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ORIGINAL PAPER

Protective effects against H_2O_2-induced oxidative damage in lung fibroblast cell by peptide isolated from plasma albumin hydrolysate

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Abstract Antioxidant peptides obtained from alcalase-hydrolyzed porcine plasma albumin (AHA, MW <3 kD) were purified by consecutive chromatographic methods, and the antioxidative effects of unpurified AHA and sub-fractions from the first step of HPLC (P4, MW <1.5 kD) and second step of HPLC (P4b, MW <1.5 kD) were evaluated in cell line. P4 exhibits the highest reducing power (0.89) than its further purified fraction P4b (0.69) and unpurified AHA (0.5). Moreover, a concentration of 100 mg/ml of P4 also demonstrates better protective effect on H_2O_2-induced oxidative damage and antioxidative enzyme activities (SOD, CAT and GPx) in fibroblast cell than AHA and P4b ($P < 0.05$). P4 is composed of seven peptides with MW of 500.19, 524.24, 550.23, 568.19, 656.18, 707.44 and 1,022.70, and their amino acids sequences are identified as LIKQ, LQHK, EQKF, PDIPK, KVPQVS, FKDLGE and EHLREKVL, respectively. All the seven peptides contain lysine, indicating that P4 is lysine-rich peptide fraction. We believe these results, with a more pronounced action of P4 than AHA and P4b plus the characterized amino sequences, would lay the foundation of understanding the antioxidative effect of albumin-derived peptides.

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Keywords Antioxidative enzyme activities · Peptide fractions · Porcine albumin hydrolysate · Purification · Sequence

Abbreviations

AHA	Alcalase-hydrolyzed porcine plasma albumin (MW < 3 kD)
BSA	Bovine serum albumin
CAT	Catalase
D	Asp
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
E	Glu
F	Phe
FBS	Fetal bovine serum
G	Gly
GSH-Px	Glutathione peroxidase
H	His
H_2O_2	Hydrogen peroxide
I	Ile
K	Lys
L	Leu
MTT	3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MW	Molecular weight
N	Asn
P	Pro
P4	Peptide fraction obtained by first-step RP-HPLC
P4b	Peptide fraction obtained by second-step RP-HPLC
S	Ser
Q	Gln
RP	Reducing power

RP-HPLC	Reversed-phase high-performance liquid chromatography
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TFA	Trifluoroacetic acid
Y	Tyr
V	Val
W	Trp

Introduction

Proteins in food not only furnish amino acids but also provide bioactive peptides after digestion or food processing [1], and it is well known that peptides derived from natural proteins are considered milder and safer than synthetic drugs and more easily absorbed [2]. Therefore, bioactive peptides produced from both animals and plants, especially those by-products during food processing (also named as non-conventional food sources), are being widely investigated [1, 3]. Animal blood from slaughter is one of the most valuable by-products among many other sources of protein in that it is abundant as well as readily available, whereas it is astonishingly underutilized as a source of protein. For example, about 1.5 million tons of porcine blood is produced in China every year, containing proteins equal to that of 2.5 million tons of whole egg [4, 5], suggesting a promising source of protein that has been overlooked over the years.

Due to the increasing interest in finding antioxidants from natural sources, which may have less potential hazard than synthetic ones, a number of natural dietary antioxidants have been revealed as potential preventative/therapeutic agents against oxidative stress [6], such as fish and a few domestic animal muscles [7, 8], by-products including chicken skin [9], fish head [10] and plant protein such as chickpea albumin [11] and hemp seed [12]. Due to the convincing evidence on their in vitro antioxidant properties [13], active protein hydrolysates and/or peptides prepared from food proteins are generally short peptides (2–20 amino acids), which can exert bioactivities and serve as ingredients of functional foods [7, 12]. Thus, food-derived peptides exerting antioxidant activity have also gained an increased interest in the past decades.

To add economic value and generate new applications for blood, recovery and extraction of bioactive peptides from blood are being actively studied [1, 3]. Some functional peptides such as angiotensin I-converting enzyme (ACE) inhibitory peptides [14], antigenotoxic peptides [4], artery relaxant peptide and opioid peptides [15] had been isolated from bovine plasma or hemoglobin. Studies also demonstrated that porcine plasma hydrolysate showed stronger radical scavenging ability than non-hydrolyzed plasma protein, including reducing power (RP), DPPH radical scavenging activity and lipidoxidation [16]. Alcalase hydrolysates of porcine plasma albumin demonstrated antioxidative abilities in vitro, such as RP, lipid peroxidation inhibitory activity and radical scavenging activities [5]. Peptide fraction of molecular weight (MW) <3 kD demonstrated antioxidative effects in the liver homogenate of mice [17]. Sun [18] purified an antioxidant peptide from peptic hydrolysate of porcine hemoglobin sequenced as ARRLGHDFNPDVQAA and identified as 115–129 residues of the β-chain. In contrast to the detailed and extensive information concerning about the antioxidant properties of blood protein hydrolysate in in vitro models, little is known about the protective effect on cells of the small peptides derived from blood against radical-initiated oxidative damage. Therefore, it is important to study the biological effects of antioxidants in food using a valid in vitro cell model, which vividly mimics the targeted site of oxidative stress in vivo and uses a simple method to determine various bioactive antioxidants [19, 20]. Hence, the specific effect of antioxidative peptides derived from porcine albumin hydrolysate needs to be identified, and further evaluation of the activities in living cell is urgently required.

The objective of this study was to identify the most effective antioxidant peptide from porcine albumin and to determine the antioxidant effect in cell line against hydrogen peroxide-induced oxidative damage and lipid oxidation. The amino acids sequences of the antioxidative peptides were identified. The ability of mixed as well as individual fractions of antioxidant peptide was investigated, and the activities of several intercellular antioxidative enzymes were assayed, to mimic the targeted site of oxidative stress in vivo.

Materials and methods

Materials

Porcine plasma albumin was prepared from whole porcine blood by cold ethanol precipitation methods as described previously [5]. Lung fibroblasts MRC-5 cell was obtained from Chinese Peking Union Medical College (Beijing, China). Testing chemicals, including GSH, NADPH, acetonitrile, trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's modification of Eagle's medium (DMEM) and fetal bovine serum (FBS), were purchased from Sigma Chemical Company (St. Louis, MO, USA). All chemicals and reagents were at least of analytical grade. High-purity water was produced by the Milli-Q plus system from Millipore Corp. (Bedford, MA, US).

Preparation of antioxidant peptide fractions from porcine albumin

Methodology for preparation of peptide fractions by ultrafiltration was as described previously [5]. Briefly, proteolytic reaction was carried out by adding alcalase (Novo Nordisk, Bagsvaerd, Denmark) at the concentration of 0.1 % (w/w) at pH 7.5, 55 °C. Hydrolysate of albumin at 12 h was withdrawn from proteolytic mixture and immediately heated in boiling water for 10 min to inactive alcalase, followed by centrifugation at 15,000×*g* for 15 min. Supernatants were used to prepare peptide fractions and those of MW <3 kD (AHA) were collected, lyophilized and desalted with dialysis bag for further study.

Determination of antioxidant capacity (reducing power)

Reducing power (RP) was selected as the indicator of antioxidant capacity during the step-by-step purification. The RP of peptide fractions was determined according to the method of Oyaizu [21]. The absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated a greater RP.

Purification of antioxidant peptides from porcine albumin hydrolysate

Sephadex G-25 gel filtration chromatography

The lyophilized AHA fractions were dissolved in distilled water to the concentration of 40 mg/ml and then filtered with a 0.45 μm filter. An aliquot of 5 ml was applied to Sephadex G-25 gel filtration column (1.6 × 100 cm), pre-equilibrated and eluted with 0.1 M phosphate buffer (pH 7.2) at a flow rate of 1 ml/min. Every 3 ml of the fraction was collected. Effluents under the same elution peak were pooled after spectrophotometric measurements. The effluents were collected into five fractions (A-E). Each elution peak was lyophilized and desalted with dialysis bag, respectively. Each fraction was then subjected to RP determination at a concentration of 0.2 mg/ml.

SP-Sephadex C-25 ion exchange chromatography

The lyophilized fraction (D) obtained from Sephadex G-25 with the highest RP was dissolved in 20 mM sodium acetate buffer (pH 4.0) at the concentration of 20 mg/ml and loaded onto a SP-Sephadex C-25 cation-exchange column adequately equilibrated with the same buffer. A linear gradient of NaCl (0–1 %, v/v) in the same buffer was maintained at a flow rate of 1 ml/min, and fractions of 5 ml were collected. Absorbance was monitored at 215 nm. The fractions eluted under same elution peak were pooled. Each elution peak (F1–F7) was desalted with dialysis bag, lyophilized and tested for RP at the concentration of 0.2 mg/ml.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

The most active fraction from cation-exchange chromatography (F5) was further separated by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Nova-Pak C_{18} (3.9 × 150 mm, 4 μm) column. A linear gradient of acetonitrile (0–40 % v/v, in 30 min) with 0.1 % trifluoroacetic acid (TFA) in Milli-Q Water was maintained at a flow rate of 1 ml/min. The eluted peaks were detected by UV absorbance at 215 nm. Five active fractions (P1–P5) were pooled, lyophilized. RP of each fraction was determined at a concentration of 0.2 mg/ml. The purified fraction showed that the highest RP (P4) was rechromatographed using a linear gradient of acetonitrile (0–15 %, v/v, in 30 min) containing 0.1 % TFA at a flow rate of 1.0 ml/min. Four active fractions (P4a, P4b, P4c and P4d) were pooled, lyophilized and tested for RP at a concentration of 0.2 mg/ml.

Peptide identification through ESI-MS2

The most active fractions of RP-HPLC were collected, digested, separated, concentrated and subjected to electrospray ionization tandem mass spectrometry (ESI-MS2) for compositional identification. The collected peptides were digested with trypsin (1:100 enzyme: sample), incubated at 37 °C overnight with shaking. Mixture was then centrifuged at 15,000×*g* to remove the insoluble materials. The digested peptides were separated using RP-HPLC. The separated peptides were analyzed with mass spectrometer, with three scans of MS/MS scans over the m/z range of 440–1,140. The sequence and MW of peptides were analyzed using Mascot [22]. The theoretical pI and hydropathicity of identified peptides were analyzed with ExPASy (http://www.expasy.org/).

Cell culture

Fibroblast cell line was cultured in Dulbecco's modified Eagle's medium, supplemented with 10 % fetal bovine serum, 100 U/ml penicillin–streptomycin, 100 μg/ml streptomycin and 2 mM of Glutamine, at 37 °C under an atmosphere of 5 % CO_2 with 90 % relative humidity. The medium was changed every other day. Cultured cells were detached and separated with trypsin (0.25 %) to make a single-cell suspension.

To evaluate the protective effect of antioxidative peptides on oxidative damage of cells, cultured MRC-5 cells

were seeded in 96-well plates (200 µl/well, for cell viability determination) or 6-well plates (1.5 ml per well, for antioxidant enzyme activity and malonaldehyde (MDA) analysis at a loading concentration of 5×10^5 cells/ml) and incubated for 24 h under same conditions. The wells were subjected to four different treatments, i.e., control (with 1 mM·H_2O_2 but without antioxidant peptides) and treatments with 1 mM·H_2O_2 and AHA, P4 or P4b (each added at 4, 20, or 100 µg/ml). For these treatments, cells were incubated at 37 °C for another 24 h. Besides, to test the toxicity of the samples to the cells, a plate of normal cell without addition of H_2O_2, was also prepared as described above.

The protective effect of peptides on H_2O_2-induced oxidative damage

Cell survival rates were determined using the colorimetric 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, which yields a blue formazan product in living cells, but not in dead cells or their lytic debris. Toxicity of the sample to cells and oxidative stressed cells in the presence or absence of antioxidative peptides, as described above, were treated with MTT (5 mg/ml) for 4 h. The dark blue formazan crystals formed in intact cells were extracted with DMSO, and optical density at 570 nm was measured using an ELISA reader (Dynex Technologies Inc., Washington DC, USA). The data were expressed as the mean percentage of viable cells in comparison with the control.

Determination of lipid peroxidation

Lipid peroxidation levels were determined by monitoring MDA content described by Zhang [17] with minor modifications. MRC-5 cells were lysed in cell lysis buffer (20 mM Tris at pH 6.5, 150 mM NaCl, 1 % Triton X-100). The reaction mixture contained 0.2 ml of cell lysate, 0.2 ml of 8.1 % sodium dodecylsulfate, 1.5 ml of acetic acid and 1.5 ml of 0.5 % thiobarbituric acid. The mixture was heated in a water bath at 95 °C for 60 min. After cooling, 5 ml of *n*-butanol/pyridine (15:1, v/v) was added and shaken well. After centrifugation at 4,000×*g* for 10 min, the absorbance of the organic layer was measured at 532 nm in a UV spectrophotometer (UNIC Equipment Co. Ltd., Shanghai, China). The level of lipid peroxidation was expressed as nanomoles of MDA per milligram of cell lysate.

Determination of antioxidant enzyme activities

The lysed cell solution was used for cellular antioxidant enzyme activity assays. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities were determined as described below.

The activity of SOD was measured according to the method of Carrillo [23] with some modifications. Two milliliters of 0.05 M, pH 7.4 phosphate buffer, which contained 100 µM EDTA, 100 µM xanthine, 40 µM cytochrome C and 0.01 U xanthine oxidase was added to 100 ml of sample. The reaction started upon adding xanthine oxidase and then conducted at 30 °C for 3 min. The absorbance was measured at 550 nm in a UV spectrophotometer. One enzyme unit was defined as the amount of enzyme required to inhibit the cytochrome C reduction by 50 %. The enzyme activity was expressed as units per milligram of cell lysate.

The activity of CAT was assayed using the method of Carrillo [23] with minor modifications. The mixture of 0.05 M, pH 7.4 sodium phosphate buffer, 1 µM·H_2O_2 and the sample was made up to a final volume of 3 ml. The decrease in absorbency was measured at 240 nm in 1 min. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 µM of H_2O_2 in 1 min. The enzyme activity was expressed as units per gram of cell lysate.

GSH-Px activity was determined adopting the method of [24] with some modifications. The mixture contained 0.6 ml of 0.25 M pH 7.0 phosphate buffer, 0.3 ml of 10 mM GSH, 0.3 ml of 10 mM EDTA, 0.3 ml of 10 mM sodium azide, 0.3 ml of 2 mM NADPH and 20 µl glutathione reductase. The mixture was added to 0.9 ml of cell lysate and then incubated at 30 °C for 5 min. The reaction was initiated by adding 0.3 ml of 2.5 mM hydrogen peroxide. The absorbance was immediately read at 340 nm. The amount of NADPH within the samples was determined by a standard curve with known amounts of NADPH.

Statistical analysis

Experiment was done in triplicate, and the results were presented as mean ± SD. One-way ANOVA was used, and the mean difference was performed using the Duncan's multiple range tests. Statistical analysis was carried out using the SAS/STAT program [25].

Results

Purification and identification of antioxidative peptides

Our previous study showed that AHA had the best antioxidant ability among all peptide fractions in the RP, DPPH radical scavenging activity and inhibition of lipid peroxidation [5]. Besides, AHA can significantly attenuate lipid peroxidation of 5 % rats liver homogenate [17], indicating that those peptides take effect in both water-soluble system and lipid-soluble system. Therefore, RP was selected as the indicator of antioxidant activity during purification and

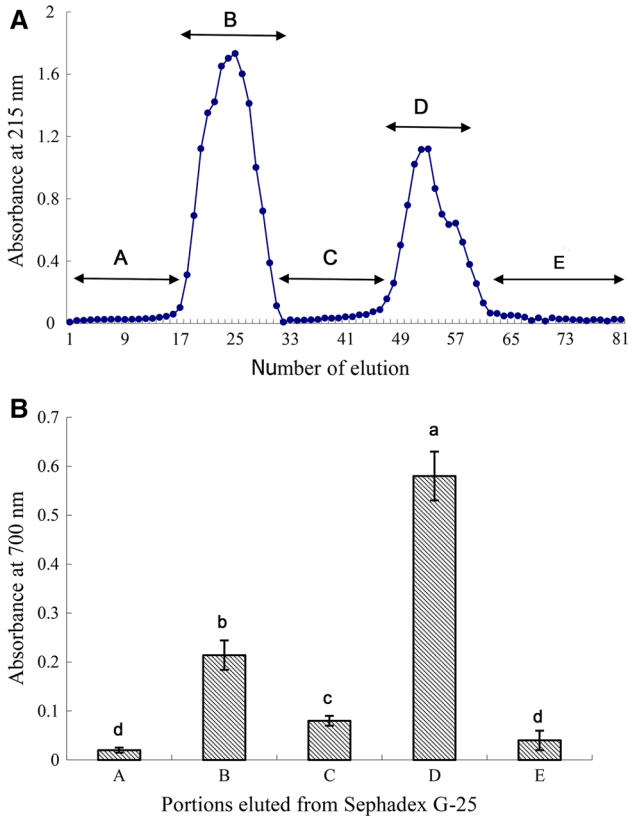

Fig. 1 Elution profiles of peptide fraction (MW <3 kDa) from the hydrolysate of porcine plasma albumin separated by Sephadex G-25 gel filtration (**a**) and the antioxidative activity determined by reducing power (**b**). Means with different letters are significantly different ($P < 0.05$)

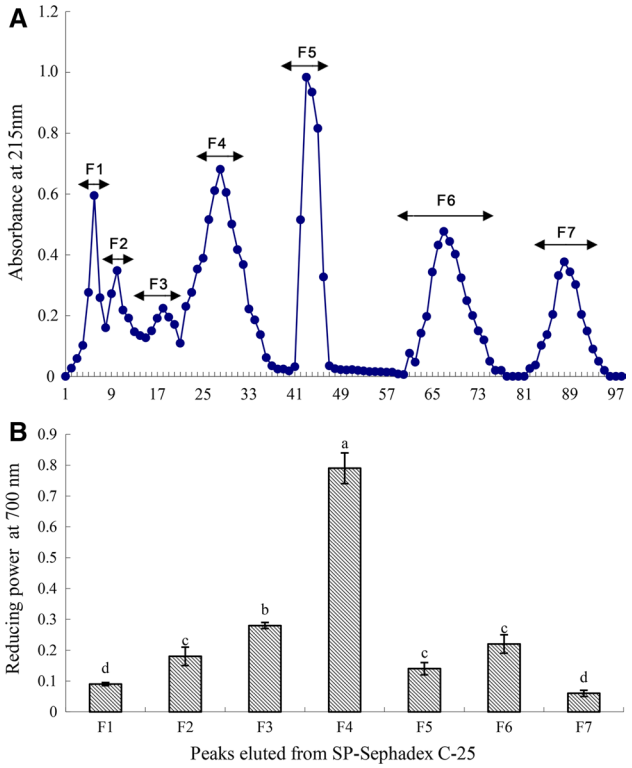

Fig. 2 Elution profiles of fraction D by SP-Sephadex C-25 cation-exchange chromatography (**a**) and reducing power (**b**). Means with different letters are significantly different ($P < 0.05$)

peptide screening in this study, following the method of Liu et al. (2010).

AHA of small MW (<3 kDa) was subjected to gel filtration, and five fractions (A, B, C, D and E) were obtained. Fraction D shows significantly ($P < 0.05$) higher RP (0.58) (Fig. 1a), and thus, it was further purified by SP-Sephadex C-25 ion chromatography. RP of sub-fractions (F1–F7) shows that fraction F4 exhibited the highest RP (0.79) compared with other peaks (Fig. 2a). Hence, it was further separated by RP-HPLC. Because of the highest RP of fraction P4 (0.89) among the five fractions (P1, P2, P3, P4 and P5) (Fig. 3a), it was further separated by RP-HPLC using a different elution solvent to yield four relatively pure fractions (P4a, P4b, P4c and P4d). P4b shows the highest RP (0.69) among the four fractions (Fig. 3a).

The obtained P4 with the highest antioxidant activity was analyzed with ESI-MS2. The MS/MS spectrum of active fraction is shown in Fig. 4a. Peptides with high identity were indicated. The observed MW of the seven peptides is 500.19, 524.24, 550.23, 568.19, 656.18, 707.44 and 1,022.70, and their amino acids sequences are identified as LIKQ, LQHK, EQKF, PDIPK, KVPQVS, FKDLGE and EHLREKVL, respectively (Table 1). The amino acids of serum albumin from *Sus scrofa*, *Homo sapiens*, *Bos Taurus*, *Ovis aries* and *Capra hircus* were aligned. As is shown in Fig. 4b, the sequence of the seven peptides is not highly conserved among five species. The sequenced peptide KVPQVS with high identity is conserved in *Sus scrofa*, *Homo sapiens* and *Bos Taurus,* while as for peptide PDIPK, conserved sequence is observed in all aligned species except Homo sapiens.

Effect of antioxidative peptides on H_2O_2-induced cells viability

It can be seen from Fig. 5 that P4 shows protective effect on H_2O_2-damaged cells in a dose-dependent manner. High concentration of P4 (100 mg/ml) could increase the survival rates of cells by 55 %, while that of P4b and AHA was 45 and 43 %, respectively. Meanwhile, 100 mg/ml of P4 showed significantly better protective effect on H_2O_2-induced oxidative damage in cells than its unpurified peptide fraction AHA and further purified fraction P4b ($P < 0.05$) in our study. In the selected concentrations (4, 20 and 100 mg/ml) of P4, P4b and AHA, there is no significant difference between the cell viability of treatments

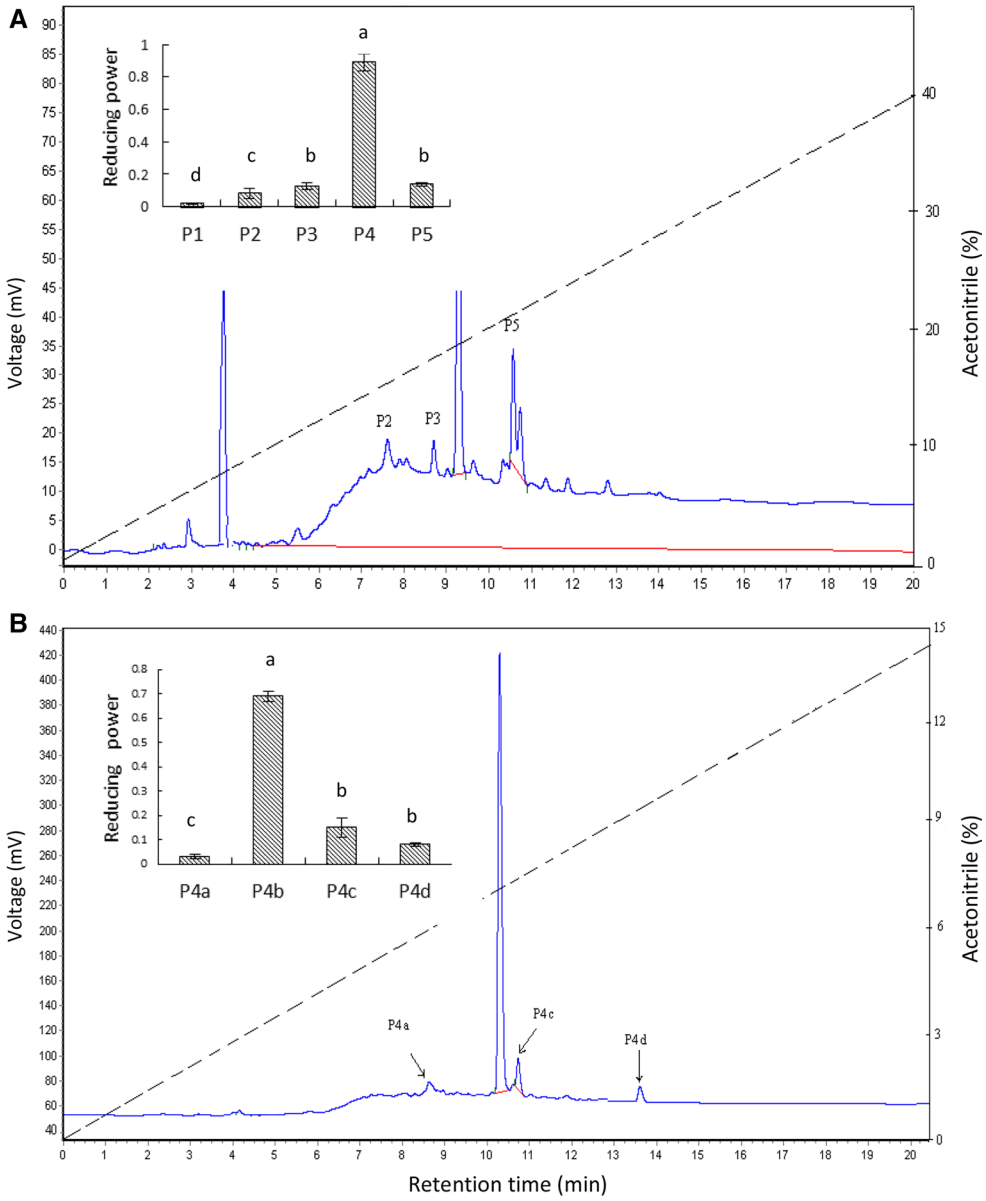

Fig. 3 Chromatograms of most active fraction separated by RP-HPLC and the reducing power. Means with different letters are significantly different ($P < 0.05$). **a** Separation with 0–40 % acetonitrile gradient at a flow rate of 1 ml/min. **b** Separation with 0–15 % acetonitrile gradient. Reducing power of each fraction was determined at a concentration of 0.2 mg/ml

and the control of normal cells (without H_2O_2), indicating that they have no toxic effects toward MRC cell (data now shown).

Effect of peptides on lipid oxidation and intracellular antioxidative enzymes

Figure 6 depicts the levels of MDA in different groups. Twenty and 100 mg/ml of AHA, P4 and P4b show significant inhibition on MDA ($P < 0.05$) in a dose-dependent manner, respectively. Moreover, the activities of SOD, CAT and GPx are presented in Fig. 6. Peptides of 20 and 100 mg/ml of P4b, P4 and AHA could increase the SOD, CAT and GPx activities in a dose-dependent manner compared with control ($P < 0.05$). A concentration of 100 mg/ml of P4 demonstrates better antioxidative enzyme activities (SOD, CAT and GPx) in fibroblast cell than AHA and P4b ($P < 0.05$).

Discussion

Purification and identification of antioxidative peptides

Our previous study showed that AHA of small MW (<3 kDa) obtained from 12-h hydrolysate of porcine plasma albumin showed the highest activity in reducing power, lipid peroxidation inhibitory and radical scavenging activities in in vitro system including liver homogenate of mice [5, 17]. Interestingly, peptide fraction P4 from the first step of RP-HPLC exhibits

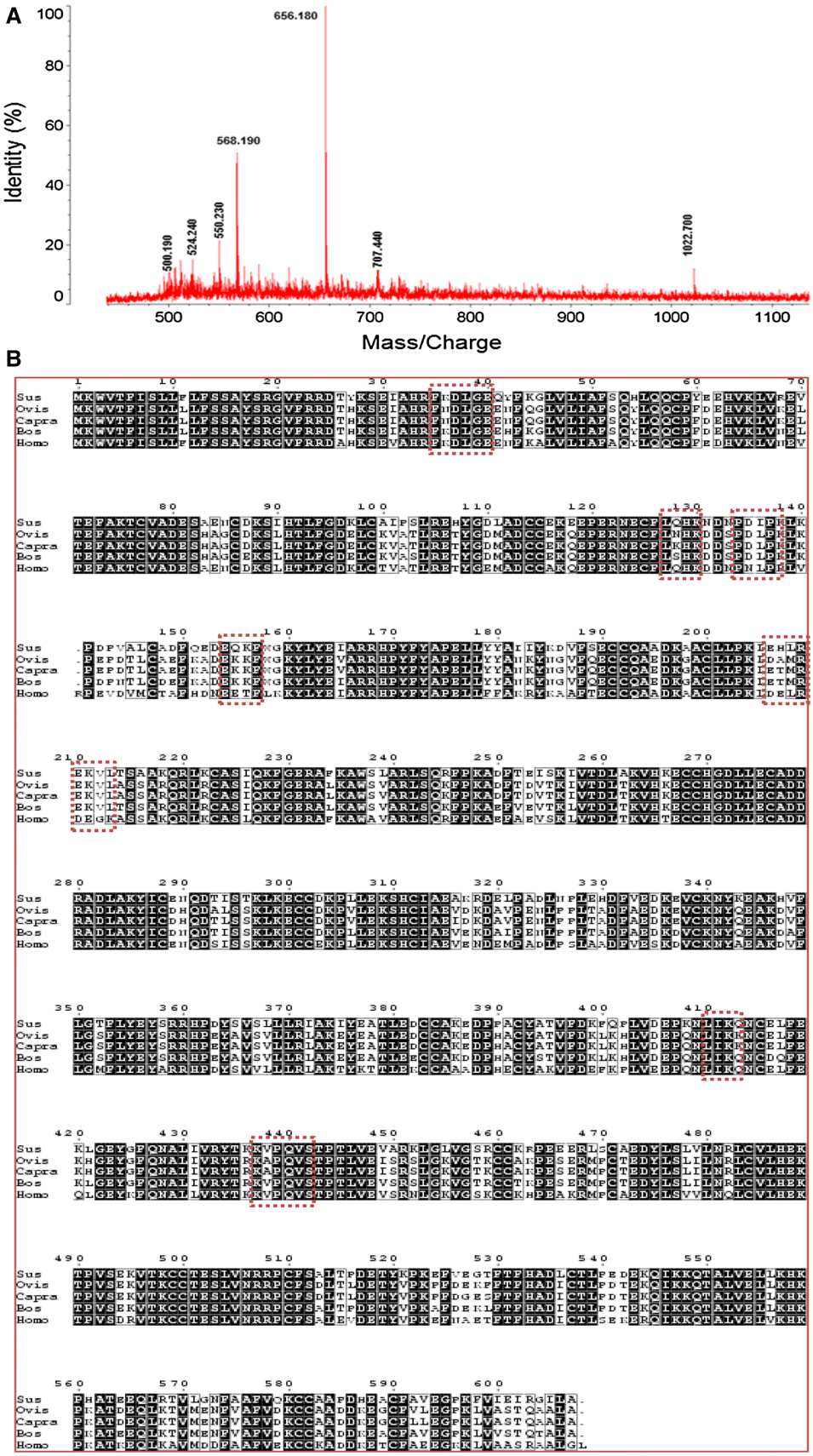

Fig. 4 **a** The MS/MS spectrum of active fraction with ESI-MS2. The active fraction was purified with Sephadex G-25 gel filtration, SP-Sephadex C-25 cation exchange and RP-HPLC. **b** Alignment of amino acid sequence of serum albumin from *Sus scrofa* (Sus, GenBank: AAT98610.1), *Homo sapiens* (Homo, GenBank: AAN17825.1), *Bos taurus* (Bos, GenBank: NP_851335.1), *Ovis aries* (Ovis, GenBank: NP_001009376.1), *Capra hircus* (Capra, GenBank: XP_005681801.1). *Boxes* indicate similar residues, and *dark shading* indicates identical residues. The peptides identified in MS/MS analysis are indicated by *dashed box*

Table 1 Characterization of identified peptides of active fractions in chromatography purification with ESI-MS2

Observed Mass (Da)	Calculated Mass (Da)	Δ mass (Da)	Peptide	Position	Theoretical pI	Hydropathicity
500.19	500.33	0.142	(N)LIKQ(N)	410–413	8.75	−0.50
524.24	524.31	0.067	(F)LQHK(N)	127–130	8.76	−0.80
550.23	550.28	0.045	(D)EQKF(W)	154–157	6.10	−2.32
568.19	568.32	0.132	(N)PDIPK(L)	134–138	6.26	−1.22
656.18	656.39	0.205	(K)KVPQVS(T)	437–442	8.75	−0.23
707.44	707.35	−0.091	(R)FKDLGE(Q)	35–40	4.37	−0.78
1,022.70	1,022.59	−0.112	(I)EHLREKVL(T)	206–213	6.86	−0.85

LIKQ Leu-Ile-Lys-Gln, *LQHK* Leu-Gln-His-Lys, *EQKF* Glu-Gln-Lys-Phe, *PDIPK* Pro-Asp-Ile-Pro-Lys, *KVPQVS* Lys-Val-Pro-Gln-Val-Ser, *FKDLGE* Phe-Lys-Asp-Leu-Gly-Glu, *EHLREKVL* Glu-His-Leu-Arg-Glu-Lys- Val- Leu-

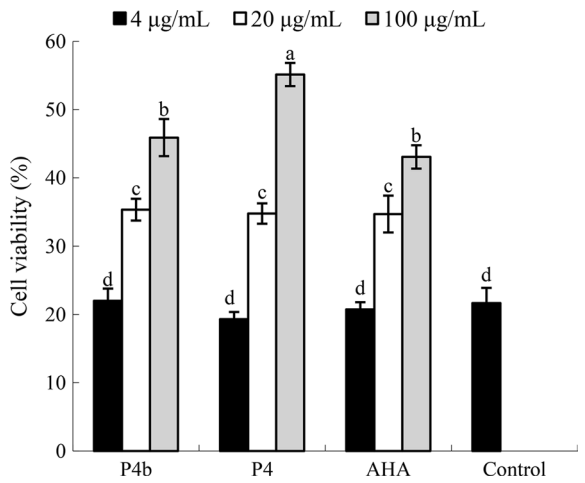

Fig. 5 The protective effect of peptides on H_2O_2-induced oxidative damage in MRC-5 cells viability. Data are expressed as average percent changes versus the control ± S.D. Means with different letters are significantly different ($P < 0.05$). P4b, P4 and AHA represent peptide separated by RP-HPLC and unpurified albumin hydrolysate (MW < 3 kD), respectively

the highest antioxidant activity indicated by RP (0.89), compared with that of further purified sub-fraction P4b (0.69) and less purified fractions D (0.58), F4 (0.79) and AHA (0.5) [5]. One of the explanations that could account for the intriguing phenomenon might be the synergistic effect of small MW peptide or amino acids, since amino acids are mentioned as synergistic antioxidants [26]. Besides, specific composition of peptides, such as the type of peptides, ratio of different concentration effectiveness and synergistic effect are also factors regarding to the antioxidant abilities of peptides [1].

To better understand the phenomenon mentioned above, we conducted further experiments to determine the composition of the active fraction. All the seven peptides contain lysine, indicating that P4 is lysine-rich peptide fraction. The theoretical pI of the indentified peptides is listed (Table 1). The peptides were purified through Sephadex G-25 gel filtration, cation exchange and RP-HPLC, and an elution buffer of pH 4 was applied in cation exchange. Ideally, the calculated pI of each peptide was higher than the pH of elution buffer, suggesting that the peptides are positively charged in the buffer of pH 4. Hydropathicity analysis showed that all the peptides are hydrophobic (Table 1), indicating that the peptides can be absorbed to the C_{18} column of RP-HPLC. Therefore, the hydrophobic property and theoretical pI of the peptides are in perfect agreement with the purification process of RP-HPLC.

To identify the corresponding peptides in similar species, the amino acids of serum albumin from *Sus scrofa*, *Homo sapiens*, *Bos Taurus*, *Ovis aries*, *Capra hircus* were aligned. These results indicate that active peptides identified could also be obtained from other animals, such as bovine and goat.

Antioxidative activity of the isolated peptides was determined by their amino acids residues and MW [27, 28]. It is generally recognized that short peptides with 2–9 amino acids express greater antioxidant potential and bioactivity than their parent proteins or larger peptides [29]. Besides, many oligopeptides can be absorbed in digestive tract and exert bioactivity [30]. For instance, Guo [31] isolated 29 antioxidative peptides from royal jelly protein hydrolysate and found that small peptides with 2–4 amino acid residues had strong antioxidant activity. Furthermore, the presence of appropriate hydrophobic amino acid residues is essential for the absorption and distribution of peptides in the cell, thereby promoting their efficacy as antioxidant agents [32]. Our results demonstrated that the seven peptides purified from AHA are composed of 4–8 amino acid residues and all of them are hydrophobic. It is consistent with those previous findings, indicating potential good antioxidant activity. On the other hand, some researchers illustrated that peptides containing histidine as the second N-terminal residue in the sequence possessed particularly strong antioxidant activity [33]. Some amino acids rich in bioactive peptide such as cysteine, serine, proline, lysine, leucine (isoleucine) tryptophane, valine have been reported with pretty good antioxidant capacity [16, 26,

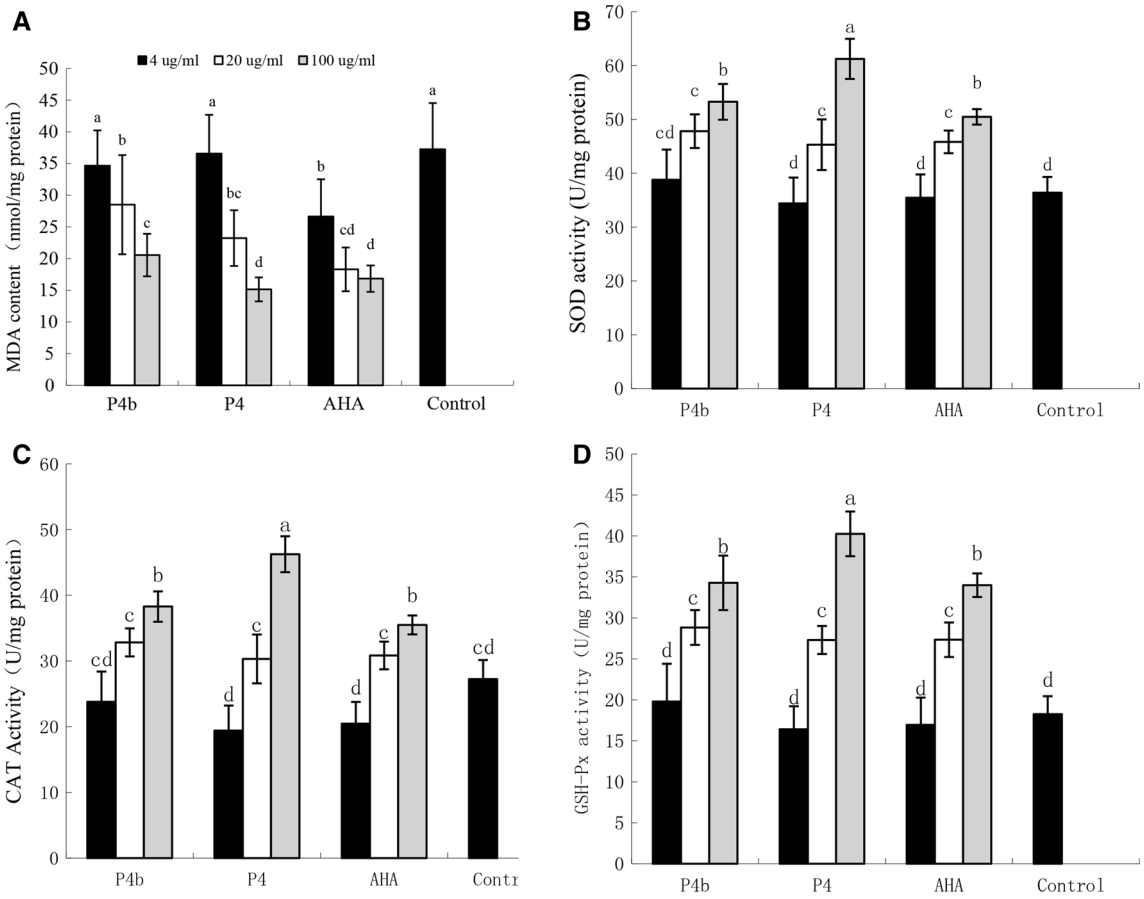

Fig. 6 The protective effect of peptides on H_2O_2-induced oxidative damage on lipid oxidation and intercellar enzymes in MRC-5. Means with different letters are significantly different ($P < 0.05$). P4b, P4 and AHA represent peptide separated by RP-HPLC and unpurified albumin hydrolysate (MW < 3 kD), respectively

34]. For example, HNGN isolated from porcine plasma [16], LARL, LHY obtained from sardinelle by-product hydrolysate [35] and WPL, VPW and VFPW released from buckwheat protein [36] all have strong radical scavenging and antioxidant activity. Contrary to those findings, only one of the peptides (EHLREKVL) isolated from AHA in the present study contains histidine, but all of the seven peptides contain lysine. The antioxidative effects of the purified lysine-rich and hydrophobic peptides P4 were further evaluated by the protectiveness on H_2O_2-induced oxidative cell damage, the clearance effect of MDA, as well as the antioxidant enzyme activities of SOD, CAT and GPx in cells. Those of P4b and AHA were also evaluated as comparison.

Effect of antioxidative peptides on H_2O_2-induced cells viability

Strong evidence indicates that oxidative stress is a main factor responsible for the death of cells. In fact, cell apoptosis is one of the most detectable and demonstrable oxidation-induced changes. H_2O_2 is generated from a variety of sources under oxidative stress, which has the ability to diffuse freely in and out of cells and tissues [37]. Similar to our findings, Kong [32] found that the survival rate of MRC-5 cell exposed to H_2O_2 can be improved to 66.1 % with 100 mg/ml of peptides from whey protein hydrolysate. It has been suggested that the antioxidant ability of biopeptides in vitro depends on peptide size, the amino acid composition of peptide and the presence of free amino acids within the hydrolysate [38]. In consistent with the of RP of P4, 100 mg/ml of P4 showed significantly better protective effect on H_2O_2-induced oxidative damage in cells than its unpurified peptide fraction AHA and further purified fraction P4b ($P < 0.05$) in our study, which should be caused by different composition of peptide and amino acids in different peptide fraction.

Effect of peptides on lipid oxidation and intracellular antioxidative enzymes

MDA can bind itself to protein, accumulating as time goes by, finally cause damage to DNA [39]. Thus, the accumulation of MDA is a sensitive index of the peroxidation of

cellular lipids in cultured cells [40]. In agreement with the RP and cell protective effects, P4b shows less inhibitory effect on MDA than that of AHA, which is composed of peptides and amino acid from hydrolysate of porcine plasma albumin. Consistently, our previous study also demonstrated that AHA can inhibit the MDA level in a simulated system with rats' liver [17].

When it comes to the activities of SOD, CAT and GPx, the result is similar to that of whey protein reported by Kong [32], suggesting that those peptides can promote the activity of intracellular antioxidative enzymes. Interestingly, the activities of SOD, CAT and GPx treated with high concentration of P4 are significantly higher than that of further purified fraction P4b and unpurified fraction AHA ($P < 0.05$). These results agree with the protective effect of peptide on cells viability damaged by H_2O_2 and RP, where the highest concentration of P4 also demonstrated better activities than P4b and AHA. Similarly, casein phosphopeptides, peptide lunasin and hydrolysate of eggshell membrane proteins have been demonstrated to exert this effect in H_2O_2-induced Caco-2 cells [20, 41], while casein and whey hydrolysates are confirmed of protective effects on H_2O_2-induced hepatic HepG2 and MRC-5 cell line, respectively [32, 42].

It is well known that oxidative metabolism is essential for the survival of cells, while the production of free radicals causes oxidation. So it is no wonder that cells have developed cooperative defense systems to reduce oxidation. The defense systems contain numerous enzymatic and nonenzymatic antioxidants, including SOD, CAT, GPx and GSH [43]. It is commonly accepted that SOD is the first antioxidant enzyme in the line of defense, which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Catalase is also an antioxidant enzyme that precipitates the decomposition of hydrogen peroxide into water and oxygen [17, 37]. And GSH-Px is the general name for an enzyme family with peroxidase activity whose main biological role is to protect the cell protein and membrane from oxidation and reduces variable hydroperoxides [44]. Therefore, only when the concentration of GPx and CAT is in equal level with SOD, the antioxidant system can clear the ROS effectively. Our results showed that the SOD is in the same level with that of CAT and GPx, indicating an efficient clearance system against radicals in fibroblast cell treated with peptides.

Seven peptides identified in P4 are mainly composed of lysine and hydrophobic amino acids, such as Phe, Leu, Ile, Val, Pro and Ala, with strong hydropathicity ranging from −0.5 to −2.3. To begin with, Phe can easily donate protons to electron-decent radicals. And pyrrolidine ring of Pro has the tendency of interrupting the secondary structure of the peptide imposing conformational constraints [45]. The antioxidative capability of peptides can be attributed to the long aliphatic side-chain groups of Leu, Ile and Val [46]. Ala also had the function of enhancing radical scavenging activity [47]. Secondly, the hydrophobicity of proteins or peptides provides easy access to hydrophobic targets and enhances the affinity and reactivity of peptides of the cell membrane in living cells [29, 48]. Finally, the small peptide size (4–8 amino residues) and the presence of amino acid residues would allow P4 to be absorbed and transported through lipid membrane, releasing its bioactivity in the MRC-5 cell [32]. Plus, specific composition, such as type of peptides and ratio of different concentration effectiveness, and synergistic effect for other antioxidants are also factors regarding to the antioxidant abilities of peptides [1]. Those information mentioned above may partially explain why P4 exhibited the highest RP, better protective effect on H_2O_2-induced oxidative damage and lipid oxidation as well as the improvement on antioxidative enzyme activities, comparing with that of further purified P4b and unpurified AHA. This study indicates a potential way to prepare beneficial ingredient for functional food from porcine blood. Continued study on the antioxidant activities of selected antioxidant peptides in Sprague–Dawley rats will be reported consecutively.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects. Sprague-Dawley rats used in the continued study of this article were maintained according to animal/human ethical committee.

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