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 provide 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 not limited to cancer, immune disorders, and cardiovascular diseases (Chalamaiah, Yu & Wu, 2018; Lammi, Aiello, Boschin & Arnoldi, 2019; Skjånes, Aessy, 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 (Aguye, 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, Guzman, Valencia, Almonacid & Fernandez, 2020), fish skin (Elaziz, Hemdan, Hassanien, Oliva & Xiong, 2017), invasive sea grass *Halophila stipulacea* (Kandemir-Cavas, Perez-Sanchez, 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 (Darot & 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 & Mjumder, 2016). 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 proteinate, the purification and identification of BP, as well as the evaluation of biological activities (Darot & 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; Zielinska 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-proteins. 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, one 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 (303-299), 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 (Darot & 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, Ramirez-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 Gobla, Jenson, & Osman, 2016; Abdel-Hamid, Osmand, El-Haladry, Romeih, Sitohy, & Li, 2020). It is important to note that the selection of the enzyme will depend on the proteins 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, Gaçagoua, 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 (IC50 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-802 and ripening time | Inhibitory activity of the ACE (90.22% after 120 days of ripening). | Baptista, Negrão, Eberlin & Gigante, 2020 |
| Parmigiano-     | β-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) ACE-inhibitory of 10 kDa permeates (25.16 %). | Martini, Conte & Tagliazucchi, 2020 |
| Reggiano cheese | αs1-CN fractions | Enzymatic digestion | Enzymatic digestion | Li et al., 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* TD5030603 | 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    | αs1-CN variant BB | Enzymatic digestion | ACE-inhibitory, anti-inflammatory, antioxidant, and anxiolytic (predicted by BIOPEP). | Sayid, Dufour, Chambon, Buffiere, Redmon, & Santé-Lhoustelier, 2018 |
| Meat            | Cooked beef       | IVAPGKGLAADESTGSIAK | In vivo gastric digestion and in *silico* analysis | Liu, Chen, Huang, Huang, & Zhou, 2017 |
| Duck meat       | < 5 kDa fractions | Post-mortem aging | Antioxidant assest at 3 days of aging by DPPH (59.83%), FRAP (greater than 300 μmol FeSO4/7H2O Eq/g sample), and ORAC (1000 μMTEq approximately) | Li et al., 2020 |
| 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 (IC50 from 81.2 μg/mL (chicken) to 238.0 μg/mL (Turkey)), and DPP-IV (IC50 from 1.88 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 (IC50 from 2.6 to 3.4 mg/mL) | Hong, Gottardi, Niggi-jimana, & Betti, 2014 |
| Tilapia skin collagens | GPAGPAGEK, DGPSPKGDR, GLPGPSGEKGR, and DGGSPGKGDRGETG | Enzymatic hydrolysis with trypsin, pepsin, neutral protease, alkaline protease and protamex | Iron-chelating (83.47% at a hydroxysate concentration of 5 mg/L) | 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 |
| Gourmay          | YYP, IP, YYP, YP  | In *silico* analysis | Opioid peptides identified using BIOPEP database | Garg, Apostolopoulos, Nugali, & Misra, 2018 |
| Maize           | 192ZP1, 192ZP2 and 192ZP3 | In *silico* analysis | ACE-inhibitory (IC50 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, Lopez-Castillo, Castorena-Torres, Serna-Saldivar, & Garcia-Lara, 2020 |
| Brown rice      | FGGSGGPGG and FGGGGAGGAGG | Enzymatic hydrolysis with bromelain | ACE-inhibitory (IC50 value of 0.20 mg protein/mL) | Selamassakul, Laohakunjit, Kerdchoechuen, Yang, & Maier, 2020 |
| Quinoas          | QPHHGILGALCAAPPST | Enzymatic hydrolysis by bromelain, chymotrypsin, and Pronase E | DPP-IV (p-value of 0.009151) and α-glucosidase inhibitory (p-value 0.06893) 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 (continued on next page) |
| Peptides Source | Protein substrate | Peptide(s) | Obtaining method | Bioactivities | References |
|-----------------|-------------------|------------|------------------|--------------|------------|
| Microorganisms  | Edible           |            |                  |              |            |
| Algae           | Red seaweed       | GGSK and ELS | Enzymatic proteolysis | β-amylase inhibitor  | Do Nascimento et al., 2021 |
| Spirulina platensis | EYFDALA | Enzymatic hydrolysis | using pepsin | Antioxidant evaluated by DPPH (EC$_{50}$ of 2.58 mM and 2.62 mM for GGSK and ELS, respectively) | Admassa, Gasmalla, Yang, & Zhao, 2018 |
| 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) | Zielinska, Baranik, & Karas, 2018 |
| 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). | | |
| Edible insects  | Schizostola gregaria | FDPPFK | 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 lipoxigenase inhibitory activity (LOX, IC$_{50}$ = 2.85 mg/mL) and Cyclooxygenase 2 inhibitory activity (COX 2, IC$_{50}$ = 7.40 mg/mL). | | |
| Gryllodes sigillatus | IIAPPBR | 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). | | Zielinska et al., 2018, |
| Tenodeph 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). | | Zielinska 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, & Lizeaga, 2018, |
| Food industry waste | Meat myofibrillar proteins | Acidic peptides (fractions 6-10) | 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 andaeus | Antioxidant evaluated by DPPH (20% of radical inhibition) and ABTS (above 15% of radical inhibition). | | Delgado-García et al., 2019 |
| Tofu whey wastewater | Lunasin | Tofo processing | Anti-inflammatory and immunomodulatory, assessed using RAW 264.7 murine macrophages (30% reduction of TNF-α with 190 µM lunasin). | | Nieto-Veloz 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, PW 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 cyano bacterium | In silico enzymatic digestion with pepsin, trypsin, and chymotrypsin. | ACE and DPP-IV inhibitors (39.1 and 47.7 % of peptides from cyanobacteria, respectively) obtained using BIOPEP database | | Ji et al., 2018 |
| Arthrospira platensis | LPSVHDLK, VLSTSFPLK | Sonicated enzymatic hydrolysis (trypsin and chymotrypsin) | Antioxidant (IC$_{50}$ = 5568 µM TE/mg and protein for VLSTSFPLK) and ACE-inhibition (IC$_{50}$ = 22.88 and 15.20 µM for LPSVHDLK and VLSTSFPLK, respectively). | | Mirzaei, Mirzamadi, Ehsani, & Aminlari, 2018, |
| Klyveromyces marxianus | 25 novel antioxidant peptides | | | | Song et al., 2020 |

(continued on next page)
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 endopeptidase, and bromelain prefers to break out proteins to poly- and oligopeptides (Holyvayka 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 (Holyvayka 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 establish 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-Nino, Rodriguez-Serrano, Gonzalez-Olivares, Contreras-Lopez, Regal-Lopez 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-Nino 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 (Zielinska 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 its exponential phase, harvested, and adjust the inoculum to the desired concentration to inoculate the protein source (Chakraborti 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

| Peptides Source | Protein substrate | Peptide(s) | Obtaining method | Bioactivities | References |
|----------------|------------------|-----------|-----------------|--------------|-----------|
| Edible mushroom (Agaricus bisporus) | Ultrafiltered fractions (1–3 kDa) | Enzymatic hydrolysis by neutral protease | Antioxidant evaluated by the total antioxidant capacity assay (0.73 μmol TE/mg). | Kimatu et al., 2017 |
| Pleurotus ostreatus | Mushroom protein hydrolysates (65% of hydrolysis degree) | Enzymatic hydrolysis by alcalase and pancreatin | Antioxidant evaluated by FRAP (0.62 of absorbance at 3 mg/mL) | Gowami, Majumdar, Das, Barui, & Bhowal, 2021 |

Table 1 (continued)
| Enzyme/substrate ratio (% w/w) | pH | Temperature (°C) | Hydrolysis time (min) | Identified peptides | Protein substrate — Bioactivity | Reference(s) |
|-------------------------------|----|-----------------|-----------------------|---------------------|-------------------------------|--------------|
| **Pepsin**                    | 1  | 2               | 37                    | No identified       | Deer velvet — ACE-inhibitory (17.57% ACE activity) | Haines, McCann, Grosvenor, Thomas, Noble, & Clerens, (2019). |
|                               | 4  | 7.5             | 37                    | No identified       | Deer velvet — ACE-inhibitory (17.57% ACE activity) | Haines et al., (2019) |
| **Pancreatin**                | 4  | 7.5             | 37                    | No identified       | No identified Deer velvet — ACE-inhibitory (17.57% ACE activity) | Haines et al., (2019) |
| **Chymotrypsin**              | 1  | 7.8             | 50                    | QHPHGLGALCAAPPST and other 34 peptides identified | Quinoa — ACE-inhibitory (IC₅₀ = 0.22 mg/mL) and DPP-IV inhibitory (IC₅₀ = 0.72 mg/mL) | Mudgil et al., (2020) |
| **Papain**                    | 0.5–4 | 6.0 – 7.0     | 37 – 50               | 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 Fe²⁺/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 (IC₅₀ from 25.1 to 31.6 μg/mL) and DPPH (IC₅₀ 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), Porcine Liver — antioxidant activity identified by SWATH and correlated with antioxidant capacity, Quinoa — ACE-inhibitory (IC₅₀ from 0.90 to 1.12 mg/mL) and DPP-IV inhibitory (IC₅₀ from 0.18 to 0.24 mg/mL) | Abdel-Hamid, et al., (2016; 2020); Borrajo et al., (2020); Cotobarren et al., (2019); Hu et al., (2020), |
| **Bromelain**                 | 1 – 100 | 5.0 – 7.0 | 40 – 50               | No identified in most investigations; 22 peptides containing the LPF tripeptide (Corn Gluten Meal) | Porcine Liver — antioxidant activity evaluated by DPPH (from 322 to 379 μg Trolox/g), FRAP (from 35.7 to 69.8 μmol Fe²⁺/100 g), ABTS (from 310 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 | 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                    | 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                    | APAAIGPYSQAVLVD 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                    | No identified       | No identified Deer velvet — ACE-inhibitory (17.57% ACE activity) | | (continued on next page) |
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 amyloliquifaciens U304, compared to that fermented with Lactobacillus acidophilus, L. plantarum or Saccharomyces cerevisiae CFI69. This could be due to the presence of a highly active protease in B. amyloliquifaciens. 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 G1, 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 have 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 ultrasounds 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 alcalase, proteamx, savinase or corollase 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 (Zielinska 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 MPAs). In addition, due to its characteristics, it does not leave residues like organic solvents, it is non-toxic and is considered a green technology (Ulgu 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.

Food 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, improved when samples

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**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 362 μg Tropolon/g), FRAP (from 43.3 to 82.9 μmol Fe²⁺/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. thermophila and L. monocytogenes | Borrajo et al., (2020); Hall et al., (2018) | | | | | |
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 CO₂. 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 proteinolysis 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 scavengers of radicals DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2, 29-azinobis (3-ethylen 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⁺. 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 Fe²⁺, 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 (Zielinska 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 (IC₅₀) (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, Yucetepete, 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 anti-microbial 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; Borrajro et al., 2020).

The antimicrobial activity of peptide 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, Karas, Rybczynska-Tkaczuk, Zielinska, & Zielinski, 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 (Borrajro 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 (Abn, 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
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, Aphabeto, Rinaldi, Anón, 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 VSK of the 115 amaranth protein (50 mg protein/kg), in order to determine the mechanism by which amaranth protein isolate hydrolysates, 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 activity 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 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 (GVSVKKEAMAPKHKEMPFPKYPVEPFTESQ), 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 Mac2 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 jejunal 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 alamine-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, Dharsouli, Boulier, Wilhelm, & Schön, 2019).

Similarly, Akazawa et al., (2018) have hypothesized that a lacto-tripeptide 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, Cermeno, Khalesi, Kleckayai, Amigo-Benavent, 2020). In this context, in silico analyses 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 experiments 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 Knowledgebase, 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 Activity tool in the BIOPEP database (http://www.chem.uw.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 thermo-lysin, followed by papain, as the appropriate enzymes to obtain the best ACE inhibitory peptides from yak milk proteins (αlactalbumin, αlactalbumin 2, β-lactoglobulin 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 (PeptideDB), from specific protein sources such as milk (MBPDB, MilkAMP), or fermented foods (FermFoolDB) (Iwaniak, Darewicz, Mogut, & Minkiewicz, 2019; Panyayai et al., 2019; Chaudhary, Bhalla, Patriy, 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, sporamin, 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 (Sayed 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, theoretical pl, aliphatic index, hydrophobicity, 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 (pl), net charge, hydrophobicity index, instability index, aliphatic index, and hydrophobicity 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, Bonnell, & 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, Gau, 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.
methods, in order to generate a new BP by a hybrid approach. Once the product has been highlighted that these interactions must be verified by conventional experiments (e.g., AlgPred), and toxicity (e.g., ToxinPred). After data analysis, the identified proteins and enzymes can be separated by molecular weight (fractions). Subsequently, molecular docking is carried out, where the interaction between peptides and receptor molecules of interest is predicted. However, it is important to obtain the new peptides and proven benefits are marketed.

In vivo 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/PeptideRanke/) 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 (YSSPHIW, 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-Rehani, 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 hydrophobic, 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.iitd.edu).
ACE through hydrogen and electrostatic bonds. In the same way, pep
molecule is given by the bioactivity targeted and is the main step to
through hydrogen bonds, hydrophobic interactions or Van der Waals
docking, and finally analyze the results. The selection of the receptor
analysis with ToxinPred tool, showing that none of the 4 iron-chelator
than 10 amino acids (Tu et al., 2018b).
fluence of BP on the virus (SARS-CoV-2) that causes COVID-19. Thus,
et al., 2018), kefir (Amorim et al., 2019a), and
preparation of the protein, preparation of the ligand, perform the
in/raghava/algpred/submission.html), tools to predict toxicity and
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, Ud-
nigwe, & 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; Madgil et al., 2020). Casein hydrolysates were also analyzed
to predict their toxicity and allergenicity, finding that none of the hy-
drolysates 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 Aller-
Top 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
allergic (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-Alli, 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, li-
gands) 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, pe-
tides obtained from edible rhizomes (Sommit et al., 2020), trout (Yu
et al., 2018), kefir (Amorim et al., 2019a), and Klyveromyces 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.
Current molecular docking has also been used to evaluate the in-
fluence of BP on the virus (SARS-CoV-2) that causes COVID-19. Thus,
Cakir, Okuyan, Şener, and Tunali-Akbay (2021) analyzed the in-
teractions 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
(spiked glycoprotein RBD, MβL, and PLαL) employing molecular
docking, mainly through hydrogen bonding and hydrophobic interaction.
Based on described results, molecular docking is increasingly popu-
lar, 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 vali-
dations 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 elimi-
nates 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 sup-
ported 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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Declaration of Competing Interest

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