Food Science of Animal Products

Review Article

The generation and application of antioxidant peptides derived from meat protein: a review

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Abstract: Meat and its products, rich in protein, are one of the important potential sources of antioxidant peptides. However, reviews on the generation and application of meat-derived antioxidant peptides are still limited. To understand the research and application progress of meat-derived antioxidant peptides, the main formation pathways and their commercial applications are exhibited, the research methods for the isolation, purification, and identification are summarized, and the influencing factors, evaluation methods, and intestinal absorption pathways are presented in this work. It is summarized that limited degradation by exogenous and endogenous enzymatic hydrolysis is the main pathway for the production of animal-derived antioxidant peptides. Traditional separation, purification, and identification techniques are also applicable to animal-derived antioxidant peptides. The formation of animal-derived antioxidant peptides is affected by many factors, and the intestinal absorption pathways of antioxidant peptides are different. Finally, insufficient and future development directions are provided.

Keywords: meat-derived antioxidant peptides; research and application; development directions

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1 Introduction

Since the theory of free radicals was put forward by Harman in 1955, the relationship between disease and reactive oxygen species (ROS) radicals has been recognized by many scientists [1]. Several ROS formations are shown in Figure 1(A), including hydroxyl radicals, hydrogen peroxide, nitroxide radicals, superoxide radicals, peroxy radicals, etc. [2]. In the field of food science, free radicals can cause the peroxidation of fats and oils, reduce the nutritional value of food, and even cause the production of toxic and harmful substances [3]. Due to the safety aspects, high efficiency, low molecular weight, easy absorption, and high stability, the research and application of antioxidant peptides have obtained more and more attention in recent years [4–9]. Figure 1(B) exhibited the number of publications with the topic "antioxidant peptides" obtained from a search of the Web of Science database from 2002 to 2020. The number of research papers on antioxidant peptides increased at a high rate in the last five years. With the advancement of science, commercial antioxidant peptide products (mainly hydrolysates of plants and animals) have been developed more recently (Figure 1(C)) [10]. It is worth noting that some antioxidant peptides naturally present in animal muscle tissue, such as carnosine, anserine, and glutathione, exhibited good antioxidant effects [11]. Moreover, the antioxidant capacity is related to the type and source of meat protein. For example, it was found that four novel antioxidant peptides (MYGAVTPVK (981.51 Da), NWEKIR (845.46 Da), APGIIPR (723.45 Da), and RWWQLR (944.52 Da)) were obtained by fermenting duck using *Bacillus subtilis*. The antioxidant capacities of the four antioxidant peptides were MYGAVTPVK (1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging rate: 89.65%) > NWEKIR (DPPH: 87.44%) > APGIIPR (DPPH: 85.44%) > RWWQLR (DPPH: 82.55%) [12]. It was also reported that the 10 and 3 kDa peptide fractions generated from the hydrolysis of bovine brisket sarcoplasmic proteins demonstrated antioxidant activities using *in vitro* assays. Among them, two antioxidant peptides AKHPSDFGADAQ and AKHPSDFGADAQA derived from myoglobin were also found [13]. Furthermore, MQIFVKTLTG and DLSDGEQGVL are two antioxidant peptides isolated from venison protein hydrolysate. The free radical scavenging activity of MQIFVKTLTG was higher than that of DLSDGEQGVL [14]. The author's research team has conducted a systematic study in recent years [6–8, 15–20]. It was found that poultry meat and its by-products peptides showed a huge potential for the activity of antioxidants (Figure 1(D)).

The generation of meat-derived antioxidant peptides can be divided into endogenous and exogenous. The endogenous antioxidant peptides are mainly derived from the meat during post-mortem aging [16]. The process of animal rigidity after slaughter and frozen storage is an important part of food processing, by which the integrity and activity of protein change through endogenous proteolysis and result in a large amount of peptide and free amino acids [21]. For example, the changes in antioxidant peptides before and after 14 days of post-mortem aging and cooking were measured using fresh beef as raw material. The results

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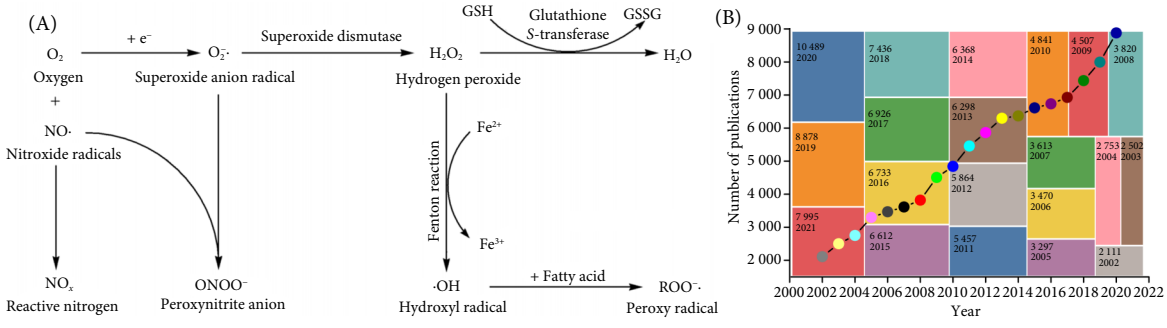

(C)

Nationality	Brand name	Manufacturer	Brand	Website	Source
Denmark	Lacprodan® HYDRO	Arla Food Ingredients		https://www.arlafoodsingredients.com/	Whey protein hydrolysate
China	Fortide	Chengdu Mytech Biotech Co. Ltd.		https://www.mytech.sc.cn/	Soybeans
New Zealand	SureStart™ 917	NZMP (Fonterra)		https://www.nzmp.com/global/en.html	Whey; Milk protein hydrolysate
UK	Beautycoll® (Peptan)	Beautycoll		https://www.beautycoll.co/	Fish collagen
Japan	Bonito peptide	Nippon Supplement Inc.		https://nippon-supplement.ru/	Bonito
USA	Seacure®	Proper Nutrition Inc.		https://www.propernut rition.com/	Pacific whitefish
Canada	Verisol	Gelita		https://www.gelita.com/en	Pork; Beef
Germany	PeptAIde™	BASF		https://www.basf.com/cn/	Brown rice
France	Prodiet F200/Lactium	Ingredia		https://www.ingredia.com/	Milk

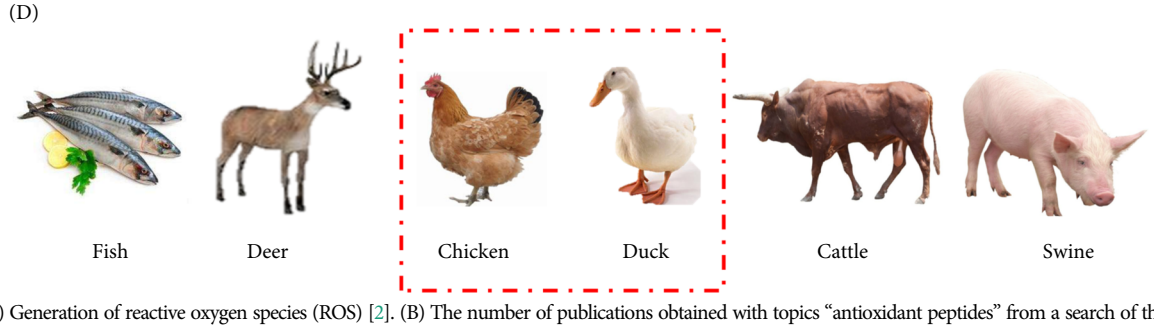

Figure 1 (A) Generation of reactive oxygen species (ROS) [2]. (B) The number of publications obtained with topics "antioxidant peptides" from a search of the Web of Science database with the year from 2002 to 2020. (C) Some commercially sold active peptide companies [10]. (D) Animal resources from which several antioxidant peptides are prepared.

showed that the peptide content increased significantly after post-mortem aging and cooking [22]. In addition to the endogenous production pathway, it is also possible to add some exogenous enzymes to hydrolyze proteins to induce potential antioxidant peptides [8]. Compared with the method of producing antioxidant peptides by endogenous enzymes, the exogenous enzymatic hydrolysis method is more rapid, convenient to control, and easy to repeat [7]. Various antioxidant peptides of animal muscle protein or processing by-products such as duck meat, pork, pig blood, beef, chicken, and chicken blood have been obtained by enzymatic hydrolysis [6, 18, 23–26]. In addition, during the long-term fermentation process in fermented meat products, such as ham and fermented intestines, the protein is strongly hydrolyzed under the action of various endogenous enzymes and microorganisms to generate abundant peptides and small peptides [27–30]. In general, the research on meat-derived antioxidant peptides obtained through various methods has become a hot topic in food science research.

Importantly, although it is possible to obtain components with concentrated molecular weights by optimizing the enzymatic hydrolysis process, the diversity and complexity of the substrate protein will lead to the complex components of the enzymatic hydrolysate. Therefore, to obtain a more uniform composition, the enzymatic hydrolysate must be separated and determined [31]. Furthermore, the activity of antioxidant peptides is affected by factors such as the molecular weight of the peptide, the composition and sequence of amino acids, and the hydrophobicity of the peptide [32, 33]. Therefore, it is necessary to comprehensively consider the factors affecting its antioxidant capacity. Additionally, to evaluate the functionality of antioxidant components, the process of separation and identification of antioxidant peptides usually uses *in vitro* free radical scavenging activity simulation, and then *in vivo* antioxidant activity verification method [34]. Due to the limitations of each antioxidant method, the use of a single detection method cannot fully reflect the function of antioxidant components [9]. Last but not the least, it requires more evidence and research that

whether antioxidant peptides can enter the body, be digested and absorbed without being destroyed, and exert antioxidant effects [35–37].

Although some excellent reviews on antioxidant peptides have been reported [5, 9, 38], there is still a lack of review in the field of antioxidant peptides derived from meat proteins. Thus, the purpose of this review aims to summarize the isolation and identification, influencing factors, evaluation method, digestion, and absorption behavior of meat-based antioxidant peptides. This work can provide references for the development and industrialization of meat-derived antioxidant peptides.

2 Isolation and identification of antioxidant peptides

With the rapid development of separation, purification, and structure identification methods, more and more methods can be applied to various peptide compounds. There are many types of peptide substances, and the separation and purification methods need to be selected according to the different sample properties. Commonly used separation and purification techniques include ultrafiltration, gel filtration chromatography, ion exchange chromatography, affinity chromatography, and reversed-phase high-performance liquid chromatography (RP-HPLC) [17, 30, 39, 40]. The structure of the polypeptides can be further identified through high-performance liquid phase chromatography-tandem mass detector (HPLC-MS) and nuclear magnetic resonance (NMR) [26]. Figure 2 summarized the methods used for the antioxidant peptides research derived from meat protein.

2.1 Isolation and purification

Ultrafiltration is a membrane separation technology that separates and purifies substances based on their molecular weight. Microporous membranes are selected with different pore diameters, according to the molecular weight cut-off size, and can separate 300–1 000 kDa substances. According to the different molecular weights of the samples, substances smaller than the membrane pores can pass through the microporous membrane smoothly, while substances with larger molecular weights are intercepted. That is because ultrafiltration is driven by pressure difference, it does not require any chemical reagents or heating during the sample separation process, so it is especially suitable for the separation of sensitive substances. Ultrafiltration also has the characteristics of simple operation, short processing time, high product recovery rate, and repeated use [2]. Therefore, membrane ultrafiltration has been widely used in the separation and purification of functional peptides. For instance, it was reported that ultrafiltration tubes with a molecular weight cut-off of 10 and 30 kDa could be used to separate duck meat antioxidant peptides, and the results showed that the components with a molecular weight of less than 10 kDa showed the strongest antioxidant activity [31]. In addition, it was also reported that an ultrafiltration tube with a molecular weight cut-off of 5 kDa was applied to obtain duck meat antioxidant peptides after post-mortem aging, and the results showed that the components with a molecular weight of less than 5 kDa showed the strongest antioxidant activity [16]. Since the amount of peptide separated in the laboratory is relatively small and ultrafiltration membranes are expensive, membrane materials and techniques must be improved to accommodate industrial production if commercial-scale production of antioxidant peptides is to be applied.

Gel filtration chromatography, also known as size exclusion chromatography or molecular sieve chromatography, is a method that uses the network structure formed by the gel to separate samples according to the difference in molecular weight and shape of the sample, and the difference in the storage time in the filler [41]. The pores of different sizes are distributed on the packing. After the sample enters the chromatographic column, it enters into different pores according to the size of the particle size [42]. Gel filtration chromatography methods need to improve the lifetime of gel filtration, and also need to develop products matching antioxidant peptides with different animal sources and molecular weights to better meet the needs of functional product design and development.

Ion exchange chromatography is a method in which ion exchange resin is used as the stationary phase, and when the component ions in the mobile phase are reversibly exchanged with counter ions on the exchanger, the separation is carried out according to the size of the binding force. According to the different charges of the exchange groups on the ion exchange resin, ion exchange chromatography is divided into cation exchange chromatography and anion exchange chromatography [31]. The active groups introduced by cation exchange chromatography include the sulfonic acid group ($-SO_3H$), phosphoric acid group

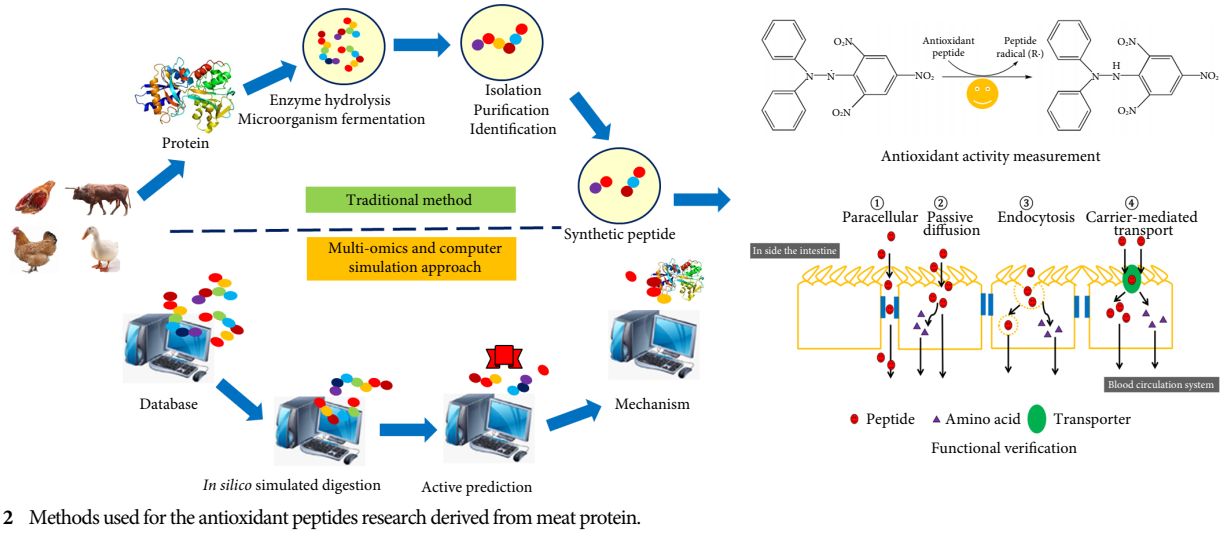

Figure 2 Methods used for the antioxidant peptides research derived from meat protein.

($-PO_3H_2$), phosphite group ($-PO_2H$), carboxyl group (–COOH), phenolic hydroxyl group (–OH), etc. H^+ can exchange with cations in the solution. The active groups introduced by anion exchange chromatography are the quaternary amine group [$-N^+(CH_3)_3$], tertiary amine [$-N(CH_3)_2$], secondary amine ($-NHCH_3$), primary amine ($-NH_2$), imino group (–NH–), etc., which can generate OH^- in water and exchange with various anions [43, 44]. This method is affected by the ionic strength and amino acid composition of the target substance, especially for peptides rich in alkaline or acidic amino acids, which can easily cause incomplete separation. Conversely, the peptides can also be selected and designed according to this method.

RP-HPLC is mainly a separation technology based on the hydrophobic effect and hydrogen bond formation of the sample. It has the characteristics of fast, high efficiency, and high recovery rate. RP-HPLC has a good separation effect on polar peptides of small molecules with a molecular weight of less than 1 000 Da [45]. For instance, it was reported that a C_{18} column was used to separate the antioxidant peptides in the chicken protein hydrolysate, and after two different gradient elution, the antioxidant peptides with higher activity were obtained [25]. It was also reported that a C_{18} column was used to separate the oyster active peptides with an antioxidant nature, after three elution with different gradients, two antioxidant peptides were obtained [46]. This method has a good selection and separation of some antioxidant peptides containing hydrophobic amino acids, and generally the antioxidant target products obtained after this process of separation exhibit several antioxidant activities.

Capillary electrophoresis uses a small capillary to replace a large electrophoresis tank, which increases the efficiency of electrophoresis by dozens of times. It is an effective technique for separating qualitative peptides and proteins. The advantages of capillary electrophoresis are short separation time, high efficiency, high resolution, and less sample volume and reagents. Compared with RP-HPLC, capillary electrophoresis has a lower total preparation volume and is suitable for the preparation of trace samples. Therefore, capillary electrophoresis has a better separation effect for a small single fraction of small molecules. According to different principles, capillary electrophoresis can be divided into capillary gel electrophoresis, micellar electrokinetic capillary chromatography, capillary isoelectric focusing electrophoresis, and capillary zone electrophoresis. For example, it was reported that capillary electrophoresis can be used to separate and purify glutamyl peptides. The results showed that capillary electrophoresis had a better separation effect for small peptides [47].

2.2 Structural identification of antioxidant peptides

Mass spectrometry has been widely used in peptide analysis. Mass spectrometry has the characteristics of high sensitivity and rapidity. In recent years, there have been relatively rapid developments in electrospray ionization mass spectrometry (ESI-MS), continuous-flow fast atom bombardment (cf-FAB), and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) [8, 44, 48, 49].

After certain processings, proteins, and peptides can be protonated to produce a series of multivalent charged ions in ESI. The proteins and peptides could be analyzed on the mass spectrometer to determine their molecular weight. ESI can perform mass spectrometry analysis of proteins with a molecular mass of 100 000 Da. In addition, ESI is suitable for online analysis of proteins and peptide, and requires vaporization or the addition of organic solvents to sensitize samples [50]. The cf-FAB is a weak ionization technique that requires the ionization of proteins or peptides first and is mainly used for the separation and identification of samples that are difficult to volatilize and have strong polarity. It is usually necessary to add polar compounds such as glycerin as a solvent in the determination to improve the sensitivity of the sample and to achieve a better separation and identification effect [51]. MALDI-TOF-MS can accurately determine the molecular mass and sequence structure of proteins and peptides. It is suitable for the determination of mixed peptide samples and has the characteristics of high sensitivity and resolution. The combined technology together with HPLC can efficiently identify peptides and proteins and plays a decisive role in the structural identification of proteins and peptides [52, 53]. NMR spectrum signals are released in a purely digital manner. If the molecular mass of the sample is too large, the overlap range will be too wide and the nuclear signal will become weak. Therefore, it is not widely used in the analysis of proteins and peptides. However, with the application of two-dimensional, three-dimensional, and four-dimensional NMR, as well as the development of molecular biology and computer processing technology, NMR has gradually become one of the main methods of protein analysis. NMR can be used to determine the amino acid sequence, quantify the composition content of each component in a mixture, and so on [54].

3 Influencing factors of antioxidant capacity

The antioxidant activity of peptides is affected by factors such as the substrate protein, the type of enzyme, the amino acid composition and sequence of the peptide, the molecular weight, and the hydrophobicity of the peptide [44, 55, 56].

3.1 Molecular weight

A large number of studies have shown that small peptides with low molecular weight may have stronger antioxidant activity [15, 31, 56, 57]. This phenomenon may be because if the peptide chain is too long, the tertiary and quaternary structure of the protein will entrap many amino acid residues with antioxidant activity, causing it to fail to exert its antioxidant activity [41]. It is generally considered that small peptides with 5–20 amino acids and molecular weight between 500–1 800 Da have strong antioxidant activity [58–61]. For example, Wang et al. [31] studied duck meat proteolysis and found that peptides with a molecular weight of less than 10 kDa have a significantly higher DPPH radical scavenging ability than peptides with a molecular weight of 10–30 kDa and greater than 30 kDa. Onuh et al. [62] digested chicken skin *in vitro* to simulate gastrointestinal digestion and then obtained 5 components by ultrafiltration. Two components (< 1, 1–3, 3–5, 5–10, > 10 kDa) with molecular weight less than 1 000 Da show the strongest DPPH radical, superoxide anion radical, hydroxyl radical scavenging rate and Fe^{2+} chelating ability. Je et al. [60] reported that peptides with a molecular weight of less than 1 000 Da extracted from the bones of Alaska cod have strong antioxidant activity. However, it is not that the smaller the molecular weight of the peptide, the stronger its antioxidant activity. For example, Wu et al. [63] found that peptides with a molecular weight of about 1 400 Da in mackerel proteolysis solution had stronger antioxidant activity than peptides of 200 and 900 Da. This may be due to the

large amount of free amino acids and small peptides with no antioxidant capacity in the smallest molecular weight component [64]. However, these small molecular weight antioxidant peptides are mainly based on *in vitro* models. Whether the small molecular weight peptides are formed after digestion and metabolism and their antioxidant stability require further evaluation.

3.2 Amino acid composition

Many amino acids and their derivatives also show strong antioxidant capacity due to their special groups. Dávalos et al. [65] measured the antioxidant activity of individual amino acids and found that methionine, tyrosine, and tryptophan have the strongest antioxidant activity, followed by phenylalanine, histidine and cysteine, other amino acids did not show antioxidant activity. These amino acids exhibit antioxidant activity because histidine, tryptophan, and tyrosine contain imidazolyl, indolyl, and free radical scavenging functions as electron donors. Sulfhydryl groups contained in methionine and cysteine also have the effect of scavenging free radicals [58]. Other amino acids such as leucine, isoleucine, alanine, and proline have no antioxidant properties by themselves, but they can increase the antioxidant activity of peptides. This is because hydrophobic amino acids such as leucine and isoleucine can increase the solubility of the peptide chain in the non-polar phase so that the peptide in the non-polar phase can better release the antioxidant activity. The R group of glycine is a hydrogen atom, which has great flexibility and can be combined with free radicals, the nitrogen heterocycle of proline will affect the secondary structure of the peptide and thus affect the antioxidant activity of the peptides [58]. For example, Wang et al. [31] reported that the peptides LQAEVEELRAALE, GYDLGEAEFARIM, and IEDPFDQDDWGAWKK separated and purified from the duck meat proteolysis solution had a higher DPPH radical scavenging rate due to the presence of leucine, isoleucine and glycine at the N-terminal. Saiga et al. [66] used papain to enzymatically hydrolyze porcine myofibril protein to obtain strong metal antioxidant peptides with the chelating ability and free radical scavenging ability, it is found that the carboxyl group of acidic amino acid side chain can passivate metal ions, weaken the free radical chain reaction, thereby improving the antioxidant capacity.

3.3 Amino acid sequence and structure

In addition to the amino acid composition, factors such as the sequence of amino acids in the peptide chain can also affect the antioxidant activity of the peptide. Chen et al. [67] used one of the antioxidant peptides (Leu (L)-Leu (L)-Pro (P)-His (H)-His (H)) obtained from the digestion of soybean protein as a prototype, artificially synthesized 28 peptides, and measured their antioxidant activities. The study found that His-His fragments have a significant effect on the antioxidant activity of the peptides. If Leu or Pro is added to the N-terminus of the His-His fragment, the newly obtained peptide has stronger antioxidant activity than His-His, and Pro-His-His has the strongest antioxidant activity. Li et al. [68] studied 214 tripeptides containing histidine or tyrosine, and the results showed that the properties of the central amino acid and the N-terminal amino acid have an important effect on the antioxidant activity of the tripeptide. When the N-terminal amino acid is a strong hydrophobic and weakly charged amino acid (such as A, G, V, and L), and the central amino acid is an amino acid with strong hydrogen bonding (such as R, K, and H), the tripeptide exhibits relatively high strong antioxidant activity, such as the antioxidant activity of the tripeptides LHX, PHX, RHX are stronger than LWX, PWX, RWX.

Since the amino acid composition and sequence have a significant effect on the role of antioxidant peptides, it is possible to design and analyze them by computer before obtaining animal-derived antioxidant peptides, so that we can make the best use of animal and its by-product resources and select different raw materials to obtain a better effect of antioxidant peptides.

3.4 Hydrophobicity

It has been demonstrated that the higher the hydrophobic amino acid content in peptides, the higher the antioxidant activity. Escudero et al. [69] used RP-HPLC to separate the antioxidant peptides in the ham and found that the first eluted components 3, 4, 12, and 17 have the strongest DPPH radical scavenging ability. It shows that the hydrophilic peptide has stronger antioxidant activity. Sacchetti et al. [70] measured the antioxidant activity of the hydrophobic and hydrophilic components of chicken and found that the hydrophilic component has stronger antioxidant activity. Additionally, the side chains of some hydrophobic amino acids can interact with polyunsaturated fatty acids, such as leucine, alanine, and valine. Zarei et al. [71] identified nine peptides from palm kernel cake with high DPPH radical scavenging rate, all of which contained more than 50% hydrophobic amino acids. Bashir et al. [72] identified and characterized 10 novel antioxidant peptides from mackerel (*Scomber japonicus*) muscle protein hydrolysates and found that IANLAATDIIF showed higher DPPH radical scavenging activity and LGTLLFIAIPI showed higher superoxide dismutase (SOD)-like activity because of the presence of hydrophobic amino acids (such as leucine (Leu) and alanine (Ala)) at the N-terminal. Wang et al. [31] isolated and purified seven peptides from duck protein digestion products and all the peptides contained also more than 50% hydrophobic amino acids.

3.5 The types of enzymes

The types of enzymes affect the antioxidant activity of peptides because different enzymes have different restriction sites, so the peptides produced have different N-terminal, C-terminal, and molecular weights. The commonly used enzymes used to prepare antioxidant peptides include alkaline protease, flavor protease, neutral protease, ficin, bromelain, papain, pepsin, and trypsin [73]. Alkaline protease has strong specificity for the hydrolysis of C-terminal hydrophobic amino acids. The specific site of papain is arginine or lysine, and the specific site of trypsin digestion, the spots are arginine, lysine, or tryptophan, while complex proteases and flavor proteases have no specific restriction sites [74]. Klompong et al. [75] use alkaline protease and flavor protease to hydrolyze yellow stripe trevally (*Selaroides leptolepis*). It was found that the DPPH radical scavenging rate of the alkaline protease hydrolysate decreased with the increase of the degree of hydrolysis, while the DPPH radical scavenging ability of the flavor protease hydrolyzate had nothing to do with the degree of hydrolysis.

For different animal sources and their by-products, we need to carefully design and select the appropriate enzyme species and optimize the enzymatic conditions. In this paper, some potential ideas are proposed, one of which is that the peptide composition and sequence of the end product can be exploited to invert suitable enzyme cleavage sites, and then commercial enzymes can be modified or targeted synthesized using biosynthesis and engineering techniques to improve the yield and production

efficiency of the target product. More, molecular dynamics simulations can be directly used to model the interaction and docking of animal-derived proteins with enzymes and to optimize the kinetic parameters and conditions of enzyme excavation to improve the use and function of antioxidant peptidases.

4 Evaluation of antioxidant peptides

The mechanisms of antioxidant peptides are summarized in the following several ways [76]: 1) Antioxidant peptides attenuate or prevent free radical chain reactions by trapping peroxyl radicals. 2) Antioxidant peptides can eliminate the catalytic effect of metal ions, thereby preventing the oxidation reaction with metal ions as coenzymes or cofactors effectively. 3) It provides hydrogen atoms for antioxidant enzymes and exerts the antioxidant effect in the presence of glutathione. To comprehensively determine the antioxidant activity of peptides, multiple determination methods should be used. The methods for determining the antioxidant capacity are mainly divided into *in vitro* experiments and *in vivo* experiments. *In vitro* experiment methods mainly include measuring free radical scavenging ability, anti-linoleic acid peroxidation ability, metal ion chelating ability, as well as cell experiments. *In vivo* experiments are mainly animal model experiments.

4.1 *In vitro* experiments

4.1.1 Determination method based on chemical reaction

According to the reaction mechanism of the chemical reaction of scavenging free radicals, the chemical antioxidant activity determination method can be divided into the reaction based on hydrogen atom transfer (HAT) and the reaction based on electron transfer (ET) [77]. The antioxidant activity determination method based on HAT focuses on detecting the ability of antioxidants to capture free radicals by donating hydrogen atoms. The reaction has nothing to do with the solvent and the pH of the reaction system and is relatively fast, usually completed within a few minutes. The most commonly used methods are the oxygen radical absorption capacity method (ORAC) and the total free radical scavenging capacity (TRAC) method. The principle of this method is that the antioxidant peptide and the substrate jointly compete for free radicals. Due to the existence of the antioxidant peptide, the probability of free radicals binding to the substrate is reduced, thereby playing a role in protecting the substrate [78]. ORAC activity is expressed in Trolox equivalent. Experiment with Trolox having different standard concentration gradients to obtain the fluorescence decay curve of the sample reaction system, and calculate the fluorescence decay. The advantage of the ORAC method is that it provides a controllable source of free radicals, which simulates the interaction of free radicals produced by antioxidants in food and physiological systems and oils.

The method based on ET mainly measures the ability of antioxidants to reduce oxidants. When the oxidants are reduced, the color of the reaction system will change and the degree of color change is related to the antioxidant activity of the sample [79]. Reaction analysis based on ET includes tocopherol equivalent antioxidant capacity (TEAC) measurement, reducing power measurement, metal ion chelating ability measurement, and free radical scavenging ability measurement (such as DPPH radical, hydroxyl radical, superoxide anion radical, and hydrogen peroxide). One of the most commonly used and most important is DPPH radical scavenging measurement. The basic principle of DPPH radical scavenging rate is based on the phenomenon in which DPPH radical generates a purple stable nitrogen-containing free radical in the solution, and has a maximum absorption peak at 517 nm of ultraviolet-visible light. When antioxidants are added, the purple color fades due to the scavenging effect of free radicals, and the intensity of the absorption spectrum decreases as the number of antioxidants added increases [2]. In addition, the chelating ability of ferrous ions is considered to be an effective indicator of the antioxidant capacity of peptides. For example, Li et al. [50] used the chelating ability of ferrous ions as the basis for the strength of the antioxidant capacity. The protein is separated and purified to obtain the peptide QGAR with a strong antioxidant capacity.

4.1.2 Cell test

Studies have shown that the mechanism of antioxidants goes beyond the scavenging effect of free radicals in promoting human health [79]. Therefore, it is particularly important to use cell culture models for antioxidant research. The cellular antioxidant activity (CAA) is expressed as the fluorescence clearance rate. Usually, the fluorescence intensity in the cell is measured within a certain period, the fluorescence growth curve of the reaction system is obtained, and the area of the fluorescence growth curve is calculated. By calculating the percentage of the area of the experimental group and the control group, the CAA value is obtained. Commonly used cells are adenocarcinoma colon cancer cells (Caco-2) [80], lung fibroblasts (MRC-5) [81], human endothelial cells (ECV304) [82], liver cancer cells (HepG2) [83], mouse macrophages (RAW264.7) [84], etc. Lee et al. [24] studied the protective effect of the antioxidant peptide (WYPAAP, 693.90 Da) derived from an enzymatic hydrolysate of duck skin by-products on *t*-BHP-induced liver cell damage in Chang cells. It was found that this novel peptide is involved in regulating apoptosis-related gene expression and contributing to antioxidative properties in the cell environment.

4.1.3 Molecular docking

Currently, molecular docking has been used to illustrate the bioactivities of plant- and animal-based antioxidant peptides. Molecular docking is based on the "lock & key principle" of the interaction between ligands and receptors, which simulates the interaction of small ligand molecules with receptor biological macromolecules. Several common peptide bioinformatics characterization websites and the basic process of the molecular docking method are exhibited in Figure 3 [9]. The interaction between ligand and receptor is a process of molecular recognition, which mainly includes electrostatic interaction, hydrogen bond interaction, hydrophobic interaction, van der Waals interaction and so on. Through calculation, the binding mode and affinity between the two can be predicted [85]. Chen et al. [86] investigated the antioxidant peptide derived from rabbit meat proteins and the molecular docking method, and the result revealed that EACF (novel bioactive peptides) established eight hydrogen bond interactions in the docking pockets. In general, the application of this method in meat-derived antioxidant peptides is still limited, and it is an innovative method that is worth developing in addition to classical *in vitro* methods.

Name	Website	Description
UniProt	https://www.uniprot.org/	Bioactive peptide database
SwePep	http://www.swepep.org/	Endogenous bioactive peptide database
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
BIOPEP	http://www.uwm.edu.pl/biochemia	Bioactive peptide database
Fragment Ion Calculator	http://db.systemsbiology.net/proteomicsToolkit/FraglonServlet.html	Calculation tools for peptide MS-MS fragment ions
ProteinProspector	http://prospector.ucsf.edu/prospector/mshome.htm	Search software for the sequence dentification of peptide and protein mass spectrometry
Mascot	http://www.matrixscience.com/search_form_select.html	Search software for the sequence dentification of peptide and protein mass spectrometry

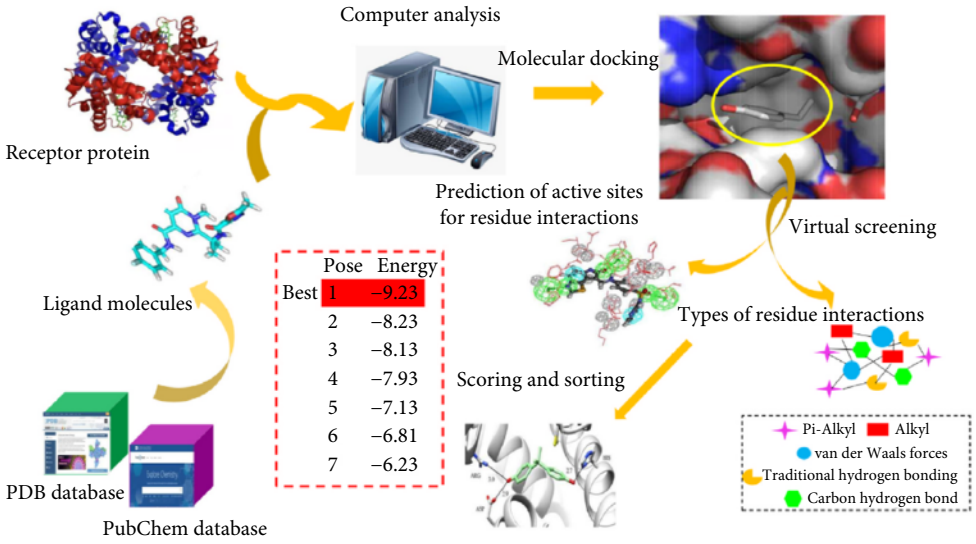

Figure 3 Several common peptide bioinformatics characterization websites and the basic process of molecular docking method [9].

4.2 *In vivo* experiments

Although there is substantial evidence that *in vitro* assays can detect the antioxidant activity of peptides, it is not known whether peptides can act as antioxidants in humans due to degradation in the intestinal, vascular, and hepatic systems. Therefore, further animal studies and *in vivo* assays in clinical trials should be performed to determine the bioavailability and functionality of peptides. However, studies on the use of animal models to assess the biological potential of protein hydrolysis products or meat protein-derived peptides are limited.

The *in vivo* test is the test method that most directly reflects the effect of antioxidant peptides in the body after being absorbed and metabolized. *In vivo* tests are commonly used to feed or inject antioxidant peptides, and put the animals to death within a predetermined incubation time, to determine the changes in the content and activity of antioxidant enzymes and mRNA levels in the blood or tissues of the animals. For example, Boukortt et al. [87] found in animal experiments that when fish protein hydrolysates were fed to mice suffering from hypertension, their overall antioxidant capacity was increased by 35% compared to the control group. Khaled et al. [88] fed sardines protein hydrolysate (SPH) to experimental mice and found that the concentration of malondialdehyde (MDA) in the blood of the mice was reduced, while the activity of antioxidant enzymes was increased. Lee et al. [89] studied the effect of oral gelatin hydrolysate on ethanol-induced oxidative stress in Wistar rats. The results showed that compared with the control group, gelatin hydrolysate can significantly increase the antioxidant enzyme activities of SOD, catalase (CAT), and glutathione peroxidase (GSH-Px). Sun et al. [25] fed *D*-galactose-treated aging mice with chicken protein hydrolysate and found that the antioxidant enzyme activities of SOD, CAT, and GSH-Px were significantly increased, while the content of MDA was significantly reduced.

5 Digestion and absorption of antioxidant peptides

In the digestion and decomposition of the digestive tract, protein substances can be degraded by pepsin and trypsin to produce

functional polypeptides. Secondly, biologically active peptides are also present in dietary supplements. The functional polypeptides obtained by the above methods are concentrated in small intestinal epithelial cells. It is absorbed into the organism through the intestinal tract. In the digestion process of the gastrointestinal tract, acid-base changes and the action of digestive enzymes are important reasons that affect the stability of biological peptide activity. The transport system of biologically active peptides is mainly located in the brush border membrane of small intestinal epithelial cells (Figure 2). Its transport methods include paracellular (the permeability of bioactive may increase by the tight junctions), passive diffusion (transmembrane transport depends on the concentration difference), endocytosis (endosomal would release the peptides after the transport), and carrier-mediated transport (transport via the intestinal H^+ and transporter) [35]. It was reported that in the early stage of small peptide absorption, the intermediate carrier is mainly carrier-mediated transport and absorbed, and then the intermediate carrier absorption activates the atresia of epithelial cells, causing the lateral glands to diffuse, and the permeability of the absorption channel of small peptides is increased. The channel began to absorb a lot. Finally, paracellular has become the main absorption method of biologically active peptides [36]. In the process of absorption of biologically active peptides, concentration-dependent passive diffusion has always played a role in the entire transmembrane transport.

In addition, the overall antioxidant capacity and performance are changing all the time when conducting cellular assays, so it is important to evaluate the antioxidant effects inside and outside the cells timely. Therefore, it is necessary to develop more real-time monitoring technologies, such as real-time sensors developed using magnetic signals, electrical signals, etc., as well as the effective combination with big data, artificial intelligence, food computing, and other cross-cutting directions, which can improve the accuracy of cellular assays.

6 Applications

Several commercial peptide preparation product manufacturers on the market were picked up, and the research papers (separation, purification, identification, antioxidant activity characterization, etc.) of meat-derived antioxidant peptides were also refined (as shown in Table 1). Among them, the bioinformatics method based

Table 1 Summary of the meat and its by-product-derived antioxidant peptides reported from 2010 to 2021.

Source	Treatment methods	Sequences	Separation and purification technique	Identification technique	Antioxidant index	Reference
Beef myofibrillar protein	Commercial enzymes (alkaline-AK and papain)	Not clear	RP-HPLC	None	DPPH and ABTS cation radical scavenging activity	[95]
Hairtail surimi	Dispase	DLYANTVLSGGTTMYPG IADR	RP-HPLC, gel filtration chromatography	HPLC-MS/MS, FT-IR	DPPH and hdroxyl radical scavenging activity	[96]
Chicken breast	Pepsin	ITTNPYDY, IGWSPLGSL, ITTNPYDYHY, and LRVAPEEHPTL	Utrafiltration	Nano-LC-ESI-MS/MS	DPPH and ABTS cation radical scavenging activity, oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP)	[23]
Duck plasma proteins	Alcalase	LDGP,TGVGTK, EVGK, RCLQ, LHDVK, KLGA, and AGGVPAG	Ultrafiltration, size exclusion chromatography, and RP-HPLC	Nano-LC-ESI-MS/MS	Superoxide anion radical scavenging activity, DPPH, ABTS, Fe^{2+} chelating ability, reducing capacity assay	[7]
Frozen duck breasts	Neutrase	AGPSIVH, AGVVGIK, DAELLR, FLLPH, GGPDF,KLLPGG, LDLGV, LDLR, LLCVAV, PVSSLK, QLELK	Ultrafiltration, gel filtration chromatography, RP-HPLC	Nano-LC-ESI-LTQ-Orbitrap MS/MS, NICOLET IS10 FT-IR and PEPstr server	DPPH radical scavenging, reducing power, ABTS cation radical scavenging activity	[8]
Forty-day-old duck (*Anas platyrhychos*)	Trypsin	Small peptides (<5 kDa)	Ultrafiltration	None	DPPH radical scavenging activity, FRAP assay	[6]
Spanish dry-cured ham	Commercial synthesised	AEEEYPDL	Size exclusion chromatography	LC-MS/MS	Oxygen radical absorbance capacity assay, ABTS cation radical scavenging capacity	[39]
Chinese dry-cured Xuanwei ham	Commercial synthesised	DLEE	Size exclusion chromatography, anion exchange colum, RP-HPLC	LC-MS/MS	DPPH, hydroxyl radical and superoxide radical scavenging activity	[97]

Table 1 (*Continued*)

Source	Treatment methods	Sequences	Separation and purification technique	Identification technique	Antioxidant index	Reference
Forty-day-old duck (*Anas platyrhychos*)	Perchloric acid	LINHA, GAPLEVQAV, LIAYLKDATAK, etc.	Ultrafiltration, gel filtration chromatography	Nano-LC-MS/MS	DPPH, FRAP, oxygen radical antioxidant capacity	[16]
Beef	Perchloric acid	WPGIL, CPSGPGTF, ISPCAMMLAL, FRSGK, etc.	Ultrafiltration	LC-ESI-MS	DPPH radical scavenging capacity	[48]
Camel meat sausages	Tris-HCl buffer (pH 8.0) containing 0.5% (*V*/*V*) 2-mercapthoethanol	FAGDDAPR, AGDDAPR,KPPDIPD, VAPEEHPT, EVHEPEEKPRP, etc.	Ultrafiltration	RP-HPLC-ESI-Q-TOF-MS/MS	ABTS and DPPH radical scavenging, hydroxyl radical scavenging activity, FRAP	[98]
Chinese dry-cured Jinhua ham	0.01 mol/L HCl	LPAHSNAVGRT, VRPPPASKSL, TPFKAGGSPK, NGMNKPLVL, GNAS, etc.	Size exclusion chromatography	LC-MS/MS	Hydroxyl radical scavenging activity, DPPH radical scavenging activity, FRAP	[99]
Fermented meat sauce	Proteolysis during fermentation	QYP	Sephadex G-10 column, HPLC	LC-MS	DPPH and hydroxyl radical scavenging activity	[30]
Duck meat	Protamex	IEDPFDQDDWGAWKK	Ultrafiltration, Gel filtration chromatography, ion exchange chromatography	LC-MS/MS	DPPH and hydroxyl radical scavenging activity, FRAP	[31]
Commercial Argentinean fermented sausages	0.01 mol/L HCl	(G)FAGDDAPRAVFPS(I), (A)VFPSIVGRPRHQG(V), (M)EKIWHHTF(Y), (L)RVAPEEHPTL(L), etc.	RP-HPLC	MALDI-TOF-MS	None	[39]
Round scad (*Decapterus maruadsi*) muscle	Alcalase, neutral protease, papain, pepsin, and trypsin	HDHPVC, HEKVC	Ultrafiltration	MALDI-TOF-MS	Reducing power assay, DPPH radical-scavenging activity assay, superoxide radical scavenging activity assay	[40]
Chinese Jinhua ham	Pepsin	Crude peptides	None	None	DPPH radical scavenging activity, hydroxyl radical scavenging activity, FRAP	[29]
Spanish dry-cured ham	0.01 mol/L HCl	TSGANPP, HNAAKLR, LGGSILIG, CQPSGNL, etc.	Sephadex G25 column	Nano-LC-MS/MS	DPPH radical scavenging assay and reducing power	[69]
Duck skin by-products	Synthesized	WYPAAP	RP-HPLC	LC-MS/MS	DPPH and superoxide radical scavenging activity, hydroxyl radical scavenging activity, alkyl radical scavenging activity, superoxide radical scavenging activity	[24]
Chicken breast protein	Papain	Not clear	Ultrafiltration, gel filtration chromatography, RP-HPLC	None	Reducing power and DPPH radical scavenging	[25]
Tilapia skingelatin	Multifect neutral and properase E	EGL and YGDEY	Gel filtration chromatography, ion exchange chromatography, and RP-HPLC	HPLC-Q-TOF-MS	Superoxide anion radical scavenging assay, DPPH radical scavenging assay, hydroxyl radical scavenging activity assay	[43]

Table 1 (*Continued*)

Source	Treatment methods	Sequences	Separation and purification technique	Identification technique	Antioxidant index	Reference
Duck skin by-products	Pepsin	HTVQCMFQ	RP-HPLC	ESI-Q-TOF-MS	Hydroxyl radical scavenging activity, DPPH radical scavenging activity, alkyl radical scavenging activity, superoxide radical scavenging activity	[100]
Croaker (*Otolithes ruber*) muscle protein	Trypsin and chymotrypsin	KTFCGNH	Ion exchange chromatography, gel filtration chromatography	ESI-MS/MS	DPPH radical scavenging activity, hydroxyl radical scavenging activity	[101]
Squid gelatin	Alcalase	GPLGLLGFLGFLGLS	Ultrafiltration and size exclusion chromatography	LC-ESI-IT-MS/MS	ABTS and FRAP assay	[33]
Duck processing by-products	Flavourzyme, neutrase, protamex and alcalase	DVCGRDVNGY	Ultrafiltration, HPLC	LC-MS	Hydroxyl radical scavenging activity	[89]
Porcine blood plasma	Porcine blood plasma	HNGN	Ultrafiltration, ion exchange chromatographyand RP-HPLC	LC-MS-MS	DPPH radical scavenging activity assay, reducing power	[44]
Sardinelle (*Sardinella aurita*) by-products proteins	Alcalase	LHY, LARL, GGE, GAH, GAWA, PHYL and GALAAH	Sephadex G-25 gelfiltration, RP-HPLC	LC-ESI-IT-MS/MS	DPPH radical scavenging activity, reducing power assay	[55]
Cornu Bubali (water buffalo horn)	Lactate dehydrogenase	YDQGV, TEDCTDCGN and AADNANELFPPN	Gel filtration chromatography, ion exchange chromatography and RP-HPLC	MALDI-LIFT-TOF/TOF-MS	DPPH radical scavenging activity	[102]

on molecular docking and the research on the digestion and absorption of antioxidant peptides may be important directions that are currently lacking and worthy of further research in the future. More, the meat-derived antioxidant peptides have been applied in some functions, which can be summarized as follows:

1) Meat-derived antioxidant peptides can be added to foods as functional ingredients to slow down the oxidative deterioration of foods. The United States and other countries have allowed some vegetable protein hydrolysates as food additives to be added to special foods. It was found that the antioxidant peptides derived from proteolysis solutions such as long tail cod gelatin, blue mussels, etc. have higher antioxidant activity than α-tocopherol, and some are even higher than butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) [32, 90, 91]. It was reported that as the flounder protein hydrolysate was added to the salmon steak, the fat oxidation process of the salmon steak was significantly reduced [92].

2) Meat-derived antioxidant peptides can also be used as functional foods and nutritional health products. LIQUAMEN®, a product derived from cod enzymatic hydrolysate that has the function of reducing oxidative stress, has been put on the market, and some other antioxidant peptides have also been verified to have good physiological functions through cell and animal experiments [10].

3) Meat-derived antioxidant peptides can also be used as cosmeceutical components. Cosmeceuticals currently on the market contain some antioxidants such as polyphenols, flavonoids, vitamin C, lycopene, vitamin E, etc. [93]. But the example of applying meat-derived antioxidant peptides to cosmeceuticals is very limited. It is only used in a few developed countries. For example, the tripeptide Cu-GHK is used in cosmetics in the United States, which is beneficial to the synthesis of collagen and elastin and the healing of wounds [94].

7 Conclusion and outlook

This work summarized the generation pathways, listed the influencing factors, and compared the analytical methods of meat-derived antioxidant peptides. However, there are still some problems in the research and application process that need to be resolved in the future, which mainly include: 1) In addition to conventional research methods, the nutrigenomics approach promotes the understanding of antioxidant peptides. Also, the field of peptidomics is relatively new and has the attractive potential to progress in the future with the advent of high-throughput MS-based technologies coupled with bioinformatics and genetic databases. By combining information from nutrigenomics, genomics, proteomics, metabolomics, peptidomics, and appropriate bioinformatics, it would be possible to reveal all properties and implications of bioactive peptides generated from food proteins including meat proteins. 2) Some new efficient separation methods of antioxidant peptides, such as synergistic treatment with physical fields (ultrasound, ultra-high voltage, pulsed electric field, etc.) can improve the separation efficiency and antioxidant capacity of antioxidant peptides, but the synergistic effect of each physical field

and its influence on exogenous enzymes need to be further investigated. 3) The stability during processing, storage, and gastrointestinal digestion and absorption still needs to be explored in depth, such as the interaction with the intestinal matrix and intestinal microbes. 4) A large number of animal and clinical trials are required to conduct a safety risks assessment, such as the dosage and the interaction with the drugs taken at the same time. 5) The absorption of active peptides from the cellular and molecular levels, and the mechanism of biological activity in the target tissues still need to be explored.

On the whole, although considerable progress has been made in the research of meat-derived antioxidant peptides, and some products have been commercialized, more investment is needed in the future.

Conflict of interest

The authors declare that they have no competing interests.

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