



Plant protein-derived antioxidant peptides: Isolation, identification, mechanism of action and application in food systems: A review

Chaoting Wen^{a,1}, Jixian Zhang^{b,1}, Haihui Zhang^a, Yuqing Duan^{a,c,*}, Haile Ma^{a,c}

^a School of Food and Biological Engineering, Jiangsu University, Zhenjiang, 212013, China

^b College of Food Science and Engineering, Yangzhou University, Yangzhou, 225127, China

^c Institute of Food Physical Processing, Jiangsu University, Zhenjiang, 212013, China

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ABSTRACT

Background: In recent years, the antioxidant capacity of plant protein-derived peptides has been depicted in a growing number of studies. Plant proteins have been considered as the new source of antioxidant peptides, which not only save energy but also strengthen treatment of oxidation-related diseases and delay the oxidation of food, thus improve the quality of life. Although reports of the preparation, structure, and activity of antioxidant peptides has increased tremendously and some antioxidant peptides have been commercialized. However, research on antioxidant peptides derived from plant proteins still lacks a systematic review and evaluation.

Scope and approach: This review summarizes recent advances in plant protein-derived antioxidant peptides, mainly including: (i) Preparation, purification, identification and application of plant protein-derived antioxidant peptides by enzymatic hydrolysis; (ii) Influencing factors, evaluation methods and mechanism of antioxidant protein hydrolysates or peptides; (iii) Future research prospects and existing problems.

Key findings and conclusions: The conventional purification and identification of antioxidant peptides are expensive and time-consuming. In order to overcome this problem, computer-aided mathematical models (molecular docking, QSAR research, etc.), bioinformatics tools and real-time updated protein databases have been widely explored and studied. In addition, future research should focus on large-scale commercial production of plant protein-derived antioxidant peptides, comprehensive study of structure-activity relationship of antioxidant peptides, deep understanding of antioxidant mechanism, and verification of health benefits *in vivo*. This review provides a timely reference for the development of new antioxidant peptides in functional foods and nutritional supplements.

1. Introduction

Excessive free radicals can produce oxidants, which may reduce the quality of fatty foods, shorten the shelf-life of food, and damage the skin (Rajapakse, Mendis, Jung, Je, & Kim, 2005). Under the normal physiological conditions, the antioxidant defense system can utilize enzymes (e.g. superoxidase dismutase, catalase, and glutathione peroxidase) and non-enzyme antioxidants (e.g. melatonin, vitamins, glutathione, co-enzymes, cofactors, tocopherol, ascorbic acid) to eliminate harmful molecular attacks (Chen, Hu, Wang, Hu, & Cui, 2016). However, the imbalance between free radicals and the endogenous antioxidant defense system can give rise to oxidative stress in the cells and damage various cellular components (DNA, proteins and membrane lipids, etc).

Oxidative damage can cause many chronic diseases, including diabetes, Alzheimer disease, arthritis, heart disease, and cancer, among others (Rice & Diplock, 1993). Synthetic antioxidants (t-butyl hydroquinone, propyl gallate and butyl hydroxyanisole) can effectively control the oxidation of food nutrients. In addition, dietary intake of antioxidants could reduce the risk of chronic diseases related to oxidative stress. However, synthetic antioxidants have the disadvantage of high costs and the potential toxicity risk. With the increasing consumer demand for high nutritional and functional protein, researchers shift their focus from traditional raw materials protein to plant proteins. Notably, natural antioxidant peptides originated from plant proteins have captured worldwide attention due to its advantages of environmental protection, sustainability, low cost and no toxic side effects. The information on

* Corresponding author. School of Food and Biological Engineering, Jiangsu University, Zhenjiang, 212013, China.

E-mail addresses: hopenwen177@163.com (C. Wen), hopenzhang177@163.com (J. Zhang), dyq101@ujs.edu.cn (Y. Duan).

¹ Chaoting Wen and Jixian Zhang contributed equally to this work and should be regarded as co-first author.

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antioxidant peptides derived from plants are summarized in Table 1.

This article summarizes the research on antioxidant peptides in plant proteins over the past 20 years (2000–2020). The preparation, isolation, purification and identification of plant protein-derived antioxidant peptides were summarized. In addition, the relationship between structure and antioxidant activities of peptide was highlighted. Moreover, the evaluation method and potential mechanism of the antioxidant peptides were concluded. Furthermore, the application of plant protein-derived antioxidant peptides in the food system was emphasized. Finally, the future opportunities and challenges of plant protein-derived antioxidant peptides are proposed.

Note: MS represents mass spectrometry; LC-MS/MS represents Liquid chromatography-tandem mass spectrometry; DPPH represents 1,1-diphenyl-2-picrylhydrazyl; TBA represents thiobarbituric acid; TEAC represents trolox equivalent antioxidant capacity; ABTS represents 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); FRAP represents ferric ion reducing antioxidant power; ORAC represents oxygen radical absorbance capacity.

2. Preparation of plant protein-derived antioxidant peptides by enzymatic hydrolysis

From the perspective of sustainable development and utilization of biological resources, protein resources in by-products or waste generated during plant processing have great potential for the production of bioactive peptides. The rational development and utilization of plant processing by-products or wastes are conducive to increasing the nutritional utilization value of wastes, saving energy, and protecting the environment, among others. In particular, wheat gluten, wheat germ, rapeseed, watermelon seed, corn gluten, rice bran, oat bran, palm kernel cake, and brewers spent grain have been used to produce antioxidant peptides. A schematic diagram of the production process of antioxidant peptides obtained from plant is described in Fig. 1. As shown in Fig. 1, plant proteins are firstly hydrolyzed by protease (animal protease, plant protease and microbial protease) to form protein hydrolysate, and then it is further purified by membrane separation, electrophoresis, and chromatography (gel permeation chromatography (GPC), ion-exchange chromatography (IEC), reversed-phase high performance liquid chromatography (RP-HPLC), etc) to obtain antioxidant peptides. The obtained antioxidant peptide was identified by MS or MS/MS and their antioxidant activity was evaluated by *in vivo* and *in vitro* experiments.

Currently, the preparation methods of peptides mainly include chemical extraction, fermentation, synthesis, and enzymatic hydrolysis. The advantages and disadvantages of the different preparation methods are summarized in Fig. 2.

Among them, the chemical extraction method has the advantages of being widely used and is a mature method. However, it has the disadvantages of large consumption of chemical reagents, environmental pollution and high equipment costs. In addition, the peptides prepared by using the fermentation method possess the advantages of good taste, low cost, high yield, but long fermentation time may cause safety problems, such as susceptibility to microbial infection (He et al., 2012). Additionally, the acquisition of peptides by utilizing synthetic methods possess the superiorities of high purity and mature, while this method is very high cost and low yields (Brandsch, Knütter, & Bosse, 2008). Compared with other peptides preparation methods, the enzymatic hydrolysis method has the advantages of easy reaction control, good repeatability, low cost and low energy consumption. Therefore, it is suitable for industrial production of peptides and is the most commonly used method. At the same time, it should be noted that the enzymolysis method also has the disadvantages of long enzymolysis time, low efficiency, and the effect of enzyme on the purity of the peptides (Di Bernardino et al., 2011).

The protein hydrolysis is mainly affected by several factors, including the types of enzyme and hydrolysis conditions (temperature, pH, the ratio of enzyme and substrate, time), among others. It is worth noting that

different types of enzymes can specifically cleave different positions of the peptide. For example, during pepsin digestion, peptide bonds between hydrophobic amino acids (preferably aromatic amino acids, including Phe, Trp, and Tyr) are cleaved, resulting in increased hydrophobicity, thereby making rice bran protein hydrolysates (RBPH) react appropriately with DPPH free radicals. However, due to further digestion by trypsin, this ability is reduced because it selectively cleaves basic amino acids (Phongthai et al., 2018). Thus, the type of peptidase is the main factor that determines the size, composition, and amino acid sequence of the peptides, and ultimately affects the antioxidant activity of protein hydrolysates (Karami & Akbari, 2019). Peptidase is mainly divided into animal, plant and microbial proteases. In general, pepsin and trypsin are the most widely used animal proteases. Suetsuna and Chen (2002) reported that the two peptides (Leu-Gln-Pro-Gly-Gln-Gly-Gln-Gly and Ala-Gln-Ile-Pro-Gln-Gln) obtained from wheat gluten protein by using pepsin have good antioxidant capacity. Delgado et al. (2016) identified antioxidant peptides obtained by pepsin and pancreatin digestion of amaranth proteins, five peptides (Ala-Trp-Glu-Glu-Arg-Glu-Gln-Gly-Ser-Arg, Ile-Tyr-Ile-Glu-Gln-Gly-Asn-Gly-Ile-Thr-Gly-Met, Tyr-Leu-Ala-Gly-Lys-Pro-Gln-Gln-Glu-His, Ile-Tyr-Ile-Glu-Gln-Gly-Asn-Gly-Ile-Thr-Gly-Met and Thr-Glu-Val-Trp-Asp-Ser-Asn-Glu-Gln) were identified. Interestingly, four peptides presented interesting antioxidant activity with ORAC assay: Ala-Trp-Glu-Glu-Arg-Glu-Gln-Gly-Ser-Arg-Tyr-Leu-Ala-Gly-Lys-Pro-Gln-Gln-Glu-His ~ Ile-Tyr-Ile-Glu-Gln-Gly-Asn-Gly-Ile-Thr-Gly-Met ~ Thr-Glu-Val-Trp-Asp-Ser-Asn-Glu-Gln.

In a related study, Beermann et al. (2009) identified seven peptides (Trp-Asn-Leu-Asn-Ala-Asn, Phe-Glu-Ser-Phe-Phe-Leu, Ser-Leu-Asp-Phe-Pro-Ala-Leu-Trp, Phe-Gln-Thr-Leu-Phe, Ser-Tyr-Leu-Gln-Gly-Phe, Thr-Thr-Tyr-Tyr and Leu-Phe-Phe) originated from soybean protein isolate hydrolysates by using pancreatic trypsin and chymotrypsin, and the antioxidant activity of peptides were tested in a Trolox-equivalent-antioxidative capacity (TEAC) assay. The results showed that fractions 5–7 showed significant antioxidant activities, while fractions 1–4 did not show any significant anti-oxidative capacity. Similarly, Vanvi and Tsopmo (2016) reported that oat bran protein was hydrolyzed with pepsin (enzyme/substrate ratio (E/S) = 1:30; time = 2 h), which showed the strongest peroxy radical clearance power (608 ± 17 μ M Trolox equivalent (TE) per gram (TE/g)). In addition, the peptide fraction (F3) separated with HPLC manifested higher hydroxyl (14%), superoxide anion (36%) and peroxy (874 μ M TE/g) radical scavenging activities in comparison with other fractions.

Papain and bromelain (plant proteases) are also used to prepare plant protein-derived antioxidant peptides. Zarei, Ebrahimpour, Abdul-Hamid, Anwar, and Saari (2012) used papain to hydrolyze palm kernel cake and the protein hydrolysates showed higher DPPH• scavenging activity ($65 \pm 0.25\%$) than that of hydrolysates prepared by trypsin (2.2%), flavorzyme (5.2%), bromelain (12.3%), chymotrypsin (15.5%), alcalase (29%), and pepsin (52%). In a similar research, the antioxidant activities of wheat gluten hydrolysates obtained by papain hydrolysis were also investigated (Wang et al., 2007). In addition, tamarind seed is the by-products of the tamarind pulp industry, which contains a large amount of protein and essential amino acids. It was reported that the processing of tamarind pulp (250,000 tons) can produce 100,000 tons of seed waste in India annually (Hiwale, 2015). It can be seen that tamarind is a reusable protein resource with great potential value. Bagul, Sonawane, and Arya (2018) reported that the radical scavenging rate of papain hydrolysates from tamarind seeds was $42.92 \pm 2.83\%$ under optimized enzymatic parameters E/S = 1:5; time = 3 h; pH = 6).

Other proteases are also widely used for the preparation of antioxidant peptides, such as microbial proteases mainly including alcalase (EC 3.4.21.62), neutral proteases (EC 3.4.24.3), and flavourzyme (EC 3.4.11.1), among others. Zhu et al. (2006) demonstrated that hydrolysates from wheat germ protein by alcalase exhibited good radical scavenging abilities. In a similar research, Cheng et al. (2006) also indicated that the wheat germ protein hydrolysate (enzyme: alcalase) displayed good DPPH radical scavenging activity (0–81.11%) with

Table 1
Antioxidant peptides from plant protein.

Raw materials	Peptide sequence	Enzyme	Separation and purification technique	Identification technique	Index	Reference
Black soybean meal byproduct	Leu/Ile-Val-Pro-Lys	Alcalase	Ultrafiltration, Sephadex G-25 chromatography, RP-HPLC	ESI-MS/MS	DPPH/Hydroxyl free radical scavenging capacity; FRAP	Chen et al. (2019)
Corn gluten meal	Not studied	Alcalase	Ultrafiltration	Not studied	The expressions of selected antioxidant genes	Wang et al. (2018)
Moringa oleifera seed protein	Not studied	Alcalase, pepsin and trypsin	Not studied	Not studied	DPPH and hydroxyl radical scavenging capacity; FRAP and metal ion chelation assays	Aderinola, et al., 2018
Oat bran protein	Trp-Gly-Tyr-Asn-Asp-Gly-Asp-Ala-Pro-Val-Val-Ala; Glu-Ala-Arg-Gln-Asn-Ile-Glu-Asn-Pro-Lys-Arg-Ala-Asp; Gly-Gln-Asn-Phe-Pro-Ile-Leu-Asn; Leu-His-Gly-Gln-Asn-Phe-Pro-Ile-Leu; Ser-Gln-Lys-Glu-Gln-Arg-Gly-Glu-Ile-Ile-Arg	Alcalase, papain, protamex, and flavourzyme	Not studied	MS	ORAC assay, ABTS/Hydroxyl/Superoxide anion radical scavenging capacity; Iron-(II) chelating assay	Esfandi, Willmore, and Tsopmo (2019b)
Oat Bran Protein	Not studied	Alcalase, flavourzyme, papain, Protamex	Not studied	Not studied	ROS scavenging activities	Esfandi, Willmore, and Tsopmo (2019a)
Oat bran Protein	Asp-Val-Asn-Asn-Asn-Ala-Asn-Gln-Leu-Glu-Pro-Arg-Gln-Lys-Glu-Phe-Leu; Thr-Ser-Leu-Val-Pro-Gln-Thr-Gln-Ser-Ile-Ala-Val; Leu-Gly-Ile-Ser-Gln-Gln-Ala-Ala-Gln-Ile-Gln-Ser-Gln-Lys-Glu-Gln-Arg-Gly-Glu-Ile; Val-Ser-His-Ile-Ala-Gly-Lys-Ser-Ile-Leu		RP-HPLC	LC-MS/MS	Iron chelating assay/Calcium binding capacity/Inhibition of linoleic acid oxidation	Baakdah and Tsopmo (2016)
Oat Bran Protein	Leu-Lys-Asn-Asn-Arg-Gly-Gln-Glu-Tyr-Leu-Leu-Leu-Lys; Tyr-Gly-Ile-Lys-Val-Gly-Tyr-Ala-Ile-Pro; Gly-Gly-Ile-Phe; Gly-Ile-Phe-Glu; Trp-Ala-Phe-Ser; Gly-Val-Gln-Glu-Gly-Ala-Gly-His-Tyr-Ala-Leu-Leu; Trp-Ala-Phe; Ala-Trp-Phe-Ser; Leu-Pro-Trp-Arg-Pro-Ala-Thr-Asn-Val-Phe	Pepsin	RP-HPLC	LC-MS/MS	ORAC Assay/Superoxide anion and hydroxyl radicals scavenging capacity	Vanvi and Tsopmo (2016)
Palm kernel cake protein	Not studied	Papain	RP-HPLC/Isoelectric focusing fractionation	LC-MS/MS	DPPH radical scavenging capacity/Metal chelating activity assay	Zarei et al. (2014)
Rapeseed	Not studied	Alcalase	Not studied	Not studied	Inhibiting activity of lipid peroxidation; Reducing power, DPPH/hydroxyl radical scavenging capacity	Zhang, Wang, and Xu (2008)
Rapeseed meal protein	Not studied	Alcalase and flavourzyme	G-25 Gel Filtration Chromatography	Not studied	Superoxide radical scavenging capacity, Rat red blood cell hemolysis inhibition test, Lipid peroxidation	Xue et al. (2009)
Rapeseed Protein	Pro-Ala-Gly-Pro-Phe	Alcalase	Anion-exchange chromatography, gel filtration chromatography and RP-HPLC	MS	DPPH radical scavenging capacity	Zhang, Wang, Xu, and Gao (2009)
Rice bran protein	Tyr-Val-Ala-Gln-Gly-Glu-Gly-Val-Val-Ala; Tyr-Leu-Ala-Gly-Met-Asn	Proteases M, N, P, S and pepsin	RP-HPLC	MS	ABTS method	Adebiyi, Adebiyi, Yamashita, Ogawa, and Muramoto (2009)
Rice bran protein	Not studied	pepsin and trypsin	Ultrafiltration	Not studied	DPPH, ABTS radicals scavenging capacity, ferric reducing antioxidant power/Metal chelating activity	Phongthai, D'Amico, Schoenlechner, Homthawornchoo, & Rawdkuen (2018)
Rice bran protein	Ala-Ile-Arg-Gln-Gly-Asp-Val-Phe; Val-Leu-Glu-Ala-Asn-Pro-Arg-Ser-Phe; Tyr-Phe-Pro-Val-Gly-Gly-Asp-Arg-Pro-Glu-Ser-Phe	Protease M, N, S, P and pepsin	Reversed-phase column chromatography and RP-HPLC	MS	Ferric thiocyanate method	Adebiyi, Adebiyi, Ogawa, and Muramoto (2008)
Rice bran protein	Not studied	Alcalase, trypsin, protamex, and flavourzyme	Not studied	Not studied	DPPH/ABTS free radical scavenging capacity and metal chelating assays	Zaky, Chen, Liu, Li, and Jia (2019)

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Table 1 (continued)

Raw materials	Peptide sequence	Enzyme	Separation and purification technique	Identification technique	Index	Reference
Rice bran protein	Not studied	Protease G6	Not studied	Not studied	Reduce oxidative stress in rats fed a high-carbohydrate and high-fat diet	Senaphan et al. (2018)
Rice bran protein	Val-Ala-Gly-Ala-Glu-Asp-Ala-Ala-Lys; Ala-Ala-Val-Gln-Gly-Gln-Val-Glu-Lys; Gly-Gly-His-Glu-Leu-Ser-Lys et al.	Papain and trypsin	RP-HPLC	LC-MS/MS	ORAC assay	Wattanasiritham, Theerakulkait, Wickramasekara, Maier, and Stevens (2016)
Rice bran protein	Tyr-Ser-Lys	Trypsin	Membrane bioreactor system, gel filtration and RP-HPLC	MS	DPPH free radicals scavenging capacity, reducing power	Wang, Chen, Fu, Li, and Wei (2017)
Soybean protein isolate	Trp-Asn-Leu-Asn-Ala-Ala-Asn; Phe-Glu-Ser-Phe-Leu; Ser-Leu-Asp-Phe-Pro-Ala-Leu-Trp; Phe-Gln-Thr-Leu-Phe; Ser-Tyr-Leu-Gln-Gly-Phe; Thr-Thr-Tyr-Tyr; Leu-Phe-Phe; Ile-Tyr; Leu-Tyr	Pancreatic trypsin and chymotrypsin	Ultrafiltration and size exclusion chromatography	LC-MS/MS	TEAC assay	Beermann, Euler, Herzberg, and Stahl (2009)
Wheat germ protein	Not studied	Alcalase	Not studied	Not studied	Linoleic acid emulsion model system; DPPH/superoxide/hydroxyl radical scavenging capacity; Reducing power and ferrous ion-chelating activity	Zhu, Zhou, and Qian (2006)
Wheat germ protein	Not studied	Alcalase	Ultrafiltration and DA201-C resin	Not studied	Inhibition of peroxidation in linoleic acid system; DPPH/superoxide/hydroxyl radical scavenging capacity; Reducing power and ferrous ion-chelating activity	Cheng, Wang, and Xu (2006)
Wheat germ protein	Not studied	Alcalase	Not studied	Not studied	DPPH and ABTS radical scavenging capacity	Wang et al. (2019)
Wheat germ protein	Thr-Val-Gly-Gly-Ala-Pro-Ala-Gly-Arg-Ile-Val-Met-Glu; Gly-Asn-Pro-Ile-Pro-Arg-Glu-Pro-Gly-Gln-Val-Pro-Ala-Tyr	Alcalase	RP-HPLC	LC-ESI/MS/MS	ABTS radical scavenging activities	Karami, Peighambari, Hesari, and Akbari-Adeghani (2019)
Wheat gluten	Not studied	Alcalase, pancreatic, pepsin, pepsin and neutrase	Ultrafiltration	Not studied	Inhibition of linoleic acid autooxidation, DPPH/superoxide/hydroxyl radical scavenging capacity	Kong, Zhou, and Hua (2008)
Wheat gluten	Not studied	Papain	Ultrafiltration	Not studied	DPPH radical scavenging capacity and TBA assay	Wang, Zhao, Zhao, and Jiang (2007)
Wheat gluten	Leu-Gln-Pro-Gly-Gln-Gly-Gln-Gln-Gly and Ala-Gln-Ile-Pro-Gln-Gln	Pepsin	Sephadex G-25 column; SP Sephadex C-25 column; RP-HPLC	MS	Assay of antioxidant activity	Suetsuna and Chen (2002)
Amaranth proteins	Ala-Trp-Glu-Glu-Arg-Glu-Gln-Gly-Ser-Arg; Ile-Tyr-Ile-Glu-Gln-Gly-Asn-Gly-Ile-Thr-Gly-Met; Tyr-Leu-Ala-Gly-Lys-Pro-Gln-Gln-Glu-His; Ile-Tyr-Ile-Glu-Gln-Gly-Asn-Gly-Ile-Thr-Gly-Met; Thr-Glu-Val-Trp-Asp-Ser-Asn-Glu-Gln	Pepsin, pancreatic	C8, Preparative RP-HPLC	LC-MS/MS	ORAC assay	Delgado et al. (2016)
Amaranth protein	Not studied	Chymotrypsin, bromelain and protease	Not studied	Not studied	DPPH and ABTS radical scavenging capacity	Mudgil, Omar, Kamal, Kilari, and Maqsood (2019)
Amaranth protein	Not studied	Alcalase	Ultrafiltration; Molecular exclusion Sepharose column	Not studied	ABTS radical scavenging capacity; Inhibition of the linoleic acid oxidation	Tironi and Anon (2010)
Amaranth Seed Proteins	Leu-Val-Arg-Trp; Asp-Pro-Lys-Leu-Thr-Leu	Alcalase, flavourzyme	RP-HPLC	MALDI-TOF MS	ABTS and DPPH radical scavenging capacity; FRAP	Ayala-Niño et al. (2019)
Quinoa protein	Not studied	Chymotrypsin, bromelain and protease	Not studied	Not studied	DPPH and ABTS radical scavenging capacity	Mudgil et al. (2019)

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Table 1 (continued)

Raw materials	Peptide sequence	Enzyme	Separation and purification technique	Identification technique	Index	Reference
Quinoa protein	Leu-Trp-Arg-Glu-Gly-Met; Asp-Lys-Asp-Tyr-Pro-Lys; Asp-Val-Tyr-Ser-Pro-Glu-Ala-Gly; Ile-Phe-Gln-Glu-Tyr-Ile; Arg-Glu-Leu-Gly-Glu-Trp-Gly-Ile	Pepsin, pancreatin, porcine bile	RP-HPLC	MALDI-TOF MS; LC-MS/MS	ORAC assay	Vilcacundo, Miralles, Carrillo, and Hernández-Ledesma (2018)
Chia protein concentrate	Not studied	Alcalase, flavourzyme, sequential alcalase and flavourzyme	Ultrafiltration	Not studied	DPPH and ABTS radical scavenging capacity; Ferric reducing antioxidant power	Coelho, Aquino, Latorres, and Salas-Mellado (2019)

concentration (0–1.6 g/L) dependent manner. The peptide (Pro-Ala-Gly-Pro-Phe) was separated from rapeseed protein hydrolysate (enzyme: alcalase), which had excellent antioxidant ability with the EC₅₀ value of 0.063 mg/mL on DPPH• scavenging rate (Shao, Wang, Xu, & Gao, 2009). Ayala-Niño et al. (2019) reported that amaranth seed proteins were hydrolyzed by alcalase and flavourzyme, two peptides (Leu-Val-Arg-Trp; Asp-Pro-Lys-Leu-Thr-Leu) were separated and showed good antioxidant activities. Besides, the combination of two or more proteases to hydrolyze plant proteins has also been studied (Aderinola, Fagbemi, Enujiugha, Alashi, & Aluko, 2018).

In addition to the aforementioned effects of the types of enzymes on the degree of proteolysis, selection and optimization of the enzymatic conditions including temperature, pH, E/S and time are also the key influence factors to obtain antioxidant peptides. Increasing temperature can speed up the enzymatic hydrolysis efficiency, while too high temperature will cause inactivation of the enzyme. In addition, a suitable pH can significantly affect enzyme activity, thereby accelerating the enzymatic hydrolysis efficiency. Appropriate ratio of enzyme and substrate is beneficial to increase the contact area between protein and enzyme, and thus affect the enzymatic hydrolysis efficiency. Therefore, it is usually necessary to screen and optimize the best reaction conditions of the enzyme through single factor experiments, orthogonal experiments, and response surface experiments. Ng, Ayob, Said, Osman, and Ismail (2013) used the degree of hydrolysis (DH) as an indicator to optimize (response surface methodology, RSM) the enzymatic hydrolysis conditions of palm kernel cake protein (PKCP), whose results showed that the PKCP hydrolysates had 50% DH and the strongest free radical scavenging ability (DPPH• and ABTS⁺• radicals) under the optimal hydrolysis conditions (pH = 8.5; T = 40 °C; concentration of protein = 0.005 g/mL; E/S = 0.035 w/w). In addition, the optimal hydrolysis parameters (T = 40 °C; E/S = 4.38 HUT/mg; t = 85 min) of pumpkin oil cake protein isolate were obtained by using RSM, and the obtained protein hydrolysates had good DPPH• scavenging rate (36%–40%) (Vaštag, Popović, Popović, Krimer, & Peričin, 2010). Similarly, Marson, da Costa Machado, Soares de Castro, and Hubinger (2019) also reported that brewer grains yeast protein hydrolysates had strong DPPH• scavenging activity (16.2–35.4 μmol_{TE} g⁻¹) and FRAP activity (5–12 μmol_{TE} g⁻¹) under suitable enzymatic hydrolysis conditions (pH = 5.5; E/S = 10%; T = 60 °C). However, the antioxidant activity and DH of protein hydrolysates are not always in a linearly proportional relationship. Excessive hydrolysis can degrade a portion of peptide fragments with antioxidant capacity into inactive amino acids or peptides. Therefore, the type of enzyme, hydrolysis conditions and other indicators need to be screened and optimized comprehensively to prepare good antioxidant peptides.

3. Purification and identification of plant protein-derived antioxidant peptides

3.1. Purification of the plant protein-derived antioxidant peptides

The enzymolysis products of protein is a mixture, including partially unhydrolyzed protein, polypeptides with different chain length, hydrophobicity and net charge, and free amino acids, among others. In order to evaluate the structure and activities of antioxidant peptides more accurately, the appropriate purification method needs to be developed. At present, the separation and purification technology of peptides mainly include chromatography (GPC, IEC, RP-HPLC), membrane separation, and combinations of these technologies. Identification of protein hydrolysates is usually accomplished by using MS, MS/MS, and bioinformatics techniques.

The first step in the purification process of protein hydrolysates is usually separated by using ultrafiltration membranes. According to the different pore size, membrane separation technique is divided into nanofiltration, ultrafiltration and microfiltration, among others. Among them, ultrafiltration membrane is widely used for the separation and purification of protein hydrolysates or peptides due to its advantage of

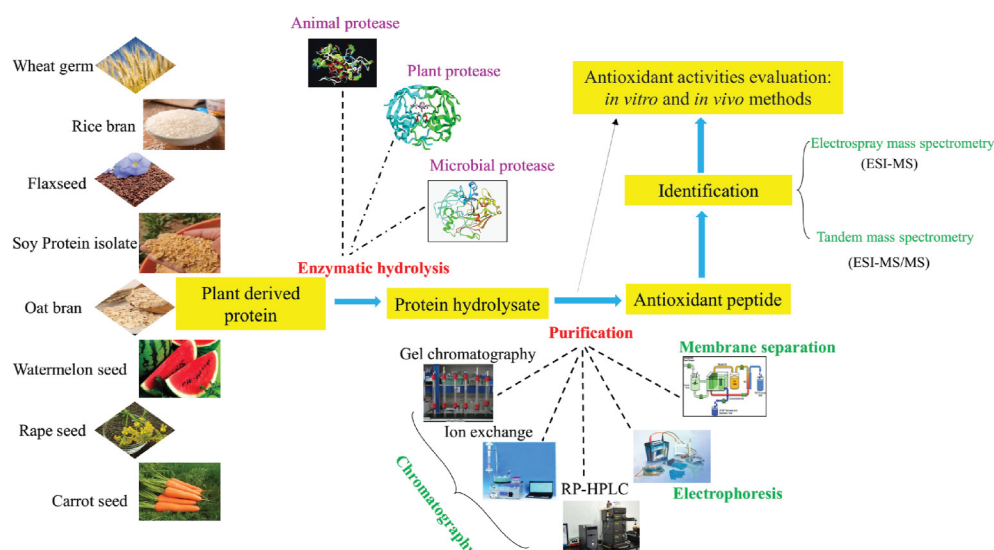


Fig. 1. Schematic representation of the production of antioxidant peptides from plant protein.

simple operation, low energy consumption and environmental protection (Doyen et al., 2014). Wang et al. (2007) used an ultrafiltration membrane (5 kDa) to separate wheat gluten hydrolysates into two components ($M_w > 5$ and $M_w < 5$ kDa), whereby the latter fraction had higher DPPH• radical scavenging ability and surface hydrophobicity ($H_0 = 324.1 \pm 26.5$) than the hydrolysates ($H_0 = 287.5 \pm 16.3$) before ultrafiltration treatment. In a similar study, four antioxidant peptide fractions were separated from wheat gluten hydrolysates by using the different ultrafiltration membranes ($M_w = 10, 5, 3$ kDa); the <3 kDa fraction showed the highest antioxidant activities (DPPH•/superoxide/hydroxyl radical scavenging capacities) (Kong et al., 2008). Phongthai et al. (2018) indicated that the membrane ultrafiltration of protein hydrolysates had a positive effect on metal chelating and ABTS•• radical scavenging activities, and peptide fraction with the strongest antioxidant ability was identified as Phe-Leu-Asn-Glu-Phe-Leu-His-Val. Although the ultrafiltration treatment can significantly improve the antioxidant activity of the peptides, this technique is difficult to obtain high pure products. In addition, the ultrafiltration membrane is easily blocked by raw materials, which causes pollution and waste of raw materials. Thus, the ultrafiltration products often need to be further purified.

Chromatography separation technology is the most common method for the purification of protein hydrolysates or peptides. The principle of chromatography technology is based on the differences in the physical and chemical properties of the mixed peptide components, which makes them differently distributed in the stationary phase and mobile phase to achieve the purpose of separation. These techniques mainly include GPC, IEC, RP-HPLC, among others.

IEC is based on the principle of the strength of the interaction between different charged peptide mixtures and ion exchange groups on the stationary phase. Suetsuna and Chen (2002) reported that wheat gluten protein hydrolysate (2 g) was separated by a cation exchange column (SP Sephadex C-25) and eluted with 3% sodium chloride at a rate of 60 mL/h. In this study, the eluted peptide fraction showed the strongest antioxidant capacity. Udenigwe et al. (2009) purified freeze-dried hemp seed protein hydrolysate by using an SP-Sepharose High Performance XK 50/20 cation exchange column. After that, the column was equilibrated with sodium acetate buffer (pH 7.5), two peptide components with strong antioxidant activities was gradient eluted by using ammonium carbonate (0.5 mol/L, 0–50%). IEC is easily affected by the eluent, and difficult to separate components with similar

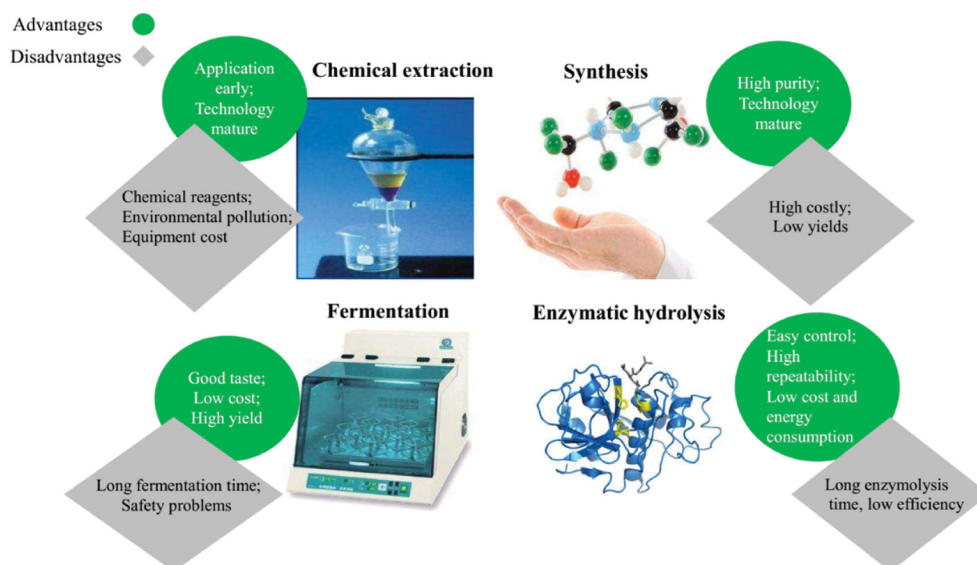


Fig. 2. Advantages and disadvantages of the different preparation methods for peptides.

charge numbers in comparison with GPC. Thus, this technology is often combined with other separation methods to obtain higher purified peptide fractions.

GPC is also known as molecular sieve chromatography or size exclusion. The separation effect of this technology is mainly affected by many factors, including the type of packing, volume of chromatography column, among others. This method is widely used to separate proteins, peptides and polysaccharides due to its advantages of easy operation, pollution-free and low cost. Rapeseed protein hydrolysates (RPHs) were separated into three subfractions by using sephadex G-25 gel filtration, and the eluted subfractions showed higher antioxidant activities (the inhibition ability of malonyldialdehyde, hydroxyl radical clearance and reducing abilities) than RPHs (Xue et al., 2009).

RP-HPLC (the polarity of the mobile phase is greater than that of the stationary phase) is based on the separation principle of the different hydrophobicity between the peptide mixture and the stationary phase. The weak hydrophobic peptide component is eluted first, then the strong hydrophobic fraction is eluted. RP-HPLC generally utilizes silica gel as a stationary phase packing for columns with ultrapure water, methanol, and acetonitrile as commonly used solvents. The rice bran protein hydrolysates were separated by RP-HPLC, and a total of 52 peptide fractions eluted with acetonitrile were isolated, of which 15 antioxidant peptides had a relative antioxidant activity greater than 1.0 (Adebiyi et al., 2008). In similarly study, Tyr-Leu-Ala-Gly-Met-Asn and Tyr-Val-Ala-Gln-Gly-Glu-Gly-Val-Val-Ala obtained from rice bran protein hydrolysates by RP-HPLC separation exhibited good antioxidant activities, and the TEAC value was 0.433 and 0.192 mM, respectively (Adebiyi et al., 2009). RP-HPLC has been widely used for the separation and purification of plant protein-derived antioxidant peptides due to its good stability, high separation efficiency, and wide applicability. It is worth noting that the separation effectiveness of RP-HPLC is influenced by many factors, such as pH, flow rate, and temperature, among others.

As mentioned above, different technologies have some advantages and disadvantages, which make it difficult to obtain ideal peptide components only by using a single technology. Therefore, the combination of a variety of separation technologies can achieve accurate classification and separation of peptide mixtures to obtain high-purity peptides. Five antioxidant peptide fractions (A-E) were separated from coconut cake protein isolates hydrolysates by using G-25 gel chromatography. Fraction E with the highest antioxidant activity was further purified by using cation exchange chromatography and RP-HPLC, the antioxidant peptide sequences (Arg-Pro-Glu-Ile-Val and Pro-Gln-Phe-Tyr-Trp) were successfully identified using MS/MS (Zheng et al., 2016). Chen et al. (2019) used a 4 kDa ultrafiltration membrane, Sephadex G-25 chromatography and RP-HPLC to separate antioxidant peptides from the black bean soybean protein hydrolysate. The most active peptide component (F2-c) was obtained after purification and identification, mainly containing two peptide fragments, Leu-Val-Pro-Leu-Lys and Ile-Val-Pro-Leu-Lys. In addition, Yang et al. (2018) studied a similar combined separation method to fractionate protein hydrolysates from perilla seed protein. In this study, two peptides (Tyr-Leu and Phe-Tyr) with strong antioxidant capacity were purified by size exclusion chromatography and RP-HPLC. The purified peptide showed strong inhibition capacity against hydrogen peroxide-induced HepG2 cell damage.

In addition to the above conventional isolation and purification methods, some emerging technologies have also been used for the purification and production of bioactive peptides. These new technologies mainly include proteomics and peptomics.

The main steps of proteomics include protein separation, MS or western blotting analysis, quantitative analysis of enzyme-linked immunoassay, virtual screening using computer software, and molecular conformation analysis, which has been widely used for biological substance separation. In addition, peptomics combined with computer technology is also used in the analysis of milk-derived peptides (Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014).

Based on the above studies, antioxidant peptides with higher purity

and activity can be obtained through different continuous purification techniques, but industrialized amplification is required to achieve the higher production efficiency of antioxidant peptides.

However, the preparation, isolation, purification and identification of these peptides are very time consuming and expensive. In addition, the relationship between the structure and activity of antioxidant peptides has not been well established and elaborated.

3.2. Identification of plant protein-derived antioxidant peptides

After a series of separation and purification protocols, the peptide structure needs to be identified. Common methods for peptide identification include DNA translation, MS, and peptide sequencer. It is worth noting that MS has been widely used in the identification of peptide sequences due to the advantages of high efficiency, sensitivity, and good reproducibility (Boja & Rodríguez, 2012). According to the different principles of mass spectrometers instrument, the type of mass spectrometers mainly includes fast atom bombardment, electrospray ionization (ESI), and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). Among them, ESI and MALDI-TOF MS are most commonly used in the identification of antioxidant peptides due to their high sensitivity and low detection limit. The mass to charge ratio (m/z) of the peptide subfractions were obtained by using the mass spectrometer, and then plugged into the online protein database to determine the amino acid sequence. If the online protein database does not contain information about the primary structure of the parent protein, the peptide sequencer can be used for *de novo* sequencing of peptide sequence. However, the peptide sequencer is not applicable for analyzing peptide samples of low purity. Moreover, liquid chromatography (LC) coupled with MS/MS is also widely applied in the analysis of peptide sequence, including LC-ESI-MS/MS, LC-MALDI-TOF-MS/MS, LC-quadrupole TOF-MS/MS, LC-FTICR (Fourier transform ion cyclotron resonance)-MS/MS, among others (Demelbauer, Zehl, Plemat, Allmaier, & Rizzi, 2004). Moreover, some online databases have also become popular for studying antioxidant peptides. The main bioinformatic analysis tools are summarized in Table 2. The BIOPEP database contains information on more than 3800 bioactive peptides, 700 food proteins, 480 taste peptides, and 130 allergic protein (Minkiewicz, Iwaniak, & Darewicz, 2019). More interestingly, this database only contains information of 640 antioxidant peptides. This may be mainly attributed to the difficulty of standardizing the evaluation methods of antioxidant activities, which

Table 2
Main tools and databases for bioactive peptide bioinformatics analysis.

Name	Website	Description
BIOPEP	http://www.uwm.edu.pl/biochemia	Bioactive peptide database
ProteinProspector	http://prospector.ucsf.edu/prospector/mshome.htm	Search software for the sequence identification of peptide and protein mass spectrometry
Mascot	http://www.matrixscience.com/search_form_select.html	Search software for the sequence identification of peptide and protein mass spectrometry
UniProt	https://www.uniprot.org/	Bioactive peptide database
SwePep	http://www.swepep.org/	Endogenous bioactive peptide database
Fragment Ion Calculator	http://db.systemsbiochemistry.net/proteomics/Toolkit/FragIonServlet.html	Calculation tools for peptide MS/MS fragment ions
PeptideCutter	https://web.expasy.org/peptide_cutter/	Peptide simulated hydrolysis
ProtParam	https://web.expasy.org/protparam/	Prediction of basic physical and chemical properties of proteins
Compute pI/Mw	https://web.expasy.org/compute_pi/	Prediction of theory isoelectric point and molecular weight of Peptide

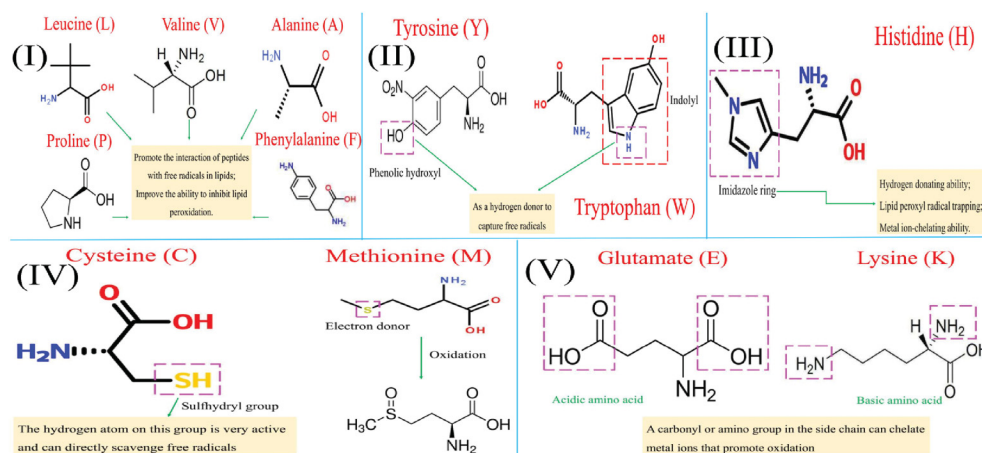


Fig. 3. Effects of different kinds of amino acids on the antioxidant activities of peptides. Note: Hydrophobic amino acid (I); Tyrosine and tryptophan (II); Histidine (III); Sulfur amino acid (IV); Acidic and basic amino acids (V).

makes the data statistics more difficult.

4. Factors that influence the characteristics of antioxidant peptides

It is very important to summarize the information about the characteristics and activities of plant-derived antioxidant peptides as comprehensively as possible, which is beneficial for guiding and predicting the relationship between structure and function. The activities of antioxidant peptides are mainly affected by amino acid composition, sequence, and molecular weight (Nwachukwu & Aluko, 2019).

4.1. Effects of amino acid composition and sequence on the antioxidant activities of peptides

Tan (2014) reported that the amount and position of hydrophobic amino acids (Leu, Val, Ala, Pro, Phe), aromatic amino acids (Tyr, Trp, His), sulfur amino acids (Cys, Met), acidic (Glu) and basic amino acids (Lys) contribute to the activities of antioxidant peptides. As shown in Fig. 3I, hydrophobic amino acid residues can improve solubility of peptides in the lipid phase, and facilitate interactions between the peptide and free radicals, thereby improving the ability to inhibit lipid peroxidation. In addition, Chen, Muramoto, Yamauchi, Fujimoto, and Nokihara (1998) also reported that hydrophobic amino acids can enhance the contact between peptides and polyunsaturated fatty chains of fatty acids in biofilms. As shown in Fig. 3II, the phenolic hydroxyl of Tyr and the indolyl of Trp can directly act as hydrogen donors to capture free radicals, thereby achieving the effect of scavenging free radicals (Hernandez-Ledesma, Davalos, Bartolome, & Amigo, 2005). In a similar study, Phe, Tyr, and Trp residues of the peptides can form a dense aromatic ring, which is beneficial to promote chelation of metal ion by peptides (Sun, Kaur, Halliwell, Li, & Bolli, 1993). Peptides contains His residue, which has a strong ability to chelate metal ions, scavenge hydroxyl radicals and inhibit lipid peroxidation (Fig. 3III). This is mainly because the imidazole group on the side chain of histidine can participate in hydrogen supply and electron transfer (Chan, Decker, & Feustman, 1994). In addition, Sun et al. (1993) reported that the addition of Pro and Leu at the N-terminus of the dipeptide (His-His) can significantly enhance the antioxidant capacity of peptides, which is mainly due to the synergistic antioxidant effects of hydrophobic and aromatic amino acids. As shown in Fig. 3IV, the thiol group of the side chain of Cys can scavenge free radicals by providing hydrogen atoms, while the S atom on the Met to be oxidized to Met sulfoxide by supplying electrons. Acidic amino acids Glu and Asp and basic amino acids can form complexes with metal ions through their charged side chain groups, thereby chelating

the metal ions (Fig. 3V).

4.2. Effects of molecular weight on the antioxidant activities of protein hydrolysates or peptides

Molecular weight can significantly affect the antioxidant activity of protein hydrolysates or polypeptides. Several studies have showed that peptides with smaller molecular weight have higher antioxidant activity, which is mainly due to the ease with which small peptides pass through the intestinal epithelial cells and become absorbed by the body (Samaranayaka & Li, 2011), and what is stated is true for *in vivo* systems, not in the case of *in vitro* tests. You, Zhao, Regenstien, and Ren (2010) compared the DPPH• and OH• scavenging ability of ultrafiltration components of loach protein hydrolysate with different molecular weights. Taking IC₅₀ value as an index, the results showed that peptide components with smaller molecular weight (Mw < 3 kDa) have higher DPPH• scavenging activity (IC₅₀ = 2.42 ± 0.01 mg/mL) and hydroxyl radical scavenging activity (IC₅₀ = 16.9 ± 0.21 mg/mL) in comparison with other peptide components (Mw > 10 kDa, 5–10 kDa, 3–5 kDa). In a similar study, Zhang, Mu, and Sun (2014) highlighted sweet potato protein hydrolysates (Mw < 3 kDa) possessed higher antioxidant activities (Fe²⁺ chelating and OH• scavenging capacities) than other peptide fractions of molecular weight.

4.3. Relationship between structural properties and activities of antioxidant peptides by using computer aided molecular modeling analysis

Currently, the relationship between structural properties and activities of antioxidant peptides was analyzed mainly by two methods. One is that the determination of amino acid composition, sequence and activity of peptides and their structure-activity relationship was established by using traditional isolation, purification and identification technique. The other is based on computer-aided molecular modeling to assess the relationship between structural properties and antioxidant activities of peptides. Molecular docking and quantitative structure-activity relationship (QSAR) are used to screen and predict the potential activity of peptides.

Molecular docking is based on the “lock-key principle” of the interaction between ligands and receptors. The molecular docking model is mainly used in the following four aspects (Meng, Zhang, Mezei, & Cui, 2011): (1) the specific action modes of receptors and small ligand molecules can be explored; (2) the target of the lead active compound can be screened; (3) the potential mechanism of bioactive compounds can be explained; (4) the molecular structure of the bioactive compound can be reasonably optimized. Fig. 4 showed the main flow chart of

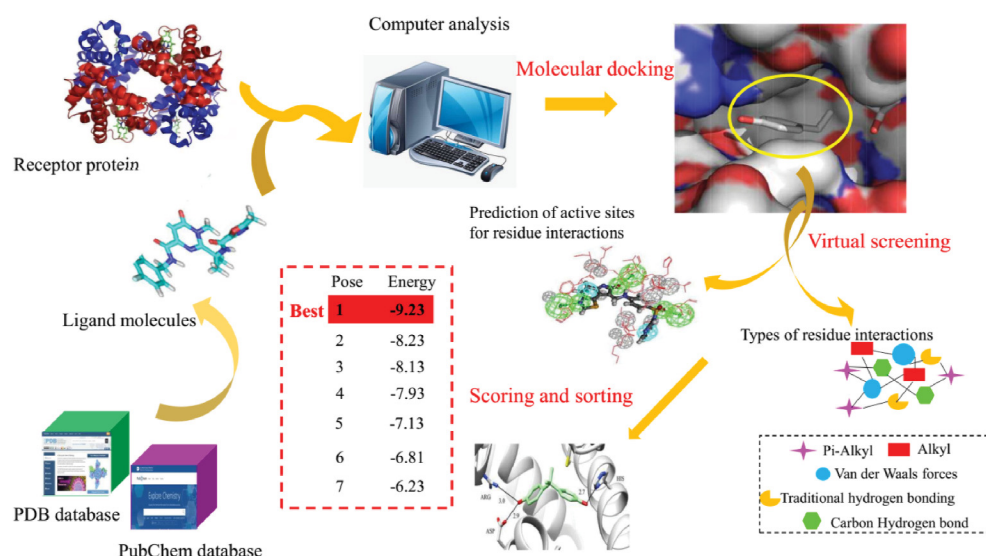


Fig. 4. Schematic diagram of the main process of molecular docking.

molecular docking. The docking process mainly includes the following steps. Firstly, the molecular structure of the receptor protein and ligand molecules are obtained from the PDB database. Secondly, the water molecules and hydrogen atoms of the receptor protein molecular are removed, and its charge is calculated. Thirdly, hydrogen atoms of the ligand molecules are also removed. Then, the interaction site, energy matching of receptor protein and ligand are determined. Fourthly, the receptor protein and the ligand molecule are docked. Finally, the obtained results are scored and sorted.

Currently, molecular docking has been widely used to elucidate the bioactivities mechanism of food-derived peptides (Ouertani et al., 2018). Tonolo et al. (2020) used molecular docking to analyze the interaction between milk-derived antioxidant peptides and Keap1. The results showed that the peptide could activate the endogenous antioxidant pathway (Nrf2-keap pathway) in the cell by binding to the Nrf2 site in keap1 pocket, thereby inhibiting binding of Nrf2 and Keap1. In addition, the IC_{50} value of DPPH free radical scavenging activity of antioxidant synthetic peptide (Glu-Asp-Tyr-Gly-Ala) from the soft-shelled turtle hydrolysate was $380.61 \pm 13.81 \mu M$, and the molecular docking results revealed the glutamate residues of Glu-Asp-Tyr-Gly-Ala could bind with

Arg 415 of the kelch domain (Wang et al., 2020). The binding of Nrf2 and Keap1 was inhibited by the novel antioxidant egg protein-derived peptides Asp-Lys-Lys and Asp-Asp-Trp (Li et al., 2017). Although molecular docking technology has been well applied into the design and development of ideal bioactive substances, the accuracy of predictions needs to be improved. In addition, the results of molecular docking need to be verified by several *in vitro* or *in vivo* experiments.

QSAR has also been applied in the study of bioactive peptides, including antioxidant peptides, bitter peptides as well as peptides that inhibit angiotensin-converting enzyme (ACE) and microbes and dipeptidyl peptidase IV (DPP-IV) (Nongonierma & Fitzgerald, 2016). QSAR is a mathematical model that establishes a quantity-dependent relationship between the structure and activity of a series of compounds. QSAR is used to evaluate the correlation between the molecular structure and activity of antioxidant peptides, which provides a good theoretical basis for peptide activity prediction, isolation and purification, and clarification of molecular mechanisms (Li & Li, 2013). QSAR modeling mainly includes the following four steps: (1) collection of peptide sequences to build the model; (2) structural parameters or descriptors for screening the peptides; (3) establishment of a QSAR model;

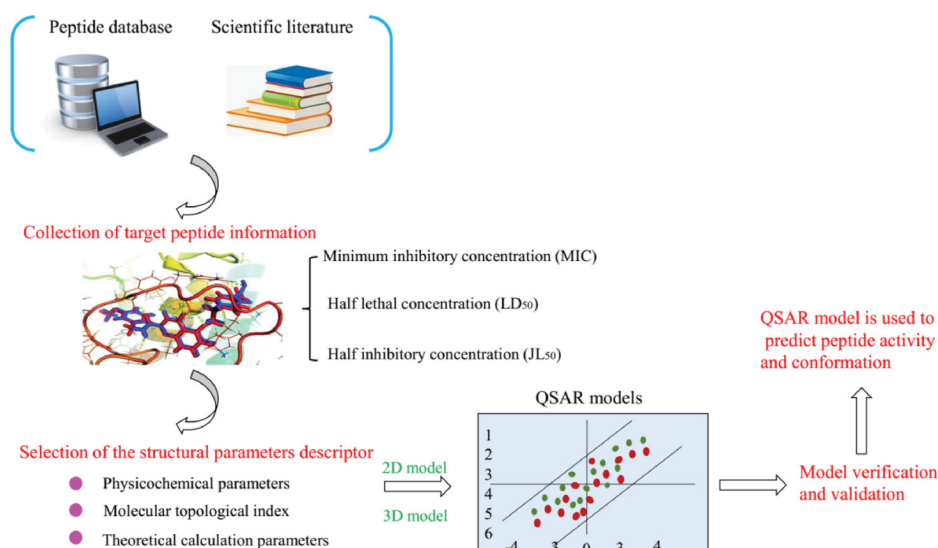


Fig. 5. Schematic diagram of the main process of QSAR model.

(4) prediction of peptide activities using the validated QSAR model (Fig. 5). For example, Li, Li, He, and Qian (2011a) reported the structure-activity relationship of antioxidative tripeptides and tetrapeptides derived from food proteins by QSAR modeling, two credible models for tripeptide and tetrapeptide databases have been built with partial least squares (PLS) regression (cumulative squared multiple correlation coefficients (R^2) for models of tripeptide and tetrapeptide were 0.744 and 0.943, cumulative cross-validation coefficients (Q^2) were 0.631 and 0.414, and relative standard deviation for calibration set (RSDc) were 0.323 and 0.111, respectively). The results showed that the electronic property of the C-terminus is beneficial for antioxidant activity. Li, Li, He, and Qian (2011b) also studied the quantitative structure-activity relationship of antioxidative peptide by using different sets of amino acids descriptors with QSAR modeling, and concluded that Divided Physico-chemical Property Scores (DPPS) is the best descriptor to describe the amino acid of antioxidative tripeptides. The DPPS descriptor revealed that the importance of the center amino acid and the N-terminal amino acid are far more than the C-terminal amino acid for antioxidative tripeptides. The hydrophobic (positive to antioxidative activity) and electronic properties (negative to antioxidative activity) of the N-terminal amino acid are suggested to play the most important significance to antioxidative activity, followed by the hydrogen bond (positive to antioxidative activity) of the center amino acid.

5. Evaluation methods and mechanism of action of antioxidant peptides

The effective prevention of oxidative stress and food oxidation by antioxidant peptides has attracted researchers to evaluate their antioxidant capacity. At present, there are three main methods for assessing the antioxidant capacity of peptides, including chemical methods, cell biological methods *in vitro*, and animal experiments *in vivo*. The potential mechanism of antioxidant peptides mainly includes the following ways: antioxidant peptides could directly (1) scavenge reactive oxygen free radicals; (2) chelate metal ions; (3) inhibit lipid peroxidation reactions; (4) activate the antioxidant defense system of the body.

5.1. Chemical evaluation method and antioxidant mechanism *in vitro*

5.1.1. Scavenging effect of radicals

The scavenge of radicals mainly depends on the type, chain length, composition, sequence, and position of amino acids of antioxidant peptides, which can promote the interaction with free radicals, and then enhance antioxidant capacities.

Based on antioxidant assessment methods *in vitro*, antioxidant

substances can deactivate free radicals through two main mechanisms: hydrogen atom transfer (HAT) and single electron transfer (SET) (Huang, Ou, & Prior, 2005). Effects of the structure of antioxidant peptides on solubility and partition coefficient can determine which mechanism plays the role of quenching free radicals. Antioxidant peptides containing Tyr residues mainly play its antioxidant role through the mechanism of HAT. However, antioxidant peptides containing Trp, Cys and His residues deactivate free radicals mainly by the mechanism of SET (Huang et al., 2005). Oxygen radical absorption capacity (ORAC), total free radical capture antioxidant parameters (TRAP) and carotene bleach analysis are based on HAT. TEAC and DPPH• scavenging ability are based on SET. The advantages and disadvantages of these evaluation methods are summarized in Fig. 6. In addition to the above evaluation methods, the scavenging ability of some free radicals (hydrogen peroxide, single oxygen, superoxide, hydroxyl and peroxyxynitrite) produced by active oxygen species and active nitrogen species is also applied for estimating the antioxidant activity of peptides.

5.1.2. Chelation of metal ions

The polypeptide (3–20 amino acids) can form a stable ring, which is contribute to chelate metal ions, thereby playing a role of antioxidant action. The mechanism of antioxidant peptides chelating metal ions mainly includes the following ways (Himaya, Ryu, Ngo, & Kim, 2012): (1) Antioxidant peptides can serve as hydrogen donors to maintain the original valence of metal elements, prevent metal ions from catalyzing the oxidation of lipids; (2) Antioxidant peptides can chelate metal ions with transport function, thereby blocking lipid peroxide formation, which is dependent on the activity of metal ions as coenzymes, especially during the Fenton reaction; (3) Antioxidant peptides could form a complex with the metal ion component of enzymes so that the catalyst is rendered ineffective, thereby blocking the process of automatic oxidation of the fat. Ferric reducing antioxidant power (FRAP) is a main method used for the reducing capacity evaluation of Fe^{3+} . Benzie and Strain (1996) developed this method to assess the activity of antioxidants in plasma. Later, the method has been widely used in measuring the antioxidant activity of food and plant extracts. The principle of FRAP method is that Fe^{3+} and triphenyltetrazole chloride (TPTZ) can form a complex under acidic condition. The Fe^{3+} -TPTZ complex can be reduced to a blue colour under the action of an antioxidant and the absorbance value of sample can be measured at 593 nm.

5.1.3. Inhibition of lipid peroxidation reaction

Lipid peroxidation is a chain reaction, which mainly includes three steps: excitation, extension and termination (Kubow, 1992). This chain reaction can cause blood vessel disease and oxidative spoilage of food, especially unsaturated fatty acids are easily oxidized due to their

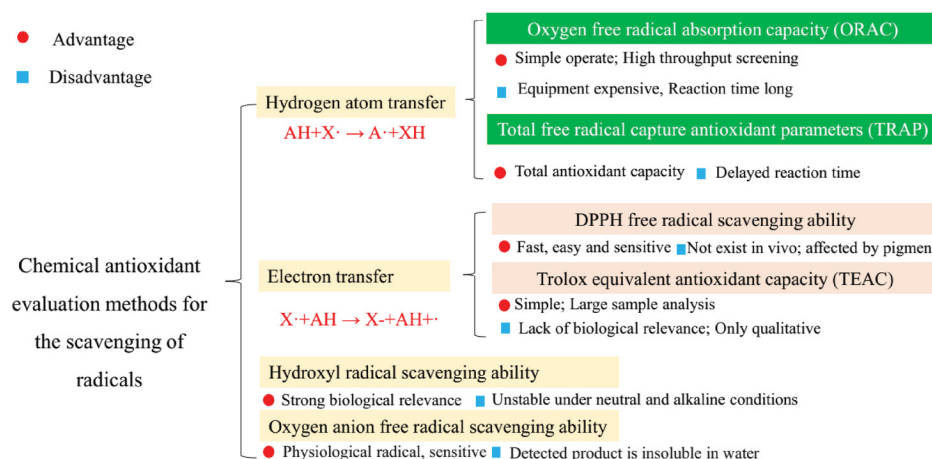


Fig. 6. Classification of evaluation methods for the scavenging of radicals.

unstable double bonds. In addition, reactive oxygen free radicals can trigger lipid peroxidation. Thus, plant-derived antioxidant peptides are used to prevent lipid oxidation, which have become the focus of food nutrition research. The main mechanisms of antioxidant peptides to inhibit lipid peroxidation are as follows (Hirose & Miyashita, 1999): (1) The strong emulsifying ability of hydrophobic amino acids can expose more active sites of the peptide, which can inhibit the chain reaction of lipid; (2) Antioxidant peptides possess strong lipase-inhibitory activity; (3) Antioxidant peptides can be well dispersed on the surface of oil molecules, blocking oil molecules from contacting the air, thereby inhibiting lipid peroxidation. β -carotene/linoleic acid system is the most widely method for the determination of lipid peroxidation. The principle of this method is that linoleic acid can self-oxidize to produce free radicals in the emulsification system and free radicals can decolorize β -carotene, while antioxidants can delay this change in colour (Luo et al., 2013). In addition, thiobarbituric acid (TBARS), peroxide value, iron thiocyanate and electron spin resonance method also are used for the lipid peroxidation inhibitory activity of antioxidants.

Overall, these antioxidant evaluation methods are very important for the characterization of peptide antioxidant mechanism. However, the lack of standardized methods makes it difficult to compare different studies. In addition, the assessment of *in vitro* antioxidant capacity cannot exactly reflect the bioavailability and stability of the polypeptide *in vivo*. Therefore, it is necessary to evaluate the antioxidant activity of peptide *in vivo*.

5.2. Biological evaluation methods and antioxidant mechanism *in vitro* and *in vivo*

5.2.1. Cell experiments *in vitro*

Compared with the *in vitro* chemical method, the cytoprotective effect of antioxidant peptides on damaged cells could more directly reflect the tension of the organism induced by oxidation. In addition, cell biological analysis is more biologically relevant than chemical assays as this method involves several aspects, including cellular uptake, distribution, and metabolism of antioxidant compounds (Sila & Bougatef, 2016). Human cell line and non-human cell line models are commonly used to evaluate the activity and properties of plant protein-derived antioxidant peptides. Among them, human cell lines mainly include neuroblastoma cells (SH-SY5Y), intestinal cancer cells (Caco-2), liver cancer cells (HepG2), human umbilical vein endothelial cells (HUVECs) and cervical cancer cells (Hela), among others. Non-human cell lines mainly include murine macrophages (RAW 264.7), rat liver cells, *Saccharomyces cerevisiae* BY4741 and rat pheochromocytoma cells (PC12). All these studies showed that plant-derived antioxidant peptides can scavenge reactive oxygen species, inhibit oxidative damage, and maintain cell redox balance. Notably, H_2O_2 is usually used to induce cell damage by establishing oxidative stress injury cell model through freely passing the biofilm and being converted into highly active hydroxyl radicals in the cell. Cell-based antioxidant protection in erythrocytes (CAP-e) assay and cellular antioxidant activity (CAA) assay are the main methods to evaluate the cell-based antioxidant level. CAP-e and CAA have advantages of simple operation, sensitive and high-throughput, while the instrument is expensive and the fluorescent marker is sensitive (López-Alarcón & Denicola, 2013). Biomarkers of oxidative damage are often used to assess the antioxidant capacity of bioactive peptides, including biomarkers of proteins (carbonyl content, 3-nitrotyrosine and antioxidant enzymes, etc), lipids (Malondialdehyde (MDA), Low density lipoprotein (LDL), etc) and DNA oxidative damage (8-hydroxydeoxyguanosine (8-OHdG)). In addition, comet assay is also used to detect DNA damage. In this context, separation of rapeseed protein hydrolysate by G-25 gel filtration chromatography produced three antioxidant peptides with the inhibition (0–26%) of hemolysis of rat red blood cells at the concentration of 0.05–2.0 mg/mL (Xue et al., 2009). In another research, corn gluten hydrolysates at the concentration of 0.01–1.0 mg/mL protect HepG2 cells from H_2O_2 induced oxidant injury by

regulating the expression of genes (such as GPX3, GPX5, SOD3, CYGB, SEPP1, MT3, and CGH1) related to reactive oxygen species (Wang et al., 2018). Protein hydrolysates (100 μ g/mL) from oat bran reduced the production of reactive oxygen species and increased the content of intracellular glutathione in the damaged HepG2 cell induced by AAPH (Esfandi et al., 2019a). However, there are also some difficulties in the *in vitro* cell experiment. For example, the cell culture process can easily cause pollution. Some cells have a long growth cycle (such as caco-2 cells). Cell experiments require a lot of expensive detection reagents, so more funding is required. In addition, most cell experiments use passaged cells, which cannot reflect the real situation in the body to a certain extent.

5.2.2. Animal experiments *in vivo*

Animal models are commonly used to assess the bioavailability of antioxidant peptides *in vivo*. However, the limited studies utilized animal models to evaluate plant-derived antioxidant peptides. Biomarkers of lipid, protein peroxidation and DNA damage are also used to monitor the changes in oxidative stress in the body. The rapeseed (*Brassica napus*) meal protein isolate was digested by flavourzyme and alcalase and the resultant rapeseed crude hydrolysate (RSCH) showed a dose-dependent reducing antioxidant power and hydroxyl radical scavenging ability. In addition, RSCH could also inhibit the MDA generation by 50% against H_2O_2 induced rat liver damage in blood serum at 150 mg/mL per day (Xue et al., 2009). Liu et al. (2015) reported that corn gluten meal hydrolysate could significantly increase the activity of SOD (plasma) and GSH-Px (liver) by 1.21- and 1.28-folds, and reduce the content of MDA by 44% at the dose of 300 mg/kg in mice of group III. In a similar research, hemp seed meal protein hydrolysate demonstrated good antioxidant capacities (increased TAC, CAT and SOD levels) when the spontaneously hypertensive rats were given gastric gavage for 8 weeks continuously (Girgih et al., 2014).

The antioxidant evaluation methods mentioned above have been widely used. However, the chemical and cell models methods *in vitro* cannot truly reflect the antioxidant capacity of active peptides under normal physiological conditions. In addition, the cost of animal experiments and human clinical trials is relatively high, which is not conducive to the screening of functional activity of peptides. Based on these, the continuous improvement of DNA chip technology and gene network database provides new ideas for the development of comprehensive, high-throughput and rapid antioxidant evaluation methods, and also provides strong support for the development of functional foods in the future. Therefore, it is very important to develop a simple, accurate, low-cost and comprehensive antioxidant evaluation method, which is still needed in the future.

5.2.3. Activation of the body antioxidant defense system

Endogenous or exogenous stimulation can lead to the production of excessive free radicals, which reduces the activity of antioxidant enzymes, damages biological macromolecules (DNA, protein and lipid, etc.), and then causes cell dysfunction and disease (Sen, Chakraborty, Sridhar, Reddy, & De, 2010). The antioxidant defense system (non-enzyme antioxidant system and antioxidant enzyme system) can maintain the dynamic balance of free radicals in the body. Antioxidant peptides can alleviate the damage of oxidative stress by activating the antioxidant defense system of body. Currently, the antioxidant mechanism of peptides is mainly summarized in two ways, including (1) regulation of the endogenous antioxidant enzymes activities; (2) regulation of the endogenous antioxidant-related signaling pathways.

5.2.3.1. Regulation of endogenous antioxidant enzyme activity. Antioxidant peptides can regulate the activity of antioxidant enzymes to eliminate free radicals and protect cells from oxidative stress. Antioxidant enzymes mainly include SOD, CAT and GSH-Px *in vivo*. According to different metal cofactors of SOD enzyme, which can be divided into three

types include Cu/Zn-SOD, Mn-SOD and Fe-SOD. These three types of SOD enzymes can catalyze superoxide anion radicals to generate hydrogen peroxide and oxygen. Hydrogen peroxide is catalyzed by two antioxidant enzymes, CAT and GSH-Px, to produce non-toxic water and oxygen (Ighodaro & Akinloye, 2018). Most of researchers mainly evaluate the action of antioxidant peptides in regulating antioxidant enzyme activities by establishing oxidative damage models *in vitro* and *in vivo*. Tilapia protein hydrolysate can significantly increase SOD and GSH-Px activities in serum and liver of D-galactose induced aging mice (Wang, Gui, Zhao, & Ren, 2014). Tao, Zhao, Chi, & Wang (2018) reported that three antioxidant peptides (Gly-Ala-Glu-Arg-Pro, Gly-Glu-Arg-Glu-Ala-Asn-Val-Met and Ala-Glu-Val-Gly) from the grise fish cartilage protein hydrolysates could significantly increase the activities of CAT, SOD, GSH-Px and GSH-Rx in the H₂O₂ induced HepG2 cell injury model. Li, Qian, Jingyun, and Bei (2014) reported that two peptides Val-Lys-Pro and Val-Lys-Cys-Phe-Arg from jellyfish protein hydrolysates could significantly improve the activities of SOD, CAT and GSH-Px in damaged rat brain microvascular endothelial cells induced by H₂O₂. Kong, Peng, Xiong, and Zhao (2012) found that whey protein hydrolysate could effectively inhibit the H₂O₂-induced toxicity of MRC-5 cell by increasing the activities of CAT, GSH-Px and SOD and reducing the content of MDA.

5.2.3.2. Regulation of antioxidant-related signaling pathways. Antioxidant peptides can clear ROS by activating the signal pathway of endogenous antioxidant damage, and then avoid oxidative stress injury of cell tissue. Nrf2-keap1-ARE is the key signal pathway of endogenous antioxidant damage. Under the normal physiological conditions, Nrf2 and its cytoplasmic inhibitory protein (Keap1) are bound in the cytoplasm. After the cells are injured by oxidative stress, Nrf2 and Keap1 are separated, and the activated Nrf2 can enter into the nucleus and bind with its specific chaperone fibrosarcoma protein (Maf) to form a dimer. Nrf2-Maf dimer can bind with the antioxidant response element (ARE), which can regulate the transcription of its downstream genes (phase II detoxification enzyme, antioxidant enzyme and cytoprotective protein gene). Finally, the expression of these downstream genes can avoid the change of cell function and enhance the resistance capacity to oxidative stress of cells (Kensler, Wakabayashi, & Biswal, 2007). It was noted that antioxidant peptides can activate this pathway and resist oxidative stress by regulating the transcriptional expression of antioxidant enzymes and phase II detoxification enzymes. For example, Tonolo et al. (2020) reported that milk-derived antioxidant synthetic peptides could protect Caco-2 cells from hydrogen peroxide-induced oxidative damage by activating the Keap1-Nrf2 pathway, and thus promote the expression of antioxidant enzymes (SOD1, Trx1, and TrxR1). Besides, Wang et al. (2020) studied the antioxidant mechanism of the soft-shelled turtle antioxidant peptide (Glu-Asp-Tyr-Gly-Ala), which mainly exerts antioxidant activity by activating the Keap1-Nrf2-ARE signaling pathway, the results showed that the peptide could up-regulate the expression of Nrf2 and down-regulate the expression of Keap1, thereby reducing the damage of HepG2 cells induced by oxidative stress.

6. Stability of antioxidant peptides under conditions of simulating gastrointestinal digestion and their bioavailability

During digestion in the human gastrointestinal tract (GIT), plant proteins can be digested into a large number of free amino acids (AAs) and small peptides by various digestive enzymes or microbial fermentation. Some of these peptides can have different biological activities and are named bioactive peptides (BAPs). The simulation of the digestion process is a very useful tool to evaluate the stability of bioactivity peptides against digestive enzymes. Simulating the digestive process requires more than confronting peptides to the action of pepsin at acidic pH and pancreatin at alkaline pH. The agreed protocol used at present (Minekus et al., 2014) involves the oral, gastric and digestive phases and at each stage the composition of the corresponding fluids is simulated,

which includes the addition of salts and other enzymes like amylase and lipase and other components like bile salts. Dai, Zhang, He, Xiong, and Ma (2017) investigated the bio-accessibility of rapeseed soluble protein (RSPs) by simulated gastrointestinal digestion, the results showed that the DH of RSPs increased from 2.6% to 8.2% in the gastric phase, while the H₀ decreased from 62.8% to 59.1%. After further digestion in the intestinal phase, the DH and H₀ reached 18.2% and 50.5%, respectively. In addition, the scavenging ability of DPPH• and OH• increased slightly after pepsin digestion, but significantly enhanced after trypsin digestion. In particular, when RSPs were incubated for 60 min at a concentration of 5 mg/mL, the absorption of RSPs from the intestine reached the maximum value (28.6%). In another study, Cian et al. (2015) reported the bio-accessibility of antioxidant peptides after simulated gastrointestinal digestion (pepsin and pancreatin), the peptides from hydrolysates presented the lower IC₅₀ value for ABTS⁺• and DPPH• scavenging activity, and the highest copper-chelating activity (CCA). Pimentel et al. (2020) showed that the *Porphyra dioica* hydrolysates still has strong antioxidant capacity after gastrointestinal digestion, which was conducive to its application in functional food.

At present, there are more and more interested in antioxidant peptides, because once they are digested and released from the parent protein and absorbed, which may positively affect the main functions of the human body. The transport of antioxidant peptides through intestinal cells is the main factor affecting their bioavailability. The hypothesis that antioxidant peptides cannot be completely digested and transported through the intestinal lumen to the blood circulation is widely accepted, which due to that the numerous studies described the *in vitro* transepithelial transport of antioxidant peptides. The Caco-2 cell model is widely used to study the bioavailability of peptides due to its similar structural characteristics and good reproducibility as human intestinal cells. For example, Zhang et al. (2018) reported the changes in the antioxidant activity of soybean protein hydrolysate (SPH) under being simulated gastrointestinal (GI) digestion and transported through the epithelium. The antioxidant properties of SPH-GI before and after Caco-2 cell absorption were investigated using the DPPH• radical scavenging, ABTS⁺• radical scavenging and Fe²⁺ chelating activities. The results showed that, after Caco-2 cells absorption from the apical site to the basolateral site, the DPPH• radical scavenging, ABTS⁺• radical scavenging and Fe²⁺ chelating activities were reduced by 25.78, 51.51 and 25.08%, respectively. However, the absorbed permeates showed no detectable reducing capacity at the tested peptide concentration. The permeability of the peptides in SPH-GI was calculated to be $19.66 \pm 0.63\%$. In addition, Ding, Wang, Zhang, Yu, and Liu (2018) investigated the transepithelial transport of two corn gluten derived bioactive peptides (Tyr-Phe-Cys-Leu-Thr and Gly-Leu-Leu-Leu-Pro-His) in human Caco-2 cell monolayers, the results show that both of Tyr-Phe-Cys-Leu-Thr and Gly-Leu-Leu-Leu-Pro-His could transport in intact form across Caco-2 cell monolayers with apparent permeability coefficient (Papp) values of $(1.10 \pm 0.16) \times 10^{-7}$ cm/s and $(1.98 \pm 0.23) \times 10^{-7}$ cm/s, respectively. Zhang et al. (2019) also studied the transport across Caco-2 cells of the wheat germ protein peptides (Gly-Pro-Phe, Gly-Pro-Glu, and Phe-Gly-Glu), the results showed that all peptides had good absorption characteristic. Besides, the ratio of apparent permeability coefficients (cm/s) both sides of apical compartment (AP) and basolateral compartment (BL) were between 0.5 and 1.0 on Caco-2 cell model, which indicated that transmembrane transportation was mainly passive transport. Moreover, clinical research on the bioavailability and bioavailability of antioxidant peptides is needed, which might be beneficial to the application of peptides in the development of functional foods.

7. Application of plant protein-derived antioxidant peptides in food systems

Numerous studies have confirmed the potential application value of plant protein-derived antioxidant peptides in the food systems. Its application mainly includes two aspects, the first is to prevent lipid peroxidation, and the other is as an additive to improve the nutritional

value of food.

As we all known, during the transportation and storage of food, the lipids constituents, colour, flavor and other nutrients in the food are susceptible to damage by the oxidation reactions, which will cause oxidative deterioration of food and the production of toxic substances (Benedict, Strange, & Swift, 1975; German, 1999). Therefore, it is an urgent problem to be solved to prevent the lipid oxidative deterioration of food. It is worth noting that synthetic antioxidants have been used in food preservation and anticorrosive. However, due to the potential harmful effects of synthetic antioxidants on health, consumers' health awareness of foods without synthetic antioxidants is creasing. Based on this, researchers in the food industry began to pay attention to new ways of obtaining natural antioxidants. In addition, because these active peptides have a certain preventive effect on non-communicable diseases, the researchers have also shown interest in adequate intake of nutrients and biologically active substances. It is reported that antioxidant peptides derived from plant proteins have better antioxidant capacity than synthetic antioxidants, and have the advantages of low price, non-toxicity, and environmental friendliness (Helkar, Sahoo, & Patil, 2016). Therefore, natural antioxidant peptides can be added to foods as additives to reduce adverse reactions caused by oxidation in food system. For example, Zhao, Wang, and Huang (2014) found that tea residue protein hydrolysates (1.0 mg/mL) could significantly reduce the oxidation of linoleic acid in linoleic acid system and inhibit the formation of thiobarbituric acid reactive substances (TBARS) in the chicken cakes in comparison with the synthetic antioxidant (BHT). Similarly, Kong and Xiong (2006) reported that zein hydrolysates strongly inhibited the peroxidation of liposome oxidizing system and the antioxidant capacities were close to that of ascorbate, R-tocopherol and butylated hydroxyanisole. Besides, soy protein isolate hydrolysate was added to cooked pork patties and stored in a refrigerator for 7 days, the results showed that the hydrolysates could effectively reduce the conjugated diene (CD) content in cooked pork patties (Peña & Xiong, 2003). Zhuang, Tang, and Yuan (2013) also highlighted that antioxidant peptides (Phe-Leu-Pro-Phe, Phe-Leu-Pro-Phe and Leu-Pro-Phe) purified from corn gluten protein hydrolysates were used as a food antioxidant to delay peroxidation of linoleic acid.

In most countries, plant-derived protein hydrolysates can be added to specific foods as food additives and functional ingredients. However, the application of antioxidant peptides as additives is still in the stage of laboratory research. However, the safety, gastrointestinal stability, potential sensory problems, bitterness and bioavailability of antioxidant peptides should be studied before they are used as functional food ingredients to produce commercial products.

8. Conclusion and future trends

As mentioned above, proteins extracted from plant products can be used to produce antioxidant peptides. Based on this, this review provides comprehensively summarized on the recent research advances in plant-derived antioxidant peptides. However, there are still some problems to be solved in the preparation of antioxidant peptides. First, the production of antioxidant peptides is still limited to the laboratory, and industrial application needs to be achieved. Second, the isolation and purification process of antioxidant peptides have the disadvantages of time consuming and high cost; therefore, more efficient separation and purification methods need to be developed. Third, most of the work only focused on the preparation, purification and characterization of antioxidant peptides, but the relationship between the structure and antioxidant activity of the peptides has not been well established. Fourth, the functional ingredients require a slow process to exert action in the body and wide diversity of antioxidant activity evaluation methods makes it difficult to compare the activities of different raw materials. Thus, standardized and long period assessment methods need to be developed. Fifth, the antioxidant mechanism of plant protein-derived peptides is not well elaborated at the molecular level. The endogenous

biomarkers, the cellular antioxidant pathway and antioxidant target genes of antioxidant peptides need to be explored. Besides, the lack of information on the absorption, distribution, metabolism, excretion, toxicology and sensitization of plant protein derived antioxidant peptides limits their production and application in the market. Therefore, the safety and quality of antioxidant peptides need to be further evaluated. Finally, the deficiency of animal model experiments and human clinical trials limits the bioavailability evaluation and market application of antioxidant peptides. The potential role of antioxidant peptides *in vivo* needs to be further investigated.

In general, the problems mentioned above need to be properly resolved in the future. Before the plant protein-derived antioxidant peptides can be used in large-scale industrial applications, their addition to foods should not cause negative effects. With the advancement of future science technology and the continuous investment research of these peptides *in vivo*, the molecular mechanism of these peptides at the entire gene level will be verified. In addition, the preparation of nanoparticles and microcapsules of antioxidant peptides and the synergy with other active ingredients is also the focus of future research. The future cooperation between scientific research field and industry field will be conducive to the long-term goal of commercial application of antioxidant peptides from plant products.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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