J. Ocean Univ. China (Oceanic and Coastal Sea Research)
https://doi.org/10.1007/s11802-021-4551-4
ISSN 1672-5182, 2021 20 (2): 421-428
http://www.ouc.edu.cn/xbywb/
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In vitro Antioxidant Effects of *Porphyra haitanensis* Peptides on H_2O_2-Induced Damage in HepG2 Cells

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(Received April 3, 2020; revised May 26, 2020; accepted November 25, 2020)

Abstract In this study, protein from *Porphyra haitanensis* was used as raw material to prepare an antioxidant peptide, and its antioxidant activity was evaluated *in vitro*. A model of H_2O_2-induced oxidative damage in HepG2 cells was established, and the effects of *Porphyra haitanensis* hydrolysates (PHHs) on superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were detected. Finally, the structure of PHHs was identified by ESI-MS/MS. The results showed that the 1,1-diphenyl-2-pyridylhydrazine (DPPH)-free radical-scavenging ability of PHHs was the strongest (59.28% at 1.0 mg mL^{-1}) when hydrolyzed with an acidic protease for 4 h. PHHs with different concentrations had protective effects on H_2O_2-induced damage to HepG2 cells, and the protective effect was enhanced with increasing concentrations. When the level was 400 μg mL^{-1}, the cell survival rate was as high as 88.62%. Moreover, PHHs can significantly reduce oxidative damage to HepG2 cells by H_2O_2, improve SOD activity, and reduce MDA content. The tetrapeptide Asp-Lys-Ser-Thr, with a molecular weight of 448 Da, was identified as an important fraction of PHHs by high-resolution mass spectrometry.

Key words *Porphyra haitanensis* hydrolysates (PHHs); antioxidant peptides; radical-scavenging activity; cell antioxidant capacity; electrospray ionization-mass spectrometry

1 Introduction

Oxidation is an important process during food deterioration that affects the safety, color, taste, and texture of food. Furthermore, oxidation during cellular metabolism can induce DNA mutations, modify the lipid components of cell membranes, and cause denaturation of cell membrane proteins. Antioxidants can prevent or decrease the harmful effects of free radicals in human body and delay the deterioration of fat, protein, and other food ingredients. Because of their potential teratogenicity, carcinogenicity, and mutagenicity, synthetic antioxidants are limited in their application. Numerous researches have been conducted on the preparation of natural and non-toxic antioxidants to replace the synthetic ones. These natural antioxidants include various plant- and animal-based extracts, such as proteins (Borawska *et al*., 2016; Yang *et al*., 2016), polysaccharides (Tang *et al*., 2016; Xu *et al*., 2016), flavonoids (Dulf *et al*., 2016; Chaaban *et al*., 2017), polyphenols (Shen *et al*., 2015; Plaza *et al*., 2016), lectins (Wu *et al*., 2015), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) (Sahari *et al*., 2017).

Antioxidant peptides, which consist of 2–20 amino acids, can be isolated from animal and plant protein by enzymatic hydrolysis. With their properties of easy absorption, intense activity, few side effects, excellent safety, and environment-friendly properties, antioxidant peptides can be used to prepare functional foods, protein supplements, and medications. Many studies have reported that hydrolyzed proteins from various animal and plant sources, such as bean (Marcela e*t al*., 2016; Carlos *et al*., 2017), corn (Jin *et al*., 2016; Wang *et al*., 2016), shellfish (Wu *et al*., 2016; Jin *et al*., 2018), shrimp (Zhou *et al*., 2014; Kleekayai *et al*., 2015), fish (Ko *et al*., 2013; Qiu *et al*., 2014), and algae (Sheih *et al*., 2009; Yu *et al*., 2016), possess antioxidant activity.

Porphyra is one of the most important cultured seaweeds and a main exported sea product of China (Liu *et al*., 2017). The annual Chinese production of porphyra is 0.20 million tons, of which *P. haitanensis* accounts for 50% (Department of Fishery and Fishery Administration, Ministry of Agriculture and Rural Areas, 2019). It is rich with proteins, polysaccharides, flavonoids, polyphenols, frames,

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lectins, DHA, EPA, and other physiologically active components. The harvest period of *P. haitanensis* is generally divided into 4–5 parts. The nutritional quality and market price of *P. haitanensis* decreases gradually with a prolonged harvest period. In the final harvest period, *P. haitanensis* usually is discarded as a waste, and leads to considerable environmental pollution. Interestingly, this waste can be used as raw material to prepare nutrients and physiologically active ingredients such as proteins, polysaccharides, DHA, and EPA. It is of considerable significance to improve the economic value of *P. haitanensis*.

In the present study, the antioxidant activities of *P. haitanensis* hydrolysates produced with different hydrolysis time was investigated, and its antioxidant mechanism was explored by measuring the radical-scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH). The antioxidant peptide was separated from the hydrolysate by gel filtration chromatography. The HepG2 cell with oxidative damage was used to further evaluate the antioxidant activity of purified PHH and explore the antioxidant mechanism. The peptide sequence was identified using an electrospray mass spectrometer (ESI-MS/MS).

2 Materials and Methods

2.1 Materials and Chemicals

P. haitanensis was supplied by the Peilong seaweed culture area in Chenghai District, Shantou City, Guangdong Province, China. *P. haitanensis* was dried at 60℃ for 4 h, then ground and filtered through a 100-mesh screen, and finally stored dry. Acid protease (5×10^4 U g^{-1}) was supplied by Hefei BoMei Biotechnology Incoporation (Hefei, China). HepG2 cells were provided by Guangzhou Yeshan Biotechnology Incoporation (Guangzhou, China). All other materials for cell culture were obtained from Hyclone (Logan, Utah, USA). Sephadex G-15 was purchased from Cool Chemical Technology (Beijing, China). Reduced glutathione (MW 307.3 Da) and bacitracin (MW 1422.69 Da) were obtained from Guangzhou Qiyun Biotechnology Incoporation (Guangzhou, China). Aprotinin (MW 6511.83 Da) and cytochrome C (MW 12400 Da) were obtained from Shanghai Maclean Biochemical Technology Incoporation (Shanghai, China). Glutathione (GSH), H_2O_2, and L-oxidized glutathione (MW 612.63 Da) were purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade.

2.2 Protein Extraction from *P. haitanensis*

The protein was extracted from *P. haitanensis* using an ultrasonic cell grinder (JY99LLDN, Ningbo Scientz Biotechnology Company, Jiangsu, China). *P. haitanensis* (1.0 g) and 100 mL of distilled water were mixed and stirred uniformly, and extracted at a power of 1260 W, room temperature for 30 min, and then centrifuged with $558.95\times g$ at 4℃ for 10 min to remove cell debris. The pH of the supernatant was adjusted to an isoelectric point of 4.2. After the solution was kept at room temperture for 1 h, it was centrifuged at $558.95\times g$ for 10 min to remove the supernatant. The precipitate was dissolved in distilled water, adjusted to neutral pH, and dialyzed in a dialysis bag with a molecular weight cutoff of 100 Da for 24–36 h. The protein solution was pre-chilled at −20℃ for 12 h, freeze-dried under vacuum at −44℃ for 36 h in an Alpha 1-4 lyophilizer (Marin Christ, Germany), and stored dry at room temperture.

2.3 Enzymatic Hydrolysis of *P. haitanensis*

The protein of *P. haitanensis* was hydrolyzed by acid protease for 2.0, 4.0, 6.0, 8.0, and 10.0 h at 54.4℃, pH 3.67, with 4240 U g^{-1} of the enzyme. Reactions were stopped by heating at 95℃ for 10 min, and samples were centrifuged to remove insoluble material. The degree of hydrolysis (DH) of *P. haitanensis* proteins was determined using the pH-stat method. *Porphyra haitanensis* hydrolysates (PHHs) were adjusted to neutral pH and dialyzed for 24–36 h in a 100 Da dialysis bag. PHHs were concentrated, and freeze-dried under vacuum at −44℃ for 36 h in an Alpha 1-4 lyophilizer (Marin Christ, Germany), and stored dry at room temperture.

2.4 Molecular Weight Distribution of PHHs

The molecular weight distribution of PHHs was determined using high-performance size-exclusion chromatography (Sallam *et al*., 2018). The column was TSK-GELG 2000SWXL (7.8 mm × 300 mm), and the mobile phase was acetonitrile containing 0.1% trifluoroacetic acid and double distilled water containing 0.1% trifluoroacetic acid. The detection wavelength was 214 nm, the sample volume was 10 μL, the flow rate was 0.5 mL min^{-1}, and the elution length was 60 min. A molecular weight standard was dissolved in mobile phase at a concentration of 0.2 mg mL^{-1}. By fitting time and molecular weight, a regression equation was obtained: $\log MW=-0.206t+6.8009$, $R^2=0.9874$. The molecular weight distribution of PHHs was calculated by normalization of the peak area.

2.5 Purification of PHHs

Purification of PHHs was performed using selective gel filtration chromatography (Mei *et al*., 1998). After lyophilization, the polypeptide was dissolved in deionized water at a concentration of 25 mg mL^{-1}. The sample was loaded onto a Sephadex G-15 column (1.6 cm × 100 cm), eluted with deionized water at a flow rate of 0.6 mL min^{-1}, and monitored at 214 nm. Fractions with strongest DPPH-free radical-scavenging ability were concentrated, lyophilized, and further analyzed.

2.6 Antioxidant Activity Assays

2.6.1 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity assay

DPPH radical-scavenging activity was measured according to a previously described method (Jang *et al*., 2016) with some modifications. The sample (1.0 mL) was combined with 1.0 mL of DPPH (0.15 mmol L^{-1} in ethanol). The solution was immediately mixed and incubated in the dark

for 30 min. The absorbance of the resulting mixture was measured at 517 nm. The control sample contained ethanol (1.0 mL) and DPPH (1.0 mL). DPPH free radical-scavenging activity was calculated as $(1-As/Ac)\times 100\%$, where As and Ac represent absorbance of the sample and control, respectively.

2.6.2 Hydroxyl radical-scavenging activity assay

Hydroxyl radical-scavenging activity was measured according to a previously described method (Chi *et al*., 2015) with some modifications. Firstly, 2 mL sample was mixed with 0.3 mL of 1,10-phenanthroline (5 mmol L^{-1} in ethanol), 0.2 mL of 0.15 mol L^{-1} phosphate buffer (pH 7.4), and 0.3 mL of 0.75 mmol L^{-1} ferrous sulfate, and the solution was stirred vigorously. Then 0.2 mL of 0.1% H_2O_2 was added to the solution and incubated at 37℃ for 60 min. Finally, the absorbance of the sample A_s was measured at 510 nm. When an equivalent volume of deionized water was used instead of the sample and H_2O_2 solution, the absorbance value A_b was applied as the control. When Ddeionized water was used instead of the sample, the absorbance value A_0 was applied as the blank. Hydroxyl radical-scavenging activity was calculated as $(1-(A_S-A_0)/A_b)\times 100\%$.

2.7 Antioxidant Activity Analysis in HepG2 Cells

2.7.1 Cell culture

HepG2 cells stored in liquid nitrogen were thawed in a water bath at 37℃. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Cells were propagated in a 25 cm^2 tissue culture flask, harvested using trypsin during exponential growth, and seeded into 96-well plates at a density of 1×10^5 cells mL^{-1}. Cells were incubated at 37℃ in 5% CO_2, and the medium was changed every 2 d.

2.7.2 Cell viability assay

HepG2 cells at Log-phase were seeded into 96-well plates with a density of 1×10^5 cells mL^{-1} and cultured for 24 h at 37℃ in a incubator containing 5% CO_2. After the cells were cultured for 24 h, 20 μL of a 5 mg mL^{-1} methyl thiazolyl tetrazolium (MTT) solution was added to each well, and the cells were cultured for another 4 h. Then the supernatant was carefully discarded, 200 μL of dimethylsulfinic acid was added to each well. After the plate was shaken for 15 min, the absorbance value was measured at 490 nm. Cell viability was calculated based on absorbance (Jang *et al.*, 2008).

2.7.3 Establishment of the cell model

In drug screening experiments, an induction concentration with a survival rate of 50% to 70% is generally selected as the optimal concentration. When the concentration of inducer is too high, the cells will be susceptible to excessive damage and the survival rate will be very low. HepG2 cells were treated with different concentrations of H_2O_2 solutions (50, 100, 200, 400, 600, and 800 μmol L^{-1}) for 4 h. Cell viability was determined using the MTT method to determine the appropriate H_2O_2 concentration and treatment time (Jang *et al.*, 2016). Using the cell survival rate as the indicator, the optimal induction condition to obtain a survival rate of 60% was culturing the cells in 259 μmol L^{-1} H_2O_2 for 4 h. HepG2 cells were divided into a blank group, a control group, and a treatment group. The blank group was HepG2 cells without H_2O_2 treatment. The control group was HepG2 cells treated with 259 μmol L^{-1} H_2O_2 for 4 h. In the treatment group, the cells were treated with GSH or PHH-III with a concentration of 100, 200, and 400 μg mL^{-1} for 24 h, respectively, and then were treated with H_2O_2. These cells were determined as low-, medium-, and high-dose groups.

2.7.4 Determination of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content

HepG2 cells at Log-phase were prepared as single-cell suspensions and seeded into 96-well plates with a concentration of 1×10^4 cells per 200 μL. Following establishment of a cell model, different concentrations (100, 200, and 400 μg mL^{-1}) of polypeptide and GSH were used to treat oxidatively damaged HepG2 cells, and the MDA content and SOD activity were determined. The SOD activity was determined using the Total Superoxide Dismutase Assay Kit with WST-8 according to the manufacturer's instructions. The MDA content in the cells was determined using the Lipid Peroxidation MDA Assay Kit according to the manufacturer's instructions.

2.8 Structure of PHHs

Structure of PHHs was identified according to a previously described method (Lin *et al*., 2019) with some modifications. PHH-III was dissolved in a mobile phase, filtered through a 0.22 μm filter, placed in a sample vial, and analyzed using ultra-high-pressure liquid chromatography-high-resolution mass spectrometry. PHH-III were precipitated with an Agilent SB-C18 RRHD column (2.1 mm × 50 mm, 1.8 μm) with a flow rate of 0.2 mL min^{-1}, 0.1% formic acid as phase A and acetonitrile as phase B, and an injection volume of 20.0 μL. The gradient elution conditions were as follows: 0–1 min, B phase remained unchanged at 15%; 1–4.5 min, volume fraction of phase B solution increased from 15% to 90%; 4.5–8 min, phase B maintained at 90%; and 8.5–10 min, phase B decreased from 90% to 15%. Scanning was performed in positive-ion mode with a scan range of m/z 100–1400.

2.9 Statistical Analysis

Results are presented as the mean and standard deviation of triplicates. Statistical analysis was performed using SPSS 20.0 software (SPSS, Chicago, IL, USA).

3 Results and Discussion

3.1 Antioxidant Activity and Molecular Weight Distribution of PHHs

PHHs obtained by different hydrolysis times were test-

ed for DPPH radical-scavenging activity. As shown in Table 1, PHHs with a hydrolysis time of 4 h were found to be more efficient than the others, and the resulting hydrolysates exhibited higher antioxidant capacity. This may result from hydrolysis producing more small peptides with antioxidant activity because of energetic electrons (Wang *et al*., 2013). However, excessive hydrolysis time increases may result in the degradation of these small fragments, which can cause down-rugulated antioxidant activity. PHHs prepared by 4 h of hydrolysis were then further isolated for the identification of antioxidant peptides.

The chromatographic data (Table 1) showed that PHHs contained a significant amount (70%−80%) of oligopeptides below 1 kDa. The results indicate that the acid proteases are effective at producing short oligopeptides and removing large peptides or undigested proteins. Hydrolysates with different molecular weights have differences in antioxidant capacity (Li *et al.*, 2013). Moreover, some research findings indicated that small molecular peptides possess more robust free radical-scavenging activity (Zhuang *et al*., 2010), further increasing the antioxidant function. Therefore, according to the molecular weight distribution of PHHs, an appropriate method can be selected to purify PHHs and further evaluate their antioxidant capacity.

Table 1 Comparison of DPPH radical-scavenging activity and molecular weight distribution of PHHs at different enzymatic hydrolysis times

Hydrolysis time	DH (%)	DPPH radical-scavenging activity (%)	Molecular weight (kDa) distribution (%)				
			>10 kDa	5−10 kDa	3−5 kDa	1−3 kDa	<1 kDa
2 h	8.40±0.08	49.81±1.00	3.37	1.11	1.66	21.87	71.99
4 h	11.40±0.07	59.29±0.74	1.44	0.83	1.55	22.72	73.46
6 h	12.81±0.14	51.46±0.92	0.00	0.00	0.19	23.01	76.80
8 h	13.46±0.04	50.42±0.29	0.00	0.00	0.19	21.29	78.52
10 h	14.89±0.13	48.04±1.34	0.00	0.00	0.00	20.02	79.98

Notes: DPPH radical-scavenging capacity is determined at a sample concentration of 1.0 mg mL^{-1}. Values are shown as mean ± standard deviation of triplicates (n = 3).

3.2 Purification of Different Fractions of PHHs

Gel filtration chromatography has been used for separation of biological extracts and protein hydrolysates (Knuckles *et al*., 2006). Considering the molecular weight of PHHs, a Sephadex G-15 column is suitable for further separation. PHHs were further separated into three fractions (I−III) by gel filtration chromatography. Each fraction was pooled, lyophilized, and tested for DPPH-scavenging ability. As shown in Fig.1, fraction III exhibited the most vigorous DPPH radical-scavenging activity with a rate of 73.32% at 1.0 mg mL^{-1}. In this research, we found that the eluted fractions' antioxidant activity increased with increasing retention time. Consequently, the fraction of PHH-III, which demonstrated the most potent activity, was selected for further analysis.

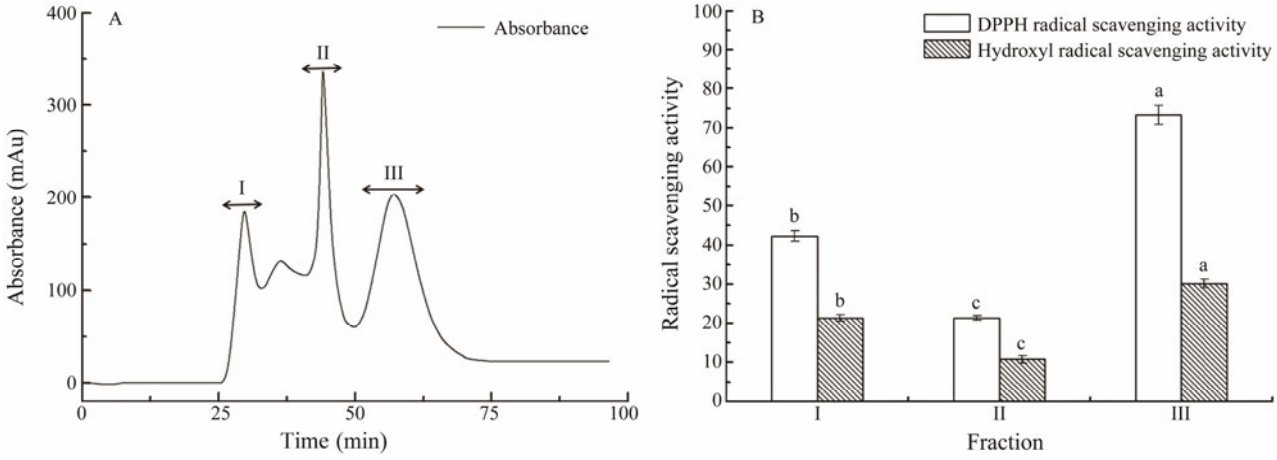

Fig.1 (A) Chromatogram of PHH fractions purified by gel filtration column; (B) DPPH radical and hydroxyl radical-scavenging ability of each fraction (samples at 1.0 mg mL^{-1}). Values within the same column, followed by the different letters (a, b, and c), are significantly different (P < 0.05).

3.3 Antioxidant Capacities of PHH-III to HepG2 Cells

3.3.1 Cell viability of the antioxidant activity cell model

Oxidative stress refers to cell damage caused by high concentrations of reactive oxygen molecules or chemical derivatives of oxygen to cells. It is an essential mediator of the occurrence and development of obesity, diabetes, lipid deposition, and chronic inflammation (Skalicky *et al*., 2008). H_2O_2 is one of the commonly used substances for establishing oxidative damage in cells and has been used in osteoblasts, nerve cells, vascular endothelial cells, and hepatocytes (Zorov *et al*., 2000). HepG2 cells can fully express antioxidant enzymes and detoxification enzymes

as in normal hepatocytes. Accordingly, They are often used for studying cytoprotection against exogenous chemicals and natural antioxidants (Lee *et al*., 2012). As shown in Figs.2 and 3, compared with healthy cells (Blank), the viability of HepG2 cells in the injury group (Control) was reduced significantly. Moreover, the injured cells showed inter-adhesion, irregular shapes, and lower cell viability. Different doses of PHH-III in each group can protect the cell and significantly increase cell viability ($P<0.05$). Together with GSH, PHH-III was better at improving the morphology and cell viability with a concentration of 100 $\mu g\,mL^{-1}$. Additionally, the viability of cells cultured with different doses of PHH-III was higher than that with different doses of GSH. The results of these experiments indicate that PHH-III can increase the viability of cells damaged by H_2O_2.

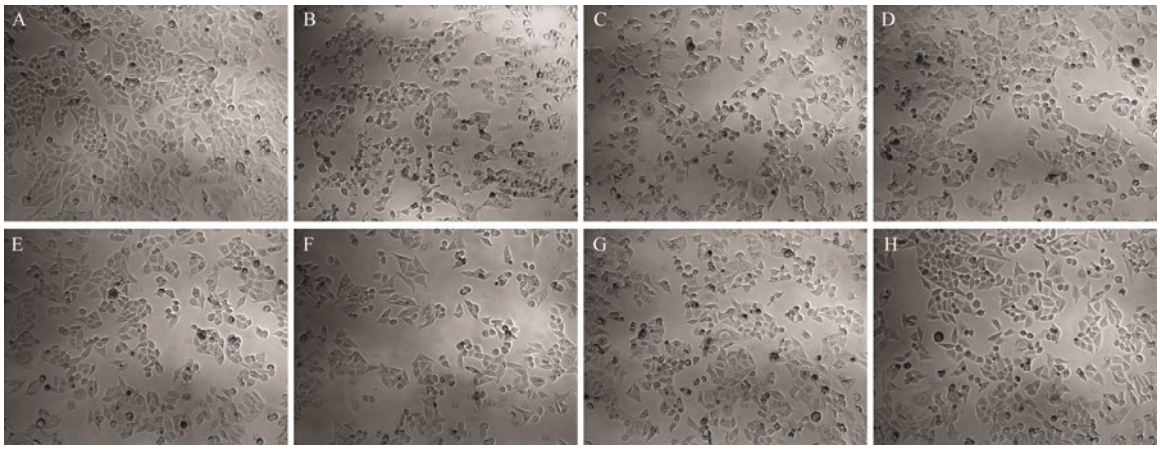

Fig.2 Effects of different concentrations of PHH-III and GSH on the morphology of HepG2 cells treated with 259 μmol L^{-1} H_2O_2 for 4 h. (A) Blank, (B) Control, (C) 100 $\mu g\,mL^{-1}$ GSH, (D) 200 $\mu g\,mL^{-1}$ GSH, (E) 400 $\mu g\,mL^{-1}$ GSH, (F) 100 $\mu g\,mL^{-1}$ PHH-III, (G) 200 $\mu g\,mL^{-1}$ PHH-III, and (H) 400 $\mu g\,mL^{-1}$ PHH-III. The blank group was HepG2 cells without H_2O_2 treatment. The control group was HepG2 cells treated with 259 $\mu mol\,L^{-1}$ H_2O_2 for 4 h.

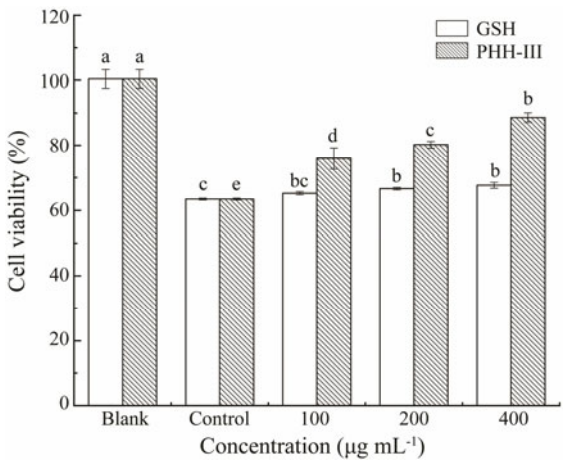

Fig.3 Effects of different concentrations of PHH-III and GSH on the viability of HepG2 cells treated with 259 μmol L^{-1} H_2O_2 for 4 h. The blank group was HepG2 cells without H_2O_2 treatment. The control group was HepG2 cells treated with 259 $\mu mol\,L^{-1}$ H_2O_2 for 4 h. The treatment group was treated with GSH or PHH-III with a concentration of 100, 200, or 400 $\mu g\,mL^{-1}$ for 24 h, respectively, as the low-, medium-, and high-dose groups. Then the H_2O_2 treatment was applied. Values within the same column, followed by different letters (a, b, c, d, and e) are significantly different ($P<0.05$). Values are shown as mean ± standard deviations from triplicates ($n=3$).

3.3.2 Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in HepG2 cells with different treatments

SOD catalyzes the disproportionation of superoxide anion to H_2O_2 and O_2 to scavenge free radicals, and its vitality plays an important role in cellular oxidation and antioxidant balance (Miller, 2004). As shown in Fig.4, compared with healthy cells (Blank), the SOD activity in injured HepG2 cells (Control) was significantly reduced. When medium- or high-dose PHH-III was added in the culture media, the SOD activity in the cells could be increased significantly ($P<0.05$), while only high dose GSH could significantly increase the SOD activity ($P<0.05$).

MDA is an oxidation end product obtained by free radicals acting on lipids in the body (Bedoya-Ramírez *et al*., 2017). The MDA content in liver cells is generally considered to be an essential indicator of the degree of lipid peroxidation (Hu *et al*., 2017). As shown in Fig.4B, compared with untreated cells (Blank), the MDA content in inhured HepG2 cells (Control) was significantly increased. The low-, medium-, and high-dose of PHH-III or GSH significantly inhibited the increase of MDA content in the cells caused by H_2O_2 in a dose-dependent manner ($P<0.05$). Compared with GSH, PHH-III was better at reducing the MDA content in cells with low-, medium-, and high-dose. Thus PHH-III can increase the SOD activity of H_2O_2-injured cells, inhibit the increase in MDA content, and enhance the antioxidant response of HepG2 cells.

3.3.3 Mass spectrometry identification analysis

Mass spectrometry can accurately determine the molecular weight and amino acid sequence of peptides and has potential applications in proteomics analysis (Li *et al*., 2007). In order to identify the peptides exhibiting major antioxidant activity, the PHH-III fractions were further se-

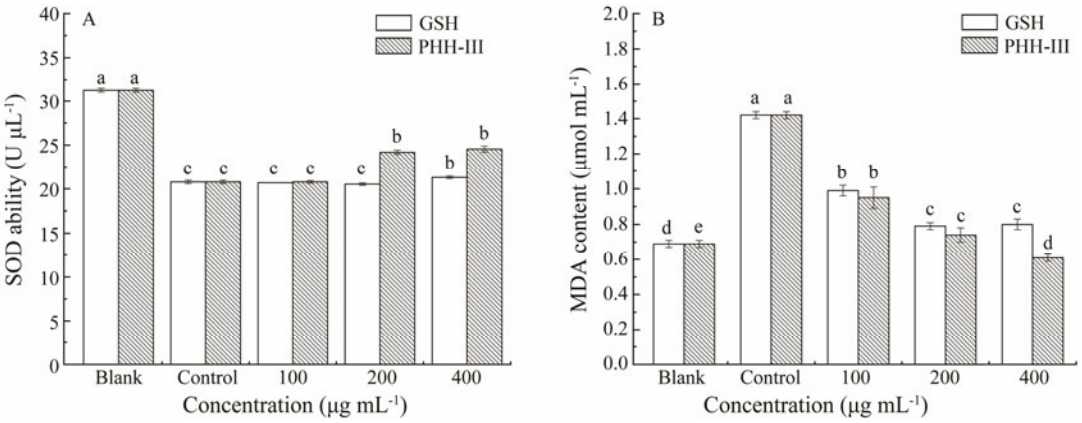

Fig.4 (A) Effects of different concentrations of GSH and PHH-III on SOD activity in HepG2 cells treated with 259 μmol L^{-1} H_2O_2 for 4 h, (B) Effects of different concentrations of GSH and PHH-III on MDA content in HepG2 cells treated with 259 μmol L^{-1} H_2O_2 for 4 h. The blank group was HepG2 cells without H_2O_2 treatment. The control group was HepG2 cells treated with 259 μmol L^{-1} H_2O_2 for 4 h. The treatment group was treated with GSH or PHH-III with a concentration of 100, 200, and 400 μg mL^{-1} for 24 h, respectively, as the low-, medium-, and high-dose groups. Then H_2O_2 treatment, was applied. Values within the same column, followed by different letters (a, b, c, d, and e) are significantly different ($P < 0.05$). Values are shown as mean ± standard deviations from triplicates ($n = 3$).

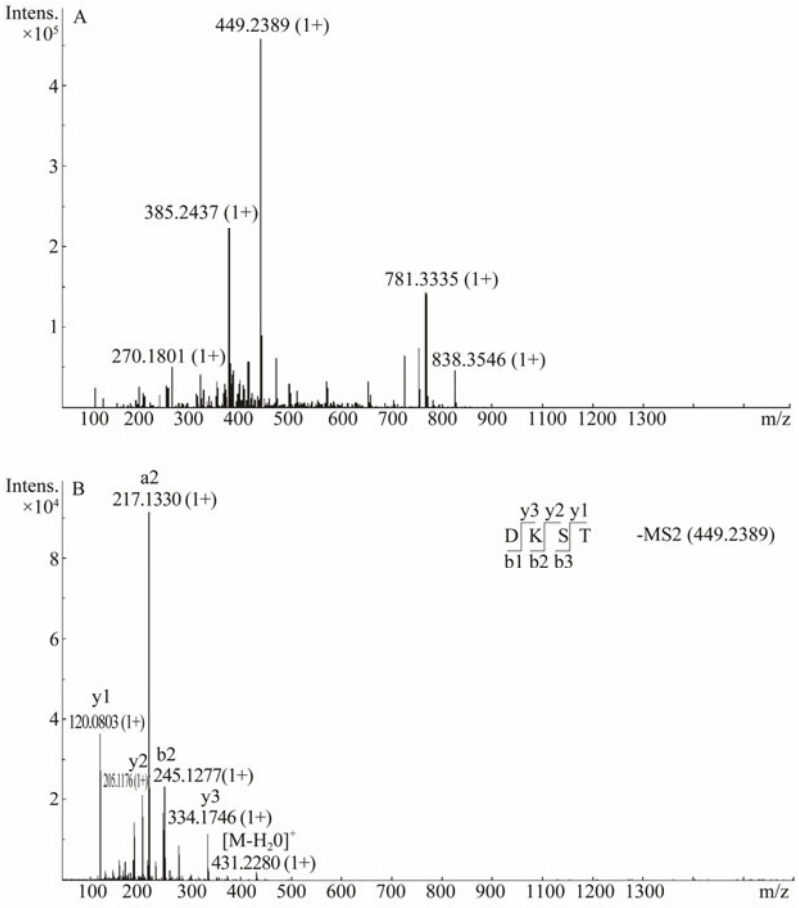

Fig.5 (A) Mass spectrum of the chromatographic peak III; (B) MS/MS spectrum of ion m/z 449.

quenced by ESI-MS/MS. The MS spectrum of this fraction is shown in Fig.5A, and the MS/MS spectrum of a single-charged ion with m/z 449 Da is shown in Fig.5B. The molecular weight of the peptide in PHH-III was determined to be 449 Da. Since each peptide matches a specific mass number and corresponding fragment map (Ma *et al*., 2012), the amino acid sequence of the peptide in PHH-III was identified as Asp-Lys-Ser-Thr. According to the naming system proposed by Roepstorff and modified by Biemann, the N-terminal fragment ions are represented by the letters a, b, and c, and the C-terminal fragment ions are represented by the letters x, y, and z (Biemann, 1992). Since the amide bond in the peptide is relatively easy to be broken, the b- and y-type fragment series may have a higher frequency of occurrence on the mass spectrum. The y3 (m/z 334), y2 (m/z 205), b2 (m/z 245), and

y1 (m/z 120) fragments are produced by cleavage of a peptide bond. The neutral molecule may also lose water or ammonia (Sun *et al*., 2008), resulting in a high abundance of m/z 431. It has been reported that residues of acidic amino acids (Asp, Glu, *etc*.) contribute to the antioxidant capacity of peptides (Saiga *et al*., 2003). Moreover, Lys contributes to the termination of a radical chain reaction, the interaction with free radicals, and the prevention of radical formation. In general, the number, type, and composition of amino acids play an essential role in antioxidative peptides.

4 Conclusions

In this study, the DPPH free radical-scavenging ability of PHHs was the strongest (59.28% at 1.0 mg mL^{-1}) when hydrolyzed with an acidic protease for 4 h. Among the hydrolyzed products, PHHs with a molecular weight range of less than 1 kDa accounted for 70% to 80% of the total PHHs. Sephadex G-15 was used to separate and purify PHHs, and it was found that the PHH-III fraction had the most vigorous activity. PHH-III could alleviate the oxidative damage of HepG2 cells caused by H_2O_2, which might be realized through increasing SOD enzyme activity and reducing the production of MDA. The tetrapeptide Asp-Lys-Ser-Thr with a molecular weight of 448 Da was identified by high-resolution mass spectrometry in the PHH-III. The specific signaling pathways and targets for the regulation of oxidative stress response in cells affected by algae peptides need to be further studied.

Acknowledgements

This work was supported by the National Key R&D Program of China (No. 2018YFD0901102), the Guangdong Provincial Special Fund for Modern Agriculture Industry Technology Innovation Teams (No. 2020KJ151), the Special Scientific Research Funds for Central Non-profit Institutes, Chinese Academy of Fishery Sciences (No. 2020 TD69), and the China Agriculture Research System (No. CARS-50).

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(Edited by Qiu Yantao)