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Review Article

Insights into the preparation of selenopeptides awaiting functional validation for fatigue mitigation: a comprehensive review

Yuxiao Guo[1], Wei Jia[1,2] ✉, Rong Zhang[1], Bo He[2], Fen Ao[1], Haizhen Mo[1] ✉

[1] *School of Food Science and Engineering, Shaanxi University of Science & Technology, Xi'an 710021, China*
[2] *Ankang Research and Development Center for Se-enriched Products, Ankang 725000, China*
✉Address correspondence to Wei Jia, jiawei@sust.edu.cn; Haizhen Mo, mohz@sust.edu.cn
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Abstract: Fatigue, a debilitating condition arising from intense physical and mental stress, significantly impairs work capacity and well-being, yet effective molecular interventions remain scarce. Selenopeptides, which incorporate the essential trace element selenium, have emerged as promising anti-fatigue agents owing to their potent antioxidant activities. However, their therapeutic development is hindered by conventional preparation methods, which yield heterogeneous mixtures with poor reproducibility, complicating purification and impeding structure-activity studies. This review emphasizes that chemical synthesis represents a critical solution to these challenges, enabling the production of highly pure, structurally defined selenopeptides. We systematically analyze recent advances in solid-phase peptide synthesis and liquid-phase peptide synthesis, highlighting innovative and sustainable approaches such as continuous flow synthesis. Although few chemically synthesized selenopeptides have been directly validated for anti-fatigue efficacy, we propose a rational design strategy based on targeted synthesis to bridge this translational gap, accelerating their application in the food and health industries.

Keywords: selenopeptide; anti-fatigue; chemical synthesis; solid-phase peptide synthesis; liquid-phase peptide synthesis

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1 Introduction

Exercise-induced fatigue has become a widespread public health concern. It indicates the body's inability to maintain the desired level of physical or cognitive performance, resulting from various factors that impair muscle function or mental capacity. The onset of this condition mainly stems from the body's stress metabolic response to exercise and related factors[1], which can serve as an early indicator of more severe health conditions, such as cancer, anemia, sudden death due to overexertion, and chronic fatigue syndrome, all of which pose significant threats to overall well-being. Consequently, identifying effective anti-fatigue agents has become a key focus of current research. Bioactive substances, e.g., polysaccharides, polyphenols, proteins, and peptides, represent major sources of anti-fatigue components[2]. In particular, peptides have shown considerable promise in fatigue reduction due to their low molecular weight, easy absorption, diverse activities, and no metabolic burden[3].

As an essential trace element, selenium functions as a vital cofactor for antioxidant selenoenzymes, underpinning numerous physiological processes. Selenium deficiency is associated with diseases such as Keshan disease and cardiovascular disorders[4]. The normal daily intake of selenium for the human body ranges from 50 to 200 μg[5]. According to the *Chinese dietary reference intakes-Part 3: trace element* (WS/T 578.3–2017), the maximum tolerable daily intake for adults is established at 400 μg. While selenium supplementation is beneficial, excessive intake can lead to chronic poisoning, manifesting as hair loss, nail loss, skin blemishes, nervous system disorders, and even death[6]. Since the human body is unable to synthesize selenium endogenously, dietary sources represent the sole means of obtaining this crucial micronutrient. Accordingly, it is essential to precisely quantify the amount of organic selenium in diets. While inorganic forms of selenium exhibit limited bioavailability, naturally occurring organic forms such as this combination demonstrate higher absorption rates and lower toxicity[7]. Once selenium is ingested, it undergoes a series of intricate metabolic transformations within the body. These primarily involve reduction, incorporation into selenoprotein synthesis, and detoxification and excretion via methylation pathways (Figure 1). However, to date, only approximately half of the identified selenoproteins have well-characterized biological functions[8]. Notably, natural autoantibodies targeting selenium transport proteins have been found in certain patients experiencing fatigue, which are linked to impaired selenium delivery to target tissues, potentially resulting in decreased selenoprotein expression[9]. This finding suggests that fatigue mitigation may represent an additional critical biological role of selenium. Since beneficial biological functions in human health are possessed by both selenium and peptides, it is reasonable to assume that the presence of selenocysteine (Sec), selenomethionine (SeMet), and other seleno-amino acids in peptides can achieve enhanced biological effects[10]. Selenopeptides, as bioactive forms of selenium, hold significant potential for dietary strategies, as they can influence energy balance, enhance immune function, and alleviate fatigue[11].

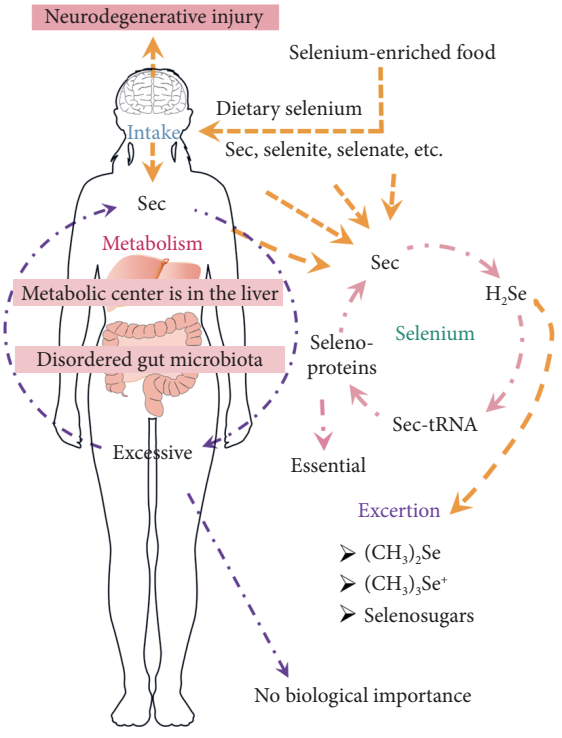

Figure 1 Metabolic activation of selenium for biological functionality. Although it is primarily involved in the synthesis of selenoproteins, selenium also participates in methylation, metabolism, and excretion pathways. Organic selenium typically exists in a reduced state (Se^{2-}), whereas inorganic selenium occurs in oxidized forms such as selenite (Se^{4+}) and selenate (Se^{6+}). Upon absorption, high-valent selenium species are reduced within the body using reducing agents such as glutathione and nicotinamide adenine dinucleotide phosphate hydrogen. Organic selenium compounds are metabolized to produce hydrogen selenide (H_2Se), which is a key intermediate. This is then converted into selenate by selenophosphatase. Selenophosphate subsequently undergoes a reaction with serine residues attached to tRNA, generating Sec-tRNA. Within this complex, Sec ultimately forms the catalytic core of selenoproteins. Synthesized primarily in the liver and secreted into plasma, selenoprotein P plays a key role in distributing selenium throughout the body to different tissues, supporting the production of additional selenoproteins. Surplus selenium undergoes detoxification through methylation, converting H_2Se into metabolites including trimethylselenonium, dimethylselenide, and selenosugars.

Current research mainly focuses on the source, amino acid composition, and anti-fatigue mechanism of common active peptides, while insufficient attention is paid to selenopeptides with more potential functions[12]. Bioactive selenopeptides are generally regarded as safe due to their production using food-grade enzymes and processes that exclude the use of synthetic or toxic chemicals. However, a major challenge lies in the limited purification capacity of conventional preparation methods, which has hindered the structural characterization and elucidation of the functional mechanisms of selenopeptides. To enable controlled synthesis and rigorous functional evaluation, it is essential to develop methods for synthesizing highly purified selenopeptides with well-defined structures and precisely controlled selenium incorporation sites. Given that these peptides have no prior history of human use, comprehensive toxicity and immunogenicity assessments are required to establish their safety for therapeutic and food-related applications[13]. This review systematically evaluates key preparation strategies, including enzymatic hydrolysis, microbial fermentation, selenium modification, and chemical synthesis, to establish a comprehensive theoretical framework and provide practical technical guidance for the efficient synthesis, functional characterization, and in-depth investigation of the anti-fatigue properties of selenopeptides. The review seeks to strengthen the scientific foundation in this field and offer novel insights into the anti-fatigue potential of selenopeptides, thereby broadening the application prospects of selenium resources and supporting the development of innovative nutritional supplements.

2 Conventional preparation strategy of selenopeptides

Selenopeptides are derived from a diverse array of sources. In addition to being extracted from selenium-enriched animal and plant proteins, as well as microbial proteins, they can also be synthesized through chemical methods. These compounds exhibit significant potential for development and possess considerable application value. Currently, the strategies for selenopeptide preparation primarily focus on three approaches: enzymatic hydrolysis, microbial fermentation, and selenium modification.

2.1 Enzymatic hydrolysis

Enzymatic hydrolysis is a mainstream natural product extraction technology that is widely used to extract selenopeptides from selenium-enriched plant and animal resources. It relies on the specific hydrolysis of selenoprotein peptide bonds by proteases. Initially, selenoproteins are extracted from raw materials through alkaline solubilization and acid precipitation, then subjected to hydrolysis by suitable proteases[14].

During enzymatic digestion, there are differences in the enzymes required for various types of proteins, and due to the different cleavage sites of different proteases, the enzymatic digests expose different peptide sequences, thus producing different hydrolysis effects. The enzymes frequently employed include trypsin, neutral, pepsin, papain, flavor protease, and so on[15] (Table 1). The dark meat of yellowfin tuna contains 18.34 mg/kg of selenium, making it an ideal natural source of selenopeptides. Five different enzymes were employed to prepare selenoprotein hydrolysates from the dark meat. Among these treatments, the protein hydrolysate generated by trypsin exhibited the highest α-glucosidase inhibition activity. Following this, selenopeptide Lys-Pro-Leu-Sec-Pro-Lys was screened via *in silico* analyzes, demonstrating a reduction in insulin resistance (IR) and enhanced antioxidant capacity in IR-HepG2 cells[16]. Selenium-enriched soybean sprouts were subjected to treatment with alcalase, trypsin, and papain protease to produce selenopeptides. Among the various substrates and enzyme concentrations tested, alcalase demonstrated superior performance, yielding selenopeptides such as Leu-Ala-Pro-Glu-Se(Met)-Ala-Leu-Pro-Val-Ala-Leu, Pro-Trp-Leu-Leu-Se(Met)-Leu-Pro-Val-Pro, and Se(Met)-Leu-Val-Pro-Leu. The bioaccessibility of the selenopeptides was measured at 28.08%, representing a 2.2-fold increase compared to that of inorganic selenium[17]. Another study focused on alkaline-soluble tea products and discovered that the hydrolysate of selenium-enriched tea alkali-soluble proteins, produced through papain catalysis, exhibited superior antioxidant activity compared to those generated by other enzymes[18]. Zhu et al.[19] prepared an immobilized alkaline protease using Fe_3O_4 nanoparticles modified with tannic acid and polyethyleneimine as a carrier, and investigated its role in the hydrolysis of *Cardamine violifolia* protein. They investigated the effect of the immobilized alkaline protease on the hydrolysis of *C. violifolia* protein and concluded

Table 1 Enzymatic hydrolysis resolves selenium-enriched proteins to produce selenopeptides.

Sources	Methods	Types of proteases	Results	Reference
Yellowfin tuna dark meat	Single-enzyme hydrolysis	Trypsin, neutral, alkaline protease, papain, and flavor protease	Trypsin fragmented the yellowfin tuna dark meat into smaller pieces, resulting in more active selenopeptides	[16]
Selenium-enriched soybean sprouts	Single-enzyme hydrolysis	Alcalase, trypsin, and papain protease	Alcalase demonstrated superior performance, yielding notable selenopeptides. The bioaccessibility and transmembrane transport efficiency of the selenopeptides were measured at 28.08%	[17]
Selenium-enriched tea	Single-enzyme hydrolysis	Trypsin, neutral, alkaline, and papain protease	Papain-catalyzed hydrolysis yielded selenopeptides with superior antioxidant activity compared to other enzymatic methods	[18]
C. violifolia	Single-enzyme hydrolysis	Immobilized alkaline protease	An immobilized alkaline protease using Fe_3O_4 nanoparticles modified with tannic acid and polyethyleneimine as a carrier, which is stable, recyclable, and reusable	[19]
Low-fat selenium-enriched egg yolk	Single-enzyme and double-enzyme hydrolysis	Alkaline, neutral protease, and double-enzyme hydrolysis, which uses a combination of alkaline and neutral proteases	Using a combination of the two enzymes produced peptides that are smaller and more homogeneous; the ability may be attributed to their wider range of cleavage sites	[20]
Selenium-enriched soybeans	Double-enzyme hydrolysis	Double-enzyme hydrolysis, which uses a combination of neutral and alkaline proteases or alkaline and neutral proteases	The soybean protein hydrolyzed first by neutral enzymes is found to be able to expose residues that could be hydrolyzed by alkaline protease, while the residues hydrolyzed first by alkaline protease could not be further hydrolyzed by neutral protease	[21]
Selenium-enriched *Pleurotus eryngii*	Single-enzyme hydrolysis	Alcalase, pepsin, trypsin, and papain protease	Trypsin is selected to hydrolyze selenoprotein following peptide content analysis of the hydrolysate, resulting in the identification of 12 selenopeptides	[22]
Selenium-enriched oyster	Single-enzyme hydrolysis	Pepsin protease	The antioxidant-active component was isolated by ultrafiltration and two-step preparative RP-HPLC, and its peptide spectrum was characterized by LC-MS/MS; three selenopeptides were obtained	[23]
Sea cucumber	Single-enzyme hydrolysis	Neutral protease	Crude protein, accounting for more than 70% of the total weight, was the predominant component in the sea cucumber body wall; sea cucumber peptides possess significant anti-fatigue activity	[24]
Selenium-enriched *Cordyceps militaris*	Double-enzyme hydrolysis	Pepsin and pancreatin proteases	Based on the advanced separation and identification technologies, two novel selenopeptides were obtained from the hydrolysate	[25]
C. violifolia	Single-enzyme hydrolysis	Trypsin protease	The selenopeptides obtained by enzymatic hydrolysis are purified by ultrafiltration, and there is a positive correlation between their anti-tumor activity and the content of organic selenium	[26]

Note: RP-HPLC, reversed-phase high performance liquid chromatogram; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

that the immobilized enzyme was stable, recyclable, and reusable, thus demonstrating the feasibility of its use in selenopeptide production.

The anti-fatigue properties of selenopeptides are intimately associated with their amino acid composition, sequence, and peptide chain length. Specifically, the amino acid composition and sequence determine the unique biological activities of selenopeptides. When the peptide chain is shorter, the active site is more fully exposed, producing diversified combinatorial effects that potentially enhance anti-fatigue efficacy. The low-fat selenium-enriched egg yolk proteins were subjected to both single-enzymatic and double-enzymatic hydrolysis. The findings indicated that the combination of two enzymes yielded peptides that were smaller in size and more homogeneous in nature. The enhanced ability of the complex protease to effectively cleave selenopeptides with anti-fatigue properties may be attributed to its broader range of cleavage sites, resulting in a greater diversity of shorter-chain selenopeptides. This, in turn, enhances the activity and bioavailability of the selenopeptides[20]. Additionally, the use of proteases was a crucial factor in determining the functionality of selenopeptides when selenium-enriched soybeans were hydrolyzed by neutral and alkaline proteases to produce these compounds. It was observed that selenium-enriched soy protein, which had initially undergone hydrolysis with a neutral enzyme, exhibited exposed residues amenable to further action by an alkaline protease. The subsequent alkaline hydrolysis resulted in the generation of additional peptides with antioxidant properties. Conversely, residues that had been hydrolyzed by an alkaline protease could not be further processed by a neutral protease. Notably, the antioxidant value of the resulting products remained largely unaffected by the hydrolysis process conducted using both alkaline and neutral proteases[21].

It is a noteworthy observation that the result of enzymatic digestion is frequently a combination of peptides and amino acids, as opposed to pure selenopeptides. To ensure the purity and quality of the final product, further separation and purification are required. Advanced techniques such as membrane separation technology, ion exchange chromatography, gel filtration chromatography, and RP-HPLC can accurately separate the selenopeptides from the mixture, ensuring that the final product meets high-quality standards. After hydrolyzing the protein from selenium-enriched *P. eryngii* using trypsin, Yu et al.[22] purified the resulting selenopeptides through ultrafiltration, gel filtration chromatography, and RP-HPLC. They identified a total of 12 selenopeptides, including Gly-Phe-Ser-Se(Met)-Pro-Gly-Leu-Lys-Gln-Asp-Leu-Val-Leu-Pro-Arg, Gln-Asn-Sec-Sec-Sec-Phe-Asp-Ser-Val-Lys, Pro-Asp-Gly-Val-Se(Met)-Leu-Val-Glu-Gly-Lys, Se(Met)-Pro-Asp-Val-Thr-Asp-Lys-Thr-Lys, among others. The antioxidant properties of these peptides were demonstrated, and there was also indirect evidence supporting their potential anti-fatigue activity. Three selenopeptides, Val-Se(Met)-Asp-Asp-Se(Met)-Leu, Leu-Leu-Val-Se(Met)-Tyr, and Met-Met-Asp-Se(Met)-Leu, isolated from the hydrolysate of selenium-enriched oysters, have significant antioxidant activity[23]. Peptides extracted from sea cucumbers have been significantly proven to have anti-fatigue properties in exhaustive swimming tests[24]. Arg-Tyr-Asn-Ala-Se(Met)-Asn-Asp-Tyr-Thr and Val-Pro-Arg-Lys-Leu-Se(Met) obtained from selenium-enriched *C. militaris* can counteract fatigue by regulating the intestinal microbiota[25]. Lin et al.[26] purified selenopeptides derived from *C. violifolia* through ultrafiltration, subsequently analyzing the fractions exhibiting the highest antitumor activity for their amino acid sequences: Lys-Asp-Ala-Sec-Tyr-Ala-Tyr-Gly-Leu-Leu, Leu-Pro-Leu-Val-Lys-Sec-Pro, Ile-Val-Ser-Sec-Thr, along with six additional fractions. Furthermore, the relationship between antitumor activity and organic selenium content was assessed.

In summary, enzymatic hydrolysis represents a significant approach for the production of selenopeptides derived from natural sources under relatively mild conditions and at a low cost, with the added advantage of scalability. This method is increasingly accepted due to its benefits in biocompatibility, safety, and alignment with consumer preferences for natural health products. However, it is important to note that the resulting products are mixtures; both the yield and purity of the target selenopeptides depend on subsequent complex separation and purification processes. Furthermore, achieving precise control over the final sequence of selenopeptides presents a considerable challenge.

2.2 Microbial fermentation

Microbial fermentation is an effective method of obtaining peptides by hydrolyzing selenoproteins using proteases produced by bacteria during growth and metabolism. Screening fermentation strains for efficient protease activity is central to this method[27]. Currently, the common microorganisms used include lactobacilli, *Bacillus*, *Pichia*, and *Aspergillus*. Nevertheless, the limited number of efficient strains available remains a bottleneck for improving productivity. Recent studies have made progress in strain optimization: for example, *Bacillus natto* fermentation effectively degraded 50%–60% of soy protein and released SeMet in its bound state, thereby improving its bioaccessibility. This process also promoted the leaching of trace elements such as iron and manganese. Furthermore, the fermentation products maintained γ-polyglutamic acid content and nattokinase activity even in environments with high levels of selenium enrichment, demonstrating process compatibility[28]. The application of composite strains also produced excellent results: the synergistic fermentation of selenium-enriched mung bean by *Lactobacillus helveticus* and bifidobacteria produced selenopeptides, which significantly enhanced free radical scavenging and demonstrated remarkable antioxidant properties[29]. Notably, Xu et al.[30] reported that the fermentation of selenium-enriched chickpeas by *B. natto* produced significantly higher levels of angiotensin-converting enzyme inhibitory peptides than regular chickpeas. It was hypothesized that selenium enrichment increased the intrinsic value of the chickpeas, corroborating our conjecture that selenium and peptides synergistically enhance the anti-fatigue effect. Additionally, the enzymatic fermentation coupling technique involving protease pretreatment followed by *Lactobacillus* fermentation can deeply hydrolyze soy protein into small-molecule peptides, enhancing bioactivity by activating signaling pathways[31]. The notable advantage of microbial fermentation lies not only in its capacity to produce the desired selenopeptides but also in its concurrent generation of other beneficial metabolites. However, this duality also presents a significant drawback. Currently, the primary challenges include the lack of efficient and specialized strains, precise regulation of the fermentation process, and ensuring product stability. In the future, advancements in both efficiency and product quality within this technology are expected to be achieved through strain engineering and optimization of fermentation processes.

2.3 Selenium modification

The selenium modification is grounded in the principles of coordination chemistry. Specifically, the 4d empty orbital of the tetravalent selenium ion (Se^{4+}) forms stable Se–N or Se–O coordination bonds with electron-donor atoms, such as the lone pairs found in nitrogen and oxygen, within naturally occurring peptide molecules. This process effectively converts inorganic selenium into biologically active organic selenopeptide compounds. Key factors influencing the chelation of selenium and peptides include reaction time, temperature, pH, and the molar ratio of selenium to peptide. The method for selenium modification is not limited to standalone applications; rather, it exhibits enhanced efficacy when integrated with other techniques, such as enzymatic hydrolysis. For instance, Qin et al.[32] combined sodium selenite with pea oligopeptide obtained via enzymatic hydrolysis, resulting in high-selenium complexes with a chelation efficiency of up to 57.23%. The chelation mechanism was analyzed using infrared and ultraviolet spectroscopy at the molecular level. Circular dichroism and fluorescence spectroscopy were employed to examine the secondary and tertiary structures of both native and selenium-enriched pea oligopeptides. Isothermal titration calorimetry was used to quantify binding sites, leading to the identification of a highly active selenopeptide (Pro-Pro-Lys-Ile-Tyr-Pro) with notable antioxidant properties. Similarly, Jia et al.[33] utilized enzymatically hydrolyzed abalone gut peptides to chelate with sodium selenite. The short peptides generated by alkaline protease hydrolysis provided more available binding sites involving –NH, –OH, –CH, C=C, C=O, and C–N functional groups. These formed selenopeptide complexes with novel crystal structures and enhanced thermal stability. *In vitro* assays demonstrated that the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical and ·OH scavenging activities of these complexes were significantly higher than those of the original peptides, confirming the

enhancing effect of selenylation on biological activity. The selenium modification method is relatively straightforward and enables the direct conversion of existing peptides into more potent selenopeptide forms. According to this theoretical framework, any bioactive peptide containing suitable functional groups has the potential to be transformed into a functional selenopeptide through selenium modification. However, significant challenges persist, including optimizing the reaction conditions to improve selenium binding efficiency and ensuring product stability. Moreover, further research is needed to precisely identify the specific selenium binding sites and their intrinsic relationship with biological activity.

Although the methods outlined above are capable of generating selenopeptide mixtures with potential anti-fatigue bioactivity, their inherent limitations, particularly the inability to yield structurally homogeneous and precisely defined molecules, pose significant barriers to rigorous biological evaluation and clinical translation. To address these challenges and enable the acquisition of single, high-purity selenopeptides with unequivocal structures, chemical synthesis represents an essential and indispensable strategy.

3 Chemical synthesis strategy of selenopeptides

Although enzymatic hydrolysis, microbial fermentation, and selenium modification methods can be employed to produce crude selenopeptides with potential anti-fatigue activity, the low purity and reproducibility of these products limit their utility in biological function research and clinical applications. Conducting systematic and trustworthy standardized animal trials is crucial for the practical translation of selenopeptides into clinical settings in order to evaluate their efficacy, safety, and mechanism of action. The capacity to produce the single selenopeptide molecule with a distinct structure and exceptionally high purity is a critical requirement for all of these endeavors. The chemical synthesis process exhibits unmatched benefits in such situations (Table 2).

3.1 Solid-phase peptide synthesis (SPPS)

SPPS is among the most commonly utilized techniques for peptide synthesis. The fundamental principle of SPPS involves the stepwise assembly of peptides utilizing amino acids that are anchored to resins, typically progressing from the C-terminus to the N-terminus of the peptide through a series of coupling, deprotection, cleavage, and washing cycles[34]. Notably, SPPS boasts remarkable scalability and can be employed to construct virtually any peptide sequence[35]. This capability is particularly significant for selenopeptides, which demonstrate anti-fatigue properties.

The success of SPPS critically depends on the appropriate protection of the α-amino group (Figure 2A). Among the various protective strategies, *tert*-butoxycarbonyl (Boc) and 9-fluorenylmethoxycarbonyl (Fmoc) represent the two primary methodologies for safeguarding the α-amino group[36]. Additionally, side-chain functional groups can be orthogonally protected, meaning that both α-amino and side-chain protecting groups can be selectively removed under distinct reaction conditions without mutual interference. The frequent application of trifluoroacetic acid (TFA) in the Boc strategy during deprotection considerably heightens the risk of premature cleavage of peptide chains from the resin support. In addition, the final cleavage step requires hydrofluoric acid (HF), a cutting reagent that is highly toxic and corrosive. This imposes stringent requirements on equipment safety and handling procedures. Furthermore, HF presents a significant risk to the stability of the critical Se–C bond in selenopeptide molecules, potentially leading to structural degradation or loss of biological activity. In contrast, the Fmoc strategy provides a milder synthetic protocol and has demonstrated significant advantages, establishing itself as the preferred method for the SPPS of functional selenopeptides. In the Fmoc SPPS, the α-amino group is protected by Fmoc, while the selenium-hydrogen structure (–SeH) of Sec is orthogonally protected by methoxybenzyl (Mob), and SeMet, being a selenium ether structure, does not require additional protection. The building block Fmoc-Sec(Mob)-OH has been effectively utilized in the synthesis of four tetrapeptides containing Sec (Gly-Cys-Sec-Gly, Gly-Cys-Sec-Ser, Ser-Cys-Sec-Gly, and Ser-Cys-Sec-Ser)[37]. In our laboratory, we synthesized the tetrapeptide Leu-Se(Met)-Ala-Lys, the hexapeptide Arg-Asp-Se(Met)-Pro-Ile-Gln, and the octapeptide Thr-Asp-Asp-Ile-Se(Met)-Cys-Val-Lys utilizing the building block Fmoc-SeMet-OH (Figures 2B–D). These successfully demonstrate the feasibility of this synthetic approach. The implementation of Fmoc SPPS is contingent upon the use of mild reagents, including *N*,*N*-dimethylformamide (DMF), the deprotection reagent piperidine (PPR), and the cleavage solution TFA.

In contrast to the Boc approach for α-amino deprotection, which demands the utilization of TFA, Fmoc elimination is generally accomplished in DMF. This solvent is less likely to hinder

Table 2 Comparison of product purity and functional verification applicability of different selenopeptide preparation methods.

Preparation method	Core advantages	Product purity	Potential of functional verification
Enzymatic hydrolysis	Natural source, mild conditions, low cost	Low, mostly mixed components	It is extremely difficult and expensive to separate and purify a single high-purity selenopeptide
Microbial fermentation	Low cost, and it may produce other beneficial metabolites	Low, mostly mixed components, with great challenges in separation and purification	It is also extremely difficult and expensive to separate and purify a single high-purity selenopeptide
Selenium modification	Easy to operate, known peptides can be converted into selenopeptides	Medium, with an unclear structure	It is difficult to obtain specific selenopeptides with uniform and clear structures
Chemical synthesis	Selenopeptides with precise amino acid sequences and clear positions of seleno-amino acids can be synthesized through stepwise synthesis and efficient purification; selenopeptides with a single structure and few impurities can be obtained; the synthesis process is highly standardized, ensuring the stability of product performance	Extremely high	It is straightforward to obtain a single, high-purity sample for subsequent functional verification experiments

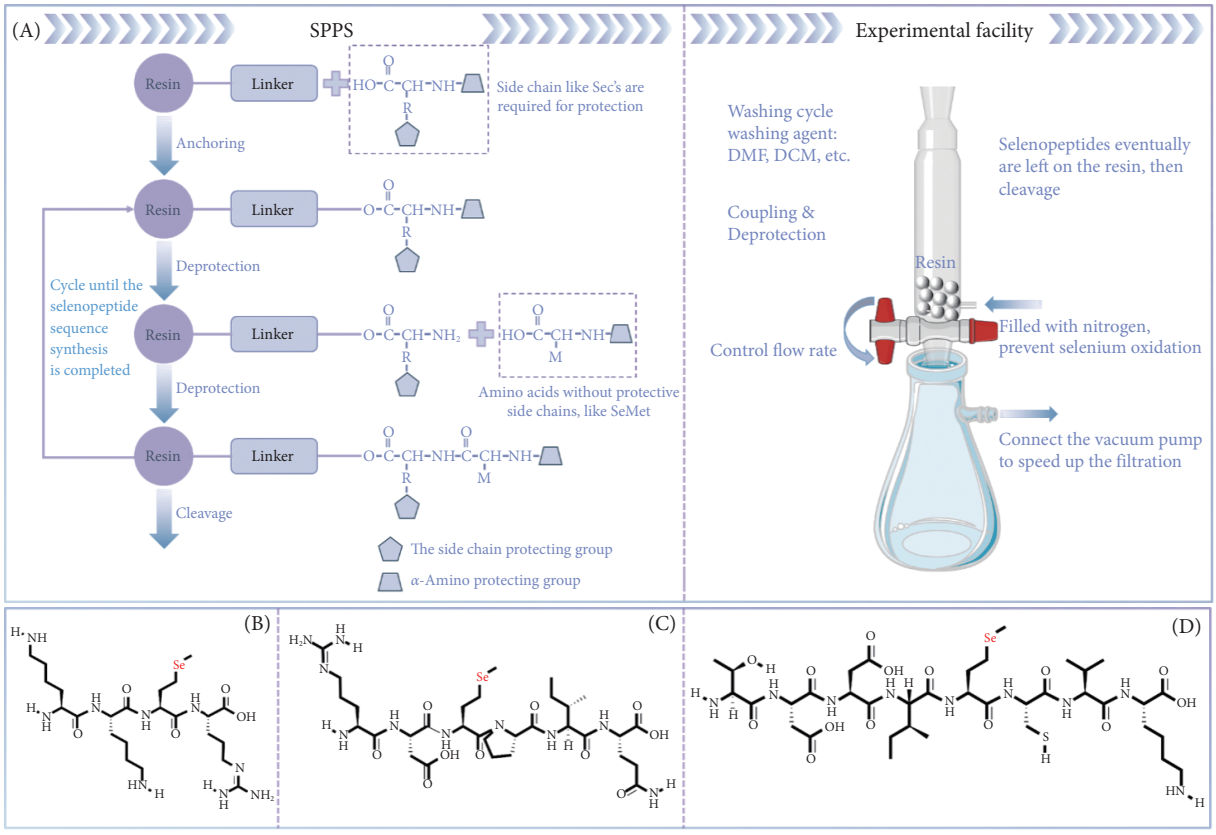

Figure 2 (A) SPPS-based design strategy and experimental facility for selenopeptide synthesis. Structural formulas for (B) Leu-Se(Met)-Ala-Lys, (C) Arg-Asp-Se(Met)-Pro-Ile-Gln, (D) Thr-Asp-Asp-Ile-Se(Met)-Cys-Val-Lys. DCM, dichloromethane.

inter-chain aggregation and is generally preferred for Fmoc SPPS. DMF, DCM, and *N*-methylpyrrolidone (NMP) are considered the gold standard solvents for Fmoc SPPS in academic and industrial research. However, these solvents are subject to regulation due to their potential health and environmental hazards. The increasing awareness of environmental concerns has prompted extensive research in recent years aimed at identifying more environmentally benign and less toxic alternatives to DMF. Various solvents considered as potential DMF replacements include ethyl acetate (EtOAc)[38], dimethyl carbonate[38], cyclopentyl methyl ether[38], 4-methyl tetrahydropyran[38], 2-methyltetrahydrofuran (2-Me-THF)[39], *N*-butylpyrrolidone[40], propylene carbonate[41], ethyl-5-(dimethylamino)-2-methyl-5-oxopentanoate (PolarClean)[42], γ-valerolactone[43], *N*-formylmorpholine (NFM)[44], dipropylene glycol dimethyl ether (DMM)[45] and triethyl phosphate (TEP)[46]. For instance, DMM exhibits comparatively minimal toxicity upon inhalation or dermal exposure and is rapidly biodegradable. TEP is nearly non-toxic, non-carcinogenic, and non-mutagenic, it degrades into harmless byproducts, positioning it as a promising environmentally friendly alternative. However, to date, no single solvent has been identified that can effectively replace DMF in SPPS. Given the challenges associated with finding a universal green solvent, Martin et al.[35] proposed the use of binary solvent mixtures as an alternative strategy. The combination of solvent polarity and viscosity was regarded as the key physicochemical parameter in SPPS. By precisely blending binary green solvent mixtures, such as dimethyl sulfoxide (DMSO) combined with 1,3-dioxolane or DMSO with 2-Me-THF, their polarity, viscosity, and solubility can be closely aligned with those of DMF. Additionally, this tunability enables the utilization of solvent mixtures with varying ratios during different synthesis stages. This approach not only serves as a replacement for DMF but also facilitates better control over side reactions and may even lead to enhanced performance outcomes. Consequently, green binary solvent mixtures are regarded as viable alternatives to DMF in SPPS, providing unprecedented opportunities for fine-tuning solvent properties[47].

DMF containing 20% PPR is the most widely used reagent for the deprotection of Fmoc groups and is recognized for its cost-effectiveness and reliable performance. Nevertheless, contemporary trends have increasingly underscored the significance of occupational health, environmental sustainability, and production safety. As peptide synthesis expands in scale, PPR finds itself under intensifying global regulatory scrutiny. Although the 20% tetrahydroisoquinoline in *N*,*N*′-dimethylimidazolidinone protocol demonstrates diminished efficacy compared to PPR/DMF in eliminating residues characterized by significant steric hindrance or in the synthesis of lengthy or aggregation-prone peptides, it presents a promising and sustainable alternative for Fmoc deprotection[48]. Pyrrolidine, when utilized as an alternative base for Fmoc removal, facilitates the execution of the entire peptide synthesis process in more environmentally friendly and less polar solvents. The synthesis of a series of challenging model peptides is accomplished with notable success. This is achieved through the utilization of pyrrolidine in conjunction with the low-polarity green solvent system of DMSO/EtOAc. The resultant peptides, when assessed in terms of purity, were found to be comparable to, if not superior to, those produced by the conventional PPR/DMF system. This development signifies a substantial advancement in the field of green peptide synthesis[49]. In addition to its toxicity, PPR is capable of inducing asparagine formation in sequences containing aspartic acid and may cause racemization at the α-carbon of chiral amino

acids, particularly histidine, serine, and cysteine. Diisopropylamine (DPA), an Fmoc deprotection reagent that can substitute for PPR, was found to markedly diminish byproduct formation in high-temperature SPPS. In the synthesis of the hexapeptide Val-Lys-Asp-Gly-Tyr-Ile, DPA reduced asparagine formation by 76.5%, achieving a product purity of 96%[50]. Concurrently, the identification of a deprotection strategy that exhibits nearly neutrality in nature utilizes 2,4-dinitro-6-phenyl-benzene sulfonyl and effectively circumvents the formation of intermediates exhibiting a proclivity for racemization. Moreover, this approach potently curtails asparaginolide formation. This method facilitates Fmoc deprotection under conditions of near neutrality and mildness, thereby circumventing damage to sensitive bonds caused by strong alkaline environments. It is especially suitable for peptides containing cysteine (S–C bond) and its analog, Sec (Se–C bond), offering a novel solution for the synthesis of challenging thiopeptides and selenopeptides[36]. In addition, a green and sustainable approach to polypeptide synthesis has been proposed, integrating aqueous-phase synthesis with the principles of a circular economy within classic Fmoc SPPS. This method transforms the conventional linear synthesis model, which encompasses material acquisition, utilization, and disposal, into a recycling model. This is achieved by replacing PPR/DMF with PolarClean/H_2O, thereby eliminating the hazards associated with toxic solvents. Waste can be filtered and recovered using mixed ion exchange resins without compromising peptide quality. This process directly reduces synthesis costs, which is particularly beneficial for peptides containing expensive amino acids, such as seleno-amino acids[51].

Following the completion of the peptide chain synthesis, the Fmoc strategy necessitates a solitary treatment with TFA for the purpose of cleaving the resin and eradicating all side-chain protecting groups. This design effectively circumvents the drawbacks associated with the Boc strategy, including repeated exposure to strong acids, minimizes the risk of premature peptide cleavage, and offers enhanced protection for the fragile Se–C bond while concomitantly reducing side reactions. Presently, no efficacious substitute for TFA exists for this step[52]. The peptide mixture obtained following the enzymatic hydrolysis of selenium-enriched *P. eryngii* was analyzed using a combination of LC-MS/MS and additional techniques. The antioxidant activities of 16 selenopeptides were found to be significantly higher than those of non-selenium peptides, and their specific amino acid sequences were identified. Subsequent analysis comprised *in vitro* antioxidant activity assays, the result of which identified Ser-Sec-Pro-Leu, Se(Met)-Pro-Gly-Pro, and Sec-Ser-Pro-Leu as the subjects of further study. The selenopeptide Se(Met)-Pro-Gly-Pro, synthesized via Fmoc SPPS, was validated through molecular docking models to exhibit strong antioxidant activity[53]. Throughout the entire synthesis process, reagents can be easily removed via simple filtration. To ensure each coupling reaction proceeds as completely as possible, 3–5 equivalents of activated amino acids and coupling reagents are typically used to drive the reaction equilibrium toward product formation. Nevertheless, the utilization of these materials results in the generation of significant quantities of toxic waste liquid, which necessitates extensive treatment. Consequently, it increases the exposure risk for operational personnel and exacerbates safety concerns, posing potential threats to both human health and the environment. To tackle these challenges, research has increasingly shifted toward green development and process optimization[54].

The Food and Drug Administration of the United States proposed the implementation of Process Analytical Technology (PAT) 20 over years ago with a view to enhancing production efficiency and quality in the pharmaceutical industry. Stager et al.[55] incorporated Raman spectroscopy into PAT to enable real-time prediction of solvent concentration during the continuous washing process using Raman spectroscopy combined with a partial least squares model. PAT relies on the inherent spectral specificity of Fmoc and PPR components detected by Raman spectroscopy. During the Fmoc deprotection stage, the extent of the reaction is monitored in real time through the concentration of solution adducts and their reaction kinetics, thereby improving the efficiency of this step. In the subsequent washing phase, Raman spectroscopy enables direct and real-time monitoring of residual PPR concentration in the reaction mixture, significantly reducing solvent consumption and waste generation.

As previously mentioned, SPPS is typically employed during the discovery phase to produce peptides at milligram to gram scales, as it is optimized for efficacy and stability. Nevertheless, the industry is still beset by challenges such as protracted processing times, substantial waste generation, and difficulties in scaling up. A novel approach, namely continuous-flow (CF)-SPPS, is reported. This approach is primarily aimed at enabling rapid and efficient synthesis of multi-gram-scale peptide fragments to support drug discovery and development. The integration of hydraulic control, online monitoring, and automation in the CF-SPPS process enables the synthesis of 19.3 g of peptides within a 3.5-h timeframe, yielding a favorable yield and a purity of 90.2%. Furthermore, solvent usage and resultant waste generation are reduced to a significant extent. This demonstrates the efficacy, environmental friendliness, and scalability of CF-SPPS as a peptide synthesis technology, enabling rapid and high-quality synthesis of SPPS-derived peptides from gram to multi-gram levels[56].

Recently, Merlino et al.[57] reported an innovative ultrasound-assisted (US)-SPPS method by comparing the synthesis of two peptides, Arg-Gln-Met-Ala-Thr-Ala-Asp-Glu-Ala and Val-Ser-Pro-Pro-Leu-Thr-Leu-Gly-Gln-Leu-Leu-Ser, using conventional SPPS and US-SPPS. US-SPPS can be considered among the current high-efficiency peptide synthesis techniques. Notably, when synthesizing Val-Ser-Pro-Pro-Leu-Thr-Leu-Gly-Gln-Leu-Leu-Ser, the crude peptide purity achieved by US-SPPS was 82%, surpassing that obtained via conventional SPPS. However, for Arg-Gln-Met-Ala-Thr-Ala-Asp-Glu-Ala, the purity was slightly lower than that achieved by SPPS. Nevertheless, repeated experiments have confirmed that US-SPPS significantly reduces the overall peptide assembly time[58].

In a recent study, Mottola et al.[59] proposed and validated a sustainable ultrasound-assisted (SUS)-SPPS method. This technique integrates coupling, capping, and deprotection steps of SPPS into a continuous process, further reducing washing steps and time. Remarkably, it can decrease solvent usage per cycle by 83%–88%, yielding crude peptides with a purity as high as 96%. This innovative, sustainable, and environmentally friendly approach represents a significant advancement in peptide synthesis and opens new possibilities for the sustainable synthesis of selenopeptides.

Although automated SPPS has been in use for several decades, existing systems still lack flexibility and often require manual intervention, such as cleavage and sedimentation steps, and struggle to accommodate complex chemical modifications. Most automated synthesizers remain limited to specific molecular types, such as oligopeptides and oligonucleotides, lacking broad applicability.

Integrating SPPS with chemical processing units (Chemputer) offers a promising solution for achieving a fully automated system from synthesis to post-modification. This platform supports non-natural amino acids, complex modifications, real-time pH control, and inert gas handling. It has successfully enabled the directed oxidative folding of disulfide bonds, and similar strategies can be directly applied to the automated formation of chemical bonds in selenopeptides. The realization of fully automated selenopeptide synthesis is anticipated through the χDL blueprint (a customizable framework for automated selenopeptide synthesis), which customizes coupling conditions for selenium-enriched amino acids, deprotection of selenoalcohols/selenoethers, diselenide bond formation, or selenium ester ligation reactions[52]. To our knowledge, green solvents have not yet been investigated using ultrasonic or continuous flow process intensified methods. A new green solvent mixture, NFM/anisole (1:1), was employed in conjunction with a safe 5-hydroxyimino-1,3-dimethyl-1,3-diazinane-2,4,6-trione/carbodiimide coupling combination US-green SPPS. Additionally, the synthetic approach was successfully employed in the construction of three complex peptide sequences and four biologically significant natural peptide compounds. The crude purity of Trp-Phe-Thr-Ile-Leu-Ile-Ser-Thr-Ile-Met was 59.7%, which was superior to the 57% previously reported in the literature. This methodology demonstrated remarkable versatility, facilitating efficient synthesis of both polypeptides and deposited peptides through on-resin esterification, as well as solution- or resin-based macrocyclization under ultrasonic conditions[60]. Additionally, peptide synthesis using green solvents in continuous flow equipment was achieved for the first time, demonstrating its potential in automated, large-scale production. SPPS, as the cornerstone of selenopeptide chemistry, is achieved through the deep integration of green chemistry and process engineering, breaking free from the dual shackles of toxicity and waste, and ushering in a new era of efficient, precise, and sustainable peptide synthesis.

3.2 Liquid-phase peptide synthesis (LPPS)

Although SPPS is the most widely used and technically mature method for peptide synthesis, the high consumption of reagents and solvents remains a critical challenge. In recent years, peptide synthesis strategies based on homogeneous reaction systems have attracted widespread attention due to their potential advantages, with significant progress being made in both methodological development and practical applications[61]. Early peptide synthesis in homogeneous solution is referred to as solution-phase peptide synthesis or classical solution peptide synthesis (CSPS). The core advantage of this system lies in eliminating the diffusion limitations of reaction reagents within resin particles in SPPS, thereby significantly improving reaction kinetics and enhancing the efficiency and uniformity of coupling reactions. For example, Dernovics et al.[62] employed CSPS, using a Sec dimer (Sec_2) as the starting material, to successfully synthesize the target selenopeptide with a 45% yield by activating the peptide segment with pentachlorophenol and coupling it with Sec_2 in DMF under controlled pH conditions. However, a significant bottleneck of this strategy is that each intermediate step requires purification operations such as column chromatography, which is not only tedious and time-consuming but also leads to oxidation and loss for sensitive molecules like selenopeptides due to multiple purification steps.

To address these issues, LPPS uses protecting groups as soluble tags emerge[63]. This method introduces tag molecules with specific solubility behaviors, enabling intermediates to be efficiently purified through precipitation or extraction, avoiding traditional chromatographic separation steps and significantly improving synthesis efficiency. Compared to SPPS, LPPS typically requires only near-stoichiometric amounts, such as 1.2–2.0 equivalents of expensive amino acids and reagents instead of the 3–5 equivalents of excess commonly used in SPPS, effectively reducing raw material costs and waste generation. These soluble tags function similarly to resins in SPPS, as their adducts with peptide chains exhibit distinct solubility differences in specific solvents, enabling rapid filtration and separation through phase separation operations. This greatly simplifies the purification process and reduces byproduct generation by up to 90%[64]. Various types of soluble tags have been developed, including polyethylene glycol (PEG), fluorous tags, ionic liquids, and hydrophobic carriers, each suitable for peptide synthesis of different lengths, structural complexities, and production scales[65]. Among these, PEG, as the earliest tag used, exhibits good solubility in common polar aprotic solvents such as DMF, DMSO, NMP, and DCM. Additionally, peptide chains can remain attached to the PEG carrier for direct use in bioactivity testing, where PEGylation not only significantly enhances water solubility but also reduces immunogenicity and extends *in vivo* half-life[66]. Fluorous tags leverage their unique solubility in highly fluorinated solvents to achieve purification through three-phase extraction, making them particularly suitable for short peptide synthesis[67]. Ionic liquids, due to their designable structures, allow for the tuning of physicochemical properties by adjusting cation-anion combinations, serving both as reaction media and recyclable carriers, demonstrating great potential for green synthesis[68].

Among hydrophobic tags, the hydrophobic benzyl alcohol tag (HBA-Tag) is compatible with both Boc and Fmoc protection strategies and enables simple and quantitative monitoring of reaction progress through acid-induced color change or light-triggered fluorescence. It has been successfully applied to the synthesis of angiotensin III selective antagonist peptide[69], β-sheet breaker peptide iAβ5[64], and thermoresponsive elastin-like peptides[70]. Furthermore, combining the biomimetic coupling reagent T3P® (propylphosphonic anhydride) with the HBA-Tag under the Fmoc strategy in LPPS efficiently synthesized Leu-enkephalin, achieving a final product purity of 99% and an overall yield of 62%, highlighting the efficiency of this combined strategy[61]. Additionally, two novel soluble small-molecule tags, TAG-1 and TAG-2, which enable flexible conversion from C-terminal carboxylic acid peptides to C-terminal amide peptides through esterification with rink amide linkers, have been designed. These tags feature concise synthesis routes, high yields (> 90%), and low-cost, readily available raw materials, demonstrating strong potential for industrial scale-up. They have been successfully applied to the large-scale green synthesis of the antithrombotic drug eptifibatide, a cyclic heptapeptide with a C-terminal amide and a head-to-tail disulfide bond[71].

To address the instability of traditional benzyl-based hydrophobic tags under hydrogenation conditions, two newly developed silyl tags (TAG4 and TAG6) exhibit stronger hydrophobicity and superior chemical stability. TAG4, containing a siloxane structure, is suitable for hydrogenation conditions and has been successfully used in the linear and convergent synthesis of the antimicrobial peptide DRGN-1. TAG6, containing an aryl silyl

group, is compatible with both Fmoc and Boc strategies. Its high C–Si bond stability, low synthesis cost, and easy recyclability make it particularly advantageous for synthesizing challenging polyalanine heptapeptides[72]. Moreover, the application of phosphonate reagents further improves the preparation efficiency of peptide compounds under assistance. These auxiliary agents can be easily modified onto various structural units, including non-classical amino acids, and enable simple and efficient purification through crystallization and filtration[73].

By rationally selecting tag types, LPPS can significantly reduce purification steps, lower solvent usage, and achieve kilogram-scale synthesis, demonstrating promising industrial applications[65]. To further minimize material loss due to multiple operations, Shimodaira et al.[74] developed a one-pot global deprotection strategy, enabling the efficient synthesis of selenoglutathione diselenide with an overall yield of 90%–98%. Such research opens new avenues for the industrial application of LPPS and promotes the integration of continuous flow synthesis technology with automated platforms, as well as the establishment of comprehensive environmental assessment systems, thereby advancing peptide synthesis technology toward greener and more scalable development.

In recent years, automated continuous flow processes, such as PEPSTAR (One-Pot Nanostar Sieving System), have combined soluble tags with organic solvent nanofiltration membrane separation technology to achieve iterative amino acid addition and peptide chain elongation in a single reactor without intermediate phase transfer or material transfer. This significantly improves synthesis efficiency and reduces solvent consumption[75]. Peng et al.[76] reported a novel CF-LPPS method based on a hydrophobic silicon-based carrier, bis(4-((*tert*-butyldimethylsilyl)oxy)-phenyl)methylamine. This system consists of amidation, deprotection, and extraction-washing modules, using environmentally friendly EtOAc as the solvent and *N*-benzyloxycarbonyl-protected amino acids instead of traditional DMF and PPR, successfully synthesizing a pentapeptide with high purity and a crude yield exceeding 90%.

In summary, LPPS, as an important development direction in peptide preparation, not only provides new ideas for the synthesis of complex structured and modified peptides but also offers a feasible path for green pharmaceutical processes. The combination of tag-assisted synthesis and continuous flow purification technology, in particular, demonstrates broad application prospects. However, there have been no public reports on the application of such advanced strategies to the synthesis of anti-fatigue selenopeptides. Therefore, systematically exploring the application of modern LPPS strategies, such as soluble tag-assisted and continuous flow membrane separation, in the efficient and precise synthesis of anti-fatigue selenopeptides will be a highly valuable research direction in the future.

Although chemical synthesis methods still require further optimization in terms of material loss, they enable the precise design and synthesis of defined sequences containing specific seleno-amino acids, yielding structurally uniform and high-purity selenopeptide products through stepwise SPPS or LPPS strategies. This lays a solid foundation for the development of anti-fatigue peptide drugs. The high degree of process standardization in chemical synthesis ensures consistency in product properties across different synthesis batches, thereby guaranteeing the reliability and reproducibility of pharmacodynamic and toxicological data in subsequent animal experiments. Additionally, chemical synthesis allows for the flexible introduction of stable isotopes or fluorescent labeling groups to produce labeled selenopeptides. Such labeled products are important tools for advanced animal experiments: isotope labeling facilitates precise tracking of the absorption, distribution, metabolism, and excretion of selenopeptides in model animals using MS, elucidating their pharmacokinetic characteristics; fluorescent labeling enables visual studies of the localization and transport mechanisms of selenopeptides in tissues or cells. This advantage of precise control throughout the sequence-structure-function chain makes chemical synthesis the gold standard for producing high-quality anti-fatigue selenopeptides for standardized animal experiments and preclinical research.

4 Conclusion and prospects

With the continuous improvement of living standards, public health awareness has been steadily increasing. Selenopeptides, owing to their unique molecular structure and significant antioxidant activity, have demonstrated considerable application potential in anti-fatigue interventions, particularly within functional foods and dietary supplements. However, their practical application still faces two major challenges. First, the safety of long-term consumption requires systematic evaluation, especially regarding the potential cumulative toxicity of selenium, as well as gastrointestinal stability and intestinal absorption efficiency following oral administration. Second, selenopeptides derived from different sources exhibit variations in amino acid sequence and composition, which may lead to differences in bioavailability and biological efficacy; however, comprehensive comparative studies remain limited. The chemical synthesis of structurally defined selenopeptides will serve as the gold standard for accurately assessing bioavailability and enabling reliable comparisons of safety and efficacy, thus facilitating the development of high-quality selenopeptide-based supplements. Future research should focus on constructing well-characterized selenopeptide libraries through chemical synthesis, systematically evaluating structure-activity relationships, pharmacokinetic profiles, and long-term safety, as well as elucidating the underlying mechanisms of action *in vivo*. Such efforts are essential to advance the scientific application and industrial development of selenopeptides in anti-fatigue interventions.

Conflict of interest

The authors declare no conflict of interest.

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