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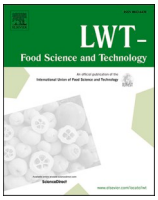

Exploring the antioxidant stability of sheep bone protein hydrolysate -identification and molecular docking

Guanhua Hu [a,b,1], Lu Dou [a,b,1], Jing Zhang [a,b], Rina Su [c], Mirco Corazzin [d], Lina Sun [a,b], Lihua Zhao [a,b], Ye Jin [a,b,*], Lin Su [a,b,**]

[a] College of Food Science and Engineering, Inner Mongolia Agricultural University, Hohhot, 010018, China
[b] Integrative Research Base of Beef and Lamb Processing Technology, Inner Mongolia Agricultural University, Hohhot, 010018, China
[c] Inner Mongolia Vocational College of Chemical Engineering, Hohhot, 010010, China
[d] Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, 33100, Udine, Italy

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ABSTRACT

The aim of this study was to obtain sheep bone protein hydrolysates (SBPHs) with antioxidant activity from sheep bone byproducts and evaluate their stability and processing characteristics under different conditions. Then, the antioxidant peptides were identified and synthesized, and their antioxidant mechanism of action was investigated using molecular docking. The results showed that SBPHs obtained by alkaline protease hydrolysis had high antioxidant capacity and a high proportion of hydrophobic amino acids (41.33%). The antioxidant activity of SBPHs was intolerant to high temperatures and weakly resistant to acids and alkalis. NaCl improved the reducing power, sugars maintained the antioxidant activity, and metal ions reduced the antioxidant activity of SBPHs. SBPHs maintained higher antioxidant activity after simulated gastrointestinal digestion. Three fractions were isolated by ultrafiltration, among which P–I (MW < 3 kDa) had the strongest antioxidant activity. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) showed that 17 peptides with Peptide Ranker >0.85 were found in P–I. Among the synthesized peptides, VYPFPGPIPN had the strongest antioxidant activity, which was mainly exerted through hydrogen bonding, π-π stacking and π-alkyl bonding with Keap1. This study provides a reference for subsequent studies on the production, storage and antioxidant function utilization of SBPHs.

1. Introduction

Reactive oxygen radicals are signaling molecules produced in living organisms and have important physiological functions, but when there is an excess of reactive oxygen species in the organism, they trigger the body to undergo oxidative stress, leading to cell death and tissue damage, which in turn causes a range of chronic diseases, such as cancer, coronary heart disease and vascular disease (White et al., 2014). Excessive free radicals also reduce nutrient and organoleptic quality and produce spoilage or off-flavors (Qiu, Chen, & Dong, 2014). In addition, free radical-mediated fat oxidation accelerates the oxidation of food during processing and storage, reducing its nutrient content and organoleptic quality, as well as producing rancidity or off-flavors that deteriorate food quality (Li, Chi, Li, & Wang, 2017). Therefore, proper supplementation with antioxidants is essential to inhibit the overproduction of free radicals in foods and organisms. Although artificial antioxidants are commonly used to reduce the levels of free radicals in food, their use has been somewhat limited due to their potential health risks. Thus, in recent years, there has been an increased focus on finding natural sources of antioxidants to fulfil this need.

It was reported that many bioactive peptides with antioxidant properties extracted from food proteins by enzymatic digestion can reduce the rate of self-oxidation, lipid oxidation and free radical formation and are an important source of natural antioxidants, with the advantages of being safe, easy to absorb and highly active (Feng, Ruan, Jin, Xu, & Wang, 2018). In addition, bioactive peptides display good physical and chemical properties, such as high solubility and heat resistance. As found in a previous study, the novel antioxidant peptides

* Corresponding author. College of Food Science and Engineering, Inner Mongolia Agricultural University, Hohhot, 010018, China.
** Corresponding author. College of Food Science and Engineering, Inner Mongolia Agricultural University, Hohhot, 010018, China.
E-mail addresses: jinyeyc@sohu.com (Y. Jin), sulin820911@163.com (L. Su).
[1] These authors contributed equally to this work and share first authorship.

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PFHPY and LLGDP isolated from bird's nest (Aerodramus fuciphagus) protein hydrolysate protected HepG2 cancer cells from H_2O_2-induced oxidative damage (Ghassem, Arihara, Mohammadi, Sani, & Babji, 2017). Novel antioxidant peptides purified from surimi hydrolysate of Hairy Tail fish ameliorated the color degradation of beef patties during storage (Wang, Liu, et al., 2021). Thus, antioxidant peptides have the potential to be used as food ingredients, animal feed, functional health products and drugs (Lorenzo et al., 2018). However, it is worth mentioning that antioxidant peptides undergo biochemical reactions such as hydrolysis, oxidation, deamidation and cyclization during production, processing and storage, which may interfere with the peptide chain structure and reduce activity (Li, Mora, & Toldra, 2022), ultimately limiting their development and application (Wang, Liu, et al., 2021). In addition, proteases in the digestive tract degrade antioxidant peptides, producing peptides of lower molecular weight and free amino acids, which can affect their stability. Therefore, to fully realize their biological effects, bioactive peptides must be digested in the gastrointestinal tract and reach their targets in an active form after absorption (Sontakke, Jung, Piao, & Chung, 2016). This means that it is important to assess the stability of antioxidant peptides in food processing systems.

The antioxidant effect of peptides can be achieved by scavenging reactive oxygen species (ROS) and chelating transition metal ions. Although the exact mechanism of peptide antioxidant activity is not known, various studies have found that certain constituent amino acids and their positions as well as peptide conformation are involved in the interaction with free radicals. Molecular docking can predict the binding modes and interaction forces of peptides with free radicals and is an effective tool currently used to explore the mechanism of peptide antioxidant action. Li, Liu, et al. (2017) used a molecular docking technique to screen 20 small peptides from egg albumin that can directly inhibit the Keapl-Nrf2 proton pump inhibitor. Among them, Asp-Lys-Lys (DKK) and Asp-Asp-Trp (DDW) showed the strongest docking binding activity to Keapl proteins and protected HepG2 cells from H_2O_2-induced oxidative damage, suggesting that egg albumin antioxidant peptides exert antioxidant effects by affecting Keapl-Nrf2 proton pump inhibitors. Agrawal, Joshi, and Gupta (2019) successfully isolated, purified and identified two peptides (TSSSLNMARGGGGGLTR and STVGLGISMRSVR) with antioxidant potential from millet proteins and found that the interaction of residues Ser and Thr with free radicals is the key to the antioxidant effect of these two polypeptides by using a molecular docking technique.

Sheep bones are rich in proteins, trace elements, and essential amino acids, and are one of the optimal sources of protein (Hu et al., 2022). However, current utilization of sheep bones is primarily focused on the development of basic products such as bone meal and bone broth, with limited processing of sheep bone protein, and there is limited information on sequence identification and reporting of antioxidant peptides derived from sheep osteoprotegerin. Therefore, in this study, protein hydrolysates with high antioxidant activity were extracted from sheep bone, and their stability was investigated under digestive system, food processing conditions and in vitro digestive conditions. Then, their antioxidant activity was evaluated by using isolation and purification, sequence analysis, and chemical synthesis. Finally, a molecular docking technique was used to predict the docking of antioxidant peptides of sheep bone with Keap1-Nrf2 pathway conformation and interaction. This study solves the problem of resource waste of sheep bone to a certain extent, improves the added value of sheep bone, and provides a theoretical and experimental basis for the production, preservation and utilization of sheep bone antioxidant peptide as a functional antioxidant.

2. Materials and methods

2.1. Materials and chemicals

Six-month-old fresh sheep shoulder blades (300 g in total) were provided by Inner Mongolia Aofeili Food Co., Ltd. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonate) (ABTS) were obtained from Sigma (St. Louis, MO, USA). Proteases including alcalase (from *Bacillus licheniformis*, activity was 3.33×10^6 nKat), papain (from unripe fruit of the papaya plant, activity was 1.33×10^7 nKat), flavor protease (from Aspergillus oryzae, activity was 5×10^5 nKat), trypsin (from porcine pancreas, activity was 4.17×10^6 nKat) and neutral protease (from *Bacillus subtilis*, activity was 8.34×10^5 nKat) were purchased from Beijing Coolpac Technology Co., Ltd. (Beijing, China). Porcine pepsin (from gastric mucosa of pigs, activity was 5.33×10^7 nKat) and trypsin (from pig pancreas, activity was 1000–2000 BAEE units/mg solid) were obtained from Sigma Aldrich Co., Ltd. (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of sheep bone protein hydrolysates (SBPHs)

Sheep bone powder (SBP) was prepared according to the method of Hu et al. (2022). The fresh sheep bones were cleaned with tap water and boiled for 10 min to remove the surface fat and then placed in an autoclave for 40 min at 0.1 MPa. Then, the sheep bones were washed repeatedly in hot water. After cooling and drying, the sheep bone was crushed evenly into powder (80 order). SBP was mixed with anhydrous ethanol (1: 10, m/v) for 24 h, and the sample solution was replaced every 8 h. The mixture was centrifuged at 3000×*g* (15 min, 4 °C), and the sediment was washed twice using cold distilled water and dried for later use. Defatted SBP was obtained. Eight percent defatted sheep bone powder was dissolved in water at a ratio of 1:15. Then, 1.33×10^5 nKat protease (alcalase, papain, flavor protease, trypsin and neutral protease) was added and agitated continuously (50 °C) for 4 h. The pH of the solution was maintained at the optimal pH. Next, the protease was inactivated by heating in boiling water for 15 min. The solution was centrifuged for 15 min (4 °C, 10,000×*g*), and the supernatant was lyophilized to obtain SBPHs.

2.3. Determination of peptide yield

Two milliliters of enzymatic hydrolysis solution and an equal amount of 100 g/L trichloroacetic acid (TCA) aqueous solution were mixed, placed at room temperature for 10 min and centrifuged at 4000 rpm/min (4 °C) for 20 min. The supernatant was collected to determine total and amino nitrogen. The total nitrogen content was determined according to the method of protein determination. The amino nitrogen in the enzymatic digest was determined using the formaldehyde titration method.

$$W = (NT\text{-}NA)*6.25/G \quad \text{Eq. (A.1)}$$

Where W: peptide yield (mg/g); G: weight of sheep bone powder (g); NT: total soluble nitrogen content of TCA in hydrolysate (mg); and NA: amino acid nitrogen content in hydrolysate (mg).

2.4. Assays of antioxidant activities

2.4.1. Hydroxyl radical (·OH) scavenging activity

The ·OH scavenging activity was determined using the Fenton reaction established by Hu, Yang, and Wang (2020). One milliliter of the sample was mixed with 1 mL of 6 mmol/L $FeSO_4$, 1 mL of 6 mmol/L salicylic acid, and 1 mL of 6 mmol/L H_2O_2 and incubated in a water bath at 37 °C for 15 min (A_1). The absorbance at 510 nm was measured. The samples were replaced by distilled water as the blank control (A_0). H_2O_2 solution was replaced by distilled water as a negative control(A_2). Other solution additions and procedures are the same as A_1. The ·OH scavenging rate was determined as follows.

$$\text{Hydroxyl radical } (\cdot\ \text{OH}) \text{ scavenging activity} = (1-(A_1 - A_2)\ /\ A_0)*100\% \quad \text{Eq. (A.2)}$$

2.4.2. DPPH· scavenging activity

DPPH· scavenging activity was determined according to the method of Locatelli et al. (2009) with some modifications. A 1.5 mL aliquot of sample was mixed with 1.5 mL of 0.1 mmol/L DPPH solution (A_1). The control group was prepared with 1.5 mL of ethanol instead of the DPPH solution (A_2). The blank was prepared with distilled water instead of the sample (A_0). The solution was left to react for 30 min at room temperature away from light.

$$\text{DPPH} \cdot \text{ scavenging activity} = (1-(A_1 - A_2) / A_0)*100\% \quad \text{Eq. (A.3)}$$

where A is the absorbance at 517 nm.

2.4.3. $ABTS^{\cdot+}$ scavenging activity

The previously mentioned methods of Agrawal, Joshi, and Gupta (2017) were applied to assess the $ABTS^{\cdot+}$ scavenging activity. Potassium persulfate (3.52 mL; 2.6 mmol/L) was added to 200 mL of ABTS solution (7.4 mmol/L), sonicated for 2 min to mix, and placed in darkness for 16 h. Next, the $ABTS^{\cdot+}$ solution was diluted using phosphate buffer until its absorbance was 0.70 ± 0.02 at 734 nm. Subsequently, 20 μL sample was reacted with 980 μL diluted ABTS radical cation solution at room temperature for 30 min, the absorbance of sample was got at 734 nm.

$$ABTS^{\cdot+} \text{ scavenging activity} = (1\text{-}(A_1\text{-}A_2)/A_0)*100\% \quad \text{Eq. (A.4)}$$

A_1 represents the absorbance of the solution in the sample group; A_2 represents the absorbance of the solution in the control group; and A_0 represents the absorbance of the solution in the blank group.

2.4.4. Reducing power assay

The method of determining the reducing power by Zamorano-Apodaca et al. (2020) was cited with some modifications. The 10 g/L sample solution, 0.2 mol/L phosphate buffer (pH 6.6), and 10 g/L potassium ferricyanide were mixed at a ratio of 1:2.5:2.5 and incubated in a water bath at 50 °C for 20 min. After adding 100 μL of 100 g/L trichloroacetic acid and 20 μL of 1 g/L ferric chloride, the solution was incubated at 50 °C for 10 min. Finally, the absorbance was determined at 700 nm. Results were expressed as the absorbance at 700 nm (A_{700}).

2.5. Determination of amino acids

Amino acids were assayed with reference to Wang, Lin, et al. (2020). A total of 0.5 g of sample was mixed with 10 mL of HCl (6 mol/L) in a 15 mL digestion tube and then hydrolyzed in an oven at 110 °C for 21 h. After removal and cooling, the hydrolysis solution was transferred to a 100 ml volumetric flask and fixed. Then, 1.0 mL of the hydrolysis product was accurately aspirated, evaporated at 60 °C, dissolved in 1 mL of 0.02 mol/L HCl, mixed fully, filtered through 0.22 μm filters and detected using an L8900 amino acid autoanalyzer (Hitachi Co., Tokyo, Japan).

2.6. Evaluation of biological activity stability

2.6.1. Stability against heat and pH treatments

The thermal stability of the SBPHs was measured as described by Yarnpakdee, Benjakul, Kristinsson, and Kishimura (2015) with some modifications. SBPHs (10 mg/mL) were incubated in water baths at 25, 40, 60, 80, and 100 °C for 2 h and cooled rapidly to room temperature. The effect of pH was assessed according to Zhu, Zhang, Kang, Zhou, and Xu (2014) with some modifications. SBPHs solutions were adjusted to pH 3, 5, 7, 9, or 11 using 1 mol/L HCl or NaOH and allowed to stand at room temperature for 2 h. The retention rates of ·OH scavenging activity, DPPH· scavenging activity, and reducing power were calculated as follows.

$$\text{The activity retention rate} = A_2/A_1*100 \quad \text{Eq. (A.5)}$$

A_1 and A_2 represent the activities of the samples before and after treatment (·OH scavenging activity, DPPH· scavenging activity and reducing power), respectively.

2.6.2. Stability against food ingredients

SBPHs solutions were generated with 20, 40, 60, 80, and 100 g/L sucrose, glucose, fructose, lactose and NaCl, respectively, and maintained at room temperature for 2 h according to Liu et al. (2017) with modifications.

2.6.3. Stability against metal ions

The effect of metal ions on the antioxidant activity retention rate of SBPHs was carried out according to the method described by Wang et al. (2017) with some modifications. $CuCl_2$ and KCl at 50, 100, 150, 200, and 250 mg/L were added to the SBPHs solution, and the antioxidant activity retention rate was determined after standing at room temperature for 2 h.

2.6.4. Stability against preservatives

Then, 0.2, 0.4, 0.8, 1.2, and 1.6 g/L sodium benzoate and potassium sorbate were added to the SBPHs solution. After standing at room temperature for 2 h, the activity retention rate was determined.

2.6.5. Simulated gastrointestinal digestion

According to the method described by Tavares et al. (2011), an in vitro model of gastrointestinal digestion was generated. Antioxidant activity as discussed in Section 2.6 was determined. Briefly, the SBPHs solution was adjusted to pH 2.0 with 1 mol/L HCl, and then 40 g/L porcine pepsin was added. The mixture was incubated for 2 h at 37 °C. The next step was to divide the solution into two parts. One part was bathed with boiling water for 15 min to stop the reaction, generating pepsin hydrolysates. The other component's pH was raised to 5.3 with 0.9 mol/L $NaHCO_3$ and then brought down to 7.5 with 1 mol/L NaOH. Then, 40 g/L trypsin was added to begin the second digestion (37 °C, 2 h), and the sample was incubated with shaking at 37 °C for an additional 2 h. This reaction was suspended in a 95 °C water bath, generating trypsinized samples. The antioxidant activity retention rates of SBPHs solutions before digestion, pepsin hydrolysis and trypsin hydrolysis were measured.

2.7. Peptide fractionation by ultrafiltration

Peptide fractionation by ultrafiltration was utilizing the approach outlined by Wang, Huang, Chen, Huang, and Zhou (2015). The lyophilized samples were prepared as a 10 g/L solution and serially fractionated using centrifuge tubes (Millipore Co., Billerica, MA) with molecular weights of 3 kDa and 10 kDa. Each fraction was collected, lyophilized, and used for determination of antioxidant activity.

2.8. Identification and synthesis of the sheep bone antioxidant peptide

Liquid chromatography–tandem mass spectrometry (LC−MS/MS) was performed on a Q Exactive Plus mass spectrometer (Agrawal et al., 2019). MaxQuant 1.6.1.0 and the UniProt website (UniProt-Ovis aries (Sheep) [9940]-63944-20210730. Fasta) were used to retrieve the MS data. Based on the HPLC–electrospray ionization–MS/MS results, the identified peptides were synthesized by the China DG Peptides Co., Ltd. (Hangzhou, China). The synthesized peptides were purified by HPLC on a Kromasil C18 column (4.6 mm × 250 mm, 5 μm) following the method of Pei et al. (2023). The allergenicity and toxicity were predicted according to http://www.imtech.res.in/raghava/algpred/and http://www.imtech.res.in/raghava/toxinpred/.

2.9. Molecular docking

The binding capacity of peptides to the Kelch domain of Keap1 was

predicted by molecular docking as reported by Wang, Ma, et al. (2021). The peptide 3D structures were created with ChemDraw 20.0 and Chem3D software. Then, the MM2 module was used to minimize energy, obtain the best structure with the lowest energy, and save it as a MOL2 file. AutoDockTools 1.5.6 was used to set the ligand and export the PDBQT format. The molecularly docked proteins were downloaded from the Protein Data Bank to determine their structures and visualized using PyMOL. Ligand−receptor docking was performed by AutoDock Vina 1.1.2. PyMOL and Discovery Studio were used for visualization.

2.10. Statistical analysis

All results were expressed as the mean values with indication of standard deviation. One-way analysis of variance (ANOVA) was used to determine the differences among treatments ($P < 0.05$). The resulting data were investigated graphically using OriginPro 2016 (OriginLab, Northampton, MA). Statistical analysis was conducted using the statistical package SPSS 20.0 (SPSS Inc., Chicago, IL, USA) for analyses of variance.

3. Results and discussion

3.1. Preparation of enzymatic hydrolysates from sheep bone and antioxidant activity analysis

Each protease has different degrees of specificity and can produce peptides with different C-terminal, N-terminal and molecular weights. Therefore, the selection of proteases is the key process step, which determines the biological activity of peptides. The hydrolysis effect of different proteases on SBP is shown in Fig. 1. It was found that the antioxidant capacity of enzymatic hydrolysates of different proteases varied significantly. The alkaline protease hydrolysates showed the highest ·OH scavenging rate of 78.37 ± 2.29%, followed by papain, flavor protease, trypsin and neutral protease. In addition, the peptide yield of alkaline protease hydrolysates (111.86 ± 0.25 mg/g) was also significantly higher ($P < 0.05$) than that of the other four proteases. This may be because alcalase is an endoprotease with a wide range of hydrolysis sites, which can randomly cut the peptide bonds in the protein molecules and release small peptides (Rui, Boye, Simpson, & Prasher, 2012). In addition, studies have suggested that alkaline protease can be used to extract antioxidant peptides from different food proteins, such as velvet antler (Ding, Ko, Moon, & Lee, 2019), chicken (Xiao et al., 2022), scallop (Wang, Liu, et al., 2021), and Atlantic sea cucumber (Zhang, He, Bonneil, & Simpson, 2020). Therefore, alkaline protease was selected as the preferred protease for the preparation of antioxidant protein hydrolysates from sheep bone in this study.

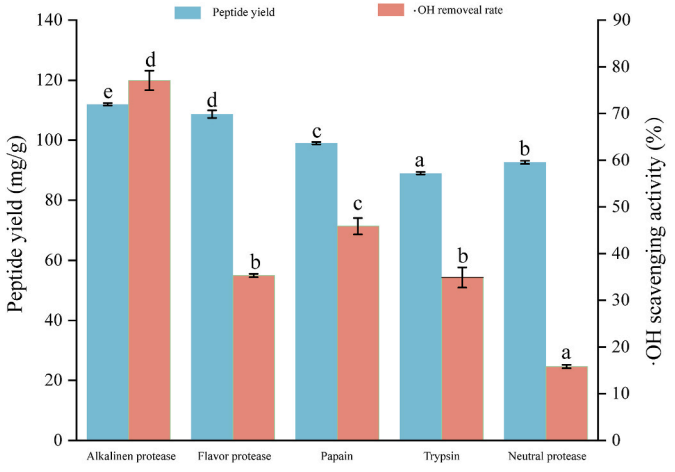

Fig. 1. Effect of different protease treatments on the peptide yield and OH scavenging activity of sheep bone protein hydrolysates (SBPHs). Values with different letters (a–e) represented significantly different ($P < 0.05$, Mean ± SD, n = 3).

As shown in Fig. 2, the ·OH, DPPH·, and $ABTS^{\cdot+}$ scavenging activities and reducing power of SBPHs increased in a dose-dependent manner ($P < 0.05$), which was consistent with a previous study of skate (*Raja porosa*) cartilage protein hydrolysate (Pan, Zhao, Hu, & Wang, 2016). Therefore, SBPHs may contribute to free radical scavenging and termination of free radical chain reactions. This may be due to the presence of side-chain groups with higher electron density and relatively high redox potential on short-chain peptides in the protein hydrolysate, promoting the scavenging of free radicals by donating electrons (Zhang et al., 2018).

3.2. Amino acid analysis of SBPHs

As shown in Table 1, the hydrophobic, aromatic, acidic and basic amino acid contents of SBPHs were significantly higher than those of SBP ($P < 0.05$), confirming that enzymatic hydrolysis can alter the amino acid pattern (Sabeena Farvin et al., 2016), which in turn enhances the content of antioxidant amino acids. A previous study suggested that hydrophobic amino acids (HAAs) had strong free-radical scavenging ability and anti-lipid peroxidation activity (Dearfield, Jacobson-Kram, Brown, & Williams, 1983) because of the better presence at the water-lipid interface (Samaranayaka & Chan, 2011). In the present study, HAAs in SBPHs accounted for 41.33% of the total amino acid content. Najafian and Babji (2015) extracted three antioxidant peptides from bardinian myofibrillar protein, and FVNQPYLLYSVHMK had the strongest antioxidant activity, and it was suggested that the hydrophobic amino acid residues Leu, Val, and Phe in the peptides contributed more to the antioxidant capacity. Aromatic amino acids (Tyr, Phe) can influence antioxidant activity by promoting the free radical scavenging activity of peptides through direct electron transfer or interaction with hydrophobic targets (Himaya, Ryu, Ngo, & Kim, 2012; Pownall, Udenigwe, & Aluko, 2010). Phenolic hydroxyl groups (-OH), such as those on Tyr, can be considered direct hydrogen donors, trapping free radicals through resonance structures and forming stable compounds and are therefore considered to have strong free radical scavenging ability. Acidic amino acids (Glu, Asp) and basic amino acids (Arg, His, Lys) contribute to the better metal ion chelating and free radical scavenging abilities of antioxidant peptides (Je, Qian, & Kim, 2007) due to their ability to form metal chelates with metal ions using the charged groups of their side chains (Egusa Saiga & Nishimura, 2013). Therefore, SBPHs may have potential antioxidant activity.

3.3. Stability of SBPHs

3.3.1. Temperature

Foods are typically sterilized by heat treatment during processing and production, so it is important to determine the effect of temperature on SBPHs antioxidant activity. The effect of temperature on the activity retention rate of ·OH and DPPH· scavenging and reducing power of SBPHs is shown in Fig. 3A. It was found that as the temperature increased (25–100 °C), the activity retention rate of ·OH scavenging and reducing power decreased gradually ($P < 0.05$); in particular, the activity retention was above 80% between 25 and 40 °C, possibly as a result of heat-induced conformational changes in the peptide. Prolonged exposure to high temperatures can lead to denaturation and aggregation of antioxidant peptides (Liu, Du, and Chen, 2020), reducing the antioxidant activity of SBPHs. Notably, the activity retention rate of DPPH· scavenging increased significantly from 25 to 40 °C ($P < 0.05$), followed by a downward trend, and decreased by 51.65 pp at 100 °C. Therefore, food processing should be carried out in a low-temperature environment whenever possible.

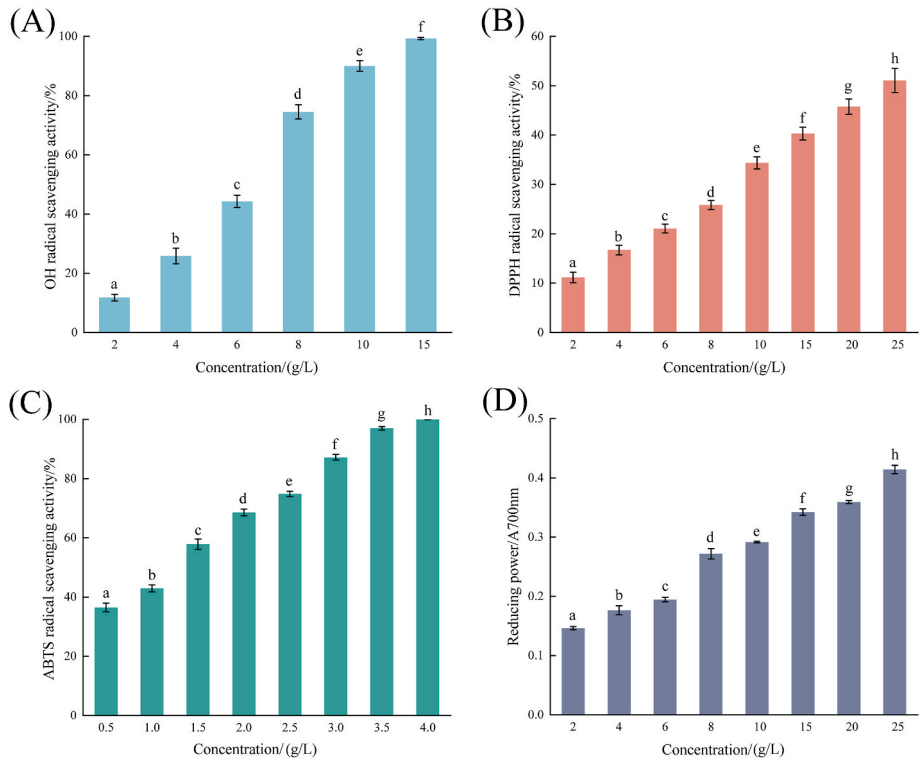

Fig. 2. Antioxidative capacity of sheep bone protein hydrolysates (SBPHs). (A) OH radical scavenging activity. (B) DPPH radical scavenging activity. (C) $ABTS^{+}$ radical scavenging activity. (D) Reducing power. Values with different letters (a–h) represented significantly different ($P < 0.05$, Mean ± SD, n = 3).

Table 1
Amino acid content in SBP and SBPHs.

Items	SBP/(g/100g)	SBPHs/(g/100g)	Items	SBP/(g/100g)	SBPHs/(g/100g)
Asp^	1.32 ± 0.10^{a}	3.6 ± 0.13^{b}	Ile*#	0.39 ± 0.03^{a}	0.96 ± 0.03^{b}
Thr*#	0.60 ± 0.05^{a}	1.55 ± 0.06^{b}	Leu*#	1.13 ± 0.09^{a}	2.73 ± 0.13^{b}
Ser	0.69 ± 0.05^{a}	1.7 ± 0.030^{b}	Tyr&	0.39 ± 0.02^{a}	0.84 ± 0.04^{b}
Glu^	2.06 ± 0.14^{a}	5.57 ± 0.29^{b}	Phe*#&	0.71 ± 0.05^{a}	1.72 ± 0.14^{b}
Gly	2.70 ± 0.12^{a}	9.23 ± 0.74^{b}	Lys#^	1.05 ± 0.08^{a}	2.44 ± 0.09^{b}
Ala*	1.46 ± 0.09^{a}	4.86 ± 0.18^{b}	His#^	0.36 ± 0.03^{a}	0.81 ± 0.02^{b}
Cys	0.11 ± 0.01^{a}	0.16 ± 0.02^{b}	Arg^	1.36 ± 0.09^{a}	3.96 ± 0.29^{b}
Val*#	0.73 ± 0.05^{a}	1.83 ± 0.09^{b}	Pro*	1.69 ± 0.09^{a}	5.84 ± 0.15^{b}
Met*#	0.16 ± 0.01^{a}	0.57 ± 0.07^{b}			
TAA	16.91 ± 1.07^{a}	48.53 ± 1.01^{b}	HAA	6.86 ± 0.44^{a}	20.06 ± 0.21^{b}
EAA	5.13 ± 0.38^{a}	12.61 ± 0.40^{b}	TAAA	6.15 ± 0.44^{a}	16.53 ± 0.59^{b}
AAA	1.09 ± 0.08^{a}	2.56 ± 0.08^{b}			

Note: * indicates that the amino acid is hydrophobic, # indicates that the amino acid is essential, ^ indicates that the amino acid is acidic/basic amino acids; & indicates that the amino acid is aromatic amino acid; TAA, Total amino acids; HAA, Hydrophobic amino acids; EAA, Essential amino acids; TAAA, Total acidic/basic amino acids; AAA, Aromatic amino acids. SBP, sheep bone power; SBPHs, sheep bone protein hydrolysates.

3.3.2. pH

Given that pH affects electrostatic interactions between charged amino acids, thereby affecting the antioxidant activity of peptides, the effect of pH on SBPHs stability was evaluated. As shown in Fig. 3B, the activity retention rate of DPPH· scavenging of SBPHs increased gradually at pH 2.0–6.0 ($P < 0.05$), stabilized at pH 6.0–8.0, and decreased at higher pH values ($P < 0.05$). Therefore, acidic and alkaline environments decreased DPPH· scavenging activity, in line with previous studies on sandfish (Jang, Liceaga, & Yoon, 2016). Extreme acid–base conditions can racemize the peptide, and the conformational change in SBPHs peptide chains inhibits its ability to bind to free radicals (Wang et al., 2017). However, pH had little effect on the activity retention rate of the reducing power of SBPHs. It is noteworthy that when the pH was 6, the activity retention rates of DPPH· scavenging, ·OH scavenging and reducing power were all above 80%. Therefore, to maintain antioxidant activity, processing, preparation, and storage should be carried out in neutral or weakly acidic environments.

3.3.3. Food ingredients

3.3.3.1. NaCl. As the mass fraction of sodium chloride increased, the activity retention rate of ·OH and DPPH· scavenging increased gradually (Fig. 3C), whereas the activity retention rate of reducing power decreased by 17% when the mass fraction of NaCl was 100 g/L compared with that without addition, but the final activity retention rate was higher than 80%. This suggested that the peptide's interaction with a food matrix containing sodium chloride maintains the peptide's biological activity, which was in line with a study on the bioactive stability of microalgal protein hydrolysates (Pereira, Lisboa, Santos, & Costa, 2019). The increase in NaCl concentration might help to expose functional groups, which are capable of releasing hydrogen ions, resulting in increased capture of the radical, thereby enhancing antioxidant activity. Therefore, an appropriate amount of salt should be added during processing and application to maintain SBPHs activity.

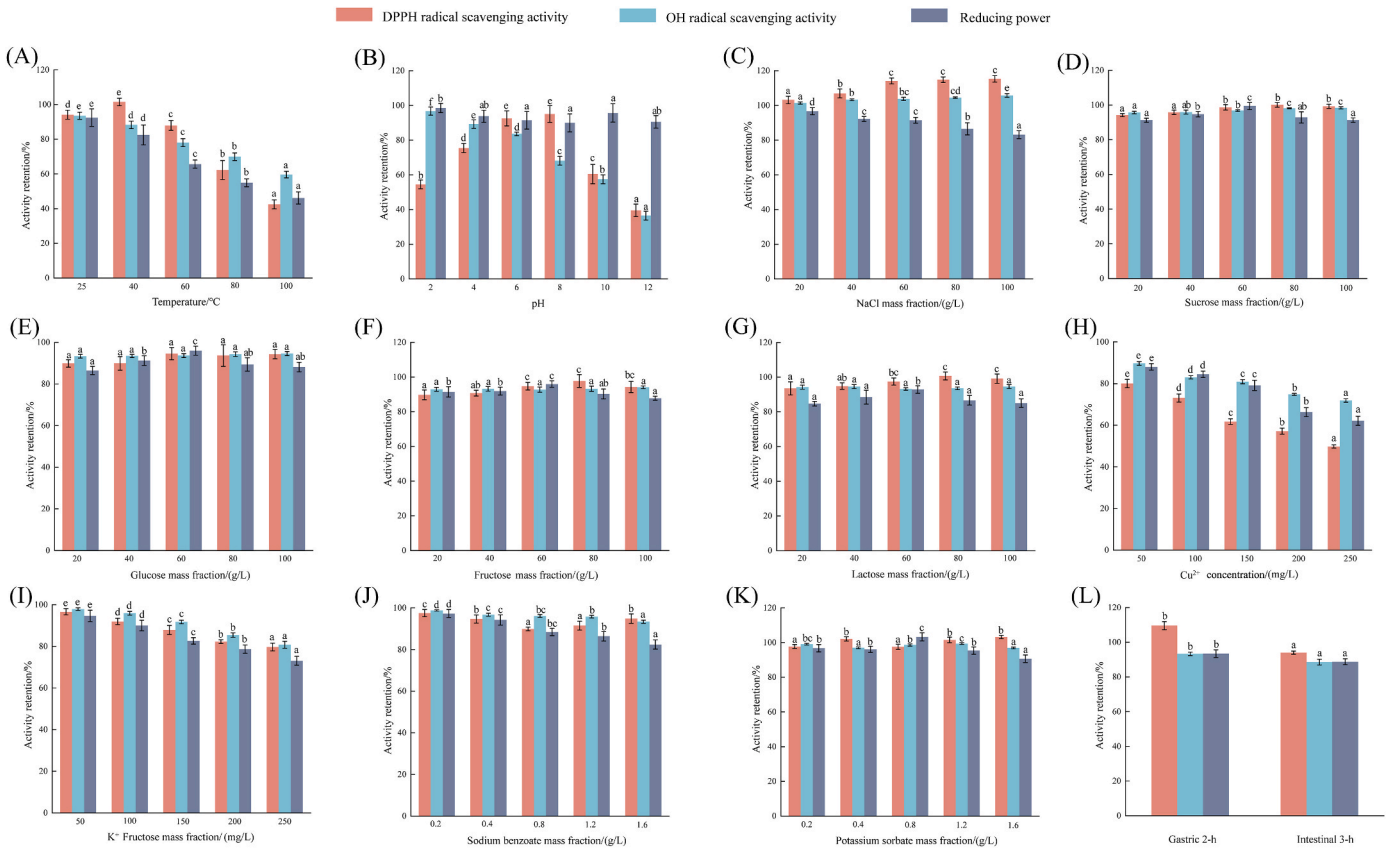

Fig. 3. Stability of sheep bone protein hydrolysates (SBPHs). (A) The effect of temperature on the antioxidant activity of sheep bone protein hydrolysates (SBPHs). (B) The effect of pH on the antioxidant activity of sheep bone protein hydrolysates (SBPHs). (C–G) The effect of food ingredients on the antioxidant activity of sheep bone protein hydrolysates (SBPHs). (H–I) The effect of metal ion on the antioxidant activity of sheep bone protein hydrolysates (SBPHs). (J–K) The effect of preservative on the antioxidant activity of sheep bone protein hydrolysates (SBPHs). (L) Stability of sheep bone protein hydrolysates (SBPHs) in gastrointestinal digestion. Values with different letters (a–e) represented significantly different ($P < 0.05$, Mean ± SD, n = 3).

3.3.3.2. Sugar. As shown in Fig. 3D, the activity retention rate of ·OH and DPPH· scavenging increased slightly as the sucrose mass fraction increased (20–100 g/L), and when the concentration was 100 g/L, their values reached 98.4% and 99.3%, respectively, whereas the activity retention rate of reducing power increased and then decreased rapidly, peaking at 60 g/L. Notably, the trends of the reducing powers of glucose and fructose were basically the same as that of sucrose. In addition, glucose and fructose both had little effect on the activity retention rate of ·OH scavenging ($P > 0.05$). For the DPPH· scavenging activity, glucose did not affect its activity; however, the activity retention increased gradually as the fructose mass fraction increased. The activity retention rate of DPPH· scavenging increased slightly with increasing lactose mass fraction as well, whereas the activity retention of ·OH scavenging was less affected by lactose ($P > 0.05$). Guérard and Sumaya-Martinez (2003) reported that the antioxidant activity of fish meat protease hydrolysate increased 20–30% after adding sugar. Peptides and amino acids undergo the Maillard reaction with reducing sugars, forming reducing substances such as aldehydes and ketones and improving the proton-donating ability of peptide liquids (Delgado-Andrade, Morales, Seiquer, & Pilar Navarro, 2010). Overall, an appropriate amount of reducing sugars improved the antioxidant capacity of SBPHs, and each sugar had a slightly different effect.

3.3.4. Metal ions

In food processing, metal ions such as K^+ and Cu^{2+} in raw materials, water, and additives are important essential metal elements. The presence of metal ions may affect product quality and efficacy. Cu^{2+} and K^+ significantly reduced the antioxidant activity retention rate of SBPHs ($P < 0.05$) (Fig. 3H–I). At a Cu^{2+} ion mass concentration of 250 mg/L, the activity retention rates of ·OH and DPPH· scavenging and reducing power of SBPHs were 71.88%, 49.78%, and 62.13%, respectively. At an identical K^+ ion mass concentration, the activity retention rates of ·OH and DPPH· scavenging and reducing power of SBPHs were 80.76%, 79.70%, and 73.10%, respectively. Therefore, SBPHs were sensitive to Cu^{2+} and K^+. Indeed, Zhao, Huang, Zhang, Chen, and Jiang (2011) reported that K^+ and Cu^{2+} inhibited the DPPH· scavenging activity of river shrimp hydrolysate, possibly because the complexing ability of metal ions was affected by the different peptide amino acid compositions, altering the free-radical scavenging ability. The addition of metal ions could disrupt the chemical interactions between peptides, reducing their solubility and exposing hydrophobic groups, thus suppressing antioxidant activity (Wong et al., 2019). Therefore, contact with copper utensils should be avoided as much as possible during the processing and storage of SBPHs, and they should not be mixed with materials rich in Cu^{2+}.

3.3.5. Preservative

Preservatives such as sodium benzoate and potassium sorbate are usually added during food processing. Therefore, the effect of preservative on the activity retention of the antioxidant activity of SBPHs was evaluated. As shown in Fig. 3J, the activity retention rate of ·OH scavenging and reducing power of SBPHs decreased as the sodium benzoate mass fraction increased, while the activity retention rate of DPPH· scavenging activity decreased (0.2–0.8 g/L) and then increased (0.8–1.6 g/L). When the mass fraction of potassium sorbate increased from 0.2 to 1.6 g/L, the activity retention rate of reducing power showed a trend of increasing and then decreasing; at 0.8 g/L sodium sorbate, the value peaked at 103.31% (Fig. 3K). Notably, when the mass fraction of

preservatives (sodium benzoate and potassium sorbate) was 1.6 g/L, the activity retention rates of ·OH and DPPH· scavenging were >90%. Overall, the antioxidant activity of SBPHs is stable to sodium benzoate and potassium sorbate, so preservatives can be added in appropriate amounts during the production of sheep bone antioxidant peptide-related products.

3.3.6. Gastrointestinal digestion stability of SBPHs

Bioactive peptides should withstand gastrointestinal digestion and arrive at their destinations in an active form following absorption to exert physio-biological effects in vivo (Espejo-Carpio et al., 2016; Wu et al., 2015). Oral antioxidant peptides are affected by digestive enzymes, including pepsin and trypsin, which affect their activities. The in vitro simulated gastrointestinal digestive model (SGM) can elucidate peptide bioavailability.

SBPHs activity after simulated gastrointestinal digestion is shown in Fig. 3L. After gastric digestion for 2 h, the activity retention rate of ·OH scavenging decreased by 6.68 pp, and the reducing power decreased by 6.56 pp. Furthermore, intestinal digestion with pancreatin decreased these values to 11.38 pp and 11.24 pp, respectively. Notably, after simulated pepsin digestion for 2 h, the activity retention rate of DPPH· scavenging of SBPHs increased by 9.58 pp, whereas trypsin (intestine) decreased the DPPH· scavenging activity of SBPHs by 6.00 pp. Overall, after in vitro simulated gastrointestinal treatment, the activity retention rates of DPPH· scavenging, ·OH scavenging and reducing power were 94.01%, 88.62% and 88.76%, respectively. Therefore, SBPHs had good gastrointestinal stability, possibly because their constituent peptides contain cleavage sites for digestive enzymes or because the small peptides obtained after digestion have good antioxidant activity. Zhan, Li, Dang, and Pan (2021) found that the scavenging activities of ·OH, $ABTS^{\cdot+}$ and DPPH· of digested peptides were 2.17-, 3.00-, and 2.91-fold those of undigested peptides, respectively, which also suggested that simulated digestion significantly increased the antioxidant activity of protein hydrolysates (Chen et al., 2021).

3.4. Antioxidant activity of the ultrafiltered SBPHs fractions

Molecular weight has a profound effect on the antioxidant activity of protein hydrolysates, and generally, low molecular weight protein hydrolysates are more effective in inhibiting free radicals (Liu, Xie, Ma, Liu, & Ke, 2021). SBPHs were ultrafiltered to obtain three fractions: P–I (MW < 3 kDa), P-II (3 kDa < MW < 10 kDa) and P-III (MW > 10 kDa), of which antioxidant activities were determined.

The scavenging activity of ·OH, DPPH, $ABTS^{+}$, and reducing power of each fraction increased gradually with increasing mass concentration, and a linear relationship was observed in the experimental concentration range, indicating that all three fractions had strong antioxidant capacity (Fig. 4A–D). It was noteworthy that the ·OH scavenging activity of each fraction exceeded 90% when the mass concentration was 10 g/L. P–I exhibited a higher ·OH scavenging rate than the other fractions ($P < 0.05$), reaching 96.88%. The DPPH· scavenging activity at the same mass concentration followed the order P–I > P-II > P-III > SBPHs, in agreement with the observation that lower-molecular-weight antioxidant peptides are stronger antioxidants (Fang et al., 2019). Moreover, the highest $ABTS^{\cdot+}$ scavenging activity of P–I was observed at concentrations of 0.5–1.5 g/L ($P < 0.05$). P–I showed higher reducing power when the mass concentration was 2, 6, 8, and 25 g/L ($P < 0.05$).

According to the analysis of the above in vitro antioxidant activity assay results, in general, it was found that the antioxidant activity of P–I was higher than that of the other three components considering DPPH· and $ABTS^{\cdot+}$ and considering, at high mass concentration, the ·OH scavenging activity. Esteve, Marina, and García (2015) reported that peptides with low molecular weights in olive oil had a greater antioxidant capacity than high-molecular-weight active peptides, which

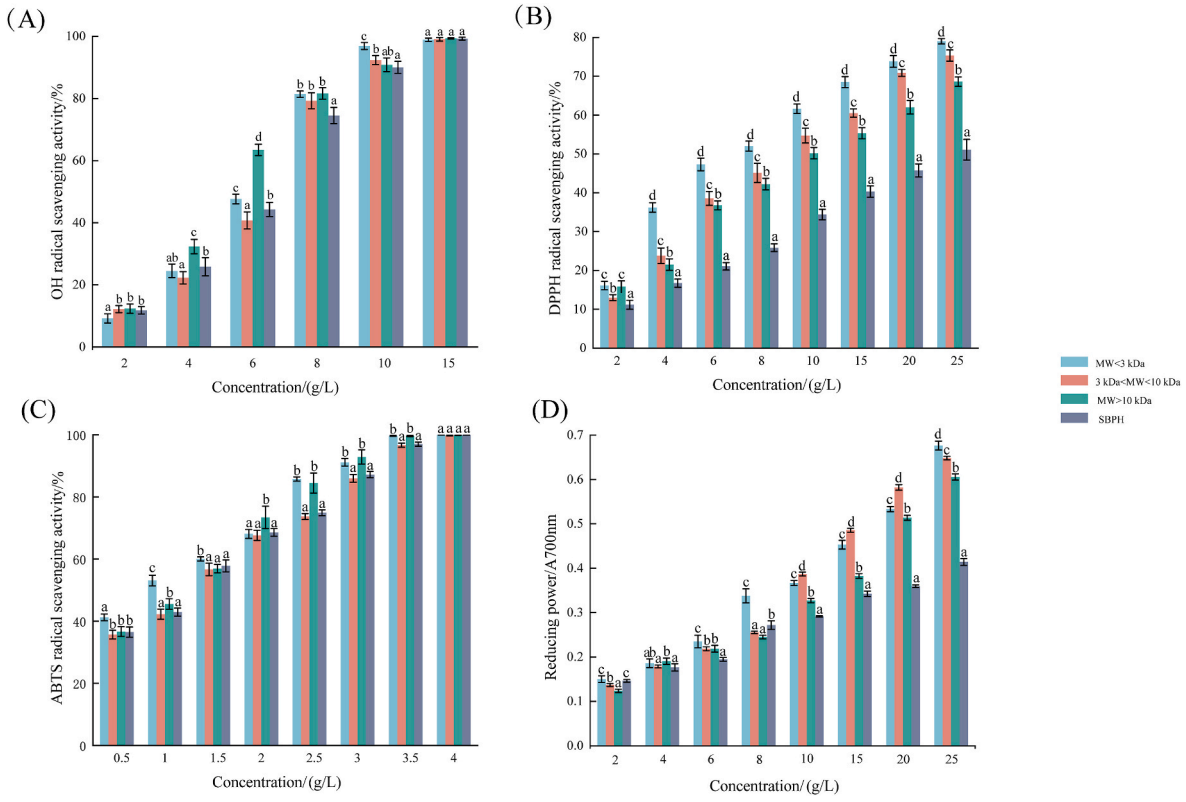

Fig. 4. Antioxidative activity of ultrafiltration fractions of sheep bone protein hydrolysates (SBPH). (A) OH radical scavenging activity. (B) DPPH radical scavenging activity. (C) $ABTS^{+}$ radical scavenging activity. (D) Reducing power. Values with different letters (a–d) represented significantly different ($P < 0.05$). MW < 3 kDa (P–I); 3 kDa < MW < 10 kDa (P-II); MW > 10 kDa (P-III). Values with different letters (a–d) represented significantly different ($P < 0.05$, Mean ± SD, n = 3).

revealed higher charge transfer efficiency to stop lipid oxidation reactions more effectively than high-molecular-weight peptides (Moure, Domínguez, & Parajó, 2006). Thus, protein hydrolysates containing low-molecular-weight peptides may display higher antioxidant capacity.

3.5. Identification of P–I

The proportions of particular amino acids and their sequences in peptides determine their bioactivity (Conway, Gauthier, & Pouliot, 2013). The P–I fraction was analyzed by LC−MS/MS to characterize the amino acid sequences of the peptides. After the MS data were retrieved, peptide-to-spectrum match FDR ≤0.01 and protein FDR ≤0.01 were used as the screening criteria to identify the peptides and proteins, respectively. A total of 468 peptides and 103 proteins were found. The peptides were 5–24 amino acid residues in length (Table 2), with sequences of 5 and 9 amino acid residues being the most abundant, at 77 and 76, respectively. There were 277 sequences of 5–10 amino acid residues, accounting for 59.18%, and 191 peptides of 10–24 amino acid residues, accounting for 40.81%, indicating that P-1 consisted mainly of short peptides (Fig. 5A). In addition, 43 fragment peptides had antioxidant activity, 308 fragment peptides had angiotensin-converting enzyme (ACE) inhibitory activity, 138 fragment peptides had dipeptidyl peptidase IV (DPP-IV) inhibitory activity, and 3 active fragment peptides had anticancer functions. Among them, 20 peptides had both antioxidant activity and ACE inhibitory activity, 11 peptides had antioxidant activity and DPP-IV inhibitory activity, 6 peptides had antioxidant activity, DPP-IV inhibitory activity and ACE inhibitory activity, and 1 peptide had all four activities. Finally, the peptide ranker was determined; a peptide ranker >0.50 indicates a relatively high likelihood of bioactivity (Tu et al., 2018). Among the identified peptide sequences, P–I contained 182 peptides with a peptide ranker >0.50 and 17 peptides with a peptide ranker >0.85, with molecular weights of 500–1300 Da (Table 3).

3.6. Sequence analysis of sheep bone peptide activity

Two common peptides exhibited antioxidant activity, ACE inhibitory activity, DPP-IV inhibitory activity, and anticancer functional activity: VYPFPGPIPN (VN-10) and SLVYPFPGPIPN (SN-12) (Fig. 5B). Previously, the peptide sequence of VN-10 was identified by Eisele, Stressler, Kranz, and Fischer (2013) in hydrolysate derived from casein hydrolyzed by *Bacillus* alkaline peptidase in an enzyme membrane reactor; the hydrolysate had ACE inhibitory and antioxidant activities, and VN-10 exhibited radical scavenging activity, with a half-maximal inhibitory concentration of 6.2 μmol/L.

SN-12 contains the complete VN-10 sequence, and the peptide ranker was 0.753363, indicative of potential biological activity. Moreover, this peptide contains Pro and Val residues or Pro and Leu residues. Cai et al. (2015) showed that the VGGRP peptide containing Pro and Val residues and the GFGPEL peptide containing Pro and Leu residues had significant DPPH·, ·OH and $ABTS^{\cdot+}$ scavenging activities. Another study suggested that peptides containing Pro, Gly, Ala, Val, or Leu amino acids presented higher antioxidant activities (Mendis, Rajapakse, Byun, & Kim, 2005). Overall, the hydrophobicity and proportion of hydrophobic amino acids affect the antioxidant activity of the peptides (Li, Li, He, & Qian, 2011). Hydrophobic residues can increase the accessibility of hydrophobic radicals and the density of peptides containing these residues at the water–lipid interface, thus enhancing the antioxidant properties of the peptide (Nikoo et al., 2014). SN-12 contains the hydrophobic amino acids Leu, Val, Pro, Phe and Ile. The Peptide Ranker for the LGFPL (LL-5) sequence was 0.94, and this peptide contained the hydrophobic amino acids Leu, Phe and Pro. Therefore, it can be predicted that the three peptides VN-10, SN-12 and LL-5 may have antioxidant activity. Subsequently, these three peptides (VN-10, SN-12 and LL-5) were selected for synthesis and further analysis.

Table 2
Statistics of protein and peptide identification results.

Sample name	Protein group	Peptide	PSM
P–I (M_W < 3 kDa)	103	468	703

PSM: peptide-spectrum matches.

3.7. Physicochemical properties and safety evaluation of the synthesized peptides

Three peptides, VN-10, LL-5 and SN-12, were synthesized via solid-phase synthesis; their purities were >96% (Figs. S1A–C). The physicochemical properties of the three peptides are listed in Table 4. The isoelectric points (PIs) of the synthetic peptides were VN-10 = 5.49, LL-5 = 5.52 and SN-12 = 5.24, indicating weak acidity. The synthetic peptides had poor water solubility and were predicted to be nontoxic. However, VN-10 showed potential allergenicity. The average hydrophilic value (GRAVY index) is the ratio of the sum of hydrophilic values of all amino acids in the peptide sequence to the number of amino acids; greater hydrophilicity is shown by more negative values, and greater hydrophobicity is indicated by more positive values. The order of hydrophilicity was VN-10 > SN-12 > LL-5. Deviations occurred in the final results, as the predicted results used in the experiment were statistically analyzed using the databases established from the peptide fragments of different species. Thus, in vivo testing is needed to confirm whether the synthetic peptides are toxic or allergenic.

3.8. Antioxidant activity of the synthetic peptides

The amino acid composition, molecular weight, hydrophobicity, and sequence of peptides all have an impact on their antioxidant effects (Mirzaei, Mirdamadi, Safavi, & Soleymanzadeh, 2020). The ·OH scavenging activity of VN-10 was significantly higher than that of SN-12 and LL-5 when the mass concentration was 8–25 g/L ($P < 0.05$) (Fig. S2A). DPPH· scavenging activity and reducing power showed the same trend (Figs. S2B and D). The antioxidant activity of VN-10 was higher than that of LL-5 and SN-12. This may be due to the combination of the Val residue at the N-terminus of VN-10, the large number of hydrophobic amino acids in the sequence, and the presence of the Tyr aromatic group. The amino acid composition analysis showed that VN-10, LL-5 and SN-12 contained 70%, 80% and 58% hydrophobic amino acids, respectively. Nwachukwu and Aluko (2019) reported that hydrophobic amino acids (Leu, Val, Met and Ala) and aromatic amino acids (Phe, Tyr, His and Trp) are important for the antioxidant activity of peptides, which is attributed to the large hydrophobic groups that facilitate contact of the peptides with hydrophobic radicals. Rajapakse, Mendis, Byun, and Kim (2005) isolated and identified two peptides containing hydrophobic amino acids (NADFGLNGLEGLA and NGLEGLK) from squid protein hydrolysis products that inhibited lipid peroxidation and scavenged free radicals. The proportion of hydrophobic amino acids in these two peptides exceeded 75%, suggesting that the high content of hydrophobic amino acids and low content of hydrophilic amino acids could help to improve the solubility of the peptides in an emulsification system and enhance the ability of the peptides to inhibit lipid peroxidation. However, when the number of hydrophobic amino acids in the peptide is too high, its antioxidant activity decreases. Chen, Muramoto, Yamauchi, and Nokihara (1996) reported that adding Leu or Pro residues to the N-terminus of His-His enhanced its ability to inhibit lipid peroxidation, but adding Leu or Leu-Leu to the N-terminus of His-His significantly decreased activity.

In the present study, VN-10 and SN-12 also contained Tyr residues. The hydroxyphenyl on Tyr acts as a direct hydrogen donor to capture free radicals while forming stable compounds using the resonance structure itself; thus, it is generally considered to have strong free radical scavenging ability. Wiriyaphan, Chitsomboon, Roytrakul, and

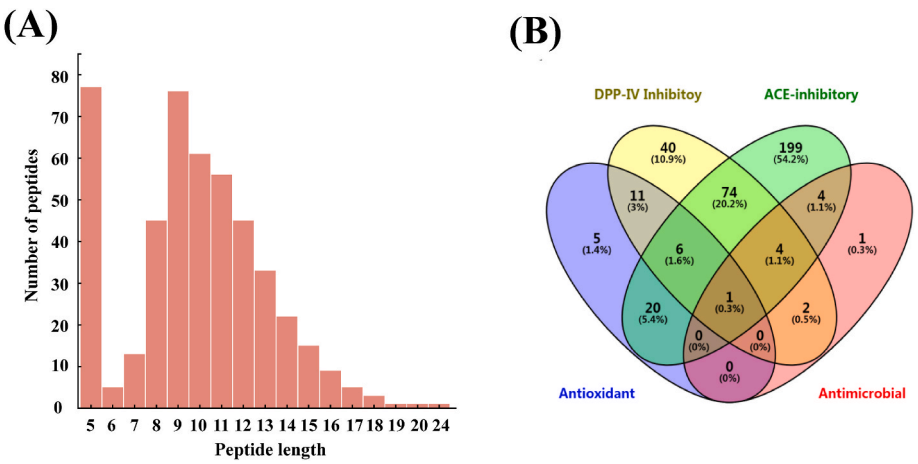

Fig. 5. Identification results of P–I. (A) Distribution of peptide length of component P–I. (B) Venn diagram of differential peptides for 4 activities.

Table 3
Peptides with a peptide ranker score greater than 0.85 in component P–I.

Sequence	Length	Peptide Ranker	Mass	Leading razor protein	Start position	End position	Charges
LWGPL	5	0.958256	584.33223	Q3T052	869	873	1
LGFPL	5	0.938085	545.32133	W5PI38	437	441	1
VLGFF	5	0.932571	581.32133	W5P4J4	207	211	1
WAIPPDPIVGF	11	0.927443	1210.6386	W5P8W6	45	55	1
LDFQLPLRF	9	0.921879	1147.639	W5QI21	563	571	2
GPAGPPGPIGN	11	0.910197	932.47158	W5P481	852	862	2
EFFGL	5	0.897731	611.29551	W5Q9H2	305	309	1
PDEYFKRF	8	0.88386	1100.5291	W5Q2E1	225	232	2
FGFDGDFYR	9	0.883352	1122.4771	W5NTT7	1108	1116	2
DFGFDGDF	8	0.882995	918.33956	W5NTT7	1107	1114	1
LSGFL	5	0.881416	535.3006	W5PGY0	123	127	1
RFFEHF	6	0.880436	881.41842	Q1KYZ8	39	44	2
GPAGPPGPIGNV	12	0.878045	1031.54	W5P481	852	863	2
RDGDRFWWQ	9	0.874371	1264.5738	W5PHQ0	673	681	2
TQRFF	5	0.872672	697.35476	Q1KYZ8	37	41	1
GFDGDFYR	8	0.870757	975.40865	W5NTT7	1109	1116	2
GFDGDFY	7	0.863626	819.30753	W5NTT7	1109	1115	1

Table 4
Physicochemical properties of synthetic peptides.

Number	Sequence	Mass (Da)	Purity (%)	Point Isoelectric	GRAVY	Instability factor	Peptide Ranker	Solubility	Toxicity	Allergenicity
P1:VN-10	VYPFPGPIPN	1100.28	97.82	5.49	−0.01	49.44	0.724837	Poor	No	Probable
P2: LL-5	LGFPL	545.68	96.44	5.52	1.68	46.52	0.938085	Poor	No	No
P3:SN-12	SLVYPFPGPIPN	1300.52	98.18	5.24	0.242	42.87	0.753363	Poor	No	No

Yongsawadigul (2013) synthesized five peptides (VELLVPK, AGNQVLNLQADLPK, LGTGTDL, FLGSFLYEYSR and NTFLFFK) derived from the hydrolytic products of surimi processing and determined $ABTS^{\cdot+}$ scavenging activity; only FLGSFLYEYSR was active, possibly because of the presence of Tyr residues. Zheng et al. (2012) isolated and identified three antioxidant peptides (VTPY, VLLY and VGTVEM) from sea cucumber gut hydrolysates and reported that all three peptides, containing Val residues at the N-terminus, had protective effects against oxidative DNA damage. VN-10, isolated in the present study, additionally contains a large amount of Pro, which has a pyrrolidone ring that enhances the structural flexibility of the antioxidant peptide due to its low ionization potential and eliminates $^1O_2$, thereby conferring high antioxidant activity.

3.9. Docking and interaction model between the peptides and Keap1

In recent years, numerous studies have suggested that the Keap1-Nrf2 antioxidant signaling pathway may be an important mechanism by which bioactive peptides exert their antioxidant effects (Han et al., 2018). Oxidative damage is reduced by activating the Keap1–Nrf_2 signaling pathway (Kensler, Wakabayashi, & Biswal, 2007). VN-10 displayed the greatest antioxidant activity among the three synthesized antioxidants (see Section 3.9). Therefore, to deeply explore the potential antioxidant mechanism of VN-10, we applied molecular docking to evaluate its interaction with Keap1 and identify the binding site. During molecular docking, small ligand molecules and receptor proteins interact via intermolecular hydrogen bonds, hydrophobic interactions, van der Waals forces, Pi bonds and electrostatic interactions, of which hydrogen bonds are the strongest.

The binding interactions between VN-10 and the Kelch domain of Keap1 are shown in Fig. S3. VN-10 interacted with ARG380, ASN382, TYR 334, ARG415 and TYR 572 of Keap1 to form five hydrocarbon bonds. VN-10 also formed a Pi stacked (π–π stacking) interaction with TYR525 of Keap1, contributing to a firmer connection. In addition, VN-10 interacted with the HIS436, ILE461 and ALA556 residues to form π–alkyl bonds. Thus, VN-10 could occupy the Nrf_2 binding site in the

Keap1 Kelch domain to inhibit the Keap1–Nrf_2 interaction. Snakehead (*Channa argus*) soup-derived antioxidant peptides (Zhang et al., 2021) and watermelon seed antioxidant peptides (Wen, Zhang, Zhang, Duan, & Ma, 2021) exhibit similar results. Moreover, Tonolo et al. (2020) predicted the interaction of antioxidant peptides purified from fermented soybean products with Keap1. In vitro cell models revealed that these peptides activated the Keap1–Nrf_2 pathway with consequent overexpression of antioxidant and phase II enzymes and were active against antioxidant stress, cell activity and ROS production. Our findings indicate that VN-10 is a potentially highly active antioxidant peptide.

4. Conclusion

SBPHs hydrolyzed by alkaline protease had high antioxidant capacity and should be stored in neutral and weakly acidic environments, avoiding contact with copper utensils and high-temperature environments during processing, storage, and administration. In addition, SBPHs can be added with appropriate preservatives during the production of related products. The SBPHs were tolerant to gastrointestinal fluids during in vitro simulated digestion, which was an important prerequisite for bioactive peptides. Three fractions were obtained (P–I, P-II and P-III) from SBPHs, among which P–I had high antioxidant activity. LC−MS/MS results revealed 17 peptides from P–I with peptide ranks greater than 0.85, which were potentially biologically active. Three peptides, VN-10, LL-5 and SN-12, were finally synthesized, all of which possessed high antioxidant activity, especially VN-10. Molecular docking results showed that carbon−hydrogen bonding, π-π stacking and π-alkyl bonding between VN-10 and Keap1 led to its high antioxidant capacity. In this study, the potency of antioxidant peptides from sheep bone was assessed by chemical analysis, and the next research focus should be to evaluate the ability of antioxidant peptides to eliminate ROS, inhibit oxidative damage, and maintain cellular redox homeostasis, and their mechanism of action, through cellular and animal models.

CRediT authorship contribution statement

Guanhua Hu: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Lu Dou:** Conceptualization, Data curation, Formal analysis, Writing – original draft. **Jing Zhang:** Conceptualization, Data curation. **Rina Su:** Writing – review & editing. **Mirco Corazzin:** Writing – review & editing. **Lina Sun:** Investigation, Methodology. **Lihua Zhao:** Investigation, Resources. **Ye Jin:** Funding acquisition, Supervision, Writing – review & editing. **Lin Su:** Funding acquisition, Resources.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115682.

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