



Article

Artificial Neural Network-Guided Discovery of Antioxidant Peptides from Peony (*Paeonia ostii*) Seed Meal: Peptidomics, Molecular Mechanism, and Cellular Validation

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Abstract

Peony seed meal (PSM), a protein-rich by-product of oil extraction from *Paeonia ostii*, represents an underutilized resource with significant potential for functional ingredient development. In this study, an integrated strategy combining artificial neural network (ANN)-guided hydrolysis, peptidomics, molecular simulation, and cellular validation was employed to identify antioxidant peptides from PSM. Neutrase was selected as the optimal protease, and hydrolysis conditions were optimized using a backpropagation ANN model ($R = 0.9935$), yielding a hydrolysate with strong radical-scavenging activity ($\text{DPPH}^\bullet \text{IC}_{50} = 0.30 \text{ mg/mL}$; $\text{ABTS}^{\bullet+} \text{IC}_{50} = 0.07 \text{ mg/mL}$). LC-MS/MS identified 364 peptides, predominantly low-molecular-weight sequences. In silico screening highlighted four candidates (FRF, WQFR, FEFR, and RWL) with favorable binding toward $\text{ABTS}^{\bullet+}$, $\text{DPPH}^\bullet$, and Keap1. Molecular docking and 100 ns molecular dynamics simulations confirmed stable peptide-Keap1 interactions, particularly for FRF. Cellular assays demonstrated that FRF and RWL significantly protected HepG2 cells against H_2O_2 -induced oxidative damage by restoring antioxidant enzyme activities (SOD, CAT, and GSH-Px). Collectively, this study establishes a systematic workflow for discovering plant-derived antioxidant peptides and supports the sustainable valorization of PSM as a functional food ingredient.

Keywords: peony seed meal; antioxidant peptide; artificial neural network; peptidomics; keap1-Nrf2 pathway; molecular docking; molecular dynamics simulation

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1. Introduction

Oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) production and endogenous antioxidant defenses, plays a crucial role in aging, metabolic disorders, and chronic inflammatory diseases [1]. Specifically, ROS, such as hydroxyl radicals ($\text{OH}^\bullet$) and superoxide anions ($\text{O}_2^{\bullet-}$), indiscriminately attack cellular macromolecules, inducing lipid peroxidation, protein carbonylation, and DNA strand breaks,

processes that are implicated in pathologies ranging from atherosclerosis to neurodegenerative disorders [2]. To mitigate oxidative damage, synthetic antioxidants like butylated hydroxytoluene (BHT) have been widely employed in the food and pharmaceutical industries. However, mounting evidence links their long-term use to hepatotoxicity and carcinogenic risks, prompting regulatory agencies like the European Food Safety Authority (EFSA) to impose stricter usage limits [3]. In response to these safety concerns, there has been growing interest in discovering natural, potent, and food-compatible antioxidants capable of restoring redox homeostasis without adverse effects, including bioactive peptides derived from food resources.

Natural antioxidant peptides, derived mainly from plant and animal proteins, have attracted considerable attention due to their safety, sustainability, and potent free-radical-scavenging activities, positioning them as promising alternatives to synthetic antioxidants for food and health applications [4]. For example, cottonseed protein hydrolysates with molecular weights below 3 kDa exhibited strong DPPH•, ABTS•+, hydroxyl radical, and ferrous-ion-chelating activities ($EC_{50} = 0.49\text{--}2.21$ mg/mL). Among the identified sequences, YSNQNGRF exhibited the strongest binding affinity to the Keap1–Nrf2 complex, as evidenced by its lowest CDOCKER energy, indicating the highest predicted antioxidant potential through hydrogen bonding and van der Waals interactions [5]. Similarly, antioxidant peptides obtained from bass (*Lateolabrax japonicus*) proteins via optimized enzymatic hydrolysis demonstrated strong DPPH• radical-scavenging capacity ($IC_{50} = 0.97$ mg/mL) and effectively protected Caco-2 cells from oxidative damage. These peptides reduced intracellular ROS levels and lipid peroxidation while enhancing antioxidant enzyme activities [6]. Notably, the peptide DDWENWAK identified from the hydrolysate was found to improve mitochondrial energy metabolism and activate Nrf2 and AMPK signaling pathways, further confirming these cytoprotective effects [7]. Despite these promising advances, the valorization of agricultural by-products, particularly from emerging oilseed crops, remains underexplored and warrants systematic investigation.

As a multifunctional oilseed crop endemic to China, peony seeds contain more than 90% unsaturated fatty acids (over 40% of α -linolenic acid), making them highly valuable from a nutritional standpoint. On this basis, “Fengdan” peony (*Paeonia ostii*) was officially recognized by the Chinese government as a novel functional food resource in 2011 [8,9]. Peony seed meal (PSM), the major by-product of oil extraction, is produced in high quantities, exceeding 100,000 tons annually in China. However, it is often underutilized as a low-grade animal feed or even discarded, leading to substantial resource waste and environmental concerns [10]. Given its high protein content (approximately 30%), PSM therefore shows great promise as a high-quality plant protein source and represents a sustainable substrate for bioactive peptide production [11].

Recently, several studies have demonstrated the potential of antioxidant peptides derived from peony seed proteins. For instance, a novel antioxidant peptide, SMRKPPG, was identified from the alcalase hydrolysates via the optimization of enzymatic hydrolysis, followed by sequential ultrafiltration and chromatographic purification. However, SMRKPPG exhibited relatively low radical-scavenging activity at 1 mg/mL (9.62% for DPPH•, 8.53% for ABTS•+, and 16.48% for •OH) [12]. By contrast, in another study, two antioxidant peptides, LPSHPPR and LHPHFR, were obtained after protease selection, ultrafiltration, and virtual screening, both of which showed DPPH• scavenging rates around 30% and ABTS•+ scavenging rates of ~70–80% at 1 mg/mL [13]. To further demonstrate their antioxidant activities at the cellular level, both peptides were tested in an H_2O_2 -induced RAW264.7 macrophage model, which reduced both intracellular ROS and malondialdehyde levels, consistent with the chemical assays. Collectively, these findings support valorizing PSM for antioxidant peptide discovery and highlight that hydrolysis

conditions govern peptide profiles and bioactivity, demonstrating that targeted optimization of enzymatic hydrolysis is pivotal to enhanced antioxidant performance.

Conventional optimization techniques such as response surface methodology (RSM) have been widely employed to improve enzymatic hydrolysis conditions for antioxidant peptide production. For example, RSM has been successfully applied to optimize the pepsin-catalyzed hydrolysis of pumpkin seed cold-pressed oil cake in order to obtain protein hydrolysates with the highest antioxidant activity [14]. However, RSM is inherently limited in describing complex nonlinear relationships among process variables due to its dependence on quadratic polynomial fitting. In recent years, artificial neural networks (ANNs) have emerged as a promising optimization methodology because of their superior nonlinear modeling and adaptive learning capabilities, allowing more accurate prediction and optimization of intricate bioprocesses [15]. For instance, ANN-based models have been used to simulate the enzymatic hydrolysis of goat milk proteins, achieving a maximal degree of hydrolysis (23.47%) under optimized temperature and enzyme–substrate ratios while improving the antioxidant potential of the hydrolysate [16]. Similarly, ANN effectively predicted antioxidant yield and radical-scavenging activities of corn distiller solubles with high accuracy, demonstrating its potential as a reliable and efficient improvement over RSM for process optimization [17]. Therefore, ANN provides a more flexible and powerful approach for optimizing antioxidant peptide production as compared with conventional RSM.

To address the limited systematic exploration of PSM as a source of potent antioxidant peptides and to overcome the limitations of conventional hydrolysis optimization, this study established an integrated discovery strategy combining intelligent process optimization and mechanistic validation. Neutrase-mediated hydrolysis was optimized using a BP-ANN model to maximize antioxidant activity, followed by LC–MS/MS-based peptidomics for comprehensive peptide identification. Candidate peptides were screened through *in silico* bioactivity prediction and molecular docking against ABTS^{•+}, DPPH[•], and Keap1, with molecular dynamics simulations evaluating complex stability. Finally, selected peptides were validated in an H₂O₂-induced HepG2 model to confirm cytoprotective effects and regulation of antioxidant enzymes. This workflow provides a rational framework for the efficient discovery of functional antioxidant peptides from agricultural by-products.

2. Results and Discussion

2.1. Protease Screening for Hydrolysis

To release peptides with higher antioxidant activity, the choice of proteases is critical due to their cleavage specificity, which determines the resulting peptides' lengths, terminal residues, hydrophobic/aromatic content, and motifs, thereby directly influencing the yield and potency of bioactivities. Based on this consideration, four commercial proteases were compared for their efficiency in hydrolyzing PSM to produce PSM protein hydrolysate (PSMH) with enhanced antioxidant activity.

Initially, degree of hydrolysis (DH) was determined to evaluate hydrolysis efficiency. As a direct indicator of peptide bond cleavage, DH reflects the extent of proteolysis and is closely related to the molecular characteristics and potential bioactivity of the peptides generated [18]. Among the tested proteases, neutrase resulted in a relatively high DH of 28.19% (Figure 1a). This observation is consistent with the catalytic properties of neutrase as an endopeptidase, which preferentially cleaves internal peptide bonds and thereby promotes the formation of short-chain peptides with relatively low molecular masses, as reported previously [19]. In comparison, the other three proteases exhibited slightly lower hydrolysis efficiencies, with DH values ranging from 21.82% to 26.80%.

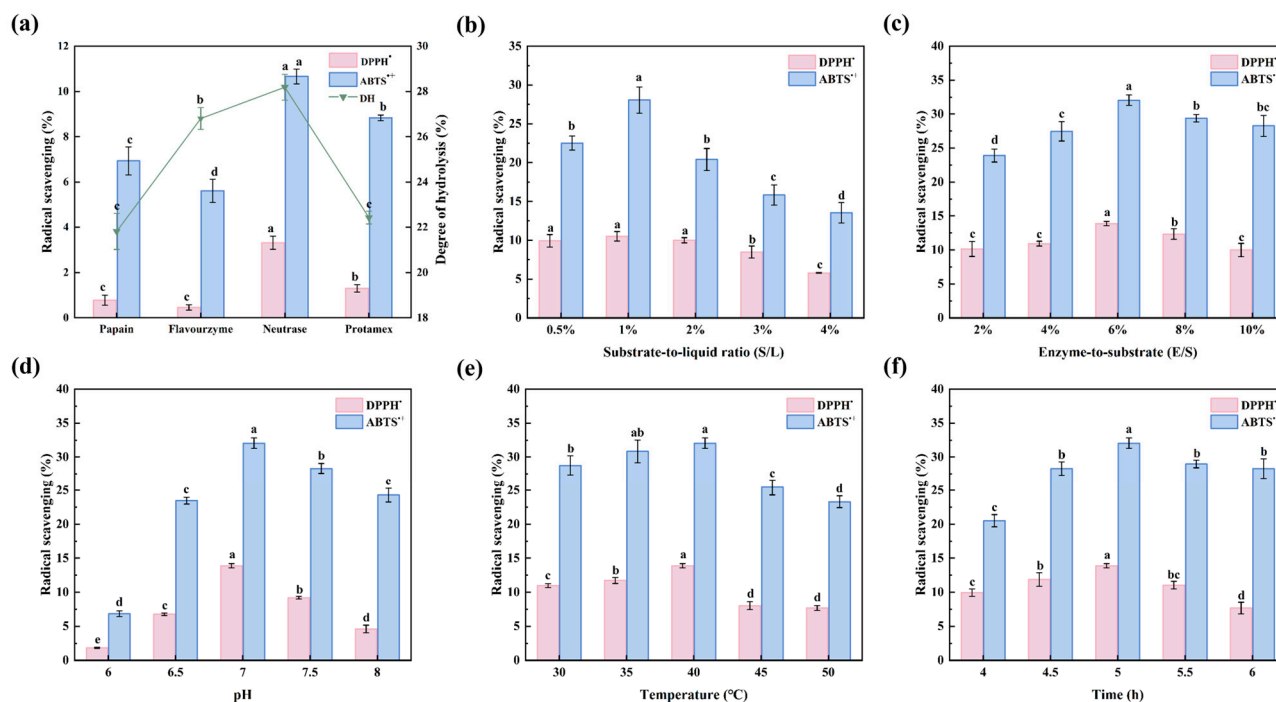


Figure 1. (a) Degree of hydrolysis and antioxidant activity of PSMH prepared by different proteases. The DPPH• and ABTS•+ radical-scavenging rates of enzymatic hydrolysate optimized at different conditions, including: (b) Substrate-to-liquid ratio (S/L); (c) Enzyme-to-substrate (E/S); (d) pH levels; (e) Temperature; and (f) Time. Results are shown as mean $\pm$ SD ($n = 3$). Different superscript letters indicate significant differences ($p < 0.05$, one-way ANOVA followed by Duncan's test).

In addition, the antioxidant capacities of the hydrolysates were assessed using DPPH• and ABTS•+ radical-scavenging assays at a concentration of 0.05 mg/mL (Figure 1a). Among them, the neutrase hydrolysate exhibited the strongest antioxidant activity. Specifically, its DPPH• and ABTS•+ scavenging rates were 3.32% and 10.66%, respectively, both of which were significantly higher than those of the other hydrolysates. Similar results have been reported previously, where neutrase hydrolysates derived from skipjack tuna milt proteins exhibited significantly higher DPPH• and ABTS•+ scavenging activities than those obtained using trypsin, papain, or pepsin [20]. This enhanced activity is likely attributed to the specific cleavage pattern of neutrase, which behaves like a thermolysin-family metalloprotease with a hydrophobic P1' preference. Such cleavage specificity tends to generate peptides enriched with hydrophobic or aromatic N-terminal residues, structural features that are consistently associated with strong radical-scavenging and metal-chelating capacities [21]. Therefore, neutrase was selected as the optimal protease for subsequent enzymatic hydrolysis optimization aimed at producing antioxidant peptides with enhanced activity.

2.2. Single Factor Optimization

To elucidate how individual processing parameters shape the generation of antioxidant peptides, the influences of substrate-to-liquid ratio (S/L), enzyme-to-substrate ratio (E/S), pH, temperature, and hydrolysis time were systematically examined (Figure 1b–f). Despite their distinct chemical natures, DPPH• and ABTS•+ assays displayed highly consistent response profiles across the examined conditions, suggesting that both assays respond similarly to variations in hydrolysis efficiency.

As shown in Figure 1b, increasing the S/L ratio from 0.5% to 1.0% markedly enhanced antioxidant activity ($p < 0.05$). This improvement likely stems from intensified enzyme–

substrate encounters, thereby accelerating proteolytic cleavage and promoting the release of peptide radical scavengers. However, once the S/L ratio exceeded 1.0%, a noticeable decline in activity was observed, indicating that excessive substrate levels may hinder the hydrolysis process and thereby reduce antioxidant performance. High substrate concentration is known to elevate system viscosity, further limiting mass transfer and reducing the effective concentration of available catalytic sites [22]. Thus, 1.0% represents a critical threshold where catalytic efficiency and substrate accessibility are optimally balanced.

Similarly, the E/S ratio exhibited a characteristic rise-and-fall pattern (Figure 1c). Antioxidant activity peaked at 6% E/S, reflecting a sufficient density of catalytic sites to drive peptide liberation. Excess enzyme, however, can overly accelerate peptide breakdown, thereby degrading structural motifs crucial for radical-scavenging activity or introducing steric hindrance around catalytic sites [23]. This observation reinforces the delicate balance between promoting hydrolysis and preserving functional peptide integrity.

The influence of pH revealed a pronounced optimum at pH 7.0 (Figure 1d), which is fully consistent with the reported catalytic profile of neutrase. Deviations from this neutral condition can modify protein surface charges, thereby weakening substrate–enzyme complementarity or disrupting the active-site conformation, ultimately impairing hydrolytic efficiency. This pronounced peak at pH 7.0 further underscores the strong pH dependence of the enzymatic reactions responsible for generating antioxidant peptides. A similar result has been reported in the production of peptides from edible bird's nest by-product using neutrase, in which the optimum pH was also determined to be 7.0 [24].

Temperature effects further underscored the importance of maintaining an appropriate catalytic environment (Figure 1e). The maximal activity observed at 40 °C reflects the optimal temperature at which neutrase exhibits sufficient structural flexibility while still preserving its catalytic efficiency. Temperatures deviating from this optimum weaken catalytic turnover, and similar evidence has been demonstrated in a study on yak whey protein hydrolysis, where moderate heating was shown to enhance enzymatic performance [23].

Hydrolysis time had an apparent impact on antioxidant capacity (Figure 1f). Activity rose steadily up to 5 h, indicating the progressive liberation of functional peptides. Beyond this point, further hydrolysis led to a modest decline in activity, a common phenomenon attributed to excessive cleavage of bioactive peptides into short fragments with diminished reactivity [25]. The optimal hydrolysis time of 5 h is particularly advantageous for industrial applications, where shorter processing time without compromising functionality is highly desirable.

2.3. Optimization and Validation of Hydrolysis Based on the BP-ANN Model

To reveal these nonlinear interactions among processing variables, a BP-ANN model was constructed and subjected to systematic optimization. As depicted in Figure 2, a network architecture containing 11 hidden neurons exhibited the strongest predictive performance ($R = 0.9935$; $MSE = 0.0001$), indicating outstanding robustness and generalization capability. The model effectively captured the intricate hydrolysis process, which conventional statistical approaches often fail to characterize with comparable accuracy.

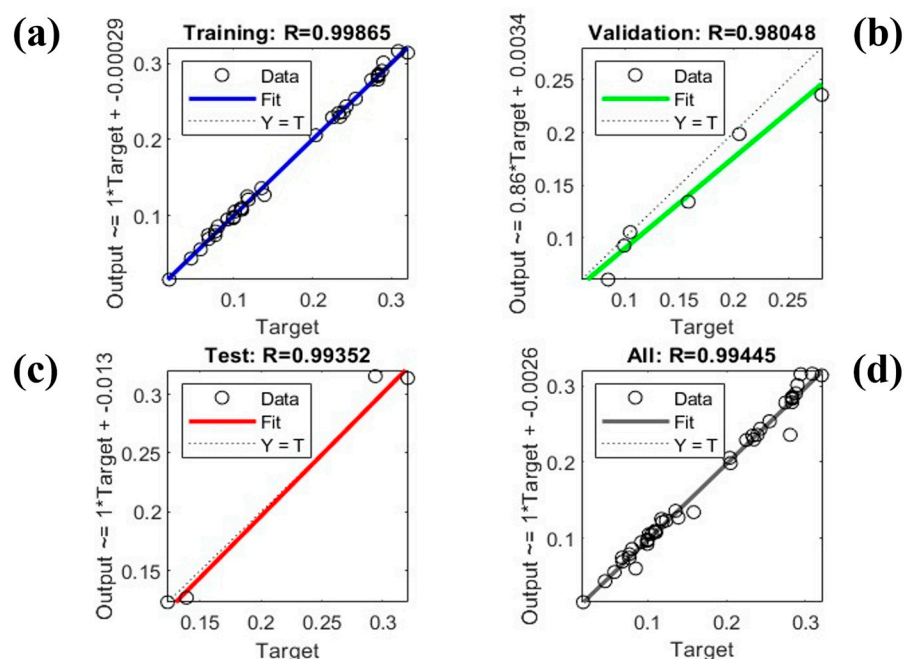


Figure 2. Regression plot observed vs. predicted results. (a) Training. (b) Validation. (c) Test. (d) All.

The optimized model predicted that maximal antioxidant activity would be achieved at an S/L of 1.025%, E/S of 6.4%, pH 7.05, 39.0 °C, and 4.85 h, with the DPPH• and ABTS•+ radical-scavenging activities reaching predicted maximum values of 14.12% and 43.81%, respectively. Experimental verification under these conditions yielded DPPH• and ABTS•+ scavenging activities of $14.08\% \pm 0.17\%$ and $43.71\% \pm 0.68\%$, respectively, aligning almost perfectly with the predicted values (error < 3%). This high accuracy confirms not only the reliability of the ANN model but also its strong capability to direct hydrolysis conditions for consistently accurate predictions of antioxidant performance.

The antioxidant potency of the optimized PSMH surpassed that of many previously reported protein-derived peptides. Its DPPH• IC₅₀ (0.30 mg/mL) was markedly lower than those of Antarctic krill peptides, such as SLPY, QYPPMQY, and EYEA (IC₅₀ ranging from 1.18 to 1.55 mg/mL) [26]. The ABTS•+ IC₅₀ (0.07 mg/mL) also dramatically exceeded those of several reported protein hydrolysates, including bovine elastin hydrolysate (0.539 mg/mL) [27], cottonseed protein hydrolysate (2.05 mg/mL) [5], and selenium-enriched *Moringa oleifera* seed protein hydrolysate (5.45 mg/mL) [22]. This comparison highlights the remarkable efficiency with which neutrase converts PSM protein into peptides of exceptionally high radical-scavenging potential, and further demonstrates the effectiveness of the BP-ANN model in identifying optimized hydrolysis conditions.

2.4. Peptidomics Analysis of the Optimized PSMH

After comprehensive LC–MS/MS analysis, a total of 364 distinct peptide sequences were identified from the PSMH obtained under the optimized conditions, revealing a peptide pool strongly enriched in low-molecular-weight components. The identified peptides comprised 2–16 amino acid residues with molecular weights ranging from 216 to 1576 Da (Figure 3a). Notably, tetrapeptides accounted for the largest proportion (103 peptides, 28.30%), followed by tripeptides and pentapeptides. Strikingly, 344 peptides (94.51%) consisted of six or fewer amino acids, a proportion considerably higher than that reported in comparable studies, where only 67.17% of detected peptides fell within this category [10]. This difference may be attributed to the higher degree of hydrolysis observed in the present study. Moreover, the enrichment of shorter peptides further illustrates the

effectiveness of the ANN-optimized hydrolysis process for promoting the release of bio-active components, since small peptides often possess increased structural flexibility, greater solvent accessibility, and enhanced electron-donating capacity, which collectively contribute to their strong radical-scavenging potential [28,29]. The predominance of these low-molecular-weight peptides provides compelling evidence that PSMH represents a highly suitable substrate for the targeted generation of natural antioxidant peptides, advancing the valorization of *Paeonia suffruticosa* meal from a low-value by-product into a high-value functional ingredient.

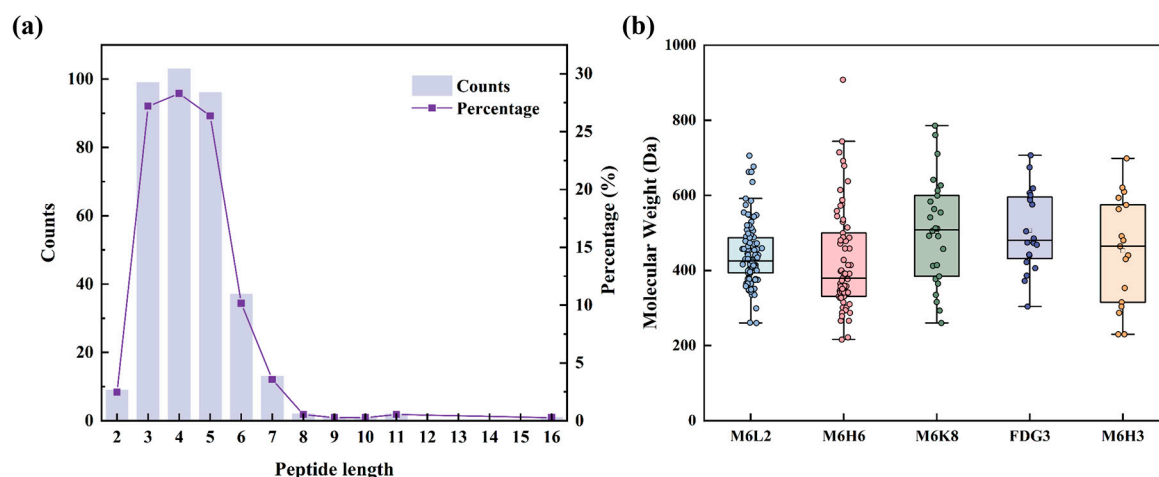


Figure 3. The peptide profile analysis of optimized PSMH. (a) Distribution of peptide length. (b) Molecular weight distribution of the peptides from the top 5 proteins with the most peptides derived. M6L2: A0A2I7M6L2; Ycf2 family; M6H6: A0A2I7M6H6, RpoC2 subfamily; M6K8: A0A2I7M6K8, TIC214 family; FDG3: A0A7D7FDG3, complex I subunit 4; M6H3: A0A2I7M6H3, RNA polymerase beta chain family.

Based on the source protein analysis, it has been revealed that 364 peptides originated from 34 distinct precursor proteins, indicating broad proteolytic coverage. Among these sources, Protein Ycf2 (UniProt ID: A0A2I7M6L2) contributed the greatest number of peptides (93 peptides, 25.55%), which is consistent with our previous report [10], in which 108 peptides (18.2%) were identified from Ycf2 (Figure 3b). Ycf2 is a chloroplast stroma-localized protein unique to *Paeonia ostii*, suggesting that chloroplast structural proteins constitute major contributors to peptide formation during PSM protein hydrolysis. Other abundant precursor proteins included DNA-directed RNA polymerase subunit β (63 peptides), Protein TIC 214 (26 peptides), NAD(P)H-quinone oxidoreductase chain 4 (20 peptides), and RNA polymerase subunit β (17 peptides). These proteins are primarily involved in energy metabolism, transcriptional regulation, and plastid-associated functions, which is consistent with their high natural abundance in seed meal cellular compartments [30].

Mass spectrometry profiling also revealed that 50 peptides lacked detectable signal intensities, suggesting that they were present at extremely low abundance and thus contributed minimally to overall bioactivity. Additionally, three peptides exhibited post-translational modifications, including cysteine alkylation and methionine oxidation. After removal of these 53 peptides, 311 peptides remained for subsequent screening and structure-activity evaluation.

2.5. Potential Bioactivity Evaluation and Physicochemical Property Analysis

PeptideRanker is one of the most widely used *in silico* tools for predicting peptide bioactivity by assigning a probability score ranging from 0 to 1 based on sequence features. Owing to its efficiency and reliability, it is particularly suitable for the initial prioritization of bioactive candidates from large peptidomics datasets [31]. Accordingly, PeptideRanker was employed as the primary screening step to systematically evaluate the bioactivity potential of the 311 unmodified peptides identified from optimized PSMH.

Using a threshold score of 0.5, as commonly adopted in previous studies [32,33], 124 peptides were prioritized as putative bioactive candidates based on PeptideRanker screening (Supplementary Table S1). These peptides were predominantly short sequences, ranging from 2 to 7 amino acids in length, with 95.16% (118/124) comprising pentapeptides or shorter. Notably, all peptides with very high predicted bioactivity ($n = 35$, PeptideRanker score > 0.9) were pentapeptides or shorter. This enrichment of short peptides among the top-ranked candidates suggests a potential association between shorter sequence length and higher predicted bioactivity. Compared with our previous study, in which only 63.2% of the peptides derived from PSMH (PeptideRanker score > 0.5) were pentapeptides or shorter [10], the present work exhibited a substantially higher proportion of short peptides following hydrolysis optimization. This marked increase in the proportion of short peptides indicates that the optimized hydrolysis conditions effectively modulated the peptidomic output toward smaller peptide species, thereby providing an improved basis for subsequent bioactivity-guided screening and validation.

Subsequently, the physicochemical properties of the optimized PSMH were systematically analyzed. From the perspective of molecular weight, all 124 PeptideRanker-prioritized peptides exhibited molecular weights below 800 Da, indicating a clear enrichment of low-molecular-weight species at this screening stage. This distribution further supports the tendency of the optimized hydrolysis conditions to generate relatively small peptide fragments. Isoelectric point (pI) analysis further showed that the peptide pool was dominated by weakly acidic to near-neutral sequences, with most peptides (79.0%) remaining electrically neutral at pH 7.0. Such a pI profile may favor structural stability and interaction compatibility under physiological conditions, since the reduction in net electrostatic repulsion allows the molecule to adopt a more compact and stable folded state, thereby facilitating precise control of noncovalent interactions, such as hydrogen bonding and hydrophobic interactions [34]. Finally, considering that aqueous solubility is generally regarded as an important physicochemical prerequisite for improved release, absorption, and bioavailability of peptides, the water solubility of these peptides was further evaluated, and 21 peptides were ultimately selected for subsequent analyses (Table 1).

Table 1. Predicted bioactivity and physicochemical properties of water-soluble peptides with PeptideRanker scores > 0.5 .

No.	Sequence	Peptideranker Score	Molecular Weight (g/mol)	pI	Net Charge at pH 7.0	Predicted Water Solubility
1	FRF	1.00	468.55	10.59	1	Good
2	DFGF	0.98	484.50	0.76	−1	Good
3	WQFR	0.97	635.71	10.72	1	Good
4	RWL	0.95	473.57	10.55	1	Good
5	GFEW	0.94	537.56	0.88	−1	Good
6	PDFL	0.92	490.55	0.69	−1	Good
7	IRGF	0.90	491.58	10.85	1	Good
8	WFDQ	0.90	594.61	0.76	−1	Good
9	RPP	0.86	368.43	10.55	1	Good
10	FEFR	0.86	597.66	6.62	0	Good

11	IWDP	0.84	529.58	0.88	−1	Good
12	LDRFL	0.84	662.78	6.64	0	Good
13	LFIR	0.74	547.69	10.84	1	Good
14	RPPI	0.71	481.59	10.55	1	Good
15	EGF	0.69	351.35	0.99	−1	Good
16	GIADF	0.67	521.56	0.76	−1	Good
17	FGEY	0.65	514.53	0.95	−1	Good
18	VSRW	0.59	546.62	10.81	1	Good
19	SLRL	0.56	487.59	10.57	1	Good
20	GIDGL	0.55	473.52	0.69	−1	Good
21	LEF	0.50	407.46	0.99	−1	Good

2.6. Binding Affinity Analysis

To further characterize the antioxidant potential of the screened peptides, molecular docking was conducted to evaluate their interactions with representative antioxidant-related targets, including the free radicals ABTS^{•+} and DPPH[•], as well as the Keap1 protein. Docking analysis revealed distinct differences in binding energies among the 21 selected peptides toward these targets (Table 2).

The interactions between the selected peptides and the free radicals ABTS^{•+} and DPPH[•] were first assessed, as these radicals are widely employed in *in vitro* antioxidant assays owing to their well-defined reactivity and pronounced colorimetric responses. During molecular docking, glutathione (GSH) was used as a positive control, exhibiting binding energies of −2.1 kcal/mol for ABTS^{•+} and −2.6 kcal/mol for DPPH[•], which are consistent with those reported in the previous study (−1.9 and −2.6 kcal/mol, respectively) [35], thereby supporting the reliability of the docking protocol. Compared with GSH, most peptides exhibited more favorable (i.e., lower) predicted binding energies toward both ABTS^{•+} and DPPH[•], with the exception of GIADF. This trend is consistent with the results of the corresponding chemical assays, which demonstrated that the optimized PSMH possessed strong radical-scavenging activity toward both ABTS^{•+} and DPPH[•]. Furthermore, compared with a previous report in which three peptides isolated from pearl shell meat hydrolysate via ultrafiltration and RP-HPLC exhibited binding energies ranging from −2.6 to −2.3 kcal/mol (ABTS^{•+}) and −2.8 to −3.5 kcal/mol (DPPH[•]) [36], 85.7% (18/21) and 76.2% (16/21) of the peptides identified in the present study displayed binding energies lower than or equal to −2.3 kcal/mol (ABTS^{•+}) and −2.8 kcal/mol (DPPH[•]), respectively, representing the most favorable values reported previously. These findings further highlight the effectiveness of the hydrolysis optimization strategy in generating peptides with enhanced antioxidant potential.

Keap1, i.e., Kelch-like ECH-associated protein 1, is a key cytoplasmic regulator of cellular redox homeostasis and serves as the primary negative regulator of nuclear factor erythroid 2-related factor 2 (Nrf2) [37]. Occupation of Keap1 by exogenous compounds can disrupt the Keap1-Nrf2 interaction, thereby facilitating Nrf2 nuclear translocation and activation of Nrf2-dependent antioxidant signaling. Given this regulatory mechanism, targeting Keap1 represents a rational strategy for enhancing cellular antioxidant defense. On this basis, the binding interactions between the selected peptides and Keap1 were further evaluated. Most of the screened peptides exhibited more favorable predicted binding energies than GSH, while only one peptide (LEF) showed a weaker interaction (Table 2).

Table 2. Binding affinities of selected peptides toward antioxidant targets.

Inhibitors	Binding Affinity with ABTS ^{•+} (kcal/mol)	Inhibitors	Binding Affinity with DPPH [•] (kcal/mol)	Inhibitors	Binding Affinity with Keap1 (kcal/mol)
FRF	−3.3	FRF	−3.9	WQFR	−10.5
WQFR	−3.3	WFDQ	−3.9	FRF	−10
RWL	−2.9	FEFR	−3.7	WFDQ	−9.7
FEFR	−2.9	WQFR	−3.6	IWDP	−9.6
PDFL	−2.8	LFIR	−3.6	FEFR	−9.5
IWDP	−2.8	VSRW	−3.5	FGEY	−9.2
RPP	−2.7	RWL	−3.4	DFGF	−9.1
LFIR	−2.7	IRGF	−3.3	RWL	−9.1
RPPI	−2.7	GIDGL	−3.3	RPPI	−9.1
EGF	−2.6	GFEW	−3.2	IRGF	−8.8
FGEY	−2.5	PDFL	−3.2	PDFL	−8.6
IRGF	−2.4	RPP	−3.2	RPP	−8.3
LEF	−2.4	RPPI	−3.2	LDRFL	−8.2
WFDQ	−2.3	EGF	−3.2	GIADF	−8.2
SLRL	−2.3	IWDP	−3.1	LFIR	−8
GIDGL	−2.3	LDRFL	−3.1	EGF	−8
GFEW	−2.2	FGEY	−3.1	GFEW	−7.9
DFGF	−2.1	SLRL	−3.1	SLRL	−7.8
LDRFL	−2.1	LEF	−2.8	GIDGL	−7.8
VSRW	−2.1	DFGF	−2.7	VSRW	−7.7
GSH*	−2.1	GSH*	−2.6	GSH *	−7
GIADF	−1.9	GIADF	−2.1	LEF	−6.9

* GSH refers to the positive control glutathione.

Generally, a binding energy of −7.0 kcal/mol has been proposed as a reference threshold for peptide–Keap1 interactions in *in silico* screening studies [38], with peptides exhibiting binding energies lower than this value considered promising candidates. In this study, the majority of the selected peptides displayed favorable binding energies toward Keap1, with 76.2% (16/21) of the peptides possessing binding affinities lower than or equal to −8.0 kcal/mol, indicating their strong capacity to interact with the Keap1 Kelch domain. These values suggest stable peptide–Keap1 complex formation within the Kelch domain binding pocket. Notably, this binding energy range was more favorable or at least comparable to previous reports. For example, five peptides screened from wheat gluten hydrolysate through enzymatic digestion, separation, and purification exhibited binding affinities ranging from −9.1 to −7.9 kcal/mol [39], which are comparable to the values obtained in the present study. In addition, antioxidant peptides identified from coix seed hydrolysates after purification and *in silico* screening exhibited binding affinities ranging from −6.1 to −2.8 kcal/mol [40], which are markedly weaker than those observed in the present study, further highlighting the potential advantage of the optimized hydrolysis strategy.

Among the 21 selected peptides, four sequences, including FRF, WQFR, FEFR, and RWL, were consistently ranked among the top 10 candidates across all three targets and exhibited more favorable predicted binding energies than GSH (Table 2). Based on their overall performance, these four peptides were selected as representative candidates for further detailed interaction analyses with the three antioxidant-related targets.

2.7. Docking Conformation Analysis

To further investigate the binding modes and interaction features of the selected peptides with antioxidant-related targets, docking conformations were analyzed.

As shown in Figure 4a–d, the binding of the peptides to ABTS^{•+} was primarily mediated by a combination of hydrophobic interaction and hydrogen bonding. Specifically, FRF established multiple noncovalent interactions with ABTS^{•+}, including four hydrophobic interactions (two π – π stacked and two π –alkyl interactions) and one π –cation electrostatic interaction. WQFR displayed the most extensive hydrophobic interaction network, comprising six π – π stacked interactions, together with two conventional hydrogen bonds, forming a mixed interaction pattern involving aromatic contacts and directional hydrogen bonding. FEFR also formed multiple noncovalent contacts with ABTS^{•+}, including four π – π stacked hydrophobic interactions and two hydrogen bonds (one conventional hydrogen bond and one carbon hydrogen bond), resulting in a mixed interaction pattern involving both hydrophobic interaction and hydrogen bonding. In contrast, RWL exhibited a more complex interaction mode, including one conventional hydrogen bond, four π – π stacked hydrophobic interactions, two π –cation electrostatic interactions, and one π –sulfur interaction, suggesting a diversified binding mechanism driven by aromatic stacking and electrostatic effects.

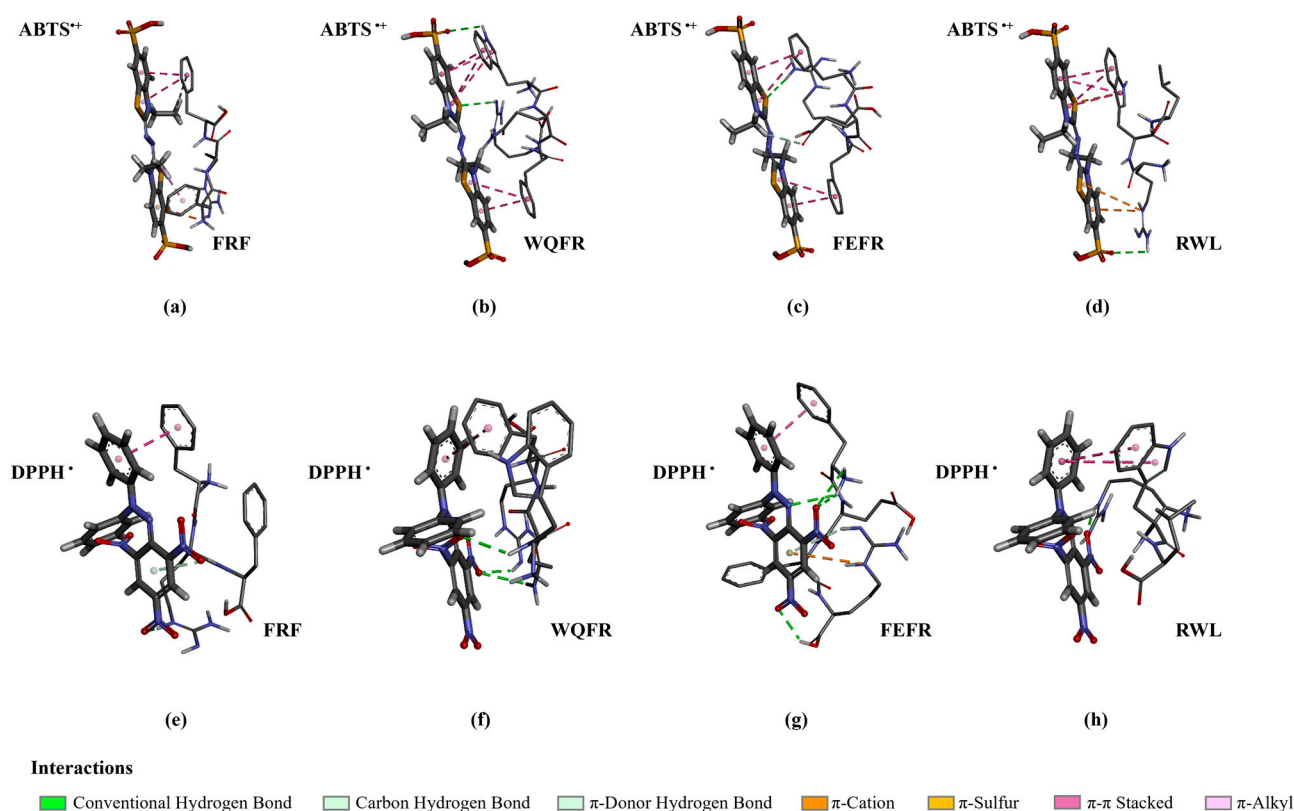


Figure 4. Molecular interactions between the selected peptides and ABTS^{•+} or DPPH[•]. (a–d) 3D docking conformations of the complexes formed between ABTS^{•+} and the peptides FRF (a), WQFR (b), FEFR (c), and RWL (d), respectively. (e–h) 3D docking conformations of the complexes formed between DPPH[•] and the peptides FRF (e), WQFR (f), FEFR (g), and RWL (h), respectively.

Similarly to ABTS^{•+}, the interactions between the peptides and DPPH[•] were also mainly mediated by a combination of hydrophobic interactions and hydrogen bonding (Figure 4e–h). However, the resulting interaction patterns were more dependent on

hydrogen bonding than on hydrophobic interactions, which differed markedly from those observed for ABTS^{••}. In particular, FRF formed a set of noncovalent contacts with DPPH[•], including one π – π stacked hydrophobic interaction and two hydrogen bonds (one conventional hydrogen bond and one π –donor hydrogen bond), resulting in a balanced interaction pattern involving both hydrophobic and hydrogen-bonding contributions. WQFR interacted with DPPH[•] predominantly through three conventional hydrogen bonds, supplemented by a single π – π stacked hydrophobic interaction, resulting in a hydrogen-bond-enriched interaction pattern. FEFR exhibited the most diverse interaction profile toward DPPH[•], combining five hydrogen bonds (four conventional hydrogen bonds and one π –donor hydrogen bond) with one π –cation electrostatic interaction and one π – π stacked hydrophobic interaction, forming a mixed interaction pattern involving multiple interaction types. In contrast, RWL formed a relatively simpler interaction pattern with DPPH[•], characterized by two π – π stacked hydrophobic interactions and one conventional hydrogen bond, indicating a comparatively lower contribution from hydrogen bonding than that observed for FEFR and WQFR.

Subsequently, the docking conformations between the peptides and Keap1 were further analyzed. Generally, Nrf2 recognition within the Kelch domain of Keap1 is mediated by a set of key residues, including Tyr334, Ser363, Arg380, Asn382, Asn387, Arg415, His436, Arg483, Tyr525, and Ser555 [41]. Among the four peptides, only FRF directly engaged the reported Nrf2-recognition residues inside the Keap1 Kelch domain, forming two π – π stacked interactions with Tyr334 and Tyr525 and one conventional hydrogen bond with Ser363. Although such an interaction is crucial, whether it translates into functional disruption of the Keap1–Nrf2 interaction will require further biochemical validation. In addition to these Kelch domain-relevant interactions, FRF formed five additional conventional hydrogen bonds with Ser508, Leu365, Ile416, and Val604 (Figure 5a,b).

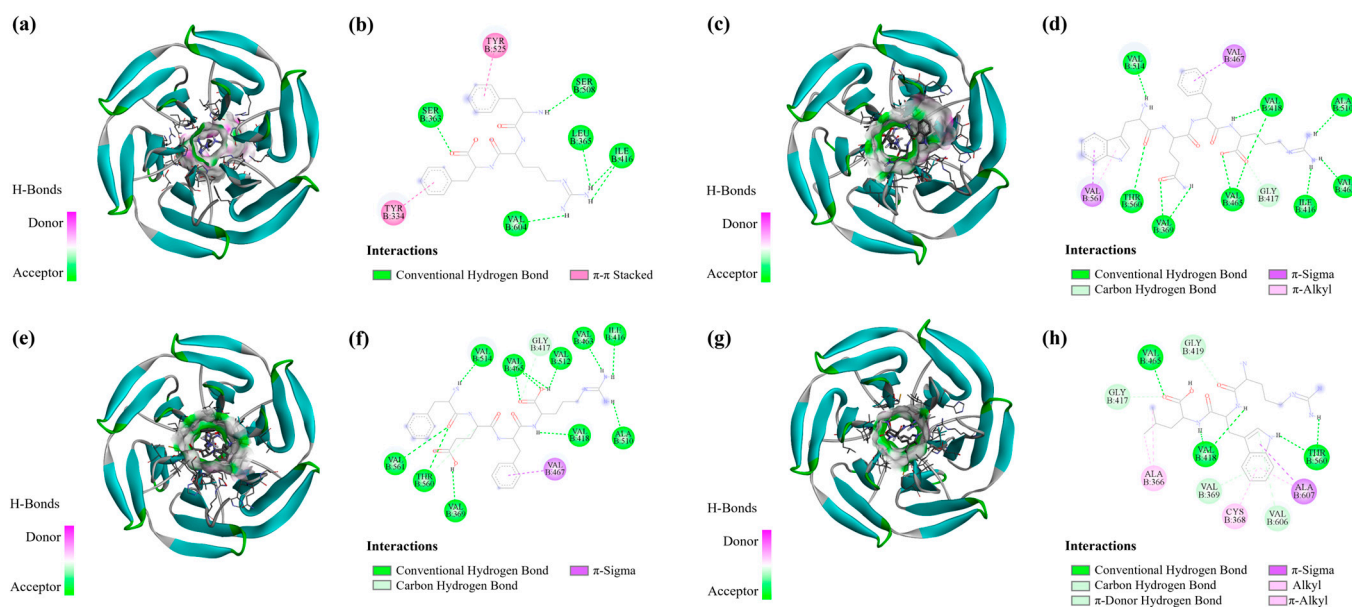


Figure 5. 3D and 2D interaction representations of the complexes formed between Keap1 and the selected peptides. (a,b) Keap1–FRF complex: 3D docking conformation (a) and corresponding 2D interaction diagram (b); (c,d) Keap1–WQFR complex: 3D docking conformation (c) and corresponding 2D interaction diagram (d); (e,f) Keap1–FEFR complex: 3D docking conformation (e) and corresponding 2D interaction diagram (f); (g,h) Keap1–RWL complex: 3D docking conformation (g) and corresponding 2D interaction diagram (h).

In contrast to FRF, the remaining three peptides did not directly engage the Nrf2-recognition residues. Instead, their interactions were primarily distributed across non-canonical regions (Figure 5c–h). Among them, WQFR formed an extensive interaction network with Keap1 dominated by hydrogen bonding, involving ten conventional hydrogen bonds and one carbon hydrogen bond, together with additional π -related hydrophobic contacts, including two π -sigma interactions and one π -alkyl interaction. Similarly, FEFR formed an elaborate hydrogen-bonding network with Keap1, comprising twelve conventional hydrogen bonds and two carbon hydrogen bonds, accompanied by a single π -sigma interaction. RWL exhibited a heterogeneous interaction pattern, involving five conventional hydrogen bonds and four carbon hydrogen bonds, together with multiple hydrophobic contacts, including four π -alkyl/alkyl interactions and one π -sigma interaction.

Overall, the docking analysis revealed that the binding between the peptides and Keap1 was predominantly driven by hydrogen bonding, accompanied by several hydrophobic interactions. FRF showed residue-specific engagement with key Nrf2-recognition residues, whereas the other peptides primarily interacted with peripheral regions of the Kelch domain. Notably, interactions occurring within the canonical Nrf2-recognition pocket may contribute more significantly to binding affinity, as FRF exhibited a relatively strong binding energy despite forming fewer total interactions with Keap1. These differences in interaction architecture provide a plausible structural basis for their differential antioxidant potential.

2.8. Molecular Dynamics (MD) Simulations

To further evaluate the dynamic stability of the peptide–Keap1 complexes, 100 ns MD simulations were conducted for the selected systems (Figure 6).

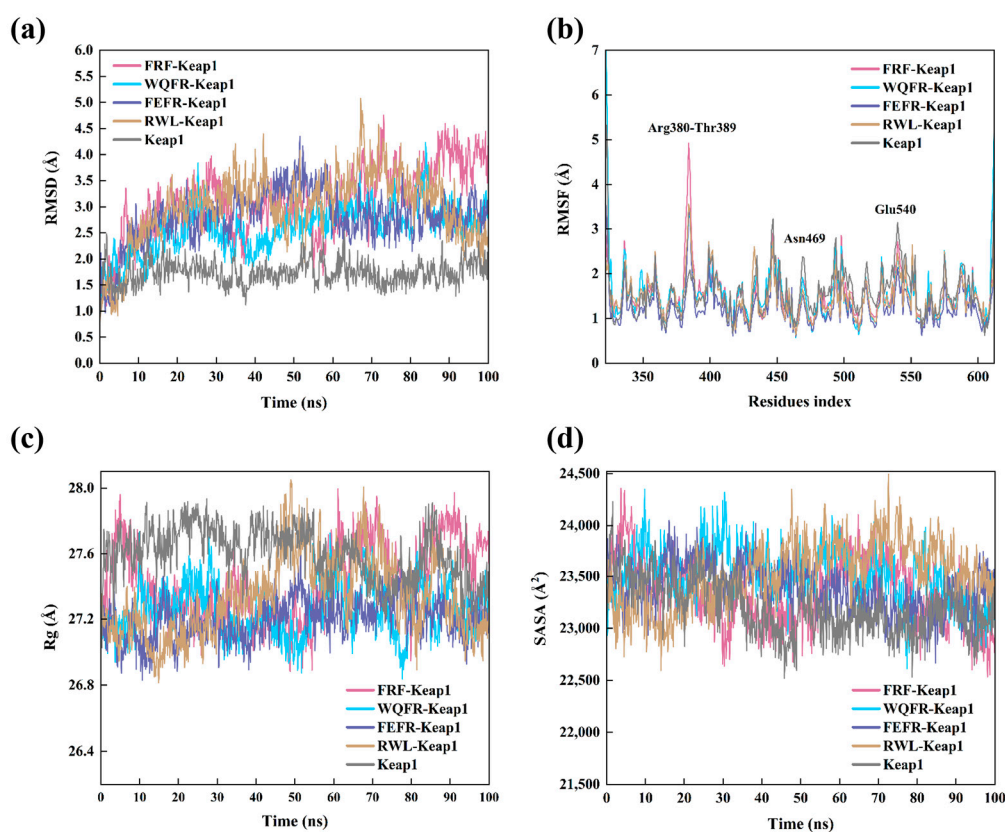


Figure 6. MD simulation of the peptide–Keap1 complex over 100 ns. (a) RMSD values; (b) RMSF values; (c) Rg values; and (d) SASA values.

In root mean square deviation (RMSD) analysis (Figure 6a), all systems exhibited an initial increase in RMSD during the early stage of the simulations (first 20 ns), followed by stabilization after equilibration. Generally, during the equilibrated phase after 20 ns, the average RMSD values of the complexes ranged from about 2.5 to about 3.5 Å. These results were similar to those reported in a previous study on antioxidant peptides derived from low-salt dry-cured ham skin, in which the RMSD values of the peptide–Keap1 complexes converged to approximately 2.0–3.0 Å after 20 ns [42]. Specifically, the average RMSD values of the FRF–Keap1, WQFR–Keap1, FEFR–Keap1, and RWL–Keap1 complexes were 3.61 ± 0.35 Å, 2.73 ± 0.16 Å, 3.00 ± 0.22 Å, and 2.56 ± 0.23 Å, respectively, which are lower than that reported for the donkey-hide gelatin peptide PGPAP–Keap1 complex (3.89 ± 0.86 Å) [43] and also comparable to or lower than the RMSD range (3.0–4.5 Å) observed for the complex formed between QPPALNDSYLYGPQ, a peptide derived from *Azumapecten farreri* meat, and Keap1 [44]. Moreover, all four complexes maintained RMSD fluctuations within a relatively narrow range, with no abrupt structural deviations observed throughout the simulation, indicating the overall structural stability of the peptide–Keap1 systems.

Root mean square fluctuation (RMSF) represents the mean deviation of individual amino acid residues from their average conformations and serves as a quantitative indicator of residue-level dynamics and structural flexibility [32]. RMSF analysis was performed in this study to evaluate changes in the flexibility of Keap1 upon peptide binding (Figure 6b). Among the four complexes, the FRF–Keap1 system exhibited pronounced localized fluctuations in the region spanning Arg380 to Thr389. This segment includes residues Arg380, Asn382, and Asn387, which have been previously reported to participate in Nrf2 recognition [45], suggesting that FRF binding induced noticeable local dynamic responses within this region of the Kelch domain, consistent with the docking conformation analysis described above. In comparison, the remaining peptide–Keap1 complexes (WQFR–Keap1, FEFR–Keap1, and RWL–Keap1) also exhibited RMSF variations within the Arg380–Thr389 segment. However, the magnitude of these fluctuations was less pronounced than that observed for the FRF–Keap1 complex. In contrast, regions surrounding Asn469 and Glu540 displayed consistently reduced RMSF values across all four peptide–Keap1 complexes relative to unbound Keap1, indicating a general stabilizing effect of peptide binding in these regions. Collectively, these findings suggest that FRF may exert a more pronounced residue-specific dynamic modulation within the canonical Nrf2-recognition region, whereas all peptides contribute to structural stabilization in distal regions of the Kelch domain.

Radius of gyration (Rg) was analyzed to assess the overall compactness of Keap1 during the MD simulations (Figure 6c). Throughout the simulation period, the Rg values of all peptide–Keap1 complexes remained relatively stable, exhibiting only minor fluctuations. The average Rg values of the FRF–Keap1 (27.44 ± 0.23 Å), WQFR–Keap1 (27.26 ± 0.16 Å), FEFR–Keap1 (27.19 ± 0.12 Å), and RWL–Keap1 (27.32 ± 0.24 Å) systems were comparable and fell within a narrow range, similar to that of unbound Keap1 (27.50 ± 0.23 Å). These results indicate that peptide binding did not induce pronounced changes in the global compactness of Keap1, and the overall structural integrity of the protein was well maintained upon interaction with the peptides.

Solvent-accessible surface area (SASA) analysis was performed to evaluate changes in the solvent exposure of Keap1 upon peptide binding during the MD simulations (Figure 6d). Throughout the simulation period, consistent with the Rg results, the SASA values remained relatively stable for all peptide–Keap1 complexes, exhibiting only minor fluctuations. The average SASA values of the FRF–Keap1 ($23,339 \pm 315$ Å²), WQFR–Keap1 ($23,516 \pm 261$ Å²), FEFR–Keap1 ($23,357 \pm 262$ Å²), and RWL–Keap1 ($23,505 \pm 315$ Å²) complexes were comparable and showed no pronounced deviation from that of unbound

Keap1 ($23,139 \pm 271 \text{ \AA}^2$). The relatively small differences among these values further suggest that peptide association did not markedly alter the solvent-accessible surface area of Keap1, and the global surface characteristics of the protein were preserved during complex formation.

While molecular docking and molecular dynamics simulations provided structural insights into peptide–target interactions, these *in silico* analyses alone are insufficient to fully characterize antioxidant bioactivity under physiological conditions. Therefore, cell-based assays were performed to further evaluate the antioxidant effects of the selected peptides.

2.9. Cellular Evaluation

2.9.1. Cytotoxicity and Cytoprotective Effects

The cytotoxicity and cytoprotective effects of four selected peptides (FRF, WQFR, FEFR, and RWL) were evaluated using HepG2 cells over a concentration range of 31.25–1000 $\mu\text{g/mL}$. As shown in Figure 7a–d, cell viability remained above 95% at concentrations up to 250 $\mu\text{g/mL}$ following treatment with each peptide, indicating no apparent cytotoxicity within this range. At higher concentrations, a modest decline in viability was observed. However, cell viability consistently remained above 85% even at the maximum tested concentration (1000 $\mu\text{g/mL}$). These results demonstrate that all four peptides exhibit favorable cellular safety profiles under the experimental conditions, thereby supporting their suitability for subsequent cytoprotective and mechanistic investigations.

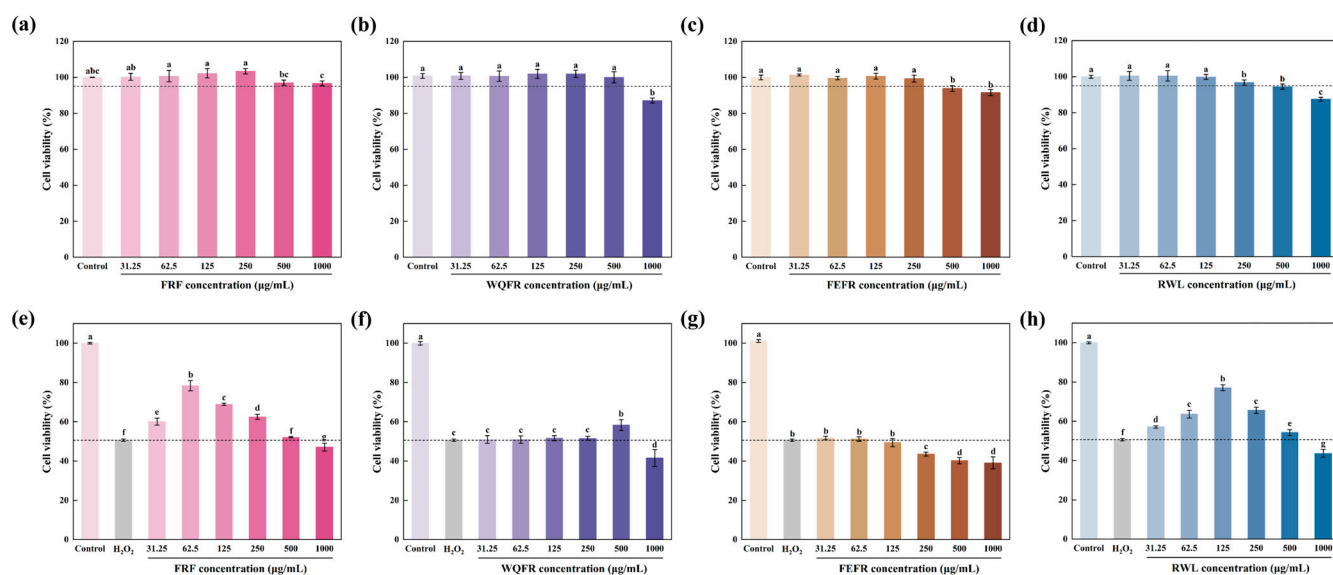


Figure 7. Evaluation of the cytotoxicity and cytoprotective effects of the selected peptides in HepG2 cells. (a–d) Cell viability of HepG2 cells after treatment with FRF (a), WQFR (b), FEFR (c), and RWL (d) at various concentrations. (e–h) Cytoprotective effects of FRF (e), WQFR (f), FEFR (g), and RWL (h) against H_2O_2 -induced oxidative stress in HepG2 cells. Results are shown as mean $\pm$ SD ($n = 3$). Different superscript letters indicate significant differences ($p < 0.05$, one-way ANOVA followed by Duncan's test). Dotted lines indicate reference lines for the non-cytotoxicity threshold (95%, (a–d)) and the 500 μM H_2O_2 group level (50.65%, (e–h)).

To further assess cytoprotective effects under oxidative stress conditions, HepG2 cells were pretreated with the peptides at the same concentration range prior to H_2O_2 exposure. Treatment with 500 μM H_2O_2 alone reduced cell viability to 50.65% relative to the untreated control (Supplementary Figure S1), confirming the successful establishment of the oxidative stress model.

As shown in Figure 7e–h, pretreatment with FRF and RWL significantly attenuated H₂O₂-induced cytotoxicity in a concentration-dependent manner. Notably, FRF exhibited its maximal protective effect at 62.5 µg/mL, restoring cell viability to 78.37%, whereas RWL showed the strongest protection at 125 µg/mL, with cell viability reaching 77.12%. These protective effects were achieved at substantially lower concentrations than those reported for antioxidant peptides derived from yak skin, which restored cell viability to approximately 80% at 500 µg/mL [46]. In contrast, WQFR displayed only a modest protective effect at 500 µg/mL, where cell viability increased from 50.60% to 58.33%, while FEFR did not exhibit a significant cytoprotective effect under the tested conditions.

These differences in cytoprotective efficacy are consistent with the molecular docking and MD simulation results described above. FRF directly interacted with key Nrf2-recognition residues within the Keap1 Kelch domain, which may underlie its superior protective performance against H₂O₂-induced damage. Similarly, the RWL–Keap1 complex exhibited the lowest RMSD value among the four systems, indicating enhanced dynamic stability, which may contribute to its relatively strong cytoprotective effect. Based on these findings, FRF and RWL were selected for further investigation of intracellular antioxidant enzyme-related responses.

2.9.2. Effects of FRF and RWL on Antioxidant Enzyme Activities

To examine whether the cytoprotective effects of FRF and RWL under oxidative stress were associated with modulation of intracellular antioxidant defenses, the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were evaluated in H₂O₂-challenged HepG2 cells. Based on the dose–response profiles obtained from the cytoprotective assays, 62.5 µg/mL for FRF and 125 µg/mL for RWL were identified as the most effective non-cytotoxic concentrations and were therefore selected for subsequent analyses.

Exposure to H₂O₂ alone significantly suppressed the activities of all three antioxidant enzymes compared with the untreated control group ($p < 0.05$, $n = 3$), indicating marked impairment of endogenous antioxidant defenses under oxidative stress conditions (Figure 8). Pretreatment with FRF or RWL significantly alleviated the H₂O₂-induced reduction in antioxidant enzyme activities. Specifically, SOD activity increased from 20.63 U/mg prot in the H₂O₂ model group to 25.65 and 24.04 U/mg prot following FRF and RWL pretreatment, respectively. Similarly, CAT activity was restored from 67.75 U/mg prot to 83.23 U/mg prot by FRF and to 78.83 U/mg prot by RWL, while GSH-Px activity increased from 161.42 U/mg prot to 195.38 and 180.43 U/mg prot after FRF and RWL treatment, respectively. Collectively, these findings indicate that the protective effects of both peptides against H₂O₂-induced oxidative damage in HepG2 cells are associated with the upregulation of intracellular antioxidant enzyme activities, consistent with previous reports [47]. Moreover, FRF elicited a more pronounced enhancement of antioxidant enzyme activities than RWL, further corroborating its superior cytoprotective performance observed in the viability assays.

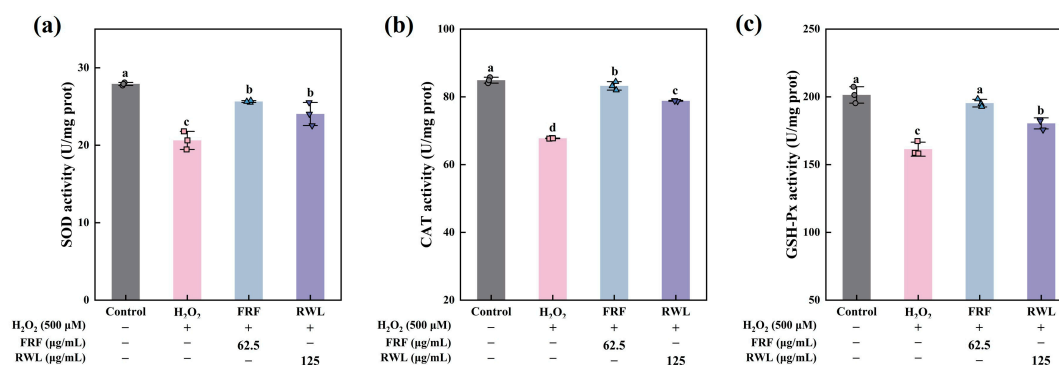


Figure 8. Effects of FRF and RWL pretreatment on antioxidant enzyme activities in the H₂O₂-induced oxidative stress model of HepG2 cells. (a) SOD activity; (b) CAT activity; and (c) GSH-Px activity. Results are shown as mean $\pm$ SD ($n = 3$). Different superscript letters indicate significant differences ($p < 0.05$, one-way ANOVA followed by Duncan's test).

3. Materials and Methods

3.1. Materials

The seed meal of “FengDan” peony (*Paeonia ostii*) was generously provided by Guohuafang Peony Biotechnology Co., Ltd. (Luoyang, China). Proteases, including flavourzyme, protamex, and neutrase (Novozymes, Tianjin, China), and papain (Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China) were used in this study.

The HepG2 cell line was obtained from Wuhan Procell Life Science & Technology Co., Ltd. (Wuhan, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Cytiva (Shanghai, China). Penicillin–streptomycin solution and trypsin–EDTA were supplied by Solarbio Biotechnology Co., Ltd. (Beijing, China), and fetal bovine serum (FBS) was obtained from Enmoasai Biological Technology Co., Ltd. (Changzhou, China). The bicinchoninic acid (BCA) protein assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Assay kits for SOD, CAT, and GSH-Px were obtained from Beyotime Biotechnology Co., Ltd. (Shanghai, China).

3.2. Enzymatic Hydrolysis Optimization

3.2.1. Screening of Proteases

PSMH was prepared by alkali extraction and acid precipitation according to our previous study [10]. To assess the influence of different proteases, PSM peptide was subjected to enzymatic hydrolysis using four proteases, including flavourzyme, protamex, neutrase, and papain, and the hydrolysates were subsequently evaluated in terms of the DH and antioxidant activities, including DPPH• and ABTS•• radical-scavenging assays. (The optimal pH and temperature conditions for each protease are summarized in Supplementary Table S2.) Before hydrolysis, the PSMH powder was thoroughly mixed with a buffer at the optimal pH of each protease, maintaining a 5% substrate-to-liquid ratio (S/L, w/v). The hydrolysis process was initiated by adding each protease at a 2% enzyme-to-substrate ratio (E/S, w/w) and conducted for 5 h at its corresponding optimal temperature. Following hydrolysis, the reaction was terminated by boiling for 10 min. The resulting hydrolysate was centrifuged at 10,000 $\times$ g for 10 min, and the supernatant was collected, lyophilized, and stored at -20°C for further analysis.

3.2.2. Single-Factor Optimization

The hydrolysis process is generally influenced by multiple parameters, including the S/L ratio, E/S ratio, pH, temperature, and hydrolysis time. To initially investigate the

effects of these factors on the antioxidant activity of the hydrolysates, single-factor experiments were designed and conducted under the following conditions based on preliminary results: S/L (*w/v*): 0.5%, 1%, 2%, 3%, and 4%; E/S (*w/w*): 2%, 4%, 6%, 8%, and 10%; pH: 6.0, 6.5, 7.0, 7.5, and 8.0; temperature: 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C; and hydrolysis time: 4 h, 4.5 h, 5 h, 5.5 h, and 6 h. The antioxidant activity of the hydrolysates was evaluated using DPPH• and ABTS•+ radical-scavenging assays, and these experimental results were subsequently used to construct the dataset for later modeling and optimization analyses.

3.2.3. ANN-Based Optimization

ANNs are multilayered computational models composed of interconnected neurons whose weights are iteratively optimized using the backpropagation (BP) algorithm to minimize prediction errors. This enables ANNs to learn complex nonlinear relationships and generalize well to new data.

To provide training data for the ANN model, a dataset was constructed using the results from the single-factor experiments described above. Each data entry comprised five input variables (S/L, E/S, pH, temperature, and hydrolysis time) and two output indicators (DPPH• and ABTS•+ radical-scavenging activities). To eliminate the influence of differing scales among input features and improve training efficiency, all input variables were normalized based on the min–max normalization method [48], using the normalization formula as follows:

$$X = \frac{X_i - X_{\min}}{X_{\max} - X_{\min}} \quad (1)$$

where X_i is the original value of the feature and $X_{\min}$ and $X_{\max}$ are the minimum and maximum values of that feature, respectively. After normalization, all values were scaled to the range [0, 1].

Based on the normalized dataset, a feedforward backpropagation artificial neural network (BP-ANN) model was established using the Neural Network Toolbox in MATLAB R2023b (MathWorks Inc., Natick, MA, USA). The network architecture included five input neurons (representing the five hydrolysis parameters as mentioned above), eleven neurons in the hidden layer, and two output neurons representing DPPH• and ABTS•+ radical-scavenging activities. The number of hidden neurons was initially estimated using the Hecht–Nielsen empirical rule and further refined through iterative testing [49].

The model was trained using the Levenberg–Marquardt backpropagation algorithm, which offers rapid convergence and is particularly suitable for small datasets. Random sampling was used to split the 25-sample dataset into a training set (76%, $n = 19$), a validation set (12%, $n = 3$), and a test set (12%, $n = 3$). Training was automatically halted if the mean squared error (MSE) of the validation set increased for three consecutive epochs [50]. Given the exploratory nature of the optimization, a limited dataset was used.

Model performance was evaluated using the correlation coefficient (R) and the MSE, which assess the agreement between predicted and actual values. A higher R (closer to 1) indicates stronger linear correlation, while a lower MSE (closer to 0) reflects better predictive accuracy. The formulas are as follows:

$$R = \frac{\sum_1^n (x - \bar{x})(y - \bar{y})}{\sqrt{\sum_1^n (x - \bar{x})^2 \sum_1^n (y - \bar{y})^2}} \quad (2)$$

$$MSE = \frac{\sum_1^n (y - x)^2}{n} \quad (3)$$

where x is actual data, y is predicted data, $\bar{x}$ and $\bar{y}$ are the average of predicted and actual data, respectively, and n is the number of samples.

3.3. Determination of DH

The DH was determined using the o-phthaldialdehyde (OPA) assay, with Gly-Gly used as the standard [51]. The DH was calculated using the following formula:

$$DH (\%) = \frac{C_2 - C_1}{C_0 - C_1} \times 100 \quad (4)$$

where C_1 and C_2 are the free amino content (mol/L) before and after hydrolysis, respectively, and C_0 is the total amino content (mol/L) determined by the Kjeldahl method.

3.4. Antioxidant Activity Assays

3.4.1. DPPH• Radical-Scavenging Activity Assay

The DPPH• radical-scavenging activity was determined according to a previously reported method with slight modifications [52]. Briefly, the PSMH solutions were diluted to 0.05 mg/mL and mixed with a 40 µg/mL DPPH• solution at a ratio of 1:2 (v/v). The mixture was incubated in the dark at room temperature for 30 min. After incubation, the absorbance was measured at 517 nm using a UV-Vis spectrophotometer (L5S; INESA Analytical Instrument Co., Ltd., Shanghai, China). The DPPH• radical-scavenging rate (%) was calculated using the following formula:

$$\text{DPPH}\bullet \text{ radical-scavenging rate (\%)} = 1 - \frac{A_1 - A_2}{A_0} \times 100 \quad (5)$$

where A_1 is the absorbance of the sample with DPPH• solution; A_2 is the sample's background absorbance without DPPH• solution; and A_0 is the absorbance of the DPPH• solution with deionized water (blank).

3.4.2. ABTS•+ Radical-Scavenging Activity Assay

The ABTS•+ radical-scavenging activity was evaluated based on a previously reported method with slight modifications [53]. Briefly, the PSMH solutions were diluted to 0.05 mg/mL and mixed with the ABTS•+ working solution at a ratio of 1:3 (v/v). The mixture was incubated in the dark at room temperature for 6 min, after which the absorbance was measured at 734 nm using a UV-Vis spectrophotometer. The ABTS•+ radical-scavenging rate (%) was calculated using the following formula:

$$\text{ABTS}\bullet^+ \text{ radical-scavenging rate (\%)} = 1 - \frac{A_1 - A_2}{A_0} \times 100 \quad (6)$$

where A_1 is the absorbance of the sample with ABTS•+ solution; A_2 is the sample's background absorbance without ABTS•+ solution; and A_0 is the absorbance of the ABTS•+ solution with deionized water (blank).

3.5. Peptidomics Analysis of the PSMH

The peptidomic analysis of the optimized PSMH was performed using nano-LC-MS/MS according to a previously established method [54]. Briefly, the analysis was carried out on an Ultimate 3000 system coupled to a Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The acquired raw data were processed using Byonic software (version 4.2.4; Protein Metrics Inc., Cupertino, CA, USA).

3.6. Virtual Screening of the Peptides from PSMH

The potential bioactivity of peptides identified from PSMH was initially evaluated using PeptideRanker, an online bioactive peptide prediction tool (<http://distill-deep.ucd.ie/PeptideRanker/> (accessed on 22 November 2025)) [55]. This tool employs a machine learning algorithm to assign a score ranging from 0 to 1 to each peptide sequence, thereby reflecting its probability of exhibiting bioactivity. In this study, peptides with PeptideRanker scores greater than 0.5 were considered potentially bioactive and selected for further analysis.

The water solubility of the selected peptides was subsequently evaluated using the Peptide Property Calculator (<http://www.innovagen.com/proteomics-tools> (accessed on 22 November 2025)). Meanwhile, key physicochemical properties, including molecular weight, isoelectric point (pI), and net charge at physiological pH (7.0), were also calculated to provide a comprehensive assessment of peptide characteristics. The peptides with both potential bioactivity and good water solubility were then subjected to molecular docking analysis.

3.7. Molecular Docking

Molecular docking was performed to investigate the interactions between the selected peptides and representative antioxidant targets, including the free radicals ABTS (ABTS^{•+}) and DPPH (DPPH[•]), as well as the key target protein Keap1 in the Keap1–Nrf2 pathway. These targets were selected to represent direct radical-scavenging mechanisms and protein-mediated antioxidant pathways, respectively. The 3D structures of the peptides were constructed using PyMOL (version 3.1.0; Schrödinger, LLC, New York, NY, USA) and saved in MOL2 format. Subsequently, the peptide structures were prepared for docking using AutoDock Tools (version 4.2.6; The Scripps Research Institute, La Jolla, CA, USA), which involved the addition of hydrogen atoms and the definition of torsion trees to allow all rotatable bonds to rotate freely. The structures of ABTS^{•+} (CID: 5360881) and DPPH[•] (CID: 2735032) were obtained from the PubChem database and converted to PDB format using Open Babel (version 2.4.1; The Open Babel Team, USA). The crystal structure of Keap1 (PDB ID: 4L7B, resolution 2.41 Å) was retrieved from the Protein Data Bank database. Prior to docking, water molecules were removed from all receptor structures using AutoDock Tools, and the processed files were saved in PDBQT format. Molecular docking simulations were conducted using AutoDock Vina (version 1.1.2; The Scripps Research Institute, La Jolla, CA, USA) with the docking settings specified (exhaustiveness = 8; num_modes = 9), and the docking grid parameters were defined as follows:

ABTS ^{•+}	Center coordination: center_x = 0; center_y = 0; center_z = 0
	Box size: size_x = 20; size_y = 20; size_z = 20
DPPH [•]	Center coordination: center_x = 0; center_y = 0; center_z = 0
	Box size: size_x = 15; size_y = 15; size_z = 15
Keap1	Center coordination: center_x = 0; center_y = 0; center_z = 22.15
	Box size: size_x = 80; size_y = 80; size_z = 80

The conformation and interactions of the complex were visualized using PyMOL and Discovery Studio Visualizer (version 17.2.0; Dassault Systèmes BIOVIA, San Diego, CA, USA) based on the docking pose with the lowest binding energy.

3.8. Molecular Dynamics (MD)

MD simulations were performed using YASARA Dynamics (version 14.10.5; YASARA Biosciences GmbH, Vienna, Austria) for 100 ns on the following systems: the WQFR–Keap1 complex, the FRF–Keap1 complex, the FEF–Keap1 complex, the RWL–Keap1 complex, and unbound Keap1 as a blank control. For each complex, the initial structure for MD simulation was selected as the lowest-energy binding conformation

obtained from molecular docking. All MD simulations were carried out following the protocol previously established by our group [35]. Briefly, MD simulations were conducted using the AMBER14 force field. The system was solvated in a simulation cell with periodic boundary conditions, utilizing the TIP3P explicit water model. To mimic physiological conditions, an ion concentration of 0.9% NaCl was maintained. The system was neutralized by replacing water molecules with counter-ions at positions of maximum or minimum electrostatic potential. After the simulations, trajectory analyses were conducted to evaluate the root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), and solvent-accessible surface area (SASA), which collectively reflect the structural stability, flexibility, compactness, and solvent exposure of the systems during the simulation period.

3.9. Peptide Synthesis

The selected peptides were synthesized by solid-phase peptide synthesis at Sangon Biotech Co., Ltd. (Shanghai, China). All peptides were purified to a minimum purity of 98%. Peptide identity and purity were confirmed by high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) prior to biological activity evaluation (Supplementary Figure S2).

3.10. Cellular Antioxidant Activity Assay

3.10.1. Cell Culture

HepG2 cells were cultured in DMEM supplemented with 4.0 mmol/L L-glutamine, 4.5 g/L glucose, 10% (*v/v*) FBS, and 1% penicillin–streptomycin, and maintained at 37 °C in a humidified atmosphere containing 5% CO₂. The culture medium was refreshed daily. Cells at approximately 80–90% confluence were detached for subsequent experiments using trypsin–EDTA.

3.10.2. Cytotoxicity Assay

The cytotoxic effects of the selected peptides on HepG2 cells were evaluated using the MTT assay, following the previously described method [56]. Briefly, HepG2 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h to allow attachment. Following incubation, the medium was replaced with fresh DMEM containing various concentrations of the selected peptides, and the cells were incubated for an additional 24 h. Subsequently, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 h to facilitate the formation of formazan crystals. After discarding the supernatant carefully, the resulting formazan crystals were dissolved in 150 µL of DMSO. Finally, the absorbance was measured at 490 nm using a microplate reader (Spark 10M; Tecan, Männedorf, Switzerland). Cell viability was calculated based on the equation below:

$$\text{Cell viability (\%)} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100 \quad (7)$$

where A_{sample} represents the absorbance of the well containing cells treated with the peptides, A_{control} represents the absorbance of the well containing untreated control cells, and A_{blank} represents the absorbance of the well containing only culture medium without cells.

3.10.3. Protective Effect Against Oxidative Stress

HepG2 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 12 h to allow attachment. Subsequently, the medium was replaced with fresh DMEM containing various concentrations of the selected peptides, and the cells were then incubated for another 24 h to assess their potential protective effects against oxidative

stress. Following peptide pretreatment, the medium was replaced with fresh DMEM containing 500 μM H_2O_2 , and the cells were incubated for an additional 12 h to induce oxidative stress. The concentration of H_2O_2 (500 μM) was selected based on preliminary experiments, which demonstrated that this concentration reduced cell viability to approximately 50%, thereby enabling the establishment of a reproducible oxidative stress model suitable for evaluating cytoprotective effects. After exposure to H_2O_2 , cell viability was determined using the MTT assay as described above. The relative cell viability was calculated based on the following equation:

$$\text{Cell viability (\%)} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100 \quad (8)$$

where A_{sample} represents the absorbance of the well containing cells treated with the peptides, A_{control} represents the absorbance of the well containing untreated control cells, and A_{blank} represents the absorbance of the well containing only culture medium without cells.

3.10.4. Determination of Antioxidant Enzyme Activities

The activities of antioxidant enzymes, including SOD, CAT, and GSH-Px, were determined in this study. Briefly, the HepG2 cells were treated with the selected peptides and subsequently exposed to H_2O_2 to induce oxidative stress, as described above. Following treatment, the cells were washed twice with pre-cooled PBS, lysed using RIPA buffer on ice, and then centrifuged at $14,000 \times g$ for 10 min at 4°C . The supernatant was collected for subsequent analyses. Total protein content in the cell lysates was determined using a BCA protein assay kit to normalize enzyme activities across samples. The activities of SOD, CAT, and GSH-Px were determined using commercial assay kits according to the manufacturer's instructions and expressed relative to the total protein content.

3.11. Statistical Analysis

All experiments in this study were conducted in triplicate ($n = 3$), and the results were expressed as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, with $p < 0.05$ considered statistically significant.

4. Conclusions

This study systematically demonstrated that peony seed meal, an abundant agricultural by-product, can serve as a valuable source of potent antioxidant peptides. By integrating ANN-based process optimization with high-resolution peptidomics and multi-level bioactivity evaluation, an efficient and reliable strategy for peptide discovery has been established. Neutrase-mediated hydrolysis optimized through the BP-ANN model significantly enhanced antioxidant activity, outperforming many previously reported protein hydrolysates. The optimized process preferentially generated low molecular weight peptides, which are structurally favorable for antioxidant function. In silico analyses revealed strong binding affinities of selected peptides toward both free radicals and the Keap1 protein, suggesting dual antioxidant mechanisms involving direct radical scavenging and modulation of the Keap1–Nrf2 signaling pathway. Among the screened peptides, FRF exhibited the most pronounced residue-specific interaction within the Keap1 Kelch domain and showed superior cytoprotective effects in HepG2 cells. Both FRF and RWL effectively restored intracellular antioxidant enzyme activities under oxidative stress, confirming their biological relevance beyond chemical assays. Overall, this work provides mechanistic insight into the antioxidant action of PSM-derived peptides and proposes an integrated discovery framework that bridges intelligent hydrolysis optimization, in silico prediction, and cellular validation. These findings support the sustainable

conversion of peony seed meal into high-value functional food ingredients with potential applications in health-promoting formulations.

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Abbreviations

The following abbreviations are used in this manuscript:

PSM	Peony seed meal
PSMH	Peony seed meal protein hydrolysate
ANN	Artificial neural network
BP-ANN	Backpropagation artificial neural network
RSM	Response surface methodology
DH	Degree of hydrolysis
ROS	Reactive oxygen species
pI	Isoelectric point
Keap1	Kelch-like ECH-associated protein 1
Nrf2	Nuclear factor erythroid 2-related factor 2
GSH	Glutathione
MD	Molecular dynamics
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
Rg	Radius of gyration
SASA	Solvent-accessible surface area
SOD	Superoxide dismutase
CAT	Catalase
GSH-Px	Glutathione peroxidase

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