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Advances in the stability challenges of bioactive peptides and improvement strategies

Jingyan Pei [a], Xinchang Gao [b,**], Daodong Pan [a,c], Ying Hua [d], Jun He [a], Zhu Liu [e,***], Yali Dang [a,*]

[a] State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of AgroProducts, College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo, 315211, Zhejiang, China
[b] Department of Chemistry, Tsinghua University, Beijing, 100084, China
[c] National R&D Center for Freshwater Fish Processing, Jiangxi Normal University, Nanchang, 330022, Jiangxi, China
[d] College of Pharmaceutical Science, Zhejiang Chinese Medical University, Hangzhou, 310053, Zhejiang, China
[e] Zhejiang Institute for Food and Drug Control, Hangzhou, 310052, Zhejiang, China

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ABSTRACT

Bioactive peptides are widely used in functional foods due to their remarkable efficacy, selectivity, and low toxicity. However, commercially produced bioactive peptides lack quality stability between batches. Furthermore, the efficacies of bioactive peptides cannot be guaranteed *in vivo* due to gastrointestinal digestion and rapid plasma, liver, and kidney metabolism. The problem of poor stability has restricted the development of peptides. Bioactive peptide stability assessments use different stability assays, so the results of different studies are not always comparable. This review summarizes the quality stability challenges in the enzymatic hydrolysis production of bioactive peptides and the metabolism stability challenges after oral administration. Future directions on the strategies for improving their stability are provided. It was proposed that we use fingerprinting as a quality control measure using qualitative and quantitative characteristic functional peptide sequences. The chemical modification and encapsulation of bioactive peptides in microcapsules and liposomes are widely used to improve the digestive and metabolic stability of bioactive peptides. Additionally, the establishment of a universal stability test and a unified index would greatly improve uniformity and comparability in research into bioactive peptides. In summary, the reliable evaluation of stability is an essential component of peptide characterization, and these ideas may facilitate further development and utilization of bioactive peptides.

1. Introduction

Bioactive peptides are protein fragments encrypted in the primary sequences of proteins, and they have a wide array of significant pharmacological effects after their degradation from proteins (Singh et al., 2021). The field of peptide therapy began in 1922 when insulin extracted from the pancreas of animals was first used for medical treatment (Banting et al., 1962), followed by corticotropin in 1949 (Elkinton and Hunt, 1949). In 1953, oxytocin became the first synthetic peptide drug (Vigneaud et al., 1953). At first, it took months to years to synthesize peptides by solution-phase chemistry until the invention of solid-phase synthesis in 1963 (Merrifield, 1965). The peptide industry has developed significantly, accompanied by the maturity of high-performance liquid chromatography (HPLC) purification technology. Due to their remarkable potency, selectivity, and low toxicity, bioactive peptides saw rapid development in the 1970s and 1980s (Muttenthaler et al., 2021). Bioactive peptides derived from foods that prevent the development of chronic diseases were included in the BIOPEP-UWM database. Today, the number of bioactive peptides registered in the BIOPEP-UWM database is 4,299, as of January 2022 (Minkiewicz et al., 2019). The functions of peptides in the BIOPEP-UWM database include antihypertensive, hypolipidemic, hypoglycemic, antioxidant, antithrombotic, immunomodulatory, antimicrobial, and anticancer.

* Corresponding author. School of Food and Pharmaceutical Science, Ningbo University, Ningbo, Zhejiang, 315211, China.
** Corresponding author.
*** Corresponding author.
E-mail addresses: gaoxc20@mails.tsinghua.edu.cn (X. Gao), zliu82@126.com (Z. Liu), dangyali@nbu.edu.cn (Y. Dang).

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In the past two decades, scientists have applied bioactive peptides to various fields, such as functional foods, biological reagents, biological fertilizers, and biopharmaceuticals. In food science, the conventional methods of producing bioactive peptides are enzymatic hydrolysis and fermentation of different precursor proteins (Iwaniak et al., 2019; Wu et al., 2019). Proteins derived from natural sources, such as plants, bacteria, fungi, and animals, have been extensively studied as sources of bioactive peptides. In addition, bioinformatics, using *in silico* analysis tools as a new method to discover bioactive peptides, has also made significant progress (Salim and Gan, 2020). Numerous food companies developed food-derived bioactive peptide products and claimed health effects from consuming these functional foods (Hartmann and Meisel, 2007). For example, Calpis Co. (Tokyo, Japan) developed a commercially available sour milk that contained peptides VPP and IPP from β-and κ-casein and declared it had a hypotensive function. The glycomacropeptide sold by Davisco (Minnesota, USA) had anti-cariogenic, antimicrobial, and antithrombotic functions. In non-food matrices, bioactive peptides also provide specific health-promoting effects. For instance, Recaldent™ and Trident™ added bioactive peptide CPP to chewing gum to help remineralize enamel subsurface lesions (Shen et al., 2001). In addition, bioactive peptides also have great potential as drug components. In state-of-the-art peptide drug discovery, display technologies, such as phage display, yeast display, and ribosome display, and integrated venomics are prominent (Muttenthaler et al., 2021). There are around 80 peptide drugs on the global market (Lau et al., 2018), and the global peptide drug market is predicted to exceed $60 billion by 2026.

However, there are stability challenges with the use of bioactive peptides as functional foods or drugs. Therefore, the evaluation of peptide stability is an essential part of peptide characterization (Cavaco et al., 2021). First, scaling up production often results in irreproducible peptides. Unguaranteed quality stability will hinder the commercial production of bioactive peptides. Second, the most convenient and acceptable delivery method of bioactive peptides is oral administration (Yap and Gan, 2020). However, gastrointestinal digestion and rapid metabolism in plasma, liver, and kidney with short half-lives ($t_{1/2}$) greatly affects the efficacy of bioactive peptides *in vivo*. Therefore, the application of bioactive peptides will be greatly hindered if high doses and frequent administration are required to ensure efficacy. As stated above, the evaluation of bioactive peptide stability has become critical to the development of this industry (Lee et al., 2019; Panchin et al., 2020).

This review summarizes the stability challenges associated with the production and administration of bioactive peptides, including i) product quality, ii) physicochemical properties, iii) gastrointestinal digestion, and iv) metabolism. In addition, we provide future directions for the improvement of bioactive peptide stability in commercial food health-enhancing compositions and pharmaceutical ingredients. Furthermore, we promote the establishment of a standard stability evaluation test and a unified stability evaluation index.

2. Quality stability of peptide products generated by enzymatic hydrolysis

An increasing number of peptide products appear on the market annually with the gradual verification of many functions of food-derived bioactive peptides. Manufacturers claim their products are functional peptides or contain functional peptides. Japan, the United States, and Europe have taken the lead in launching bioactive peptide products with various functions, forming an industry with great commercial prospects. At the same time, the evaluation of the quality of bioactive peptide products has become an urgent problem.

The most common peptide production method is enzymatic hydrolysis, followed by fermentation of different precursor proteins. Generally, bioactive peptide products derived from natural products, especially food, are not single peptides with high purity. The separation and purification of bioactive peptides from by-product proteins are currently receiving extensive attention. In particular, the by-products of fish and poultry are inhomogeneous, and the ingredients vary greatly, leading to complex protein hydrolysates with variable composition (Mage et al., 2021). In order to improve the quality stability of peptide products, greater consistency from batch to batch is needed. For example, the sequence and content of the peptides obtained by enzymatic hydrolysis should be approximately the same to ensure stable biological activity. Therefore, ensuring the stable quality of different batches of peptide products is worthy of further research. In addition, our experience and the production practices of cooperative companies have demonstrated the challenges in producing precisely the same peptide products generated by enzymatic hydrolysis, even under the same conditions and raw materials. This has been attempted in products, such as yak bone peptides, broccoli stem and leaf peptides, and sea cucumber peptides. Processing conditions, such as temperature, fermentation, and hydrolysis time, can affect the reproducibility of peptides, especially when protein mixtures exist in the substrate (Singh et al., 2021). Even when these factors are controlled and the process is consistent, minuscule and intrinsic batch-to-batch heterogeneity is still unavoidable. Therefore, it is necessary to explore the quality evaluation indicators, such as the content of bioactive peptides, activity stability, and the mechanism of activity stability. The traditional quality evaluation of bioactive peptide products is mainly carried out from the following aspects: the color and appearance, the molecular weight distribution of the peptide, the type and content of amino acids, and the purity and extraction methods for the peptide. However, studies on the functional stability of bioactive peptides are lacking. The functional stability of peptides is related to the quantity and content of specific functional peptides in bioactive peptide products. Therefore, the current research hotspots and difficulties lie in the repeatability and accurate quantification of the characteristic active peptide sequences.

3. Physicochemical property stability of peptides

Proteins are generally composed of a complex network of stable interactions, including hydrophobic interactions, salt bridges, and hydrogen bonds, that result in a unique 3D structure that is significantly stable, compared to other conformations (D'Addio et al., 2016). The bioactive peptides formed after protein degradation appear more "dynamic" in that the energetic barriers to transition among different conformations are usually small, compared with rigid proteins. The link between peptide structure and stability is unique to each individual peptide sequence. Different amino acids vary in their sensitivity to environmental factors, such as heat, pH, and salt. Heat treatment would change the secondary structure of peptides and make the peptide inactive, which might be due to the aggregation (Enciso et al., 2015). pH conditions could cause changes in the ionization properties of peptides and lead to electron transfer. For instance, threonine, serine, and cysteine are unstable under alkaline conditions (Lopez-Sanchez et al., 2016; Wang et al., 2017). The concentration of salt may affect the structure of the bioactive peptides due to changes in the side chains of amino acid residues under high salt concentrations that may result in a decrease in biological activity (Zhu et al., 2014).

The sequences of peptides greatly influence their physicochemical property stability. Guruprasad et al. (1990) reported the correlation between the stability of a protein and its dipeptide composition, which revealed the occurrence of certain dipeptides was significantly different in the unstable proteins, compared with that in the stable ones. The ProtParam tool of the Expasy database was used in this method to estimate the stability of peptides (Gasteiger, 2005). Peptide VLSTSFPPK was purified and identified from *Kluyveromyces marxianus* protein hydrolysate. If the proline in the antepenultimate position was replaced with cysteine, tyrosine and histidine, the new peptides VLSTSFCPK, VLSTSFYPK, and VLSTSFHPK would have lower stability against the NaCl treatment in DPPH assay (Mirzaei et al., 2020a,b). As a result of

exposure to high concentrations of salt, a conformational change in the side chains of cysteine, tyrosine, and histidine may explain the lower stability of VLSTSFCPK, VLSTSFYPK, and VLSTSFHPK (Mirzaei et al., 2020a,b). Peptide KLTWQELYQLKYKGI (KI-15) had exceptional thermal stability, but the stability decreased when the 10th-position leucine was replaced by phenylalanine and valine (T_m from 78.3°C to around 40°C). Computational and experimental studies showed heat resistance was based on the hydrophobic interaction of leucine-7 and leucine-10; therefore, the substitution of leucine in the 10th-position would lead to a substantial decrease in T_m (De Rosa et al., 2021). By replacing the dehydroalanine with dehydrobutyrine at position 5 of antimicrobial peptide Nisin, the resistance to acid-catalyzed chemical degradation of the peptide was enhanced.

In addition, the amino acid sequences affect the structural characteristics of the bioactive peptides, such as α-helices and β-sheets. Structural characteristics play an essential role in the stability of bioactive peptides. For example, the reduction in the peptide-folded helix group was highly correlated with a loss in thermal stability (Diana et al., 2010). Asaduzzaman and Chun (2015) and Udenigwe and Fogliano (2017) also reported bioactive peptides with a high proportion of β-sheet structures would be more sensitive to heat treatments. Mirzaei et al. (2020a,b) believed the higher percentage of β-sheet structures in peptide VLSTSFCPK was the reason for its higher sensitivity to heat treatment, compared with other similar peptides. The stability of the physicochemical properties of the peptide itself is the basis for its development and utilization.

4. Gastrointestinal digestion stability

Currently, clinically mature peptide therapy is most commonly administered via intramuscular injection, sustained-release subcutaneous injection, and intranasal delivery. As a typical delivery, hydrophobic depots could increase the duration of action (Mitragotri et al., 2014). However, oral administration is the most convenient and acceptable delivery method of bioactive peptides, especially for the application of peptides as daily therapy for chronic disease management. Many peptides will be digested into inactive peptide fragments or free amino acids and lose activity *in vivo* after oral administration; however, these same peptides may have significant biological activity *in vitro*. For example, Li et al. (2018) identified a novel H1N1 virus neuraminidase inhibitory peptide, PGEKGPSGEAGTAGPPGTPGPQGL, from cod skin hydrolysates, and this peptide was almost inactivated after simulated gastrointestinal digestion. Most bioactive peptide drugs, such as insulin, have to be administered by parenteral routes, which is inconvenient and painful (Di, 2015). Therefore, the ability to resist digestion by gastrointestinal protease is critical to bioactive peptides.

4.1. Gastrointestinal digestion of peptides

Susceptibility to the action of gastrointestinal proteases, such as pepsin, trypsin, and chymotrypsin, is one of the important reasons for the short half-life of bioactive peptides (Wang et al., 2019). The cleavage specificity for pepsin (EC 3.4.23.1) lacks specificity, preferentially before or after Trp, Tyr, Leu, or Phe, with other restrictions in hydrophobic amino acid sites. Trypsin (EC 3.4.21.4) was found in pancreatic secretions, which specifically hydrolyzes amino acid residues Lys and Arg of the C-terminal. The chymotrypsin (EC 3.4.21.1) belongs to the serine protease family, and the hydrolysis side chains are composed of Phe, Tyr, and Trp, as well as other large hydrophobic side chains with specificities, such as Met and Leu. The presence of these gastrointestinal cleavage sites on the peptide is an important cause of its gastrointestinal instability. At present, the methods for evaluating the gastrointestinal resistance of bioactive peptides are mainly *in silico* simulation and *in vitro* enzymatic hydrolysis assay.

4.2. *In silico* simulation of gastrointestinal digestion stability

The "Enzyme(s) Action" application in the BIOPEP-UWM database (Minkiewicz et al., 2019) and "PeptideCutter" application in the ExPASy database (Wilkins et al., 1999) are commonly used to predict the hydrolytic actions of gastrointestinal proteases. The activity of chicken breast muscle hydrolysate-activated alcohol dehydrogenase was significantly reduced after intestinal digestion, and *in silico* assessments subsequently revealed the degradation of peptides KDLFDPVIQ, YPGIADRM, VAPEEHPTLL, and ADGPLKGIL might be responsible for the reduced activity (Xiao et al., 2020). However, there are differences between simulated digestion *in silico* and actual gastrointestinal digestion. Simulating gastrointestinal digestion in the database, based on the specificity of an enzyme, assumes that all bonds theoretically susceptible to a given proteinase are hydrolyzed (Minkiewicz et al., 2008, 2019). The proteolysis is often incomplete under actual conditions. *In silico* hydrolysis might not include the conditions, such as pH, temperature, time, the nature of protease-protein interactions, the complexity of the gastrointestinal environment, and other characteristics of enzyme application (Udenigwe, 2014; Vermeirssen et al., 2004). Moreover, the availability of peptide bonds also influences the effectiveness of enzyme action (Vermeirssen et al., 2004). Therefore, there are cases in which sites that could not be cut are cut, and *vice versa*.

4.3. *In vitro* simulation of gastrointestinal digestion stability

In vitro simulation of gastrointestinal digestion provides simple and inexpensive way for researchers to estimate the gastrointestinal stability of bioactive peptides. Peptide stability has been evaluated by revealing the peptide sequence and the role of each amino acid (Mirzaei et al., 2021). The consensus is that high molecular weight peptides are more prone to hydrolysis than smaller ones. Chen and Li (2012) proved casein-derived peptides with molecular weight >3 kDa are more likely to be degraded than peptides <3 kDa during two-stage *in vitro* gastrointestinal digestion. Compared with gastric digestion, peptides are more prone to hydrolysis in the intestine. In addition, bioactive peptides with more acidic amino acids (Picariello et al., 2010) and higher hydrophobicity (Xie et al., 2015) would be more stable during gastrointestinal digestion. In addition, the stability of peptides is influenced by peptide sequences (Mirzaei et al., 2020a,b). A lack of cleavage sites for gastrointestinal proteases is essential for improving gastrointestinal stability. Proline and glutamic acids are conducive to improving the resistance of bioactive peptides to pepsin and pancreatin (Savoie et al., 2005). However, experiments performed by different teams vary in their methods of simulating digestion.

For gastric digestion, Hao et al. (2020) adjusted the pH to 3 and incubated the sample (1 mg/mL) with pepsin (1000 U/mL) at 37°C for 120 min. Differently, Yuan et al. (2018) adjusted the pH of pepsin (enzyme/substrate of 1:35, w/w) to 2.5 and incubated the mixture at 37°C for 60 min. When simulating the gastrointestinal digestion of casein-derived peptides, peptides were adjusted to pH 2 and hydrolyzed with pepsin (2% w/w) at 37°C for 90 min (Chen and Li, 2012). Marseglia et al. (2019) mixed sodium and potassium salts with pepsin (25,000 U/mL) to prepare simulated gastric juice and incubated the sample with simulated gastric juice at 37°C for 2 h after adjusting to pH 3. The digestion stability of peptides is commonly assessed by dividing the remaining amount of peptide after digestion by the amount of peptide before digestion. Different amounts of enzyme added and different reaction conditions (such as pH, incubation time, and solution constituent) will inevitably lead to different results.

In summarizing the reported methods of intestinal digestion, differences still exist among the digestion methods. Furthermore, the source of the enzymes is crucial, but porcine and bovine trypsin were used in different studies to evaluate the digestion stability of peptides. Baptista et al. (2020) prepared pancreatic juice mixed with pancreatin (from porcine pancreas, 800 U/mL) and bile solution *in vitro*. After adjusting to

pH 7, the samples were digested at 37°C for 2 h. Jin et al. (2016) used trypsin (1:100, w/w) from bovine pancreas to simulate the intestinal digestion of yogurt.

Different experimental reagents and methods will affect the results. Ahmed et al. (2022) summarized 31 articles about the simulated digestion stability of peptides, including 27 stable peptides and 66 unstable peptides. However, due to the differences between evaluation methods, it is difficult to directly compare these peptides. For example, if under the same experimental conditions, a peptide that was reported to be unstably incubated for 2 h may be more resistant to gastrointestinal digestion than a peptide that was reported to be stably incubated for 1 h. In addition, a simple mixture solution is different from actual body fluids, which may lead to differences between digestion *in vitro* and *in vivo* (Cavaco et al., 2021). Fortunately, more and more studies have reported digestion simulation methods closer to the real human body conditions in recent years, such as the studies by Brodkorb et al. (2019) and Minekus et al. (2014) Moreover, some investigators have developed methods of *in vivo* digestion in mice instead of *in vitro* stimulation (Fan et al., 2019; Tu et al., 2019).

4.4. Stability of peptides on the transepithelial transport

The extensive enzymatic system in the gastrointestinal tract, epithelial cells, and mucosa constitute a significant barrier. Whether bioactive peptides can resist hydrolysis by brush border membrane enzymes and travel intact through intestinal epithelial cells is important. Caco-2 cells, human colon cancer cells, are widely used to simulate the transport and stability of bioactive peptides across intestinal epithelial cells (Ohsawa et al., 2008). The apparent permeability coefficient values of bioactive peptides were mainly between 10^{-8} and 10^{-6} across the Caco-2 cells monolayer. Pep T1-mediated transport, paracellular diffusion, and transcytosis are primary transfer methods across intestinal epithelial cells (Fig. 1). Several metabolic enzymes on the Caco-2 cells membrane may reflect drug absorption (Teng et al., 2012). The resistance of different peptides against Caco-2 enzymes varies widely. For example, 96% of the ACE-inhibitory peptide QIGLF keep stable after incubation with a Caco-2 cells membrane for 2 h (Ding et al., 2014), while the peptides AAATP, AAPLAP, KPVAAP (Gallego et al., 2016), and YAEERYPIL (Miguel et al., 2008) were almost entirely degraded after 2 h.

5. Metabolism stability

Bioactive peptides show great potential in function foods and pharmaceutical engineering, due to their high potency and low toxicity. However, most peptides have short $t_{1/2}$ of 2–30 min because of the broad distribution of peptidases in the body (such as plasma, kidney, and liver). After absorption, bioactive peptides are often rapidly degraded by proteases or rapidly cleared in plasma, kidney, and liver. For example, the $t_{1/2}$ of bioactive peptides VP, VPY, VPYP, and YPQ were less than 5 min (Zheng et al., 2019). Giapreza® (DRVYIHPF), approved by the Food and Drug Administration (FDA) in 2017, is metabolically unstable in the human body and its estimated $t_{1/2}$ is 30 s. The short $t_{1/2}$ means that an increase in dosage or repeated administration is required for efficacy, which significantly limits the development of bioactive peptides.

5.1. Metabolism stability in blood

Digestion in the gastrointestinal tract can still be avoided by changing the route of administration, but metabolism after entering the bloodstream is an unavoidable problem. Peptidase genes account for 3.1% of all encoded human proteins, making peptidases one of the most abundant classes of enzymes and widely distributed in the body (Klein et al., 2018). When the bioactive peptides are absorbed into the bloodstream through the epithelial cells of the small intestine, a large number of proteases and peptidases present in the blood will act on the peptides (Fig. 1). Peptidases commonly found in the blood include carboxypeptidase N (cleavage site specificity: Lys, Arg, Ala, and Gly), aminopeptidase N (cleavage site specificity: Ala, Leu, Arg, Trp, Leu, and Phe), DPP4 (cleavage site specificity: Pro and Ala), plasmin (cleavage site specificity: Arg and Lys), furin (cleavage site specificity: Arg, Ser, and Lys), and neprilysin (cleavage site specificity: Phe, Leu, Try, Ile, and Val) (Lai et al., 2021). Bottger et al. (2017) incubated eight peptides with blood, plasma, and serum. Except for one stable peptide, the degradation products of the remaining seven peptides were detected in blood, plasma, and serum. When assessing the serum/plasma stability of bioactive peptides, the assays vary from different study to study, such as the species source of the plasma or serum, commercial plasma or serum and fresh acquisition, peptide/plasma or serum ratio, pH value, and buffer. These all affect the results. For example, to assess its stability, Yang et al. (2017) incubated an antithrombotic peptide RGDWR (200 ng/mL) with rat plasma. Low et al. (2020) used complete human EDTA blood, obtained from the Red Cross Blood Donor Service, to evaluate the plasma stability of a proopiomelanocortin-derived peptide (2 μM). Wang et al. (2021) mixed physiological saline-containing peptide (1 mg/mL) with fresh human plasma (1:1, v/v) to investigate the effect of structural modification of antimicrobial peptides on their plasma stability. Moreover, commercial human serum is to be used with caution because a lack of homogeneity between batches can lead to variable activity (Cavaco et al., 2021).

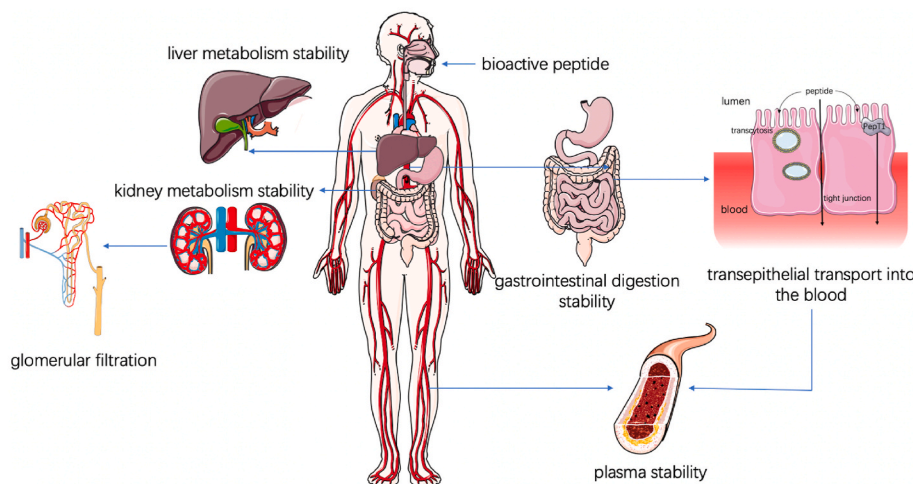

Fig. 1. Stability challenges encountered by bioactive peptides *in vivo*, including gastrointestinal digestion and metabolism.

5.2. Metabolism stability in kidney and liver

Although there are over 44 kinds of tissues, the enzymatic breakdown of bioactive peptides mostly happens in the kidney and liver after reaching organs throughout the body (Hall, 2014). In addition, because the pore size of the glomerulus is about 8 nm and the molecular weight of bioactive peptides is less than 25 kDa, peptides are easily filtered and rapidly removed from systemic circulation by the kidney (Fig. 1) (Katsila et al., 2012). Subsequently, bioactive peptides enter the bladder and excrete from the body because the renal tubules cannot reabsorb (Chia, 2021). The liver is the organ with the most active metabolism and plays an essential role in peptide metabolism. The metabolic rate of bioactive peptides in the liver depends on the amino acid sequence and the specific intake of hepatocytes (Meibohm and Zhou, 2012). At present, there are still few studies on liver and kidney metabolism of food-derived bioactive peptides, as the majority focus on mature peptide drugs. Cetrotide®, a decapeptide containing five unnatural amino acid residues approved by the FDA in 2000, was hydrolyzed by peptidase between Ser-4 and Tyr-5 and became a tetrapeptide before renal elimination (Chia, 2021). Firmagon®, a decapeptide, was degraded in the liver and excreted mainly as tetrapeptide and pentapeptide fragments from the urine (Sonesson and Rasmussen, 2013). Firazyr®, a decapeptide, was metabolized into two inactive fragments through the action of plasma and liver peptidase and excreted by the kidney (Bork et al., 2007; Leach et al., 2015).

6. Strategies to ensure the quality of peptide products

Reproducibility and stability are vital for the entrance of peptide products into the market. Therefore, it is crucial to establish quality control indicators to evaluate product stability. Lopez-Morales et al. (2019) used size exclusion chromatography (SEC), mass spectrometry (MS), and nuclear magnetic resonance spectrometry (NMR) to evaluate the peptide profile, and determined the safety of collagen hydrolysates as a quality indicator. By determining the average molecular weight and polydispersity index with MS and NMR, Vazquez-Leyva et al. (2019) used structural and mass mobility orthogonal analysis to profile complex peptide mixtures. In addition, Mage et al. (2021) studied the potential of using a large Fourier-transform infrared (FTIR) fingerprinting database to obtain new insights on the quality of peptide products.

Traditional Chinese medicines (TCM) are small molecule mixtures with complex component. The fingerprint of TCM is a quality control method for extracts and preparations, which has become an international consensus (Tao et al., 2017). The FDA accepts TCM-style chromatographic fingerprints in application materials. The World Health Organization (WHO) also stipulated in the 1996 guidelines for the evaluation of herbal medicines that if the active ingredients of any given TCM are not clear, chromatographic fingerprints can be provided to prove the product's quality. Chromatographic, spectroscopic, mass spectrometry, and capillary electrophoresis are widely used in fingerprinting. The application of fingerprints may solve the problems of the quality assessment of TCMs, which often have complex ingredients, unidentified active ingredients, and differences in quality across product batches. Taking the *Ginkgo biloba* extract preparation as an example, these fingerprints reflect the 33 chemical components (mainly flavonoids and lactones) contained in the preparation and their respective concentrations. Research on the correlation between chemical composition and drug efficacy has shown that extracts composed of about 24% ginkgo flavonoids and about 6% ginkgolide have the best curative effects. The reproducibility of the fingerprint spectrum is good. Crude peptide products generated by enzymatic hydrolysis of protein usually contain different peptide sequences. Learning from the quality control experience of TCM, qualitative and quantitative characteristic function peptide sequences combined with fingerprints may play an essential role in the quality control of crude peptide products.

7. Strategies to improve the stability of bioactive peptides

7.1. Modification

At present, chemical modification is one of the primary measures to improve the stability of bioactive peptides, including D-amino acid or unnatural amino acid substitution, increased molecular mass, cyclization, and N/C-terminal modification or substitution (Fig. 2 & Table 1) (Yao et al., 2018). D-amino acid substitution is a common strategy to improve the stability of peptides. Mao et al. (2021) reported the introduction of D-amino acid promoted the stability of antimicrobial peptides against plasma and liver S9 metabolism when all amino acids of IRIKIRIK were in D configuration. Jia et al. (2017) reported the same D-amino acid substitution strategy to improve the stability of an antimicrobial peptide polybia-CP. A20FMDV2 is a 20-mer peptide, and D-amino acid substitutions could significantly prolong its serum stability (Cardle et al., 2021). Substituting L-amino acid in cationic AMP peptide KRLFKKLLKYLRKF with D-amino acid and unnatural amino acids, the stabilities of the peptides toward proteases were enhanced (Lu et al., 2020). Chia (2021) identified 25 peptide drugs approved by the FAD, 20 of which contain unnatural amino acids. Unnatural amino acid substitution could significantly extend the systemic half-life of bioactive peptides. Not many peptide drugs are composed of natural amino acid residues, as represented by vasopressin, angiotensin II, plecanatide, linaclotide, and oxytocin.

Based on the size-dependent mechanism of renal clearance, increasing the molecular mass of bioactive peptide is an excellent strategy to increase their blood circulation times and extend their half-lives (Maack et al., 1979). Therefore, linking bioactive peptides to large polymers was widely used to avoid renal filtration because polypeptides with molecular mass of 50 to 70 kDa would be retained longer in circulation. Conjugation to polyethylene glycol (PEG) has widely been employed. The $t_{1/2}$ of bioactive peptides attached to PEG could be extended 10- to 100-fold. A PEGylated analogue of the gut hormone oxyntomodulin is in phase I trials for treating obesity (Bianchi et al., 2013), which has long-term antihyperglycemic, insulinotropic, and anorexigenic effects. Zhou et al. (2009) used methoxy-PEG-aldehyde to modify the N-terminal of an RGD-modified endostatin–derived synthetic peptide, and the $t_{1/2}$ was prolonged by 5.86-fold. Conjugation to lipidation, plasma proteins, and albumin-binding molecules was used to increase the size of bioactive peptides (Pollaro and Heinis, 2010). Albumin ligand-conjugated thymopentin (RKDVY) became more stable in plasma and had better immune-modulating activity (Tan et al., 2017).

Cyclization is another common method of modification of peptides to improve their stability and activity. Endogenous opioid peptides have great potential in treating pain. However, natural opioid peptide receptors have poor specificity and unstable metabolism, and cannot reach the brain after systemic administration. The cyclized opioid peptide has a longer half-life and higher metabolic stability while retaining its analgesic activity (Piekielna et al., 2013). Vernen et al. (2019) demonstrated backbone cyclization of tachyplesin peptides improved their stability and properties of antimicrobial and anticancer. Methods of cyclizing bioactive peptides have also been reported more in recent years. For example, Zhang et al. (2019) reported a highly chemoselective and simple method of cyclizing bioactive peptides. The reaction was readily accomplished and was found to be capable of tolerating different functionalities. Yamagami et al. (2021) demonstrated the peptide cyclization strategy by combining a soluble-tag-assisted liquid-phase peptide synthesis with a backbone amide linker. In addition to chemical peptide ligation, naturally occurring and engineered enzymes are also used for peptide cyclization (Nuijens et al., 2019). Toplak et al. (2016) reported a peptiligase that could efficiently catalyze head-to-tail peptide cyclization. Thioesterase SurE could cyclize two distinct non-ribosomal peptides (Matsuda et al., 2019).

N/C-terminal modifications are widely used to protect bioactive peptides against endogenous exopeptidases. An antimicrobial peptide

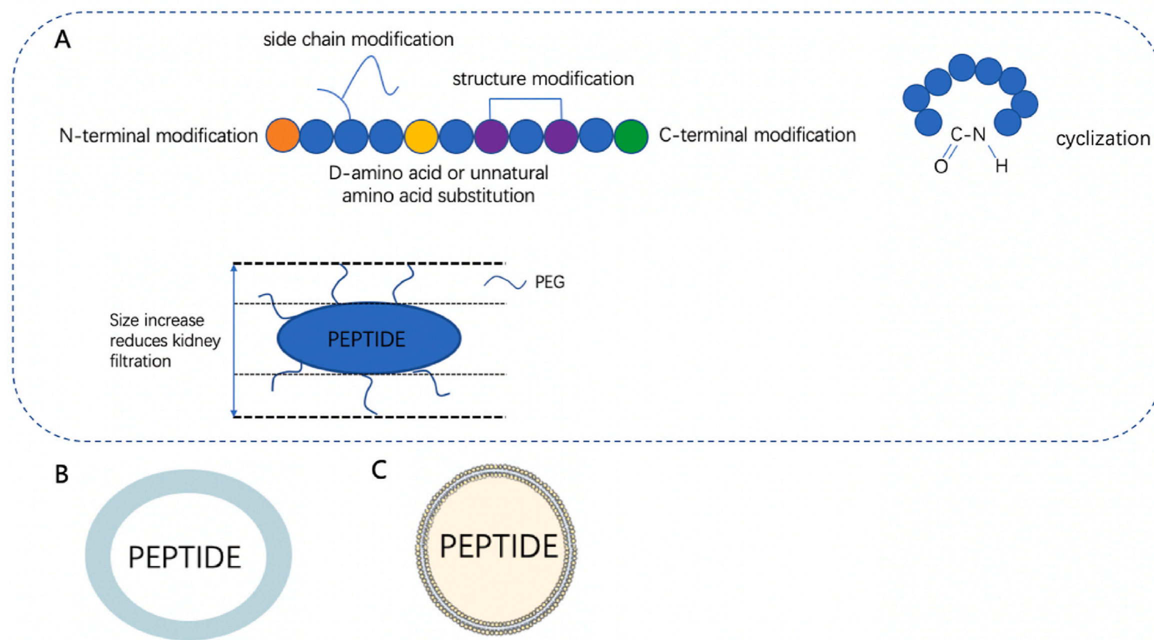

Fig. 2. Different strategies to improve the stability of bioactive peptides (A) Various chemical modification, (B) Microcapsules, and (C) Liposome.

Table 1
Summary of the strategies to improve the stability of bioactive peptides.

Strategies	Applications	Examples	Ref.
Chemical modification	D-amino acid substitution	IRIKIRIK	Mao et al. (2021)
		Polybia-CP	Jia et al. (2017)
		A20FMDV2	Cardle et al. (2021)
		KRLFKKLLKYLRKF	Lu et al. (2020)
	Unnatural amino acid substitution	Afamelanotide Bivalirudin Cyclosporine Pasireotide	Chia (2021)
	Increase molecular mass	Endostatin-derived peptide	Zhou et al. (2009)
		RKDVY	Tan et al. (2017).
	Cyclization	Cyclized opioid peptide	Piekielna et al. (2013)
		Tachyplesin peptides	Vernen et al. (2019)
	N/C-terminal modification or substitution	Antimicrobial peptide	Li et al. (2021)
		PMAP-36PW	Liu et al. (2020)
		RLYE	Yun et al. (2019)
		A20FMDV2	Hung et al. (2017)
Encapsulation	Microcapsules	Antimicrobial oyster peptides	Zhang et al. (2009)
		Phaseolus lunatus L. peptides	Cian et al. (2019)
		α-helical polypeptide	Morikawa et al. (2005)
		Insulin	Aiedeh et al. (1997)
	Liposome	Antioxidant peptide	Ramezanzade et al. (2021)
		RLSFNP	Zhang et al. (2019)
		Insulin	Cui et al. (2015)

reported by Li et al. (2021) was easily degraded in the plasma, and acetylating its N-terminal improved the stability of peptides against pH, plasma, and trypsin degradation. Liu et al. (2020) demonstrated N-terminal myristoylation enhanced the antimicrobial activity of PMAP-36PW. N-terminal acetylated peptide RLYE showed better anti-tumor activity, due to a decrease in the susceptibility to serum peptidases (Yun et al., 2019). C-terminal modification could impact bioactive peptide stability, membrane permeability, and activity (Arbour et al., 2020). Through generating C-terminal peptide amides of antibacterial peptides, a head-to-tail cyclized antibacterial peptide was synthesized, which exhibited higher proteolytic stability (So et al., 2021). A modified 20-residue peptide A20FMDV2, with N-terminal D-Asn and C-terminal D-Thr, showed improved activity by reducing susceptibility to plasma degradation (Hung et al., 2017).

In addition, side-chain modifications, backbone modification, and alkylation of amide nitrogen are all chemical modifications widely used in bioactive peptides (Pernot et al., 2011). The biochemical modification has received more and more attention in recent years (Gao et al., 2018). Some unmodified bioactive peptides have astonishing resistance to proteolysis, but the determinants of this unusual phenomenon remain mysterious (Bottger et al., 2017; Werner et al., 2016). Demystifying the mechanism behind it is an important direction of future efforts.

7.2. Encapsulation

Modifications are often used for target bioactive peptides that have the potential to be developed as drugs. However, encapsulation methods are more acceptable and inexpensive for bioactive peptides as functional foods.

Microcapsules and microgels are ideal for bioactive peptides delivery applications. The bioactive peptides embedded in microcapsules have a range of advantages, including resistance to digestion in the gastrointestinal system and prevention of chemical and enzymatic degradation to ensure biological activity (Malmsten, 2006). They can also control the release rate and reduce adverse immune side effects (Frokjaer and Otzen, 2005). Zhang et al. (2009) reported alginate/chitosan/starch microcapsules that could deliver antimicrobial oyster peptides to the intestine, and the microcapsules showed a sustained-release effect.

Phaseolus lunatus L. peptides embedded in maltodextrin/gum arabic microcapsules preserved their α-glucosidase, α-amylase, and dipeptidyl peptidase-IV inhibitory activity through gastrointestinal digestion (Cian et al., 2019). Morikawa et al. (2005) prepared α-helical polypeptide microcapsules using emulsion-templated self-assembly of amphiphilic poly(γ-benzyl L-glutamate)s (PBLG), which was stable in a dry environment. Chitosan microcapsules could ensure the stable release of insulin over a long period (Aiedeh et al., 1997). The wall material is the primary determinant of microcapsule performance. Natural polymers (such as sodium alginate, chitosan, and cyclodextrins), fully synthetic polymers (such as polylactic acid/glycolic acid copolymer and polycaprolactone), semi-synthetic polymers (such as ethyl cellulose), and inorganic materials (such as calcium carbonate, silicate, double metal hydroxide, phosphate, and clay) have been used to prepare microcapsules. The release of microcapsules depends on pH values, temperature, ionic strength, particle sizes, and additives. At present, the preparation technology of microcapsules focuses on improving the encapsulation efficiency and prolonging the drug release. Furthermore, safer and non-toxic wall materials derived from food ingredients can provide new directions for microcapsules. The development of strategies to better ensure the biological activity of bioactive peptides in microcapsules, particularly those stored at room temperature for extended periods, is also needed in future efforts.

Liposomal packaging is another strategy used to improve bioactive peptide stability. Liposomes, with a microvesicle structure, consist of an aqueous core and an amphiphilic bilayer. Niu et al. (2014) found liposomes containing bile salts showed longer residence time and stronger transmembrane permeability *in vivo*. Ramezanzade et al. (2021) prepared liposomes coated with chitosan cross-linked with sodium tripolyphosphate and embedded fish-purified antioxidant peptide. The bioavailability of milk-derived ACE-inhibitory peptide RLSFNP after liposome encapsulation was improved and had better intestinal absorption. Parmentier et al. (2011) investigated tetraether lipid liposomes, which could deliver oral peptides. The plant-derived ergosterol liposomes screened by Cui et al. (2015) could significantly improve the oral bioavailability of insulin. Liposomes should maintain their vesicular morphology and avoid early leakage of encapsulated material by resisting destruction by bile salts, pancreatic enzymes, and acidic conditions to improve the oral bioavailability of encapsulated bioactive peptides in the gastrointestinal tract.

8. Conclusions

Bioactive peptides have been developed and advanced over the past two decades and have applications in novel foods and pharmaceuticals. However, their stability continues to be an important challenge to overcome. For the quality control of peptide products, we suggest the method of fingerprint for qualitative and quantitative analysis of specific peptide segments as a quality control index. In order to ensure the efficacy of bioactive peptides, peptide products must have stable physicochemical properties, resistance to gastrointestinal digestion, and specific metabolic stability, so as to achieve an appropriate retention time in the body. Chemical modification and encapsulation in microcapsules or liposomes are two mainstream methods to improve the stability of bioactive peptides. We believe stability studies are essential and will prove highly beneficial to bioactive peptide development. Moreover, we found that different researchers used various methods for mainstream evaluation tests, such as *in vitro* gastrointestinal digestion and plasma stability, making it difficult to compare the data between different reports. Therefore, establishing a standard stability evaluation test and a unified index are necessary.

CRediT authorship contribution statement

Jingyan Pei: reviewed the literature and wrote the manuscript. **Xinchang Gao:** with manuscript revised and discussions. **Daodong Pan:** contributed with discussions. **Ying Hua:** contributed with discussions. **Jun He:** contributed with discussions. **Zhu Liu:** contributed with manuscript revised and discussions. **Yali Dang:** contributed with manuscript revised and discussions.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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