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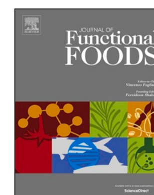

Identification of a new antioxidant peptide from porcine plasma by *in vitro* digestion and its cytoprotective effect on H_2O_2 induced HepG2 model

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ABSTRACT

Antioxidant peptide, referred as Trp-Gly-Pro-Gly-Val-Glu (WGPGVE), was isolated and identified from porcine plasma which was hydrolysised 5 h by alkaline protease. The sequence of WGPGVE had hydrophobic amino acids G, V, aromatic amino acids W, and acidic amino acid E, as well as its values of hydroxyl, ABTS, DPPH radicals scavenging rates and iron chelating rate were improved by 30.15%, 10.05%, 38.69%, 51.40% respectively, compared to glutathione (29.32%, 81.06%, 25.43%, 17.82%). Moreover, under *in vitro* digestion model, it exhibited stable antioxidation (50.15%, 92.49%, 24.29%, 35.35%) after the hydrolysis effect of pepsin and trypsin. Additionally, WGPGVE could protect HepG2 cells against H_2O_2 by promoting the expression of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and decreasing reactive oxygen species (ROS), malondialdehyde (MDA) contents. Thus, the new sequence antioxidant peptide could be potentially used in pharmaceuticals or functional foods and promote the application of porcine plasma in food.

1. Introduction

Pig blood, as one of the raw materials of slaughterhouse, is often discarded as garbage except being made low level by-products, which not only pollutes the environment, but also wastes resources. In fact, it is useful sources as it contains certain proteins, such as fibrinogen, albumin, hemoglobin, etc. Moreover, hydrolysis of pig blood will produce bioactive peptides containing 2–20 amino acids in length, which had some functions, such as antioxidant properties (Jin, Choi, & Kim, 2020). At present, many studies have reported that the hydrolysate obtained from pig blood has antioxidant function, Liu, Kong, Xiong, and Xia (2010) had reported that plasma protein hydrolysates trended to have stronger radical-scavenging ability than non-hydrolysed plasma protein. The increase in hydrolysate level could enhance the antioxidation of protein hydrolysate, which was reported by Verma, Chatli, and Mehta (2018) as well.

Antioxidation is an important function in human health. The oxidation reaction in human body would damage cellular structures and functional molecules by producing free radicals and reactive oxygen species, leading to some diseases such as Parkinson, Alzheimer et al (Xue et al., 2019). Additionally, it also could cause the decrease of nutrient component in foods, the reduction of shelf life, and the production of some toxins (Girgih et al., 2015). While the artificially synthesized antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) would harm human health (Chi, Wang, Hu, 2015; Chi, Wang, Wang, 2015). Therefore, there is growing interests among researchers for natural and security antioxidant peptides from natural food sources (Liu, Ren, & Li, 2018). The effectiveness of peptide to act as antioxidant and the mechanism of activity are mainly determined by peptide structure, that is, the sequence and location of the amino acids (Sarmadi & Ismail, 2010).

However, some peptides showing antioxidant fail to exert effect after *in vitro* digestion (trypsin and pepsin) due to the influence of enzymes. Gallego, Mauri, Aristoy, Toldrá, and Mora (2020) had reported that gastrointestinal digestion could decrease DPPH radical scavenging activity and ferric-reducing antioxidant power of polypeptides from dry-cured ham. Hence, it is also a necessary evaluation method to determine the antioxidant capacity of samples after *in vitro* digestion.

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Nevertheless, most studies on porcine plasma hydrolysate antioxidant peptides were carried out by DPPH radical scavenging activity, ABTS methods, and hydroxyl radical scavenging experiments, which cannot characterize its effect in oxidative damage cells or after *in vitro* digestion. On the other hand, hydrogen peroxide (H_2O_2), as an oxidative stimulant of intra and extracellular, is stable and diffusible in many different cell membranes (Devi et al., 2017). Therefore, H_2O_2-induced oxidative stress model could be served as a potential tool for screening antioxidative compounds.

In this study, HPLC and LC-MS were used to isolate and identify antioxidant peptides from porcine plasma hydrolysates. The hydroxyl, ABTS, DPPH radical scavenging rates and iron chelating ability were used to represent the antioxidant properties of peptides. Additionally, after synthesis, *in vitro* digestion model was used to determine the antioxidant stability of peptide after digestion and oxidative damaged cell model induced by H_2O_2 was established to evaluate the cytoprotective effect of the peptide.

2. Material and methods

2.1. Materials and chemicals

Pig blood added 5% sodium citrate was obtained from Slaughterhouse in Ningbo and was quickly transported to the laboratory with ice pack. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-aAzino-bis (3-ethylbenzothiazo line-6-sulfonic acid) diammonium salt (ABTS), α-deoxyribose, H_2O_2, trypsin, pepsin were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). HepG2 cells were obtained from Boster Biological Technology Co., Ltd (Wuhan, China). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum, and penicillin–streptomycin were purchased from Shanghai Bridge Du Biological Technology Co., Ltd (Shanghai, China). Lipid peroxide malondialdehyde (MDA), reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) assay kits, Ferrozine and Human nuclear factor-erythroid 2 (Nrf2) kit were obtained from Shanghai Biyuntian Biotechnology Co., Ltd (Shanghai, China).

2.2. Methods

2.2.1. Enzymatic hydrolysis of pig plasma

The pig blood was centrifuged at 4500 rpm·min^{-1} for 20 min at 4 °C to separate plasma and blood cells. The plasma was adjusted to pH 8.0 after 95 °C water bath of 15 min. Then 0.2% alkaline protease was added to hydrolyze plasma at 55 °C for 7 h with samples collected at 1, 2, 3, 4, 5, 6, and 7 h respectively, with reaction immediately terminated by heating (100 °C, 10 min). After that, the samples were freeze-dried as polypeptide powder.

2.2.2. Determination of proteolysis degree

According to the method of Li, Wang, and Wang (2017) with some modifications, 1 mL enzymatic hydrolysis solution, 10 mL deionized water and 3 drops of phenolphthalein solution were mixed. The mixture was adjusted to pH 8.2 using 0.01 M NaOH, and the volume of NaOH was V1. Then 5 mL of neutral formaldehyde was added to it, using NaOH to adjust pH to 9.2 and recording the NaOH volume as V2. The enzymatic hydrolysis solution was replaced by deionized water as blank and the volume difference between two additions of NaOH is V0. The amino nitrogen and proteolysis degree are calculated according to formulas (1) and (2):

$$M1 = (V2 - V1 - V0) \times C_{NaOH} \times 14 \times 25/(1000 \times 2.5) \quad (1)$$

$$Degree\ of\ protein\ hydrolysis\ (DH,\ \%) = M1/M2 \times 100\% \quad (2)$$

where M1 is the amino nitrogen of enzymatic hydrolysis solution (g/g); M2 is the total nitrogen of the raw material (g/g).

2.2.3. Determination of molecular weight

The molecular weight of hydrolysates were determined by gel permeation chromatography (GPC). A sample of 0.30 g was added to 5 mL sample bottle, filling it to the mark by ultrapure water containing 0.1 M sodium nitrate. Then the mixture was tested after ultrasound dispersion. The standard sample was the narrow distribution polyethylene glycol provided by Polymer Standards Service-USA Inc., and the molecular weight was 330,000, 176,000, 82,500, 44,000, 25,300, 20,600, 12,600, 7130, 4290, 1400, 633, 430 Da respectively. The peak area of the standard samples with different contents were used as control, and the relative content of the sample was calculated by comparing the peak area of the sample with that of the standard sample, which represented by Mw.

2.2.4. Determination of structure

According to the methods of Li et al. (2020), the structural properties of proteins were analyzed using Fourier Transform Infrared Spectroscopy (FT/IR-4700, Jasco Corp., Tokyo, Japan). The dried protein was mixed with KBr powder in a ratio of 1:20. The FT/IR spectra were collected over the range of 500–4000 cm^{-1} with a resolution of 4 cm^{-1}.

2.2.5. Determination of antioxidation

2.2.5.1. Hydroxyl radical scavenging assay.

According to the method of You, Zhao, Regenstein, and Ren (2010) with some modifications, 0.2 mL sample was mixed with 0.1 mL 10 mM $FeSO_4 \cdot 7H_2O$, 0.1 mL 10 mM EDTA-Na_2, 0.5 mL 10 mM deoxyribose, 0.9 mL sodium phosphate buffer (pH 7.4). Then hydrogen peroxide (0.2 mL, 10 mM) was added to the mixture, incubating for 1 h at 37 °C. 1 mL 2.8% trichloroacetic acid (TCA) and 1.0 mL 1.0% thiobarbituric acid (TBA) were added to the mixture and boiled for 15 min. The absorbance was measured at 532 nm (A1). The sample was replaced by Na_3PO_4 (pH 7.4) as blank (A0). Hydroxyl radical scavenging assay (HRSA) was calculated as following.

$$HRSA\ (\%) = [(A0 - A1)/A0] \times 100\% \quad (3)$$

2.2.5.2. DPPH radical scavenging assay.

According to the method of Locatelli et al. (2009), DPPH solution was prepared using ethanol as solvent. Then, 1, 2, 4, 6, 8 mg/ml polypeptide solutions and glutathione solutions were prepared, respectively. The liquid in the centrifuge tube was shaken sharply and incubated in dark environment for 30 min. The absorbance was measured at 517 nm, and the scavenging rate was calculated by DPPH radical scavenging assay (DPPH RSA) formula.

$$DPPH\ RSA\ (\%) = \left[1 - \left(OD_{Sample} - - OD_{Control}\right)/(OD_{Blank} - OD_{Control})\right] \times 100\% \quad (4)$$

2.2.5.3. ABTS radical scavenging assay.

The ABTS radical scavenging assay (ABTS RSA) was determined according to the method of Li, Wang, Li, Li, and Wang (2008). ABTS solution of 7.4 mM and potassium persulfate solution of 2.6 mM were prepared, and 88 mL potassium persulfate solution was added to 5 mL ABTS solution to react for 12–16 h, making ABTS working solution. The working solution was diluted with PBS buffer until the absorbance was between 0.7 ± 0.02. Then the 200 μL diluted working solution was mixed with 10 μL sample and the absorbance was measured immediately after standing for 6 min in the dark.

$$ABTS\ RSA\ (\%) = \left(OD_{Working\ fluid} - - OD_{Sample}\right)/OD_{Working\ fluid} \times 100\% \quad (5)$$

2.2.5.4. Determination of iron chelating ability.

According to the method of Zhu (2015), 1 mL sample solution was mixed with 0.05 mL 2 mM $FeCl_2$ and 0.2 mL 5 mM Ferrozine solution. The mixture was allowed to

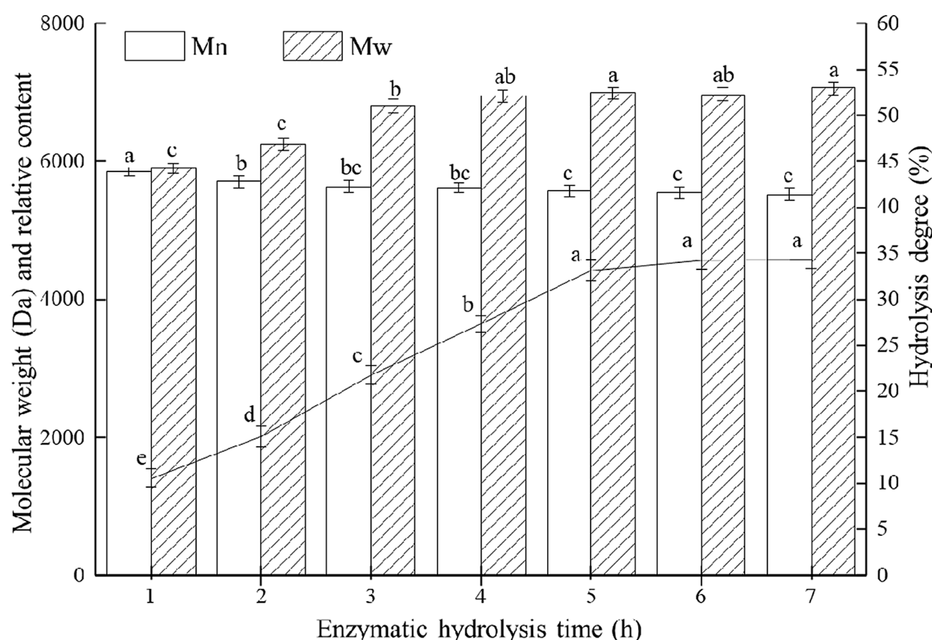

Fig. 1. Hydrolysis degree and molecular weight of different enzymatic hydrolysis time (%). Note: Mn and Mw represented average molecular weight and relative content respectively; Different letters represent significant difference (P < 0.05).

stand for 10 min at room temperature (25 °C) after shaking. The solution absorbance value was measured at 562 nm. The control group replaced the sample with deionized water sample. The inhibition of Ferrozine-Fe^{2+} complex was used to represent iron chelating ability and calculated by the formula:

$$\text{Iron chelating rate } (\%) = \left[\left(A_{Control} - A_{Sample}\right)/A_{Control}\right] \times 100\% \quad (6)$$

2.2.6. Purification and identification of hydrolysates

The hydrolysate solution was firstly separated by G-15 gel chromatography. The peptide solution was divided into multiple components according to the chromatographic peaks. The collected components were selected to purify again by HPLC through determining its antioxidant function. The obtained components which had the best antioxidant property, was identified by LC-MS/MS. The results of peptide sequences with strong signals and reliable protein sources were selected to synthesis in Sangong Biotech Co., Ltd (Shanghai, China). The purity of the synthetic peptides was higher than 98%.

2.2.7. In vitro digestion

According to the method of Li et al. (2020) with some modifications. 2 g of pepsin (15 U/mg) was mixed with 50 mL of 0.1 M HCl and 0.5 g of trypsin (0.25 U/mg) was mixed with 50 mL of PBS (pH 7.0). 1 mg synthetic peptide was mixed with 1 mL deionized water and the pH was adjusted to 2.0 by 0.1 M HCl, followed by adding 1 mL pepsin solution to the mixture, incubating at 37 °C for 1 h. Then the pH of reaction solution was immediately adjusted to 7.0 followed by adding 0.3 mL of trypsin solution and incubated at 37 °C for 2 h to begin the second digestion process and terminated by heating at 100 °C for 10 min. The antioxidant property was determined by the method depicted in Section 2.2.4.

2.2.8. Oxidative damage HepG2 cells model induced by H_2O_2

2.2.8.1. Establishment of oxidative damage cells model. The HepG2 cells were cultured in DMDM supplemented with 10% (v/v) fetal bovine serum, 4 mmol L-1 L-glutamine, 1% (v/v) penicillin/streptomycin at 37 °C/95% humidified air/5% CO_2. The cells were passaged every 4 d, with the passage ratio of 1:3 when the cells grown to more than 80% confluence (afterwards, medium could be replaced every 1–2 d according to cell growth). The cells viability containing different H_2O_2 (100 mM, 400 mM, 700 mM, 1,000 mM, 1300 mM) in the DMEM medium, were determined by CCK-8 Kit after 4 h incubation to ensure its half inhibitory concentration (IC_{50}) of H_2O_2.

2.2.8.2. Determination of the content of ROS. The cells growing with the DMEM was control groups. The DMEM containing H_2O_2 (IC_{50}) were set as model group. The H_2O_2 (IC_{50}) and different peptides (0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL) mixing in the DMEM were set as peptide groups. After incubation of 4 h, the ROS content of cells were detected according to ROS assay kits.

2.2.8.3. Determination of the content of antioxidase. The SOD, CAT, GSH-Px of control group, model group and peptide group were detected according to different assay kits.

2.2.8.4. Determination of the content of MDA. The MDA of control group, model group and peptide group were detected according to MDA assay kits.

2.2.8.5. Determination of the content of Nrf2. The Nrf2 of control group, model group and peptide group were detected according to Nrf2 assay kits.

2.2.9. Statistical analysis

All tests were performed in triplicates and the data were presented as mean ± standard deviation (mean ± SD). Statistical analysis was performed using one-way ANOVA, followed by Duncan multiple comparisons, using SAS8.1 (SAS Campus Drive, Cary, NC USA). Differences with $P < 0.05$ were considered to be statistically significant.

3. Results and discussions

3.1. Optimization of enzymatic hydrolysis time

Enzymatic hydrolysis would destroy the structure of protein, releasing some active amino acid residues which enhanced its functions. Moreover, different enzymatic hydrolysis changed the hydrolysis degree of samples, affecting the length and contents of small molecule peptides

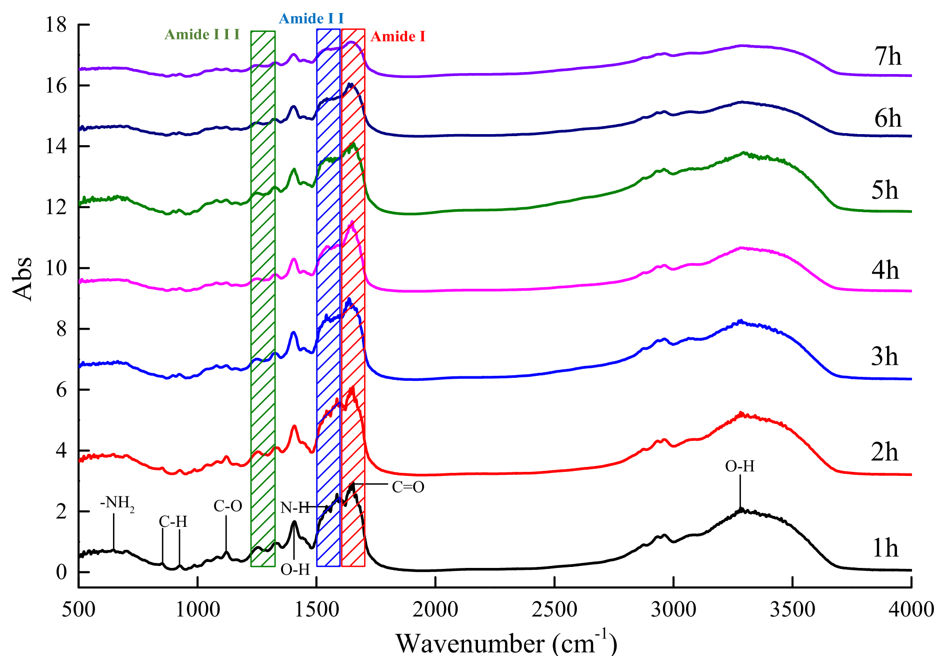

Fig. 2. FT/IR spectrum of different enzymatic hydrolysis time.

Table 1
The antioxidant properties of porcine plasma hydrolysates.

Content (mg/mL)	Hydroxyl radical (%)		ABTS free radical (%)		DPPH free radical (%)	
	Glutathione	hydrolysates	Glutathione	hydrolysates	Glutathione	hydrolysates
0.10	16.88 ± 0.98^{d}	42.82 ± 0.81^{e}	42.12 ± 1.11^{d}	63.25 ± 1.23^{c}	10.12 ± 1.32^{d}	81.43 ± 1.26^{c}
0.25	22.37 ± 1.03^{c}	44.19 ± 1.11^{d}	66.23 ± 1.65^{c}	82.11 ± 1.18^{b}	13.84 ± 1.21^{c}	85.22 ± 1.14^{b}
0.50	26.23 ± 0.72^{b}	48.94 ± 1.41^{c}	79.23 ± 1.07^{b}	83.25 ± 1.24^{b}	21.14 ± 1.33^{b}	86.77 ± 1.10^{ab}
0.75	28.23 ± 1.11^{a}	51.33 ± 1.27^{b}	80.96 ± 0.88^{ab}	85.69 ± 1.07^{a}	24.28 ± 1.25^{a}	87.28 ± 0.99^{ab}
1.00	29.51 ± 1.07^{a}	52.07 ± 1.35^{a}	81.22 ± 0.81^{a}	86.12 ± 1.18^{a}	25.89 ± 1.19^{a}	88.11 ± 1.06^{a}

Note: Different letters represent significant differences ($P < 0.05$).

with special groups such as C-O, O-H groups (Fig. 2). Increased hydrolysis degree would break peptide band and shorten peptide chain length, leading to the reduction of molecular weight distribution and the increase of free amino acid content, thereby influencing its functions, especially in antioxidant property (Shahi, Sayyed-Alangi, & Najafian, 2020; You, Zhao, Cui, Zhao, & Yang, 2009). To better purify antioxidant peptides with low molecular weight, low cost, high activity, the enzymolysis time should ensure to produce high hydrolysis degree and more small peptides. As shown in Fig. 1, the hydrolysis degree of hydrolysates increased with the enzymatic hydrolysis time increasing and had no significant differences after enzymatic hydrolysis for 5 h. Moreover, more small peptides that less of 8 kDa could be produced, as the enzymolysis time increased until it reached 4 h. Though hydrolysis could form peptide by destroying the protein structure, especially in secondary structure, which were shown in Fig. 2 that the peaks of amide I, II, III representing protein secondary structure changed significantly. While

Table 2
The antioxidant properties of peptides.

		Hydroxyl radical scavenging rate (%)	ABTS free radical scavenging rate (%)	DPPH free radical scavenging rate (%)	Iron chelating rate (%)
Glutathione (1 mg/ml)		29.32 ± 1.12^{h}	81.06 ± 0.93^{c}	25.43 ± 1.17^{f}	17.82 ± 1.57^{g}
Peptides after chromatography (1 mg/ml)	P1	35.19 ± 1.11^{g}	89.77 ± 1.26^{a}	81.52 ± 0.96^{a}	48.93 ± 1.88^{a}
	P2	26.23 ± 1.19^{i}	69.16 ± 1.37^{f}	75.74 ± 1.24^{b}	44.53 ± 1.66^{b}
	P3	21.87 ± 1.07^{j}	80.54 ± 1.14^{c}	71.36 ± 1.08^{c}	41.96 ± 1.47^{b}
Peptides after HPLC (1 mg/ml)	P1-1	56.25 ± 1.28^{c}	45.18 ± 0.82^{h}	16.21 ± 1.33^{h}	33.64 ± 1.33^{de}
	P1-2	49.41 ± 1.44^{c}	61.39 ± 1.15^{g}	11.88 ± 1.25^{i}	30.22 ± 1.55^{ef}
	P1-3	47.86 ± 1.22^{e}	78.42 ± 1.07^{d}	11.76 ± 1.37^{i}	31.92 ± 1.41^{e}
	P1-4	53.41 ± 1.37^{d}	74.22 ± 1.24^{e}	10.68 ± 0.89^{i}	28.32 ± 1.22^{f}
	P1-5	47.33 ± 1.02^{e}	72.66 ± 1.18^{e}	14.26 ± 1.05^{h}	27.35 ± 1.49^{f}
	P1-6	55.72 ± 0.82^{c}	45.83 ± 0.99^{h}	22.19 ± 1.17^{g}	32.27 ± 1.37^{e}
	P1-7	60.44 ± 1.34^{b}	36.45 ± 1.03^{i}	23.11 ± 1.26^{fg}	35.42 ± 1.57^{d}
	P1-8	59.07 ± 0.99^{b}	83.29 ± 1.12^{b}	31.34 ± 1.33^{e}	38.42 ± 1.29^{c}
	P1-9	65.29 ± 1.27^{a}	88.71 ± 1.08^{a}	37.59 ± 0.98^{d}	42.55 ± 1.61^{b}
	P1-10	42.09 ± 1.88^{f}	80.51 ± 0.91^{c}	24.62 ± 1.07^{f}	36.58 ± 1.97^{cd}

Note: Different letters represent significant differences ($P < 0.05$).

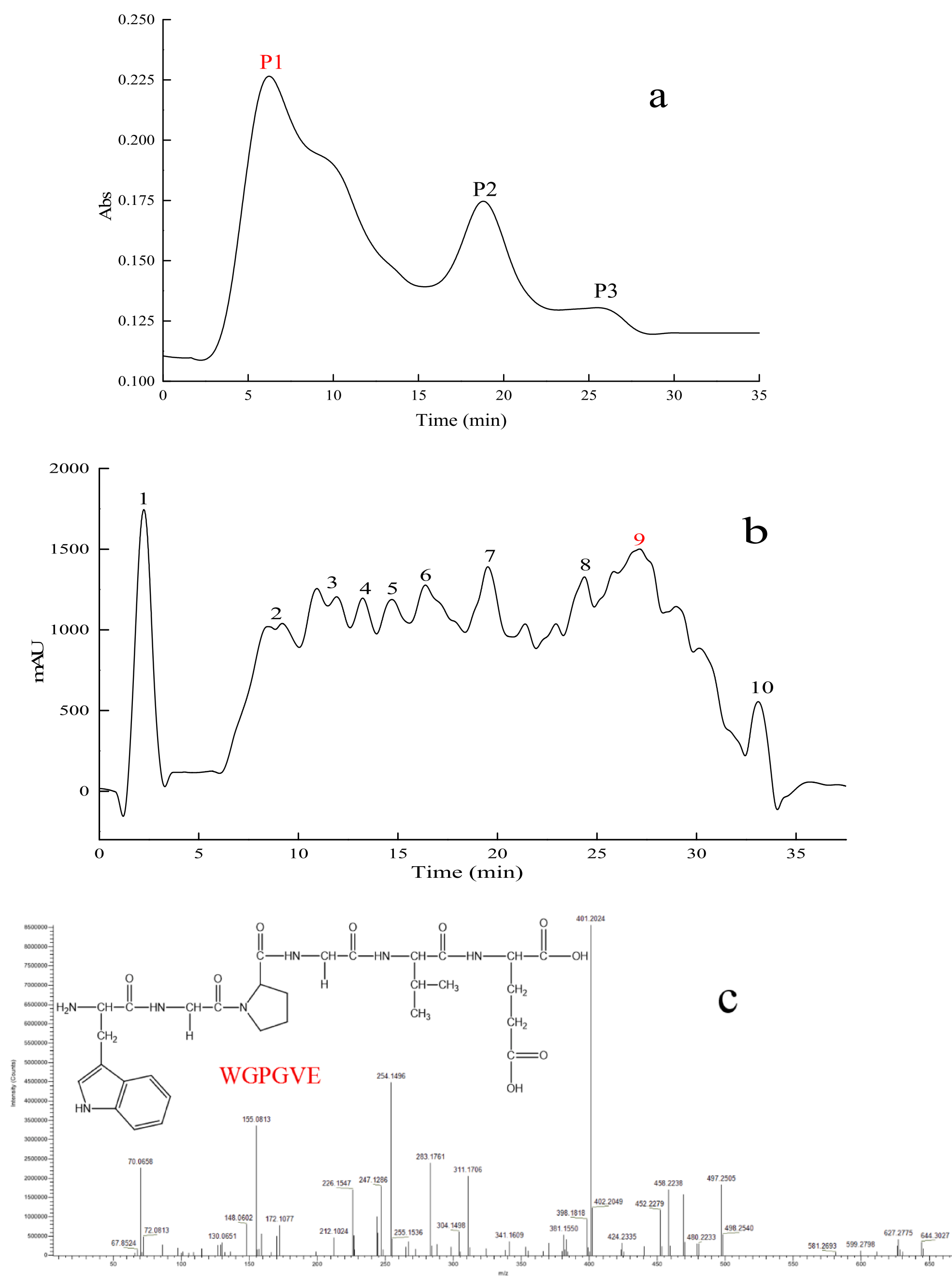

Fig. 3. Chromatography, HPLC and LC-MS/MS of sample.

the peaks which represented functional groups O-H (3300 cm^{-1}, 1406 cm^{-1}), C-O (1128 cm^{-1}), $-NH_2$ (650 cm^{-1}) still existed after hydrolysis (Fig. 2), indicating that hydrolysis did not eliminate these active groups (Li et al., 2020). Moreover, hydrolysis also did not eliminate the absorption peak of 840–920 cm^{-1} representing C-H substituent group in benzene ring, meaning that the hydrolysate had some amino acids with these functional groups. Therefore, the hydrolysate of 5 h enzymolysis was used to separate antioxidant peptide.

3.2. Antioxidant properties of hydrolysate

Results in Table 1 shown the hydrolysates with different contents all could better scavenge hydroxyl radical, ABTS radical, DPPH radical compared with glutathione. The hydroxyl radical, ABTS radical, DPPH radical scavenging rates of samples were 1.76–2.54, 1.06–1.50, 3.40–8.05 times of that of glutathione, respectively. Moreover, the antioxidant effect was the best (HRSA 52.07%, ABTS RSA 86.12%, DPPH RSA 88.11%) when the hydrolysate content reached 1.00 mg/mL, especially in DPPH radical scavenging, suggesting the hydrolysate from porcine plasma had excellent antioxidation. Hydrolysis could reserve certain O-H, C-H groups (Fig. 2), decreasing the steric hindrance caused by the two benzene rings of DPPH radical, providing H^+ to react with hydroxyl radicals and supplying electrons to reduce DPPH radical and $ABTS^+$ (Hu, Song, Mao, Wang, & Zhao, 2016; Locatelli et al., 2009). Additionally, hydrophobic amino acid produced by hydrolysis could also influence its antioxidant properties (Shazly et al., 2017). To further determine the functional peptides, chromatography, HPLC and LC-MS/MS were used to isolate and identify the specific functional fragments.

3.3. Isolation and identification of antioxidant peptide WGPGVE

According to the chromatography results (Table 2 and Fig. 3a), P1

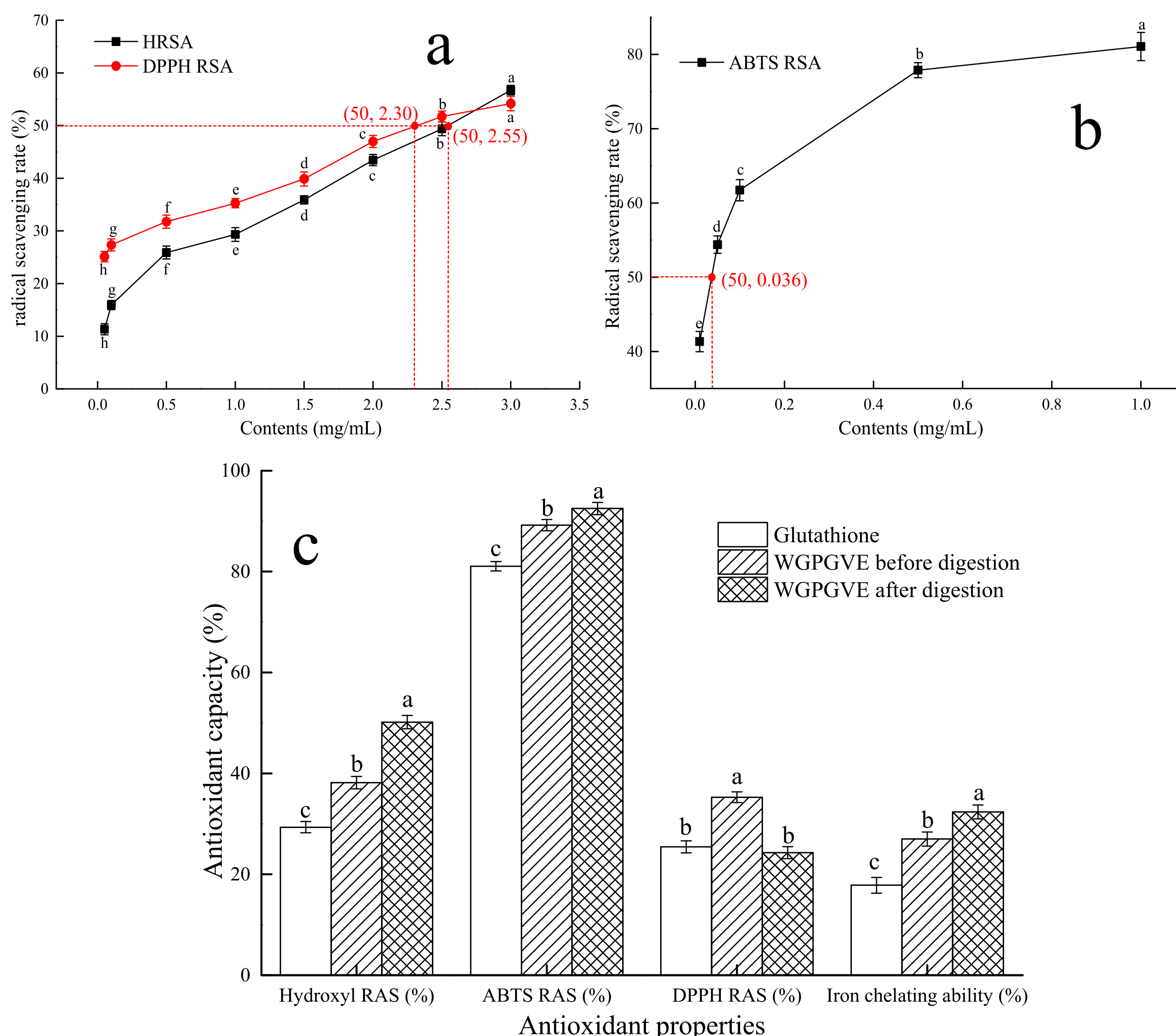

Fig. 4. Antioxidant properties of WGPGVE before and after digestion. Note: Different letters represented significant differences ($P < 0.05$); C was the antioxidant properties of samples (1 mg/mL).

fragment had best antioxidant properties among the 3 fragments of P1-P3. Compared to glutathione, HRSA, ABTS RSA, DPPH RSA and iron chelating rate of P1 improved by 20.02%, 10.75%, 220.57% and 174.57% respectively, while HRSA and ABTS RSA of other two fragments were both lower than glutathione. Therefore, based on the results, fragment P1 was considered to be further isolated by HPLC to ensure the antioxidant component. From P1 fragment, 10 different components (P1-1 to P1-10) were obtained, according to the results of Table 2 and Fig. 3b. Through the determination of its antioxidant properties (HRSA, ABTS RSA, DPPH RSA, iron chelating rate), the P1-8 and P1-9 components both displayed more significant antioxidant properties (more than 60%, 80%, 30%, 38%) than others (less than 60%, 80%, 30%, 38%). Moreover, the antioxidant properties of the two components both exceeded those of glutathione (29.32%, 81.06%, 25.43%, 17.82%). While those of P1-9 component were 6.22, 5.42, 6.25 and 4.13 higher than those of P1-8 component respectively, indicating that P1-9 component had the best antioxidant properties. Therefore, P1-9 was identified to be more suitable to explore the relationship between structure and antioxidant properties.

LC-MS/MS can make samples ionize to generate band gap ions with different charge-to-mass ratios, forming an ion beam through the action of accelerating electric field and entering the mass analyzer to form the mass spectrum in the electric field and the magnetic field (Shazly et al., 2017). The component P1-9 was analyzed by LC-MS/MS to identify its amino acid sequences and the antioxidant properties of synthetic peptide with identified sequence were determined. As shown in Fig. 4a and b, the EC_{50} value of HRSA, ABTS RSA, DPPH RSA of synthetic peptide trp-gly-pro-gly-val-glu (WGPGVE) contents were 2.550 mg/mL, 0.036 mg/mL, 2.300 mg/mL respectively. Moreover, HRSA (38.16%), ABTS RSA (89.21%), DPPH RSA (35.27%), iron chelating rate (26.98%) of WGPGVE were all more than those of glutathione (29.32%, 81.06%, 25.43%, 17.82%) at the content of 1 mg/mL (Fig. 4c). These meant WGPGVE as a hexapeptide had better antioxidant properties. Chen, Muramoto, Yamauchi, and Nokihara (1996) had reported that the antioxidant peptides containing only 5 to 16 amino acid residues (about 450 to 1360 Da) were more likely to react with free radicals to act as an antioxidant. Antioxidant peptides mainly worked to scavenge radicals through two mechanisms: hydrogen atom transfer (HAT) and single electron transfer (SET), which provided hydrogen atom and electron to reduce radicals respectively, inhibiting the oxidant reaction (Huang, Ou, & Prior, 2005). Chelating metal ions was another important method to play the role of antioxidant capability. Peptides could chelate with metal ions to maintain the valence state of metal ions by supplying hydrogen, preventing them from generating hydroxide ions through redox reactions (Wen, Zhang, Zhang, Duan, & Ma, 2020; Zhu, 2015)). Hydrophobic amino acid glycine (G) and valine (V) were identified in the synthetic peptide (Fig. 3c), providing H^+ and enhancing synergy with other amino acids, playing the role of antioxidant properties (Chen et al.,

1996). Wen et al. (2020) had reported that peptides with hydrophobic residues could provide H^+ to exert antioxidant properties when they researched antioxidant peptides derived from plant protein. Zhang, Huang, and Jiang (2014) also identified antioxidant peptides with the sequences CATTA consisted of hydrophobic residues alanine (A). Moreover, V as a polar amino acid could interact with non-polar amino acids and its side chain could inhibit the oxidation reaction of radicals through chelation (Rajapakse, Mendis, Jung, Je, & Kim, 2005). Saiga, Tanabe, and Nishimura (2003) also had reported that antioxidant peptides with polar amino acid obtained from porcine myofibrillar hydrolysate could exert good radical scavenging ability. Additionally, the imidazole ring on aromatic amino acids tryptophan (W) at the N-terminus and proline (P) could donate hydrogen, trap radicals, interact with other amino acids to improve hydrophobicity and enhance antioxidant capacity (Chen et al., 1996). As an acidic amino acid, glutamine (E) had carboxyl, which could chelate metal ions to form complexes and enhance antioxidation by preventing the oxidation–reduction reaction of metal ions (Wen et al., 2020). Je, Qian, and Kim (2007) reported that the antioxidant properties of peptides containing acidic amino acids were related to the side chain carboxyl chelating metal ions. These antioxidant amino acids had appeared in the sequences of many reported antioxidant peptides, such as Pro (P) -Arg derived from Salmon milt (Wang, Zhu, Han, & Wang, 2008), Gly (G) -Gly-Glu (E) derived from *Sardinalla aurita* (Bougatef et al., 2010), Trp (W)-Glu-Gly-Pro-Lys derived from bluefin leatherjacket (Chi, Wang, Hu, 2015; Chi, Wang, Wang, 2015), Pro-Met-Asp-Tyr-Met-Val (V)-Thr derived from Thunnus tonggol (Hsu, 2010), thereby WGPGVE could exert good antioxidant effect due to the combination of the amino acids.

3.4. Antioxidant stability of WGPGVE after in vitro digestion

Synthetic peptide WGPGVE still had antioxidant effect after *in vitro* digestion, and its HRSA, ABTS RSA, iron chelating rate also increased from 38.16% to 50.15%, 89.21% to 92.49%, and 26.98% to 32.35% respectively, compared to undigested peptides (Fig. 4), indicating that the antioxidant capacity of peptide WGPGVE was stable in the digestive environment of gastrointestinal tract. Xiao et al. (2020) had reported the similar conclusion when they studied the effect of *in vitro* digestion on antioxidant peptide from chicken breast muscle. Zhu (2015) also had reported that the digested peptides from Jinhua ham had better iron chelating ability than undigested peptides. Pepsin mainly influenced the peptide bond composed of aromatic amino acids or acidic amino acids, and the main part of trypsin action was on the peptide bonds composed of arginine (R) and lysine (K) carboxyl groups (Wen et al., 2015). Peptide WGPGVE had acidic amino acid glutamine (E) with carboxyl, which supplied pepsin site of action, producing smaller peptide and exposing residues. Tryptophan (W) with imidazole ring in WGPGVE could combine with remaining or novel amino acids, increasing the antioxidant ability. Moreover, the exposure of neutral and acidic amino acid side chains, such as amino-groups and carboxyl groups, could better chelate with metal ions to enhance iron chelating ability (Fig. 4), passivating the oxidation of metal ions and weakening radicals chain reactions (Je et al., 2007). Moreover, tryptophan with imidazole ring could better provide H^+ to reduce hydroxyl radical and ABTS radical when it was released by gastrointestinal digestion. Zheng, Zhao, Dong, Su, and Zhao (2016) also found that peptides containing tryptophan showed strong hydroxyl radical and ABTS radical scavenging capabilities. However, the decrease of DPPH RSA might cause by the partial degradation of antioxidant peptides diluted the active sequence donating electrons, weakening DPPH radical scavenging ability (Xiao et al., 2020).

3.5. Antioxidant properties of WGPGVE in HepG2 cells

HepG2 cell isolated from human hepatoma is a highly differentiated, virus-free cell, which has similar metabolism and biotransformation activity to human primary hepatocytes. HepG2 cells can be damaged by oxidative damage inducers, while this damage also can be repaired by suitable antioxidants (Javitt, 1990). H_2O_2 penetrates cell membranes very easily and has strong oxidizing. It can react with Fe^{3+} of cells through Fenton to form highly active radicals, stimulating cells to produce oxidative stress and attacking unsaturated fatty acids in phospholipid membranes to trigger lipid peroxidation chain reaction (Li, Ma, & Wang, 2015). Therefore, oxidative damage cell model was induced by H_2O_2 in this study. With the increase of H_2O_2 content, the cell viability of HepG2 cells gradually decreased and gradually was close to the half inhibitory concentration (50%) when the H_2O_2 content reached 800 mM (Fig. 5a). Moreover, in the verification experiment, cell viability was 50.15% when the H_2O_2 content was 800 mM, thereby the oxidative damage model could be established by adding 800 mM H_2O_2.

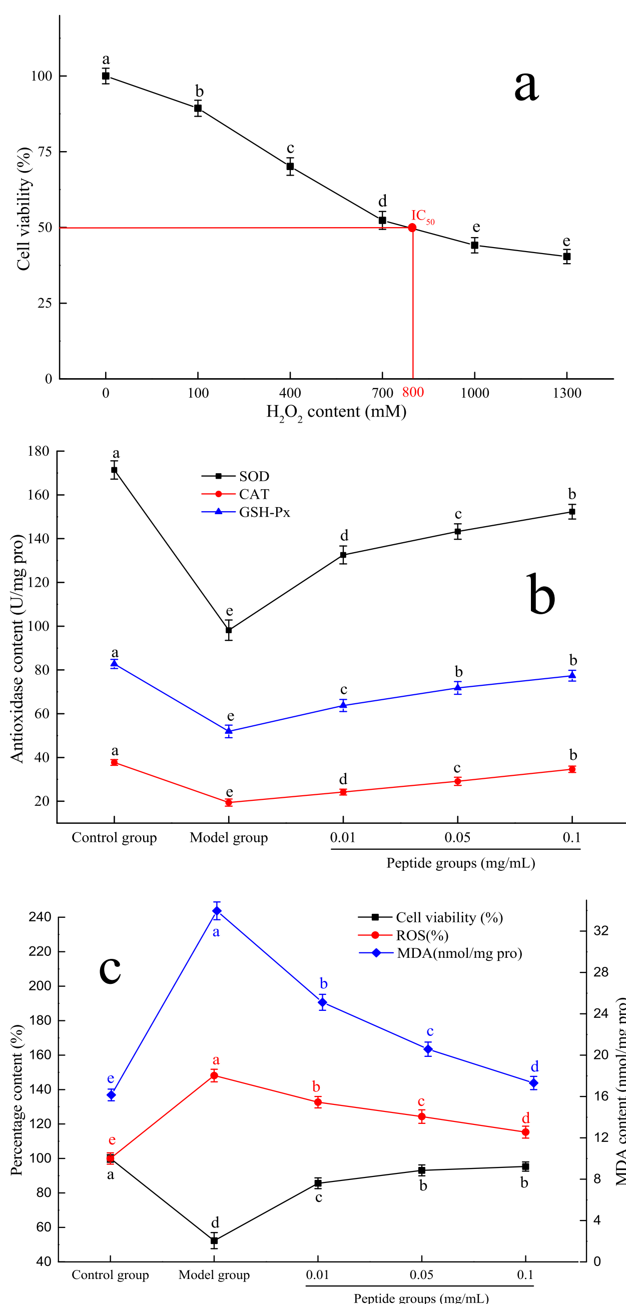

Fig. 5. Antioxidant properties of WGPGVE in H_2O_2 induced oxidative damage cells model. Note: Different letters represent significant differences ($P < 0.05$).

Antioxidases (SOD, CAT, GSH-Px), ROS and MDA were the

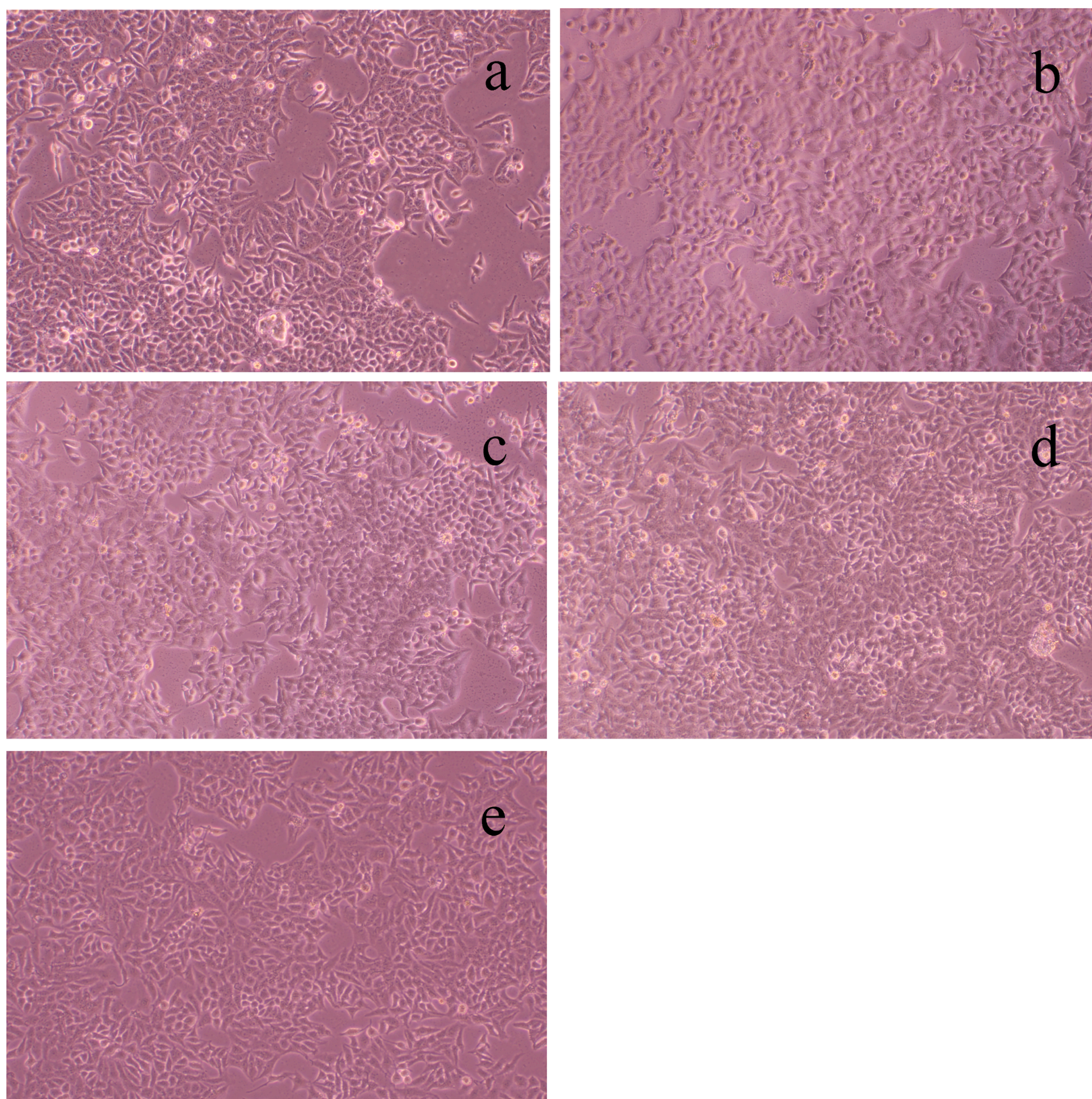

Fig. 6. HepG2 cells in different groups. Note: a, b, c, d, e represented control group, model group, peptide group (0.01 mg/mL), peptide group (0.05 mg/mL), peptide group (0.1 mg/mL) respectively.

substances most closely related to antioxidant properties of HepG2 cells. SOD was the first line of defense for cells against oxygen free radicals, which could keep cells from oxidative damage and prevent lipid peroxidation by scavenging radicals (Bafana, Dutt, Kumar, Kumar, & Ahuja, 2011). GSH-Px was based on GSH, which specifically catalyzed the reduction reaction of GSH to H_2O_2, and oxidized GSH to oxidized glutathione (GSSG), protecting the integrity of cell membrane structure and function (Zhao, 2018). CAT was an important enzyme system for removing hydrogen peroxide in the body, combining with mitochondria and peroxidase in the cell. It could quickly remove the toxic substance H_2O_2 produced by cell metabolism, thereby co-protecting the role of sulfhydrylase, membrane protein and detoxification with GSH-Px (Cimen, 2008). MDA generated by the formation of lipid peroxidation due to the attack of ROS to polyunsaturated fatty acid in the biofilm and the higher the MDA content, the more serious the cell oxidative damage (Zhao, 2018). Under the H_2O_2 induced (800 mM) oxidative damage cells model, the same trends appeared in the results of the content of SOD, CAT and GSH-Px. They all increased when different peptide contents were added to model group containing 800 mM H_2O_2 (Fig. 5b). Though the antioxidase contents of peptide groups were much lower than those of the control group, they were rather higher than those of the model group. The cell viability also had the same trends with antioxidases, while ROS and MDA were opposite of them (Fig. 5c). These results indicated that peptide WGPGVE could protect HepG2 cells to resist the oxidative damage induced by H_2O_2. The results of Fig. 6 also shown the same conclusion. H_2O_2 could destroy the cell morphology through oxidative stress after H_2O_2 passed through the cell membrane, which could be seen from Fig. 6b, compared with the control group (Fig. 6a). Different contents of WGPGVE could weaken the damaging effect of H_2O_2 on cell morphology from Fig. 6c-e that the cell morphology treated by H_2O_2 gradually approached the normal morphology with the increase of WGPGVE content. These results shown that WGPGVE had cytoprotective effect on H_2O_2 induced HepG2 cells.

The increase of ROS caused by H_2O_2 could affect the Keap1-Nuclear factor-erythroid 2-related factor 2-Antioxidant response element (Keap1-Nrf2-ARE) signaling pathway, weakening the antioxidative

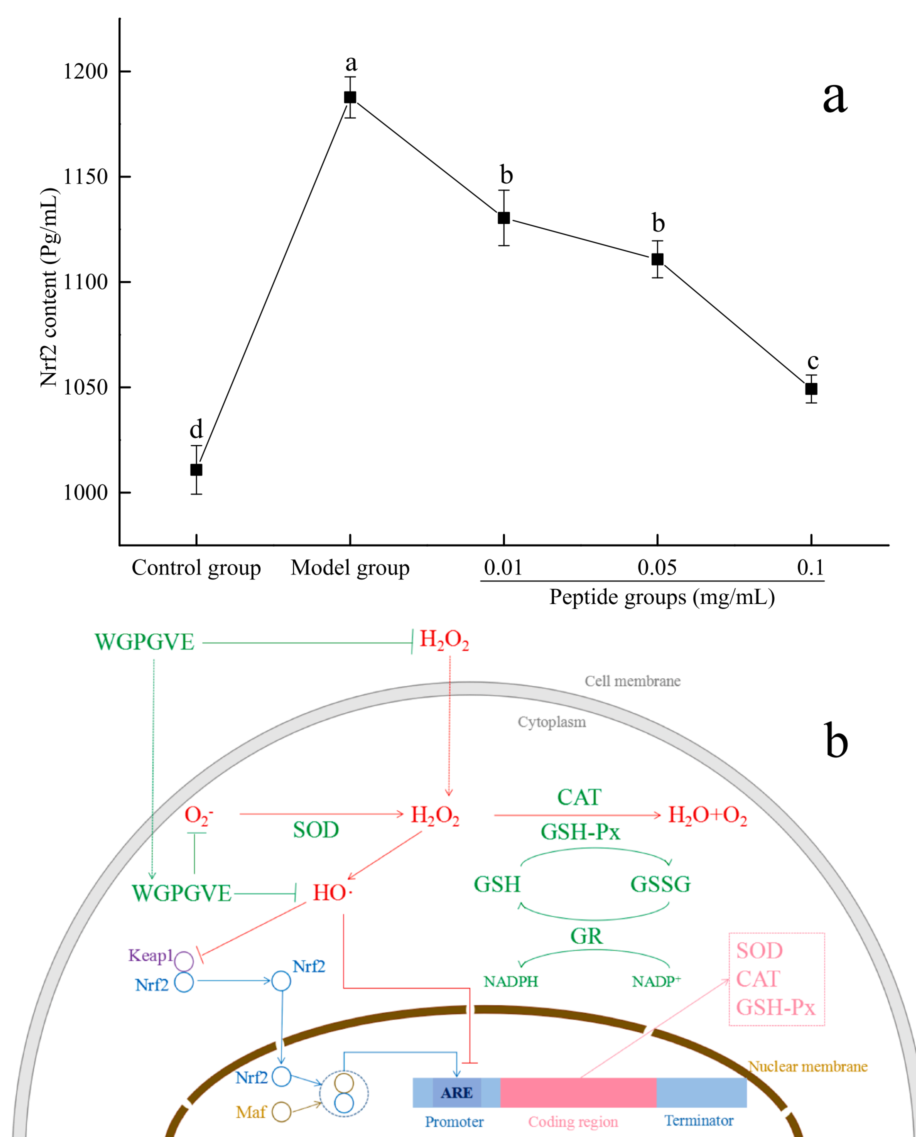

Fig. 7. Nrf2 contents and schematic diagram of ROS scavenging and antioxidase regulating effect of WGPGVE in oxidized HepG2 cells induced by H_2O_2. Note: Different letters represent significant differences (P < 0.05).

defense ability in HepG2 cells, leading to cell oxidative damage and apoptosis (Ho, Cheng, & Chau, 2012). Nrf2 is an important redox sensitive transcription factor, including Neh1 to Neh6, among which Neh2 has a site that can bind to Keap1. Nehl can combine with ARE by recognizing the base sequence (GCTGAGTCA) on ARE through forming a heterodimer with the small molecule protein Maf. ARE called electrophilic reaction element, is a *cis*-reactive element that can encode many detoxification enzymes and cell protection protein genes in the promoter region. The combination between Nrf2 and ARE can start the transcription of target genes to express some antioxidases to improve the ability of cells to resist exogenous stimuli. Keapl is a polypeptide containing 624 amino acids and its N-terminus can interact with the Neh2, negatively regulating the function of Nrf2 (Tkachev, Menshchikova, & Zenkov, 2011). Nrf2 binds and interacts closely with Keap1 in the cytoplasm at normal physiological conditions, maintaining free Nrf2 at a low level. While when the cells are attacked by ROS or electrophiles, the combination of Nrf2 and Keap1 dissociates to produce free Nrf2, which quickly translocates into the nucleus through the nuclear pore, and combines with Maf to form a heterodimer, recognizing the site on ARE to bind to it, transcribing and activating antioxidase gene regulated by Nrf2 (Ho et al., 2012). The Keap1-Nrf2-ARE pathway was known to play an important role in HepG2 cells antioxidant effect as reported by Jung and Kwak (2010), Stepkowski and Kruszewski (2011) and Heiss, Daniel, and Kristin (2013). Though H_2O_2 could produce O_2^- and HO^- which could stimulate the release of Nrf2 from complex to enter nuclear, increasing Nrf2 content (Fig. 7a) to promote the expression of antioxidases. While the excessive ROS also could consume antioxidase and damage DNA fragments, affecting the expression of antioxidase (Stepkowski & Kruszewski, 2011), reducing the level of antioxidase in cells (Fig. 5b), leading to the cell death. However, peptide WGPGVE weakened the oxidative stress and decreased work intensity of signal pathway, leading to that the Nrf2 and antioxidase content gradually returned to normal levels from Fig. 7a and 5b respectively. These mainly caused by that WGPGVE might chelate with substance of cells such as metal ions with transport function and metal ion component of enzymes to form a barrier, protecting the activity of Nrf2, Keap1 and preventing transcription genes from being destroyed by ROS. Moreover, the peptide also could provide H^+ to maintain the original valence of metal elements, prevent metal ions from catalyzing the oxidation of lipids and inhibit the oxidation–reduction reaction of metal ions to produce OH^- to enhance the oxidative stress (Wen et al., 2020). Furthermore, WGPGVE as a hydrogen donor could react with ROS to decline the level of ROS,

reducing oxidative stress signal, preventing impaired gene expression and improving the level of antioxidases (SOD, CAT, GSH-Px) in Fig. 7b. Additionally, the increase of antioxidases could better help HepG2 cells to resist ROS. SOD could convert O_2^- to H_2O_2 and CAT could promote the decomposition of H_2O_2 into H_2O and O_2, inhibiting the production of MDA through the reaction between H_2O_2 and lipid, enhancing the antioxidant defense ability of cells (Li et al., 2015; Wang et al., 2016). Zhao (2018) also had reported antioxidant peptides from bass muscle could exert the effect of antioxidation by decreasing ROS, MDA and increasing SOD, CAT, GSH-Px. Based on the results and its characteristics, the peptide WGPGVE from porcine plasma had stable antioxidation and could be used in the development of functional foods.

4. Conclusion

The component P1-9 of P1 with the best antioxidation was isolated from porcine plasma hydrolysate with 5 h hydrolysis. It showed better antioxidant properties for scavenging hydroxyl, ABTS, DPPH radicals and iron chelating ability. Moreover, the antioxidant peptide WGPGVE was further identified from P1-9 by LC-MS/MS, which could better scavenge hydroxyl radical (38.16%), ABTS radical (89.21%), DPPH radical (35.27%), iron chelating rate (26.98%) compared to glutathione (29.32%, 81.06%, 25.43%, 17.82%) due to some specific amino acids. Hydrophobic amino acids G and V in WGPGVE could provide H^+ and enhance synergy with other amino acids. Aromatic amino acids W also could provide H^+ and keep function stable by resonance. Acidic amino acid E could chelate with metal ions to decrease the redox of metal. Additionally, antioxidant properties (50.15%, 92.49%, 24.29%, 32.35%) of WGPGVE also were shown after *in vitro* digestion, indicating that the peptide had high stability of antioxidation. Furthermore, WGPGVE also could reduce ROS to enhance the effect of Keap1-Nrf2-ARE signaling pathway, promoting the expression of antioxidases to increase the content of SOD, CAT, GSH-Px, decreasing H_2O_2 content to protect HepG2 cells against oxidative damage induced by H_2O_2. The obtained novel antioxidant peptide had stable antioxidant capability and showed great potential in formulating functional foods as a natural antioxidant.

Ethical statement

We declare that this research does not involve animal or human experiments.

CRediT authorship contribution statement

Gaoshang Li: Writing – original draft. **Junqi Zhan:** Data curation, Methodology. **Lingping Hu:** Software. **Chunhong Yuan:** Writing – review & editing. **Koichi Takaki:** Supervision. **Xiaoguo Ying:** Supervision. **Yaqin Hu:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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