from digested proteins, or that existing peptides lost their bioactivity (Chakrabarti et al., 2018). For this reason, it is necessary to expose the new peptides to simulated digestive conditions in order to establish whether the peptide could generate a health benefit.

In silico approaches can also mimic the digestive process and obtain results easily and rapidly. For instance, *in silico* method has been used to predict and estimate the resistance of BP obtained from dairy products to simulated digestive conditions. Some results have showed that 1066.07 μmol of peptides resistant to digestion presented high bioactivity (Barati et al., 2020). In addition, the *in silico* analysis may display the possible peptides that will be formed after protein source ingestion, and thus associate them with some possible benefits. In this sense, plant tuber proteins (patatin, sporamins, dioscorins, tarins, and globulins), were exposed to a digestion process mimicked by *in silico* analysis with pepsin (pH 1.3), chymotrypsin, and trypsin, with the help of the BIOPEP database. After that, 387 peptides with diverse bioactivities, such as inhibition of DPP-IV and ACE, antioxidant, antithrombotic, antimicrobial, and anticancer properties, were obtained (Ibrahim et al., 2019). However, it should keep it in mind that all these studies need to be validated through *in vitro* and *in vivo* assay.

Alternatively, *in silico* methods may be combined to conventional approaches, this combination is known as hybrid or integrated methods (Fig. 1). Overall, conventional techniques are used after performed the peptide prediction to validate the results, although this is not always the case. In this context, an *in vivo* gastric digestion study was performed by mini pigs with gastric cannulas; mini pigs were fed with cooked beef as source of protein. A total of 203 peptides obtained from gastric digestion were then identified and quantified. Later *in silico* digestion was also performed, mimicking the digestion with intestinal enzymes, from which 255 potential bioactive peptides were obtained. Authors pointing to cooked meat as a source of antioxidant and DPP-IV and ACE inhibitor peptides (Sayd et al., 2018).

In silico digestion is a suitable method to predict BP release and resistance. Nevertheless, the use of integrated methods can improve the prediction of promising new BP.

4.3. Peptide characterization

Peptides obtained after simulated digestion have been characterized according to their physicochemical, biological, and sometimes sensory properties. The former includes molecular weight, theorical pI, aliphatic index, hydropathicity, among others. While the later include bioactivities, toxicity, and allergenicity (Agyei et al., 2018).

Several authors have used *in silico* tools, such as the PepDraw (http://www.tulane.edu/~biochem/WW/PepDraw/), to calculate the hydrophobicity and net charge of peptides. However, more complete tools such as ExPASy ProtParam (https://web.expasy.org/protparam/) have also been used. These tools led to can calculate the molecular weight, isoelectric point (pI), net charge, hydrophobicity index, instability index, aliphatic index, and hydropathicity of peptides. The higher the value, the higher the hydrophobicity (Ji et al., 2019; Jakubczyk et al., 2020). The hydrophobicity of the peptides could indicate the presence of hydrophobic amino acids, such as Met and Trp, which could act as hydrogen donors or acceptors, improving the antioxidant capacity of peptides (Zhang, He, Bonneil, & Simpson, 2020).

Additionally, the structure of the peptides is an important characteristic, since the bioactivity of the hydrolysates depends on it and the peptide sequence (Hu et al., 2020). For instance, most antimicrobial peptides present an α-helical structure, with a cationic and amphipathic nature. The cationic part is the responsible for the formation of pores in the cytoplasmatic membrane of microorganisms due to the electrostatic interaction with the anionic phospholipids of the cell wall (Bhandari, Rafiq, Gat, Gat, Waghmare, & Kumar, 2019).

Additionally, it has been reported that BP are sometimes not suitable for use as therapeutic agents, as they can be unstable, tend to aggregate, and have a short half-life in plasma. Nevertheless, knowledge of the structure can compensate for these disadvantages, since it would be possible to identify essential amino acids and the sites where an amino acid could be substituted through the construction of the structure–function relationship, generating an appropriate design of a therapeutic peptide (Wang et al., 2018). Subsequently, databases, which houses important information about the structure of peptides, such as StraPep (http://isyslab.info/StraPep/), are important to calculate the structure–function relationship and to the design of therapeutic BP.

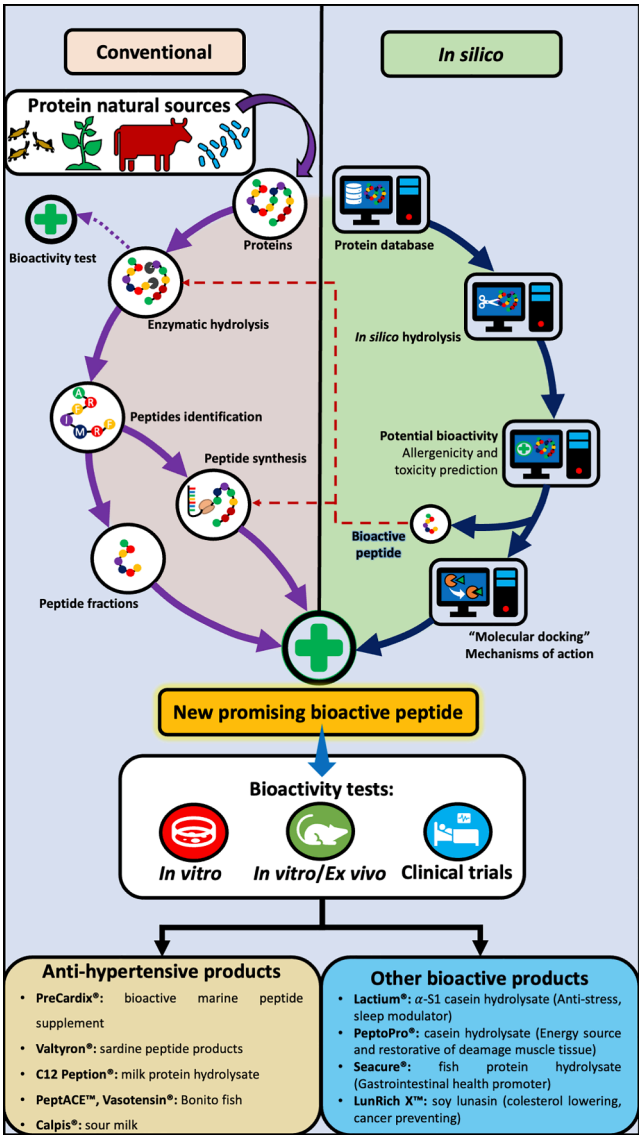

Fig. 1. Hybrid approach for the generation of new bioactive peptides from food-derived proteins. Conventional methods consist of a series of *in vitro* studies. First, the protein source is subjected to an *in vitro* hydrolysis with an enzyme selected according to its known specificity. Afterward, the hydrolysates are assessed by *in vitro* tests for the bioactivity of interest. Then the peptides are identified and can be synthesized or separated by molecular weight (fractions). Subsequently, the *in vitro* bioactivity is evaluated again in the peptide previously identified. In case of finding positive results, a promising new peptide should be validated through *ex vivo* and *in vivo* studies and, finally carry out clinical trials. While the *in silico* approach consists of a series of bioinformatics methodologies. Previously known proteins of interest are downloaded from a database (e.g., UniProt), to be loaded into specialized databases on BP (e.g., BIOPEP), where hydrolysis can be performed with different enzymes at the same time. As a result, peptides are obtained, which are evaluated using computational tools to predict their bioactivity (e.g., PeptideRanker), allergenicity (e.g., AlgPred), and toxicity (e.g., ToxinPred). After data analysis, important information can be obtained that indicates the way forward (i.e., proper selection of proteins and enzymes to obtain BP) and, in the case of hybrid approach, these conditions are replicated by *in vitro* hydrolysis, but also within this approach can synthesize peptides and follow conventional strategies. Subsequently, molecular docking is carried out, where the interaction between peptides and receptor molecules of interest is predicted. However, it has been highlighted that these interactions must be verified by conventional methods, in order to generate a new BP by a hybrid approach. Once the product has been evaluated through *in vivo* studies followed by clinical studies, products with the new peptides and proven benefits are marketed.

4.4. *In silico* prediction of potential biological activity profile (Structure-function analysis)

In silico methods are used to predict the performance of BP from food proteins. Several methods have been developed, such as the online tool PeptideRanker (http://distilldeep.ucd.ie/PeptideRanker/) and Quantitative Structure-Activity Relationship (QSAR) (Tu, Cheng, Lu, & Du, 2018a).

PeptideRanker shows a score with values between 0 and 1, the closer the score is to 1, the more bioactive the peptide is. According to the developers, the threshold is 0.5, hence peptides with scores above this are considered bioactive (Mooney, Haslam, Pollastri, & Shields, 2012). This is currently one of the most used tools to predict bioactivity. Garg et al., (2018) used PeptideRanker to rank peptides obtained from wheat gluten, and to compare them with known opioid peptides from wheat (exorphins). Eleven peptides from wheat gluten were selected because they have Tyr and Pro, amino acids detected in opioid peptides. Following a hybrid approach, three peptides (YPG, YYPG, and YIPP) which obtained a score greater than 0.77 (probability of being bioactive) were selected to confirm their bioactivity (inhibition of cyclic adenosine monophosphate production in cells), indicating a binding to opioid receptor (κ and μ), and hence opioid activity.

Similarly, in a study carried out with pea protein hydrolysates with antioxidant activity, only three peptides obtained a score greater than 0.5 (YSSPIHIW, ADLYNPR, and HYDSEAILF), These three peptides showed to contribute to antioxidant activity (Ding et al., 2020). Therefore, bioinformatics tools prove to be fast, reliable, and useful in the prediction of peptide bioactivity regardless of its protein source or targeted bioactivity.

On the other hand, the quantitative model that establishes the relationship between physicochemical or structural properties and biological properties, also called QSAR, has been used to determine the relationship between sequence/structure and biological activity. This method involves the use of bioinformatic techniques to predict the physicochemical properties of peptides as well as to perform chemometric analysis – least squares regression, component analysis, and artificial neural networks (Agyei et al., 2018; Iwaniak et al., 2019). In other words, QSAR is a model that uses mathematical functions that determine the relationship between biological activities and characteristics of the structure, for which a set of numerical descriptors is generated from the desired compounds that will help to describe the properties of peptide (Mahmoodi-Reihani, Abbasitabar, & Zare-Shahabadi, 2020).

Studies with QSAR models have focused on different bioactivities, such as antioxidant. For instance, a QSAR model was constructed with 91 antioxidant tripeptides. Based on the analysis, 19 peptides were selected based on the model and validated *in vitro*, showing an antioxidant activity higher than that predicted by the QSAR model (Chen, Chen, Yao, & Li, 2018). The antihypertensive capacity of peptides has also been evaluated using QSAR models. Deng et al., (2017) built a QSAR model with 141 ACE-inhibitor dipeptides, the results showed that the hydrophobics, steric, and electronic properties, as well as C-terminal amino contribute to the ACE inhibitory activity. Five peptides were selected and evaluated *in vitro* to be validated, the results demonstrated that the model is a reliable prediction to evaluate the inhibitory activity of ACE in peptides.

4.5. *In silico* prediction of allergenicity and toxicity

Peptides are considered bioactive because they have a positive physiological effect; therefore, undesirable properties such as toxicity and allergenicity must be evaluated to prevent or limit their presence or, if necessary, change the protein-enzyme combination (Agyei et al., 2018). In the same way that bioactivity, toxicity, and allergenicity can be evaluated by *in silico* methods, which are considered effective.

Many studies have used the ToxinPred (https://webs.iiitd.edu.

in/raghava/toxinpred/) and AlgPred (https://webs.iiitd.edu.in/raghava/algpred/submission.html), tools to predict toxicity and allergenicity, respectively. ToxinPred is a method that predicts the toxicity of peptides or toxic regions in the protein, by a hybrid model based on the composition of dipeptides and motif scanning (Ji, Udenigwe, & Agyei, 2019). While AlgPred predicts the allergenic proteins; however, it has the limitation that can only analyze peptides with more than 10 amino acids (Tu et al., 2018b).

A study with conventional approach was complemented by a toxicity analysis with ToxinPred tool, showing that none of the 4 iron-chelator peptides obtained from tilapia skin collagen presented toxicity (Lin et al., 2021). Similarly, ACE-inhibitory peptides obtained from yak milk casein showed no toxicity (Lin et al., 2020). Peptides from other protein sources, such as tubers and cereals, also showed no toxicity (Ibrahim et al., 2019; Mudgil et al., 2020). Casein hydrolysates were also analyzed to predict their toxicity and allergenicity, finding that none of the hydrolysates presented toxicity or allergenicity. However, concerning allergenicity, the AlgPred tool has a limitation since only sequences greater than 10 amino acids can be evaluated, so the smaller ones are out of the analysis and could present potential allergenicity (Tu et al., 2018b).

Therefore, other tools have been used to assess allergenicity, such as AllergenFP v.1.0 (https://ddg-pharmfac.net/AllergenFP/), and AllerTop v.2.0 (https://www.ddg-pharmfac.net/AllerTOP/). AllergenFP v.1.0 is a platform where the allergenic capacity of proteins is predicted through the transformation of amino acids properties (hydrophobicity, size, abundance, behavior of hydrogen bridges) into fingerprints, which via Tanimoto similarity searches with the allergenic profile of known proteins, and it has been used to evaluate the allergenic potential of flaxseed proteins, where 21 of 23 proteins are classified as probably allergenic (Ji et al., 2019). On the other hand, AllerTOP v.2.0 is a powerful tool that classifies allergens and non-allergens, using the k-Nearest Neighbors method, with an accuracy of 87% (Aminnezhad, Abdi-Ali, Ghazanfari, Bandehpour, & Zarrabi, 2020). This tool has been used to predict the allergenicity of quinoa seed proteins, in which only 1 of 8 peptides showed probable allergenicity (Wong, Ong, Kumar, & Chai, 2021).

The *in silico* tools, used to predict allergenicity and toxicity, seem to be fast and appropriate. However, they show some limitations, and in most cases, the results are not confirmed by conventional approaches, especially the undesirable effects such as the allergic reactions that need to be tested by *in vivo* methods.

4.6. Molecular docking

Molecular docking is a widely *in silico* strategy used to illustrate the biological mechanisms of food-derived peptides (Tu et al., 2018a). This method predicts the mode of binding of peptides (small molecules, ligands) with their respective receptors (e.g., enzymes). This allows for the planning and creation of peptide therapies that are better tailored to the receptors (Iwaniak et al., 2019). To achieve a successful molecular docking, a series of steps must be followed, including the selection and preparation of the protein, preparation of the ligand, perform the docking, and finally analyze the results. The selection of the receptor molecule is given by the bioactivity targeted and is the main step to follow in this process (Tu et al., 2018a).

Auwal et al., (2019), observed that stone fish peptides interact with ACE through hydrogen and electrostatic bonds. In the same way, peptides obtained from edible rhizomes (Sompinit et al., 2020), trout (Yu et al., 2018), kefir (Amorim et al., 2019a), and *Kluyveromyces marxianus* protein hydrolysates (Mirzaei et al., 2018) can interact with ACE through hydrogen bonds, hydrophobic interactions or Van der Waals and electrostatic forces. Ponting to an antihypertensive ability of peptides.

Currently, molecular docking has also been used to evaluate the influence of BP on the virus (SARS-CoV-2) that causes COVID-19. Thus, Çakir, Okuyan, Şener, and Tunali-Akbay (2021) analyzed the interactions of β-lactoglobulin peptides with the main protease involved in the replication of SARS-CoV-2, as well as with the spike proteins of the virus and its receptor binding site. The peptides ALPMHIR, IPAVFK, and GLDIQK showed a predicted inhibitory effect of the main protein of SARS-CoV-2, and therefore an inhibition in its replication. While only ALPMHIR showed interactions with the spike proteins of the virus, pointing to possible prevention of the binding of SARS-CoV-2 with the host cells. Similarly, Wong et al. (2021) identified the ability of seven peptides from quinoa seed proteins to bind three targets of SARS-CoV-2 (spike glycoprotein RBD, M^{PRO}, and PL^{PRO}) employing molecular docking, mainly through hydrogen bonding and hydrophobic interaction.

Based on described results, molecular docking is increasingly popular, as it can predict the mechanisms of interaction between the peptide and the receptor molecule and, if required, provide a solution to diseases of global importance, such as hypertension, cancer or even COVID-19. However, as promising as the results may be, *in vitro* and *in vivo* validations are still necessary.

5. Conclusions and future outlooks

Bioactive peptides have long attracter attention because they represent a promising alternative against epidemic of non-communicable diseases that global population is currently suffering. The constant search for bioactive peptides has led to establish that their bioactivities are directly related to the amino acids that constitute them; hence, a correct selection of the protein source promises a good start in the search for new bioactive peptides.

The search for bioactive peptides through *in silico* techniques is promising since it avoids the economic cost, waste of time and eliminates the guessing factor that conventional methods bring. Therefore, they indicate the way forward with the only limitation that databases present since important peptides could remain in the shadow if they are not capture in a database. Nevertheless, this drawback is being amended because more and more researchers are adopting *in silico* studies to select the appropriate protein source and protease, to obtain peptide sequences after hydrolysis, test their biological activity, or to test their mechanisms of action (molecular docking), which will generate efficient bioactive peptide production with potent health benefits. It is important to point out that the biological effects predicted by bioinformatics must be supported based on *in vitro* and *in vivo* studies. Therefore, the combination of conventional and *in silico* approaches, known as hybrid or integrated methods, is a potential way to obtain new and promising bioactive peptides, regardless of the protein source, quickly and without wasting time and resources.

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CRediT authorship contribution statement

Audry Peredo-Lovillo: Investigation, Visualization, Writing – original draft. **Adrián Hernández-Mendoza:** Conceptualization, Writing – review & editing. **Belinda Vallejo-Cordoba:** Writing – review & editing. **Haydee Eliza Romero-Luna:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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