Food-derived bioactive peptides-methods for purification and analysis

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Abstract. Bioactive peptides attract the attention of researchers thanks to their high potential to beneficially influence human health. Various activities are reported, and some of these peptides are commercialized as therapeutic agents. Food-related proteins represent an excellent source in this regard. However, the identification, purification, and characterization of bioactive peptides require a complex approach. The full range of analytical techniques is used in combination with the chemical and biological properties of the peptides. The emerging "omics" techniques and "in silico" methods have given a new direction to peptide analysis in recent years. Developing new methods, rapid and low-cost, for the identification, characterization and purification, is a challenging task because of the complexity of food samples. However, bioinformatics is a promising technique for their exploration. These new strategies can predict different types of peptides, their properties and represent a new horizon for releasing their potential. That is why, in this review, we summarize information about methods used for purification and analysis of food-derived bioactive peptides so far, as well as present our point of view about the role of bioinformatics in this process.

1 Introduction

It is expected that the world population will reach 9.6 billion by 2050, which require increasing the amount of food and, more particularly, proteins. Proteins are dietary macronutrients that exert various structural and functional properties, essential for the survival of living organisms. Recent scientific evidences show that protein in the diet not only acts as a nutrient, but can also regulate the physiological functions of the body. Therefore, the protein quality is strongly related to the amino acid content, ratios of essential amino acids, susceptibility to hydrolysis during digestion, the impact of processing and the physiological use of the amino acids after digestion and absorption [1]. In addition, the encoded in the protein structure different amino acid sequences may exert various health promoting properties. Over the past decades, a wide range of peptides with biological activity in foods has been identified [2-5], Antihypertensive, antibacterial, antithrombotic, immunomodulatory, opioid, antioxidant, and mineral binding properties are only a few of the many functions that these peptides can influence. Moreover, the peptides can act as neurotransmitters and hormones *in vivo* [6]. Bioactive peptides are frequently multifunctional [7] and development of new techniques for their identification will be helpful in the discovery of new molecules with beneficial functions.

Bioactive peptides can be isolated from a variety of plant and animal sources [8-10], The review of Henchion et al. [10] examines future protein markets and consumption trends, as well as the positive and negative characteristics of animal, vegetable, marine, and innovative protein sources such as insect, algal, and others. At present, cow's milk, cheese, and dairy products appear to be the largest source of bioactive proteins and peptides [11]. When compared to alternative protein sources, edible insects can diversify diets (high quality protein, vitamins, and amino acids), contribute to food and nutrition security, and have a reduced ecological impact. Several insects have shown the potential to be employed as a source of bioactive peptides, such as *Alphitobius diaperinus* [12]. All these examples demonstrate the enormous potential of food proteins as a carrier of health benefits.

Because bioactive peptides are normally inactive within their precursor proteins, it has been estimated that protein hydrolysis is required for peptides to generate bioactivity. Digestive proteolysis *in vivo*, chemical or enzymatic hydrolysis *in vitro*, bacterial fermentation, gene expression, and chemical synthesis are all popular processing methods for producing bioactive peptides [11, 13]. In their essence, these methods are identical, based on proteolytic hydrolysis. One can use pure enzymes, and this way possesses more control of the process and obtain reproducible results [12]. However, the drawback is that pure enzymes are very expensive. When microbial fermentation is applied, the proteolytic enzymes of selected microorganisms are used. Lactic acid bacteria (LAB), such as *Lactococcus lactis*,

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Lactobacillus helveticus, and *Lactobacillus delbrueckii* ssp. *bulgaricus*, have a well-known proteolytic system [14].

The proteolytic potential of the microorganism depends on certain growth parameters such as inocula conditions and protein content of medium [15]. The liberation of bioactive peptides *in vivo* is done by the gastrointestinal enzymes. Peptides, which are generated during digestion or food processing and have a variety of bioactivities, play a significant role in metabolism. The proteases pepsin, trypsin, and/or chymotrypsin, can liberate bioactive peptides in the stomach and small intestine [11]. The digestion of dietary proteins is heavily influenced by a number of parameters, including gastric and intestinal pH, endogenous secretions, protein type, and digestive enzyme activity [16].

The complexity of food matrix and the resulted protein hydrolysates, of which the bioactive peptide may represent only a minor constituent turns their identification, characterization, and purification in difficult and labour-intensive task.

Different methods have been used to obtain better separation and purification, influencing peptides' chemical and biological properties.

New technologies represent a new horizon with the appearance of omics and *in silico* techniques. They can be helpful in the analysis of food-derived bioactive peptides, which could lead to a new era in biotechnology and food chemistry.

That is why it is important to provide different isolation and purification methods that will increase protein, detect a higher rate of peptide synthesis, and provide better strategies related to food safety at lower risk.

2. Methods for purification and analysis of bioactive peptides

Separation and purification are one of the most important steps considering the bioactivity of the protein. Food protein hydrolysis may lead to hundreds of peptide fractions in a complex environment. Molecular weight of peptide, charge, hydrophobicity and solubility are only a few factors that influence the separation and purification process. Isolation of a single sequence may not be easy, that's why different techniques could be used in order to overcome the challenges.

2.1 Physico-chemical methods

Salting out and solvent extraction represent conventional approaches for separating and purifying proteins and peptides [17]. Fractionation methods include precipitation steps such as the addition of organic solvents, acids, the use of salts (ammonium sulphate) or just the adjustment of the pH to the isoelectric point of the peptide. The solubility of peptides in the precipitating agent determines their fractionation [18]. However, caution should be exercised due to the possible degradation of peptides and fluctuations in the physical and biological properties.

2.2 Membrane-based methods

Membrane techniques in peptide separation are one of the most used in the food industry. Membranes are pressure-sensitive barriers that enable some feed components to pass through while keeping others out. Pore size, concentration, and filtration time all play a key role in bioactive peptide separation and it is considered that particles larger than the membrane pores are kept, while smaller particles or molecules flow through. There is a wide variety of membranes with different sizes. Their selectivity allows pressure-driven membrane processes, such as microfiltration (MF), reverse osmosis (RO), nanofiltration (NF) and ultrafiltration (UF), as the last two methods are mainly used to release bioactive peptides from protein hydrolysis, which have minor differences in their physiological parameters. Pre-treatments to eliminate interfering components and concentrate peptides for separation and identification are usually required due to the complexity of the hydrolysates [19].

Membrane processes represent an efficient tool for the separation of bioactive peptides in the food industry due to their advantages. Its use is simple, not related to the addition of chemicals, it is cost-effective, the process lines are well arranged, and it uses mild working conditions compared to chromatographic techniques [16, 18]. The membranes have been widely used to fractionate the hydrolysis of milk proteins to improve their biological or functional properties [18]. Therefore, to increase their separation potential, it is recommended to combine with other technologies [20].

2.2.1 Ultrafiltration

Like all the membrane techniques, the ultrafiltration is used for concentrating, purifying, fractionating, and clarifying a variety of solutes. It features a high throughput, low operating costs, energy-saving options, and is simple to clean.

Ultrafiltration can be used to remove insoluble substrates, large molecular weight proteins, and peptides from polypeptide separation, resulting in bioactive peptides with lower molecular weights with an increased bioactivity [16, 21]. This method is also used to separate peptides less than 7 kDa [18]. Then, the fractions are collected and filtered in two/ three flows to obtain peptides of various sizes [18]. Some peptides and amino acids can be divided into four molecular mass ranges at pH 4.6 [22]. For example the great majority of dietary protein ACE inhibitory peptides were short sequences of 2 - 20 amino acids and were filtered through a 3 kDa membrane [16, 23]. Another positive point of ultrafiltration is that in a comparison to concentrations generated through dissolving and precipitation, ultrafiltration improved the solubility behaviour of soy protein concentrates and isolates [24]. It has been also reported as a useful technique in the preparation of phosphopeptide-enriched mixtures from casein. A study by Tavares et al. [25] used various filtration modes linked with enzymatic hydrolysis of whey protein to obtain a peptide-rich product with

antihypertensive features. Many studies prove that more efficient is using one or more chromatography techniques after ultrafiltration to increase the purity of peptide fractions [26].

2.2.2 Nanofiltration

The principle of nanofiltration is to separate bioactive peptides based on the charge interaction with membranes. Selectivity of peptides with nanofiltration is related to their charge interactions with membranes and size separation as most of them contain charged functional groups at given pH. The type of membrane and the feed composition are also of crucial importance. For example Pouliot et al. [27] used this type of method with a range of molecular weight cut-off (MWCO) between 1000 and 5000 Da to separate whey peptides. A combination of ultrafiltration and nanofiltration was used to improve the separation efficiency [21]. First, ultrafiltration of hydrolysate is proceeded to remove the intact proteins and intermediate peptides altogether. The permeate obtained is fractionated by NF, and then the peptide with a molecular mass < 1 kDa is isolated using the membranes. Butylina et al. [28] applied it to fractionate sweet whey peptides.

2.2.3 Electrodialysis and filtration membrane (EDFM)

Electrodialysis, which is part of the membrane techniques, has been found used in improving the yield and selectivity of the peptide separation, along with electronanofiltration and electrofiltration (combination of electrical potential gradients and pressure) [26]. On milk hydrolysates samples, a method combining ultrafiltration and high-performance liquid chromatography (HPLC) was also used to improve peptide separation.

The two processes – hydrolysis and separation of produced peptides with identical molecular weights based on their net charge-were carried out consecutively in a novel type of ultrafiltration technology that combined electrodialysis and filtration membrane (EDFM) [29]. The EDFM principle is to attract molecules based on their charge as they pass through filtration membranes with the appropriate MWCO range for the fraction of interest. A fundamental advantage of EDFM is the simultaneous separation of acid, neutral or basic peptides while carrying out the process [29]. This technique was developed and effectively used to produce bioactive peptides from various protein hydrolysates [30-32]. An important point is the use of commercial electrodialysis cells, whose design may influence on the separation process. The workload of electrodialysis (ED) cell is less involving because the charge selectivity of ED is combined with the size exclusion properties of porous membranes (NF, UF, and MF). In 2013, Doyen et al. [33] used this method to hydrolyze enzymatically milk proteins and isolate them in one step. The potential applications of EDFM were increased by performing simultaneous hydrolysis and fractionation of the generated bioactive peptides [32] and stacking filtration membranes with variable molecular weight cut-offs [34].

2.3 Chromatographic-based methods

Chromatography is one of the most versatile techniques for the isolation and purification of biologically active peptides. Different chromatographic techniques have been developed depending on the peptide properties [35].

Differences in peptide size, net charge, or hydrophobicity are used in the three principal modes of HPLC used in peptide separations [size exclusion chromatography (SEC)-, ion-exchange chromatography (IEC)-, reversed phase high-performance liquid chromatography (RP-HPLC), resp.] [36]. Mobile phase parameters can be modified in these modes to enhance the separation capability of a particular HPLC column. In addition, in the recent years, an unique mixed-mode peptide separation technique known as hydrophilic interaction chromatography (HILIC)/cation-exchange chromatography (CEX) has demonstrated outstanding potential as a companion to RP-HPLC [36].

2.3.1 Size exclusion chromatography (SEC)

Size-exclusion chromatography is a technique for separating components depending on their molecular size. The sample molecules are separated by differential exclusion from the pores of the packing material as they move through a bed of porous particles. This method is one of the most used in separating food-derived bioactive peptides, because of the use of mild conditions to separate size-based molecules and the easiest to perform [17]. Solutes are separated at SEC based on their physical size. In SEC stationary phases, organic gels and silica-based particles are utilized. Polymers of cross-linked styrene-divinylbenzene or polyacrylamides are commonly used to make gels. The size of the pores is determined by the degree of cross-linking. Pore diameters in silica particles range from 40 to 2500 A on average. Different pore diameters are designed for each type of stationary phase to accommodate separations in different molecular weight ranges. Compared to membrane fractionation, SEC is faster and with higher resolution [17]. The principle lies in the entering of small molecules into the pores of the solid-phase matrix. Thus, a long time is needed to travel across the media. Larger molecules do not interact with the pores due to their size and get out of the column first [37]. In recent evidences, SEC has been demonstrated as efficient in the fractionation of hydrolysates from agro-industrial wastes, rice bran, and peanut meal [38].

2.3.2 Reversed-phase liquid chromatography

For peptide separations, RP-HPLC remains the most extensively utilized HPLC mode [38]. It leads among the other HPLC methods, in terms of speed and efficiency. It's also useful for analytical and preparative separations due to the availability of volatile mobile phases.

Chemical bonding of various groups to a silica surface produces the most extensively used column pickings. In protein RP-HPLC, trifluoracetic acid (TFA), a strong acid, is often employed to lower the pH to around 2. It is an excellent solubilizer, and even at low concentrations, it protonates peptide carboxyl groups, boosting their affinity for reversed phases. To take advantage of acidic volatile mobile phases (especially aqueous trifluoroacetic acid [TFA]/ acetonitrile [CH_3CN] systems), the most of studies have preferred to perform HPLC below pH 3.0 [36]. The peptides that are first eluted are these which molecular weight and hydrophobicity are low and possessing low retention time.

However, the stability of macromolecules such as peptides in RP- HPLC reduces dramatically as the amount of organic modifier increases [39]. Because species with similar structures can exhibit highly varied adsorption behavior at a given mobile phase composition, gradient elution is recommended for macromolecules.

Different types of peptides were found using this type of chromatography. For example, lunasin, a 43 amino acid residue peptide, with an established cancer chemopreventive effects soybean hydrolysate was purified using this technique combined with isoelectrofocusing [38]. Another significant peptide isolated and purified thanks to this method was a synthetic peptide, part of the luteinizing hormone releasing hormone (LHRH) sequence [36]. It was also proved that peptides which eluted last from the reverse phase column showed the strongest angiotensin-converting enzyme (ACE) inhibitory action, as previously demonstrated with chickpea and sunflower protein hydrolysates [40]. ACE inhibitory peptides are thought to be high in hydrophobic amino acids, resulting in better retention in hydrophobic chromatography columns [40].

2.3.3. Hydrophilic Interaction / Cation-Exchange Chromatography

The name "hydrophilic interaction chromatography" was developed to characterize separations based on solute hydrophilicity [36] with solutes eluted from the HILIC column in order of increasing hydrophilicity, the inverse of RP-HPLC elution behavior. Cation exchange chromatography is a type of ion exchange chromatography (IEX) that uses net surface charge to separate molecules. HILIC/CEX combines the features of two distinct separation methods, namely, a separation based on peptide hydrophilicity/hydrophobicity variations placed over a separation based on net charge [36]. It was demonstrated by Alpert [39] that, while the separation mechanism is mostly dependent on ion-exchange at low concentrations of acetonitrile (ACN), retention increases considerably at high concentrations of ACN. Hydrophilic interactions are responsible for this. Electrostatic effects, on the other hand, are becoming less important.

This techniques has many applications and could be used in the detection of antioxidant and ACE inhibitory peptides, combined with electrospray-mass spectrometry- mass spectrometry (ESI/MS/MS) [41]. This type of chromatography is also commonly used in combination with RP-HPLC. The breakdown of algae protein waste yielded a potent antioxidative peptide. Ammonium sulphate precipitation, gel filtration, and ion exchange chromatography were used to isolate the peptide [26]. Peptides from *Chlorella vulgaris* waste, exerting antioxidant and anticancer activities, were purified using ion exchange chromatography [42]. Indeed, for specific peptide mixes, it has approached or even exceeded RP-HPLC [36]. The breakdown of alga protein waste yielded a potent antioxidative peptide.

2.4 Electrophoretic methods

Electrophoretic techniques, in addition to chromatographic procedures, are recognized to create a vast and powerful family of technologies capable of separating, visualizing, and quantifying single proteins or peptides. A significant portion of these approaches is automated, allowing for the processing of a large number of samples in a short amount of time. Although RP-HPLC remains the preferred peptide separation method, capillarity electrophoresis (CE) has also considered to be successful in peptide separation [36]. This method is gaining popularity as a result of its ability to combine the advantages of traditional gel electrophoresis and HPLC, while generally avoiding their drawbacks. CE provides separation mechanisms similar to those seen in gel electrophoresis, but without the time-consuming gel preparation and staining methods. Instead of staining, detection is done in real time, similar to HPLC, and automation is simple. Furthermore, separation efficiency and resolution outperform those of HPLC. Capillary electrophoresis, more specifically capillary zone electrophoresis (CZE), takes advantage of the mass-to-charge ratio of the analyte. This review [43] focuses on applications that are directly relevant to food systems, such as the study of proteins and peptides from dairy products, cereals, and other foods.

For example, methods for the analysis of caseins and whey proteins have been reported in papers on the CE analysis of milk proteins [43] . Capillary HPLC was also found valuable in identifying immunostimulant soy peptide “MITLAIPVNKPGR” [38]. Moreover, capillary zone electrophoresis (CZE) was also used to separate maize polypeptides by Righetti et al. [43].

3. The role of ‘’omics’’ techniques in the peptide analysis

Peptidomics, a branching field from proteomics, could be defined as a systematic, comprehensive, qualitative/ quantitative multiplex study of all peptides in a sample and their functional qualities, allergenicity, sensory properties, and biological activities found in food and raw materials [44]. Many proteomics techniques are used in peptidomics analysis. However, the target is different.

Instead of determining whether complete proteins are present in a sample, peptidomics searches for endogenous protein fragments. Nutritional peptidomics is concerned with discovering and quantifying nutritionally essential peptides that may exert bioactivity singly or in combination. The various physicochemical properties of proteins, the large number of peptide sequences that can become available, and the large dynamic concentration range of chemicals in the real samples are the key challenges in protein and peptide analysis.

Furthermore, despite recent advancements, the availability of relevant technologies still limits protein hydrolysis, separation, and purification methods [45]. For this reason, modern separation, analytical and computation technologies are employed. Peptidomics has shown to be a reliable and large-scale approach of identifying dietary peptides using high-resolution technology such as mass spectrometry and chromatography.

Mass spectrometry is a technique frequently used in identifying various metabolites and is also involved in the quality and safety of the food. Ionization, separation, detection, and analysis are the four main functions in mass spectrometry. The mass of the peptide precursor, as well as its related product ions, are precisely measured to detect peptides. This method can be done once with a single analyser or multiple times with separate mass analysers. Tandem mass spectrometry (MS/MS or MS2) is a term used to describe the process of performing two or more reaction steps on selected ions at the same time [46]. Normally, the mass spectrometry (MS) analysis is preceded by other purification techniques.

For MS analyses of peptides, there are two basic ionization methods that are used. Because most of the peptides contain fewer than ten amino acids, electrospray ionization (ESI) is the most popular one [47]. Matrix-assisted laser desorption/ionization (MALDI) is the alternative approach employed for longer sequences. Analysis and validation of proteomic data generated by tandem mass spectrometry have been recently reviewed in detail by Nesvizhskii et al. [48].

Antihypertensive, antithrombotic, antioxidant, anticancer, and antimicrobial peptides have been identified using mass spectrometry methods [45]. Peptidomics has been found useful in the investigation the qualities of a variety of food samples, including human, cow, and other mammalian milk. Dallas et al. [47], for example, discovered over 300 peptides in human milk. Peptiomics of milk has been proposed as a way to identify mastitis at the clinical and subclinical levels, in addition to its bioactivity. [49]. Moreover, LC/ESI–MS/MS-based methods with triple quadrupole mass analyser have been found to detect allergic particles from almonds, nuts, soy, milk etc. [50]. The celiac disease belongs to the group of autoimmune diseases that occur in people, who have a genetic predisposition to digest gluten leading to damage of the small intestines [51]. Mass spectrometry has its role in identifying tTG-mediated modifications of peptides (when the disease occurs, secretion of Ig A and autoantibodies are induced to this type of peptides) [52]. The fingerprints of all potential peptide peaks in antihypertensive salmon protein hydrolysate fractions were obtained using peptididomics with an ESI- MS technology [53]. In other circumstances, the powerful tool has been also utilized to facilitate the identification of antioxidative peptides which purpose is to combat oxidative stress, related to cellular damage which can lead to chronic health disorders such as inflammation, atherosclerosis, diabetes, and cancer [47]. For instance, the peptides WVYY and PSLPA, isolated from hydrolysed hemp seed protein fraction were discovered to be free-radical-scavenging antioxidants after peptidomic analysis [54]. Furthermore, by means of the combination (liquid chromatography–MS/MS analysis) and bioinformatics, researchers were able to analyze the peptide composition of an albumin hydrolysate (molecular mass less than 100 Da) with potential antidiabetic activity (using BIOPEP tools) [55]. Moreover, hundreds of peptides have already been identified using peptididomic methods in a variety of cheeses, including Parmigiano-Reggiano, Emmental, Gouda and Chedda [56].

MS has become a crucial analytical method for the identification, quantification, and bioavailability of these health-promoting peptides due to its specificity and sensitivity, especially when combined with HPLC.

However, small peptides are difficult to be detected on mass spectrometry because they are very often singly charged [57]. The classic proteomics methods are not relevant in this situation, and the identification of peptides shorter than six amino acids is not possible using bioinformatics software. *De novo* peptide identification, another method to investigate the peptide sequence, was found very useful in obtaining newer fragmentation techniques. When database information is insufficient, this technique helps to determine the structure of peptides [58]. Recent advances in this domain have coincided with the advancement of MS equipment, allowing for de novo peptide sequencing for novel fragmentation techniques including electron transfer dissociation and electron capture dissociation spectra [38]. The combination of *de novo* peptide identification and traditional database search appears to be robust in investigating, for example, vegetable sequence and an unspecific protease [38].

However, it may also be used in non-sequenced organisms [59]. Hence, due to the de novo sequencing approach, together with the MS data analysis is possible to identify peptide sequencing correctly [38].

4. Bioinformatics - *in silico* tools and databases - driving force in the discovery of new bioactive peptides

Bioinformatics is an interdisciplinary scientific field that analyses and interprets biological and biochemical data, stored in databases and cloud storage, developing appropriate software tools and algorithms.

Currently, an abundance of biologically active peptides information derived from experimental in vivo

and in vitro protocols is being stored in a large number of public databases, summarized in Table. 1 [60]. The

Table 1. Currently available databases of food-derived bioactive peptides [60]

Database	Website [a]
FeptideDB Database of food-derived bioactive peptides	http://www4g.biotec.or.th/FeptideDB/
BioPepDB Database of bioactive peptides of food origin	http://bis.zju.edu.cn/biopepdbr/
AHTPDB Database of food-derived AHTPs	http://crdd.osdd.net/raghava/ahtpdb/info2.php
BIOPEP-UWM DB Database of bioactive peptides of food origin	http://www.uwm.edu.pl/biochemia/index.php/en/biopep
MBPDB Milk Bioactive Peptide database	http://mbpdb.nws.oregonstate.edu
FermFooDb Database of peptides derived from fermented foods	https://webs.iiitd.edu.in/raghava/fermfoodb/

[a] All websites indicated in the table were accessed in September 2021.

existing databases are of two categories - comprehensive and specialized databases. Comprehensive ones are those that include peptides with different functionality, while the specialized ones cover a specific type of peptides, for example anticancer. From Table 1, AHTPs database belongs to the category of specialized databases, as they include only antihypertensive peptides. Predicting peptide function is a very important and challenging task in bioinformatics. Until recently, most bioactive peptides have been isolated mainly by in vivo and in vitro experimental protocols. However, they are too expensive, time-consuming, and require enormous experimental and human effort. Recently, in silico approaches have been used to predict bioactive peptides in a more efficient way. They refer to methods or predictions using computational approaches such as machine learning (ML) or artificial intelligence (AI) algorithms. In recent years, ML-based technology for predicting peptide activity has become extremely popular and used. Various ML techniques have already proved that they successfully cope with the task of predicting the activity of the peptides while providing high-performance accuracy and saving time. Among them isan Artificial Neural Networkork (ANN), Random Forest (RF), Support Vector Machines (SVM), Deep Learning (DL) algorithms, k-Nearest Neighbors, Naive Bayes etc. Numerous studies have provided web server programs that can be freely used to build an effective predictive model and to predict bioactive peptides. A small part of them is summarized in Table 2 [60].

Despite their successful application for predicting bioactive peptides, ML predictive models have some drawbacks. Primarily, the dataset utilized for training the model should be of precise, curated, and of high quantity and quality. Typically, the data must be precleaned or pre-processed before construction of the models, which is additional challenge. Also, the biological data are usually imbalanced with the positive samples being largely outnumbered by the negative ones. The ML models (especially ANN and DL) have very high complexity, which makes them difficult to interpret. In addition, they have a huge number of learning parameters which is often computationally demanding and, in some cases, can lead to overfitting the training data and even to inability generalize well on the testing data.

Table 2. Web server ML predictors

Prediction Tool	Type of predicted peptides	Description	ML Alghoritm	Website[a]
ACPred-FL	anti-cancer	A sequence-based predictor for identifying anti-cancer peptides from proteins	SVM	http://server.malab.cn/ACPred-FL/
ADAM	anti-microbial	ADAM provides two computational tools built to predict whether the target peptides are antimicrobial	SVM, HMM	http://bioinformatics.cs.ntou.edu.tw/ADAM
AHTpin	antihypertensive	AHTpin is an *in-silico* method developed to predict and design efficient antihypertensive peptides	SVM	http://crdd.osdd.net/raghava/ahtpin/
AIPpred	anti-inflammatory	AIPpred is web-based prediction server for Anti-inflammatory peptides	SVM-PVP, RF	http://www.thegleelab.org/AIPpred/
AntiBP	anti-microbial	Antibp server predicts the antibacterial peptides in a protein sequence	ANN, SVM, QM	http://crdd.osdd.net/raghava/antibp/
Antifp	anti-fungal	Antifp is an *in-silico* method, which is developed to predict and design antifungal peptides	SVM, RF, NB	https://webs.iiitd.edu.in/raghava/antifp/
CAMP	anti-microbial	CAMP (Collection of Anti-Microbial Peptides) is tools for prediction for antimicrobial peptides	ANN, RF, SVM, DA	http://www.bicnirrh.res.in/antimicrobial
CellPPD	cell penetrating peptides	CellPPD is an *in-silico* method, which is developed to predict and design efficient cell penetrating peptides	SVM	http://crdd.osdd.net/raghava/cellppd/
ClassAMP	anti-microbial	Prediction tool for classification of AMPs	SVM, RF	http://www.bicnirrh.res.in/classamp/
DBAASP	anti-microbial	Prediction of only linear peptides which are active against some bacterial strain	SVM, ANN	https://dbaasp.org/
HAPPENN	therapeutic peptides	HAPPENN is a novel tool for haemolytic activity prediction for therapeutic peptides which employs neural networks	SVM, RF, ANN	https://research.timmons.eu/happenn

[a] All websites indicated in the table were accessed in September 2021.

6. Conclusion and future perspectives

Due to the broad-spectrum action of bioactive peptides, they attract considerable interest from researchers. They have a growing application in many fields, and exploring more their activities and creating new methods for their analysis will bring to a new era in the food and plant industry, biomolecular sciences and medicine. However, there is a lack of research related to food-derived bioactive peptides' safety, and it is a challenging task to develop effective strategies. *In silico* analysis and 'omics' approaches combine different benefits due to which is possible to make peptide analysis, prediction and validation.

Proteomic, peptidomic, bioinformatics, and chemometric technologies will provide the biotechnological expertise required to manufacture and commercialize functional foods containing bioactive peptides at a low cost.

This is why future developments in food proteomics are needed, and it is expected to increase more and more because of their ability to potentially characterize food products on a molecular level which can lead to the creation of new food [1].

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