# Bioactive Peptides Derived from Hen Egg Proteins

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Hen eggs, characterized by high protein and digestibility, are an excellent source of food-derived bioactive peptides. Some peptides comprising fewer than six amino acids may resist gastrointestinal digestion and remain intact as they cross the intestinal epithelium.

Keywords: egg-derived peptides ; enzyme inhibitors ; intestinal health

## 1. Introduction

Hen eggs are a high-demand food on the global market. In 2019–2021, the average annual production of hen eggs reached 90,513 million tons and was consumed at a rate of 10.6 kg per capita per year [1]. Hen eggs have been described as near-perfect because of their nutritional balance, high digestibility, and affordable price [2]. Proteins are the primary nutrient in hen eggs. Hen egg proteins have high digestibility and absorption rates, and are called complete proteins [3]. Proteomic analyses identified 371, 428, and more than 500 proteins in egg white, yolk, and eggshell membrane proteins, respectively [4][5]. Egg white proteins mainly include ovalbumin, ovotransferrin, ovomucin, ovomucoid, and lysozyme [6]. Egg yolk proteins mainly include lipovitellin, globular protein, phosvitin, and low-density lipoprotein [7]. Eggshell membrane proteins mainly include collagen, keratin, agrin, and laminin [8]. Hen egg proteins possess many functional activities, including antibacterial, immunomodulatory, and antioxidant [9][10][11]. In particular, the antioxidant activity has attracted considerable attention. Moreover, ovalbumin, ovotransferrin, and lysozyme often exhibit significant antioxidant properties [10]. After different enzyme hydrolysis, hen egg proteins generate bioactive peptides that demonstrate significantly enhanced biological activities compared to the original proteins, or even new biological activities. Yeon Cho et al. (2023) found that enzymatic hydrolysis improved the nutritional and functional characteristics of the proteins [12]. Studies have shown that peptides exhibit the higher absorptivity and carrier saturation in recent years than proteins [13]. They are easily absorbed by enterocytes in the small intestine and enter the portal vein through capillary vessels in the small intestine [14]. Some peptides comprising fewer than six amino acids may resist gastrointestinal digestion and remain intact as they cross the intestinal epithelium [15]. Animal and plant proteins are the primary sources of food-derived bioactive peptides. However, bioactive peptides derived from plants are always deficient in one or more essential amino acids, leading to a reduced nutritional value and bioavailability [16]. An increasing interest has been shown to bioactive peptides of animal origin, particularly milk and egg origins. Hen eggs contain various proteins and provide a comprehensive nutrition for the development of hen embryos [17]. Therefore, an increasing body of research suggests that hen egg-derived peptides (HEPs) may exhibit a superior biological activity than their protein counterparts in many cases [18]. For example, phosvitin (PV) has a strong binding affinity to iron, making it difficult to release its ion, which results in a decreased intestinal absorption [19]. Feng et al. (2005) proposed that phosvitin phosphopeptides (PPPs), obtained by the enzymatic hydrolysis of PV, could promote absorption and bioavailability in the monolayer model of Caco-2 cells [20]. Moreover, ovomucin (OM) does not possess antioxidant activity, whereas the hydrolysates exhibit more [21]. Ibrahim et al. (2001) confirmed that the antibacterial activity of lysozymes improved greatly after enzymatic hydrolysis, attributed to the exposure of the antibacterial active sites of the protein [22]. Beyond that, HEPs possess a range of regulatory properties, such as angiotensin-I-converting enzyme (ACE) inhibitory, immunomodulatory, and anti-inflammatory activities [23][24]. Recent reviews have summarized the preparation of HEPs from a specific part of the egg, as well as certain biological properties and potential applications in the food and pharmaceutical industries. Moreover, there are few summaries of HEPs based on preparation methods, and a limited discussion of the potential applications and biological activities.

## 2. Bioactive Peptides Derived from Hen Egg Proteins

The eggshell membrane (ESM) is a polymeric fibrous meshwork, and the primary component is protein, which accounts for approximately 90% [24] ESM proteins mainly consist of collagen, keratin, agrin, and laminin [8]. However, the eggshell membrane is difficult to separate from hen eggs [25]. Microwave-assisted separation, chemical separation, and vacuum treatment are widely used to separate eggshell membranes [4][26][27]. Moreover, it has been reported that hydrolyzed ESM

are used more widely than ESM, improving the bioavailability of the active components [4]. The main component of the whole egg is egg white, accounting for 63% of the whole egg weight, of which the content of protein is 10.5% [28]. Egg white proteins (EWPs) mainly contain ovalbumin (OVA, 54%), ovotransferrin (OTF, 12%), OM (11%), ovomucin (OV, 3.5%), lysozyme (LM, 3.4%), globulin (8%), and avidin (0.05%) [28]. Therefore, EWPs have a high nutritional value, and its hydrolysates have various biological properties after hydrolysis. The egg yolk has 16% proteins, mainly including low-density lipoprotein, high-density lipoprotein, livetins (α, β, and γ), and PV [29]. PV and gamma-livetin are the most studied egg yolk proteins. Gamma-livetin mainly consists of immunoglobulin Y (Ig Y), which can react with serum immunoglobulins. Because it is relatively easy to obtain and purify, it is used as a substitute of mammalian antibodies [30]. In particular, yolkin is a polypeptide complex accompanied by Ig Y, consisting of several peptides and proteins [31]. PV is a highly phosphorylated protein with excellent emulsification, antioxidant, and metal-chelating properties, which has a great application potential [32].

EWP, OVA, OVTF, OM, LM, PV, and ESM proteins have most commonly been used as precursor proteins. HEPs are mainly prepared via enzymatic hydrolysis, microbial fermentation, chemical synthesis, and chemical hydrolysis. However, after chemical hydrolysis, the resulting molecules may become non-absorbable and have poor nutritional qualities or low functionalities [33]. Therefore, there is little research on the preparation of HEPs using chemical hydrolysis.

## 2.1. Peptide Preparation

### 2.1.1. Enzymatic Hydrolysis

Enzymatic hydrolysis is a process that involves the cleaving of protein structures using proteases, thereby releasing bioactive peptides [34]. The enzyme type and reaction conditions play a vital role in the preparation of HEPs, determining the composition and sequence of amino acids [35]. There are two main ways to utilize enzymatic hydrolysis for the preparation of bioactive peptides: in vivo gastrointestinal digestion and in vitro enzymatic hydrolysis. In vivo gastrointestinal digestion entails the proteins entering the human body orally and being degraded by gastrointestinal (GI) enzymes, such as pepsin, trypsin, and chymotrypsin. However, only some studies have focused on the preparation of HEPs by in vivo gastrointestinal digestion due to the complexity of organisms and the limitations of genotype information. Currently, most studies have prepared HEPs by in vitro simulated GI digestion [36]. Zhang et al. (2023) reported that EWPs were subjected to in vitro gastrointestinal digestion simulation using pepsin and trypsin, and found that EWP peptides (EWPP) were released abundantly in the stomach, with 307 peptides being released in the stomach and 160 peptides released in the intestine [37]. Palika et al. (2015) reported that food peptides, released during the in vitro gastrointestinal digestion of hen egg whites, could bind and solubilize ferric iron [38]. In vitro enzymatic hydrolysis entails bioactive peptides being prepared by non-GI proteases derived from plants or microorganisms. The proteases commonly used for this purpose include bromelain, papain, alcalase, thermolysin (EC 3.4.24.27), and neutral proteases (EC 3.4.24.4) [36][37].

Moreover, the biological activities of peptides obtained from some non-GI proteases are stronger than those from GI protease hydrolysis, due to differences in the hydrolysis specificity. Young et al. (2010) presented a comparison of the phosphate content of egg yolk peptides (EYP) from alcalase (EC 3.4.21.62) and protease S, protease N (EC 3.4.24.28), protease A (EC 3.4.24.39), protease P, and trypsin (EC 3.4.21.4) digestion, and found that EYPs from protease P had the highest phosphate content (43.95 μg of $PO_4$/mg of protein), thus exhibiting a higher antioxidant stress bioactivity [39]. Five proteases (alcalase, trypsin, neutrase (232-752-2), papain, and pepsin (EC 3.4.23.1)) were used as hydrolytic enzymes to produce antioxidative peptides from ESMs, and found that the highest free radical scavenging activity belonged to alcalase hydrolysates (degree of hydrolysis, DH of about 28%) [25]. Abeyrathne et al. (2016) evaluated the inhibition of ACE by ovomucin-derived peptides (OMPs) obtained using trypsin, papain, and alcalase under their optimum conditions, and found that OMPs from alcalase and papain (EC 3.4.22.2) exhibited the highest ACE inhibitory activity (>70%) [21]. Alcalase mainly acts on the peptide bonds of Ser, Thr, Asp, and Glu. Papain cleaves the peptide bonds of amino acids, including Leu, Gly, and Cys. Trypsin breaks down peptide bonds containing Lys and Arg. Memarpoor-Yazdi et al. (2012) proposed that the antioxidant activity of egg white lysozyme peptides (LMPs) obtained from papain were better than that of trypsin. The DPPH and ABTS radical scavenging activities of the LMPs were 37.2 ± 1.69%, 50.4 ± 2.1%, 1.91 ± 0.13, and 2.57 ± 0.19 μmol Trolox equivalents (TE)/mg protein for trypsin and papain, respectively [40].

ESMs have been hydrolyzed by alkaline protease from *Bacillus altitudinis* GVC11, resulting in protein hydrolysis with a significant antioxidant activity [41]. The enzyme produced by the Pseudomonas aeruginosa strain ME-4 can hydrolyze the ESM and produce two water-soluble peptides containing proline and the free amino acid tryptophan [42].

Furthermore, the biological activity of peptides is related to the length of the amino acid sequence. The DH and peptide chain length of HEPs are improved by the hydrolysis of two or more enzymes. Currently, there are two critical parameters used to assess the extent of enzymatic proteolysis: the DH and molecular weight distribution (MWD) of peptides. Thus,

the combination of different enzymes may be another viable option. Shi et al. (2014) prepared ESMP with higher antioxidant activity by single or the combination of enzymatic hydrolysis (alcalase, protease A, protease N, protease P, and protease S); the DH and total nitrogen recovery from combinatorial enzymatic hydrolysis were higher than those from single enzymatic hydrolysis, 26.39 ± 0.46% (the hydrolysate of alcalase and protease P) and 65.60 ± 0.43% (the hydrolysate of alcalase and protease S), respectively [43]. Then, three groups of peptides with different molecular weights, isolated and purified from the hydrolysate of alcalase and protease S, showed excellent antioxidant activity, which improved the cellular redox state of Caco-2 cells by inhibiting IL-8 secretion and increasing the GSH concentration [43]. OVAPs (ovalbumin-derived peptides) were obtained by hydrolysis with 1% of pepsin, trypsin, α-chymotrypsin (EC 3.4.21.1), papain, and alcalase singly or in combination at 37 °C, for 4 h, and found that the Fe-chelating capacity of the hydrolysates differed among the enzyme treatments: the alcalase + trypsin and chymotrypsin (232-671-2) treatment was the best, followed by the pepsin +papain and pepsin + alcalase treatments [44]. Papain, alcalase, trypsin, and thermolysin are widely used to prepare HEPs. At present, HEPs mainly act as functional additives or functional drinks. For instance, Zheng et al. (2020) proposed that HEPs could be used to prepare peptide with hypotensive effects [45] EWPPs could act as antioxidants and anti-inflammatory additives to be applied in food and drugs [46]. Moreover, HEPs were also incorporated into a functional beverage to enhance the nutritional value of foods [47].

Currently, enzymatic hydrolysis is the most common method used to generate HEPs. It is milder than other methods, and the obtained proportions of some essential amino acids were higher than those in previous studies [48]. HEPs, obtained through proteolysis by food-grade commercial enzymes, have been generally regarded as safe (GRAS). However, conventional enzymatic hydrolysis has a low efficiency because of the infrequent contact between the substrate and enzyme. Thus, studies have reported that some assistive technologies or pretreatments can be applied to enhance the efficiency and bioactive peptide yield, including pulsed electric field, ultrasound treatment, thermal denaturation, and high-pressure treatment [49]. Among them, the preparation of PPPs is dramatically affected by enzymatic hydrolysis. Early studies have shown that PV containing phosphorylated amino acid residues showed an antagonistic effect on the protease activity, significantly reducing the efficiency of enzymatic hydrolysis [50]. In order to improve the efficiency of enzymatic hydrolysis, some auxiliary technologies have been used as pretreatment, such as NaOH treatment, phosphatase treatment, heat treatment, and pressure treatment [51]. Jiang et al. (2000) applied the alkali treatment method using various concentrations of a NaOH solution for the dephosphorylation of PV, resulting in an improved DH [52]. Samaraweera et al. (2013) reported that acid or alkali pretreatments could improve the hydrolysis process of PV, thereby enhancing its functionality [53]. Moreover, proteases were used to hydrolyze the high-pressure (600 MPa) and phosphatase-pretreated PV to obtain a relatively large molecular weight of peptides [54]. Huang et al. (2019) reported that high-temperature and mild-pressure (HTMP, at 100 °C for 60 min) pretreatment could help the hydrolysis of PV further, and the DH values of enzyme combinations (trypsin and multifect 14 L) were the highest compared to those of other combinations, with a DH of 26.01 ± 1.23% [55]. PV dephosphorylated by alkaline phosphatase (E.C.3.1.3.1) was digested using trypsin, pepsin, or thermolysin (EC 3.4.24.27), and low-molecular-weight peptides (<5 kDa) in trypsin hydrolysates and alkaline hydrolysis accounted for 23.59% and 21.22%, respectively [56]. The enzymatic hydrolysis of other proteins in eggs can be significantly enhanced by assistive technologies. Jain et al. (2016) found that ESMPs, prepared through a combination of ultrasound enzymatic hydrolysis (alcalase and papain), exhibited superior physicochemical properties (emulsifying properties, foaming properties, and water-holding capacity) than conventional enzymatic hydrolysis, and the optimal preparation conditions were amplitude, time, and solid-to-solvent ratio values of 95.74%, 28.06 min, and 1:30 (g: mL), respectively [57]. Lei et al. (2011) demonstrated that ultrasound pretreatment could increase the reactive sulfur groups in a 5% ovotransferrin solution by 50% [58]. Shen et al. (2010) also reported that ovotransferrin-derived peptides (OTFPs) were obtained using ultrasound pretreatment-assisted thermolysin hydrolysis, whose antioxidant value was further increased from 0.49 to 1.95 μmol of TE/mg [59]. This might be because microwave energy could prevent protein aggregation to improve enzymatic hydrolysis by penetrating inside the samples. In addition, pulsed electric field (PEF) is a green pretreatment technology with a low energy consumption and high efficiency, which is widely applied in multiple domains. Li et al. (2023) found that PEF-assisted alcalase hydrolysis increased the DH (46.81%), surface hydrophobicity (decreased by 13.60%), and free sulfhydryl group content (increased by 79.85%) [60]. Liu et al. (2018) reported that the PEF treatment (653–695 kJ/kg, 1.4–1.7 kV/cm) had an enhancing effect on the enzymatic hydrolysis of ovomucin. However, a significant enhancement only arose from the PEF treatment (695 kJ/kg, 1.7 kV/cm) of ovomucin at acidic (pH 4) conditions, which showed a similar DH compared to ovomucin heat-treated at 80 °C [61]. This can be due to the fact that the PEF treatment may alter the conformational space of proteins to expand the secondary and tertiary structures. Thus, the optimization of these factors, such as the protease type, hydrolysis conditions, and other factors, is effective in obtaining target bioactive peptides.

#### 2.1.2. Microbial Fermentation

Microbial fermentation is a method that catalyzes the preparation of HEPs under specific conditions using enzymes derived from certain microorganisms. Microbial fermentation produces a complex array of enzymes to form a complex enzyme system. Microbial fermentation has the advantage of relatively low production costs, but it has the disadvantages of a low peptide yield and a lack of specificity in peptide formation [62]. Lactic acid bacteria (LAB) fermentation is the simplest and safest technology, which affects the protein structure by enzymatic action and acidification. Nahariah et al. (2020) reported that Lactobacillus sp (LAB), consisting of *L.bulgaricus, L.achidopillus*, and Streptococcus thermophilous, egg proteins were used in egg protein fermentation to obtain HEPs with antioxidant activity [63]. PPPs were prepared by lactic acid bacteria (*S. thermophilus: L. bulgaricus* 1:1) fermentation at 1% *w*/*w* PV, 42 °C, for 9 h, and found that its calcium-binding capacity (NaOH consumption of 0.9 mL) was enhanced compared to the PPPs produced by enzymatic digestion (NaOH consumption of 2.85 mL) [64]. In another study, ESMPs were hydrolyzed by *Lactobacillus plantarum* fermentation under the optimal environmental conditions (pH 8.0 and 36 h), resulting in the maximum protein concentration (177.3 mg/g) and DH (25.1%) of the hydrolysates [65]. In addition, during fermentation, LAB can produce a little lipase to be applied in fat breakdown. Proteases derived from LAB are commonly used in the preparation of bioactive peptides that act as natural feed additives in animal nutrition. However, microbial metabolites are complex and diverse, making the isolation and purification of peptides difficult. Therefore, further research is necessary to address the issues.

### 2.1.3. Chemical Synthesis

The chemical synthesis method entails preparing bioactive peptides based on known amino acid sequences, and the commonly used chemical synthesis includes liquid-phase synthesis and solid-phase synthesis (SSPS). SPSS is one of the most common techniques used in peptide synthesis, and the general process involves adding amino acids one by one from the C-terminus to the N-terminus of the sequence to synthesize the target peptides [66]. Moreover, SPPS is the preferred method for synthesizing peptides in laboratories and the industry due to its simplicity, effectiveness, and characteristics of automated synthesis. However, the amino acid sequence of the target peptide is essential for synthesizing peptides. Thus, most studies combine enzymatic digestion and SSPS, and the general process is: HEPs are prepared by protease; then, the peptides of simple components are identified by isolation and purification, and finally, the target bioactive peptide is synthesized according to the requirements. For instance, Liu et al. (2015) reported that the hydrolysates of egg white proteins were isolated and purified to synthesize EWPPs (DHTKE, FFGFN, and MPDAHL) by SSPS [11]. Zhang et al. (2021) isolated and solid-phase synthesized a potent calcium-binding peptide (DEEENDQVK) from the high-phosphorus protein hydrolysate of yolks (calcium-binding capacity increase to 151.1 mg/g) [67]. Si et al. (2023) also reported that PPPs with a high calcium-binding capacity were identified and synthesized from yolk high phosphoprotein hydrolysates (Glu-Asp-Asp-pSer-pSer) and found that the maximum calcium-binding capacity of EDDpSpS was 468 ± 2.80 mg/g [32]. However, egg-derived synthetic peptides are mainly used for laboratory experiments. For instance, Sun et al. (2017) investigated the binding mechanism of Asp-His-Thr-Lys-Glu (DHTKE) from egg whites to calcium and the calcium absorption of a peptide–calcium complex by a Caco-2 cell model, and found that this complex increased calcium absorption capacity by 7 times [68]. And the KPHAEVVLR (KR-9) peptide, derived from hydrolyzed egg whites, was reported to accelerate the wound healing of the palatal mucosa in rats by promoting human gingival fibroblast proliferation [69].

The various methods of preparing HEPs discussed here are summarized in **Table 1**. Enzymatic hydrolysis is a mature method with high safety, specificity, and activity, making it suitable for production. The low content and purity of HEPs obtained from enzymatic hydrolysis do not contribute to the application of their related products. SSPS has the advantages of a short synthesis cycle and high production, which can effectively overcome the deficiency of natural HEPs. However, synthesis is only suitable in laboratories, due to its low yield and high cost. Beyond that, genetic engineering strategies for the production of peptides have been a research hotspot. Feng et al. (2010) reported that the bovine lactoferricin derivative peptide LfcinB-W10 was successfully expressed by *Escherichia coli*, and found that it exhibited growth inhibition activity against *Staphylococcus aureus* ATCC25923 [70]. At present, some studies have reported that some hen egg proteins can be successfully expressed using genetic engineering strategies. For instance, with the assistance of a xylanase fusion partner, hen egg-white lysozymes were recombinantly expressed in *Pichia pastoris* [71]. Therefore, a new research trend in the preparation of HEPs is genetic engineering strategies.

**Table 1.** Preparation methods of HEPs.

| Methods | Protein | Preparation | Evaluation Indicator | Results | References |
|---|---|---|---|---|---|
| Enzymatic hydrolysis | Eggshell membrane protein | Alcalase: E/S 1% *w*/*w* at pH 10, 55 °C for 4 h.<br>Proteases S: E/S 2% *w*/*w*, pH 10 at 55 °C for 12 h.<br>Alcalase + Protease S | Total nitrogen recovery<br>DPPH radical scavenging activity<br>Hydroxyl radical scavenging activity | 65.60 ± 0.43%<br>42.74%<br>25.5% | [43] |
| | | Alcalase from *Bacillus licheniformis:* E/S (*v*/*v*) 2.2%, pH 7.6, 55 °C, 6 h.<br>Viscozyme L: E/S (*v*/*v*) 1.90, pH 4.6, 50 °C, 6.61 h.<br>Protease from *Bacillus amyloliquefaciens:* E/S (*v*/*v*) 5.3%, pH 6.6 h, 50 °C, 2.90. | ACE inhibitory activity | $IC_{50}$ (μg/mL:<br>43.0 ± 8.5<br>63.0 ± 4.2<br>43.0 ± 8.5 | [72] |
| | | Recombinant LasB_ME4 (*Pseudomonasaeruginosa*) E/S 3, pH 6.5, 50 °C for 24 h, with shaking at 140 rpm. | Soluble peptides and proteins (mg/mL) | 0.502 ± 0.016 | [73] |
| | | Papain: pH 6.0, 50 °C, 5 h. | DPPH scavenging activity<br>The recovery of the 3 h papain hydrolysate | 73.14%<br>50.62% | [11] |
| | Egg white protein | Protease P: E/S (*w*/*w*) 25:1, pH 7.5, 45 °C, 3 h. | DH<br>ORAC (μmol TE/mg)<br>ABTS (μmol TE/mg) | 93.3%<br>1.28 ± 0.06<br>1.61 ± 0.00 | [74] |
| | | Ultrasound-assisted alkali treatment: pH 8.5, 55 °C, 20 min.<br>Alcalase: E/S 5% (*w*/*v*), pH 8.5, 55 °C, 5 h. | Foaming ability<br>Foam stability<br>Oil absorption capacity<br>Water absorption capacity | Compared to unpretreated enzymolysis, these properties increased by 88.5%, 228.7%, 102.6%, and 67.4%, respectively | [75] |

| Methods | Protein | Preparation | Evaluation Indicator | Results | References |
|---|---|---|---|---|---|
| | | Promod 278P: E/S (*w*/*w*) 1:50, 45 °C, 3 h. | ACE-inhibitory activity<br>Cytotoxic activities | 76.82 ± 1.28%<br>$IC_{50}$ (mg/mL)<br>MCF-7 cells: 10.05 ± 1.55<br>HeLa cells: 3.45 ± 0.94<br>HepG2 cells: 4.43 ± 1.87<br>HT-29 cells: 4.92 ± 0.63<br>oVo cells: 10.43 ± 3.91 | [76] |
| | | Sonication pretreatment (60 Hz for 30 s)<br>Thermolysin: 5% (*w*/*v*), pH 8, 60 °C, 3 h.<br>Thermolysin with sonication | ORAC (μmol/mg) | 1.95 ± 0.02 | [59] |
| | Ovotransferrin | Promod 278P + Thermolysin: E/S 1:50 (*w*/*w*), 6 h. | Cytotoxic activity | $IC_{50}$ (mg/mL)<br>AGS, LoVo, HT-29, and HeLa: 0.79, 0.78, 0.92, and 0.78, respectively | [77] |
| | | Papain: E/S (*w*/*w*) 5%, pH 7.0, 50 °C for 6 h. | Biofilm eradication activities | Compared to the control, the treatment of OTFP (500 μg/mL) reduced the biofilm formation rates by 13.76%, 19.18%, and 39.80% and inhibited the metabolic activities by 27.84%, 57.85%, and 65.71% in *L. monocytogenes* ATCC 15313, H7962, and NADC Scott A, respectively | [78] |
| | | Elastase: E/S 1% (*w*/*v*), pH 7.8, 25 °C, 24 h.<br>α-chymotrypsin: E/S 1% (*w*/*v*), pH 6.5, 37 °C, 3 h. | $Fe^{3+}$-chelating activities | 1.06 ± 0.88%<br>1.25 ± 0.24% | [79] |
| | Lysozyme | Alcalase: E/S (*w*/*w*) 0.5%, pH 7.0, 37 °C, 6 h. | Oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay<br>DPPH radical scavenging activity | $IC_{50}$<br>0.463 μmol TE/moL<br>1.698 μmol TE/mg | [80] |
| | | Trypsin + Papain<br>E/S (*w*/*w*) 1:20, pH 7.5, 37 °C, 2 h. | DPPH radical scavenging activity<br>Radical scavenging assay (μmol (TE)/mg) | Trypsin + Papain<br>64.2 ± 2.95%<br>2.82 ±0.14 μmol TE/mg | [40] |

| Methods | Protein | Preparation | Evaluation Indicator | Results | References |
|---|---|---|---|---|---|
| | | Enzymatic treatments: Phosphatase: pH 4.8, 37 °C for 15 h. Pancreatin: pH 7.5. Pepsin A: pH 2.3. | The degree of dephosphorylation Angiotensin-converting enzyme (ACE)-inhibitory antioxidant activity | 63% Dephosphorylated phosvitin: increased by 52% Protease-treated phosvitin: increased by 39% | [54] |
| | | Alkaline treatment: 0.1 mol/L NaOH, at 37 °C for 4 h. Trypsin: E/S (*w*/*w*) 1:50, pH 8.0, 37 °C, 24 h. | Amino acid peptides Phosphate retention of phosvitin Phosphopeptides | 10~20 35% | [52] |
| | | Trypsin: pH 8.0, 40 °C, 6 h. Multifect 14 L: pH 8.0, 70 °C, 6 h. Trypsin + Multifect 14 L: The substrate/enzyme ratio was 50:1. | DH Number of peptides | 26.01 ± 1.23% 164 | [55] |
| | Phosvitin | High-temperature and mild pressure (HTMP): 121 °C at 0.1 MPa, 30 min. Trypsin: pH 8.0, 37 °C. Thermolysin: pH 8.0, 68 °C. Trypsin + thermolysin: The same enzyme: E/S (1:50, m/m) and incubation time (8 h). | DH The calcium-binding rate of phosvitin Phosphopeptides | 45.55% 43.01% | [81] |
| | | HTMP: 121 °C and 1.5 atm for 60 min. Trypsin: pH 8.0, 37 °C. Trypsin—sterilization hydrolysis: The same enzyme: E/S (1:50, m/m) and incubation time (6 h)- | Effects of phosvitin phosphopeptides on the melanin synthesis in α-MSH-stimulated-B16F10 cells Effects of phosvitin phosphopeptides on the elastase activity Effects of phosvitin phosphopeptides on the NO production in LPS-stimulated RAW 264.7 cells | HTMP-T-S at the concentration of 3 mg/mL suppressed the melanin content by 38.58% HTMP-T-S at a concentration of 50 mg/mL inhibited the elastase activities by 70.67% HTMP-T-S at the concentration of 5 mg/mL reduced NO production by 76.69% | [82] |
| | Eggshell membrane protein | *Lactobacillus plantarum*: E/S (*W*/*V*) 5%, 30 °C at 120 rpm initial pH 8.0, 30 °C, 30 h. | DH Foaming capacity Emulsification activity | 25.1% 36.7% 94.6 $m^2$/g | [65] |
| Microbial fermentation | Egg white protein | Lactobacillus sp E/S (*v*/*v*) 1:1, 37 °C. Addition of different-level milk powder 2% and fermentation times, 24 h. | Antioxidant activity | 3.92 ± 0.56% | [63] |
| | Phosvitin | *Streptococcus thermophiles* to *Lactobacillus bulgaricus* was 1:1. E/S (*w*/*w*) 1%, pH 6.7, 42 °C, 6 and 9 h. | Surface hydrophobicity Emulsifying properties The consumption of NaOH | Fermentation time: 6 h 2.04 60.49 min Fermentation time: 9 h 0.90 mL | [64] |

| Methods | Protein | Preparation | Evaluation Indicator | Results | References |
|---|---|---|---|---|---|
| Chemical synthesis | | AAPPTEC 396 Automated Peptide Synthesizer. The 9-Fluorenylmethoxycarbonyloxy (Fmoc)-protected amino acid synthesis method peptide (RVPSLM). | α-glucosidase inhibitory activity | $IC_{50}$ 23.07 μmol/L | [83] |
| | Egg white protein | Fmoc-protected amino acid synthesis method. AAPPTEC: Apex 396 automated peptide synthesizer. Peptide QIGLF. | ACE-inhibitory activity | $IC_{50}$ 75 μmol/L | [84] |
| | | Fmoc solid-phase peptide conditions. AAPPTEC: Apex 396 peptide synthesizer. Peptide MIR. | Dipeptidyl peptidase IV-inhibitory activity | $IC_{50}$ 24.97 ± 0.80 mmol/L | [85] |
| | Ovotransferrin | Solid.phase peptide procedure. FMOC-protected amino acids synthesis method. Peptide RVPSL (328–333). | ACE-inhibitory activity | $IC_{50}$ 20 μmol/L | [86] |
| | Ovalbumin | AAPPTEC: Apex 396 peptide synthesizer. Peptide EEK. | XO-inhibitory activity | $IC_{50}$ 141 μmol/L | [87] |
| | Lysozyme | AEERYP | ORAC (μmolTE/mg) | $IC_{50}$ 4.35 ± 0.09 | [74] |
| | Phosvitin | Solid-phase peptide synthesis method. Peptide DEEENDQVK. | Calcium-binding capacity | 151.1 mg/g | [67] |

## 2.2. Purification and Identification of Peptides

Enzymatic hydrolysis has the disadvantages of high cost, low peptide yield, and difficult separation and purification of the mixed peptides. Microorganisms are relatively inexpensive sources of proteases, and microbial fermentation uses proteases that can be produced by microbial strains to hydrolyze substrate proteins. HEPs are a complex mixture, mainly containing hen egg proteins, peptides of various molecular weights, free amino acids, and salts. To further investigate the biological properties and the structure–activity relationship, it is necessary to obtain highly purified/single fractions of HEPs. The methods for the isolation and purification of peptides are mainly membrane separation, electrophoresis, and chromatography [51]. Moreover, the purification scheme for HEPs consists of multiple combined purification procedures, including ion-exchange chromatography, gel filtration chromatography, affinity chromatography, and reverse phase-high-performance liquid chromatography (RP-HPLC). Sun et al. (2014) reported that EWPPs fractions with different molecular weights (>5, 2–5, 1–2, and <1 kDa) were obtained using ultrafiltration, and found that the fraction (2–5 kDa) exhibited stronger antioxidant activity than of other fractions [88]. Jahandideh et al. (2018) showed that two papain-generated antioxidant EWPPs (Tyr-Leu-Gly-Ala-Lys and Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe-Gln) were purified sequentially by ultrafiltration, gel filtration, and RP-HPLC. Adipogenic-differentiating peptides were purified sequentially from egg white proteins after ultrafiltration, C18 Sep-Pack cartridge, and cation-exchange chromatography [89]. Eckert et al. (2019) reported that the decapeptide QSLVSVPGMS (Gln-Ser-Leu-Val-Ser-Val-Pro-Gln-Met-Ser) was fractionated from EYPs by a series of purification procedures, including membrane filtration, gel filtration chromatography, and RP-HPLC, and its ACE-inhibitory and DPPH radical scavenging activities were almost two-times and over three times higher compared to those of the initial EYPs, respectively [90].

Moreover, the purified HEPs must be further identified by peptide sequencer techniques, including Edman degradation methods, mass spectrometry (MS) methods, and bioinformatics analysis. The Edman degradation method has some limitations, such as blocking N-terminal amino acids, forming a blank cycle, and being susceptible to interference [91]. MS methods are used to separate ions by their mass to charge ratio (*m*/*z*) using mass analyses, and they can also be combined with chromatography for excellent analytical accuracy and specificity [92]. The mass analyzer is the core component of MS analysis and identification, and there are commonly used mass analyzers, including quadrupole, time of flight (TOF), ion trap, and electrostatic field orbital trap (Orbitrap) [93]. Two or more mass analyzers are combined in series to enhance the speed and accuracy of the analyses. MS methods have gradually replaced the Edman degradation

method as a powerful tool for peptide sequencing due to their high sensitivity and high- throughput sequencing. Currently, the most common strategies for confirming peptide sequences include matrix-assisted laser deionization time-of-flight (MALDI-TOF), liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS), electrospray ionization mass spectrometry (ESI-MS), and triple quadrupole mass spectrometer (TQ-MS) [23][55][56][89][94]. Zambrowicz et al. (2015) reported that some isolated peptides, deprived from egg yolk proteins, were identified using MALDI-TOF [95]. WEKAFKDED, QAMPFRVTEQE, ERYPIL, VFKGL, AEERYP, DEDTQAMP, and RVPSL were identified and isolated from EWPs by LC-MS/MS [74][86][89]. KGGDLGLFEPTL (Lys-Gly-Gly-Asp-Leu-Phe-Glu-Pro-Thr-Leu), DEEENDQVK AEFGTEPDAKTSSSSSSASSTATSSSSSSSSSPN (Asp-Glu-Glu-Glu-Asn-Asp-Gln-Val-Lys-Ala-Glu-Phe-Gly-Thr-Glu-Pro-Asp-Ala-Lys-Thr-Ser-Ser-Ser-Ser-Se-Ser-Ser-Pro-Asn), KPMDEEENDQV (Asn-Pro-Met-Asp-Glu-Glu-Glu-Asn-Asp-Gln-Val), and SGHLEDDSSSSSSSSVLSKIWG (Ser-Gly-His-Leu-Glu-Asp-Aspr-Ser-Ser-Ser-Ser-Se-Ser-Ser-Ser-Val-Leu-Ser-Lys-Ile-Trp-Gly) were also identified and isolated by LC-MS/MS [9][96]. Moreover, Samaraweera et al. (2014) identified ten PPPs from trypsin EYPs [56]. Additionally, a variety of databases, web resources, and software are used in bioinformatics techniques, commonly referred to as in silico analysis, to predict the bioactivity of peptides. This is accomplished by analyzing the type and quantity of amino acid residues using statistical and computational methods [97]. Currently, peptidomics and bioinformatics collaborate to provide a cost-effective and efficient approach to screening, analyzing, and predicting. UniportKB, BIOPEP, PeptideCutter, and PeptideRanker are databases dedicated to analyzing the functionality of peptide sequences [98]. Marcet et al. (2022) used LC-MS/MS to identify the EYPPs released from EYPs. Subsequently, they utilized the BIOPEP database, ExPASy ProtParam, AHTpin, and PeptideRanker to analyze the discovered EYPPs and predict their bioactivities [99]. Mohd Adam Salim et al. (2020) utilized a comprehensive integrated bioinformatics technique (PeptideCutter, PeptideRanker, and Pepsite2) to study the structure–activity link between OVAPs and ACE/DPP-4. They found eight peptides with particular activity [100]. With the development of technology, bioinformatics is gradually being utilized to study the structure–activity relationship of bioactive peptides. For peptides with well-defined structures, molecular docking and molecular dynamics simulations are commonly used to examine the biological activity mechanisms. When the peptide structure cannot be identified, the quantitative structure–activity relationship (QSAR) is employed to examine the association between the biological activity and chemical structure. Majumder et al. (2010) found that three ACE-inhibitory peptides were successfully isolated from OTFP after screening the virtual enzymatic hydrolysis products predicted by the QSAR model [101].

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