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Chemiluminescence Evaluation of Antioxidant Activity and Prevention of DNA Damage Effect of Peptides Isolated from Soluble Eggshell Membrane Protein Hydrolysate

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A new kind of soluble eggshell membrane protein (SEP) was prepared from eggshell membrane (ESM). The extraction rate of SEP could rise to 90% by two times, basically accomplishing the complete utilization of the whole ESM. Five proteases were employed as hydrolytic enzyme for the preparation of antioxidative peptides from SEP, and the antioxidative activities of the hydrolysates were investigated using a chemiluminescence method. Among the hydrolysates, alcalase hydrolysates with the highest free radical scavenging activity were further separated into three fractions using Sephadex G-25 gel filtration chromatography of the 4 h hydrolysate (SP_1, SP_2, and SP_3). Among these three fractions, SP_2 with an average molecular weight of 618.86 Da possessed the strongest fraction of scavenging activity. The IC_{50} values of the superoxide radicals, hydroxyl scavenging activities, and protective effect on DNA damage caused by hydroxyl radicals generated were 0.10, 0.18, and 0.95 mg/mL, respectively. These results demonstrate that inexpensive ESM waste could be a new alternative in the production of antioxidative peptides.

KEYWORDS: Eoluble eggshell membrane protein; peptides; chemiluminescence method; antioxidant activity

INTRODUCTION

Free radicals and active oxygen can induce oxidant damage. Lipid peroxidation, which involves a series of free radical mediated chain reaction processes, is also associated with several types of biological damage. The role of free radicals and active oxygen is becoming increasingly recognized in the pathogenesis of many human diseases, including cancer, aging, and atherosclerosis (*1*,*2*). Free radical scavengers are preventive antioxidants. Because the use of synthetic antioxidants must be under strict regulation due to potential health hazards (*3*), the search for natural antioxidants as alternatives is of great interest among researchers (*4*). The free radical scavenging activity of fruit (*5*), vegetable (*6*), and medicinal plant (*7*) extracts has been widely studied before. Recently, several studies have already observed that some peptides derived from different protein sources possess antioxidative effects (*8*).

It is estimated that > 68 million tons of eggs were generated in the world, and the annual yield of eggs was about 29.46 million tons in China, accounting for 43% (*9*). As much as 30% of all eggs consumed today are used to manufacture processed egg products or ingredients. The eggshell membranes (ESM) are largely disposed of as byproduct, which is unfavorable to environmental safety. Therefore, the disposal of eggshell membranes poses a significant challenge to the industry. ESM is a thin and highly collagenized fibrous membrane comprising inner and outer layers (*10*). It is mainly formed by types I, V, and X collagen, making up 88−96% of its dry weight (*11*). The presence of other proteins, such as osteopontin, sialoprotein, and keratin, has also been reported (*10*). Collagen is widely used as a bioactive and nutritional ingredient in health and food products. Previous studies have shown that collagen hydrolysates contain a broad range of antioxidant activity or free radica -scavenging activity (*12*). In addition, from amino acid analysis, ESM contains cysteine, histidine, tryptophan, lysine, arginine, leucine, valine, glutamic acid, proline, and β-hydroxyl tryptophan, etc. (*13*), which have been verified to exhibit antioxidant activities (*14*).

Because of its high sensitivity and rapidity, chemiluminescence (CL) has been widely used as a sensitive assay for monitoring free radicals and reactive metabolites from enzyme, cell, or organ systems (*15*). The effects of antioxidants are measured by the depression of the signal from its uninhibited level and expressed as a percentage attenuation of the maximum CL. The superoxide anion ($O_2^{\bullet-}$) scavenging ability of samples can be determined by a CL method in the pyrogallol−luminol system (*16*). Pyrogallol was autoxidized in alkaline conditions to generate $O_2^{\bullet-}$. In this reaction, luminol was excited by $O_2^{\bullet-}$, and the decay from the excited state back to the ground state is accompanied by the emission of light (luminescence). The scavenging ability of samples on hydroxide radical ($^{\bullet}OH$) can be measured in a $FeSO_4$−luminol−H_2O_2 CL system (*16*). It is a typical Fenton reaction that generated hydroxide radical. Similar to that mentioned above, peak values of CL are inversely proportional to the hydroxide radical scavenging activity of analytes. Preventing

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Table 1. Optimum Conditions for the Hydrolysis of SEP

enzyme	temperature (°C)	pH	time (h)	buffer
alcalase	50	8	2	0.1 M PB[a]
trypsin	50	8	2	0.1 M PB
Neutrase	50	7	2	0.1 M PB
caroid	50	6.5	2	0.1 M PB
pepsin	37	2	2	0.1 M GHB[b]

[a] Phosphate buffer. [b] Glycine−HCl buffer.

DNA damage effect of samples can be determined by a $CuSO_4$−Phen−Vc−H_2O_2−DNA CL system (*16*).

Although raw ESM can be utilized to some degree, it is not dissolved in the general condition because of its highly crossed bonds, limiting its applications. Therefore, soluble eggshell membrane protein (SEP) is highly desirable. The preparation of SEP has been reported (*13*); however, reports on the bioactivities, such as antioxidant activity or free radical scavenging activity, of SEP and its peptide are few (*17*). In this paper, the dissolution process was carried out before purification to prepare SEP innovatively, which was the key step of the preparation of SEP. Furthermore, SEP was hydrolyzed by five different proteases, and the antioxidative activities and protective effect against DNA damage of the hydrolysates were investigated using a CL method for the first time. Among the hydrolysates, alcalase hydrolysates (AH), which had the highest free radical scavenging activity, were further separated into three fractions using Sephadex G-25 gel filtration chromatography of the 4 h hydrolysate (SP_1, SP_2, and SP_3); SP_2 exhibited the strongest free radical scavenging effects, which was evidenced by CL, and the IC_{50} values of the superoxide radicals, hydroxyl scavenging activities, and protective effect against DNA damage caused by hydroxyl radicals generated were investigated, respectively.

MATERIALS AND METHODS

Materials. ESM waste, a byproduct of egg manufacturing of China, was obtained by manual peeling from Chinese commercial eggshells and composed of both inner and outer membranes.

Alcalase, trypsin, Neutrase, caroid, and pepsin were purchased from Novo, Denmark. Calf thymus DNA, pyrogallol, luminal, vitamin B_{12}, cytochrome *c*, reduced glutathione, and disodium ethylenediaminetetraacetate (EDTA) were purchased from Sigma, St. Louis, MO; 3-mercaptopropionic acid was purchased from Merck & Co. Inc., United States. Acetic acid, Na_2CO_3, $NaHCO_3$, $FeSO_4$, H_2O_2, Na_2HPO_4, NaH_2PO_4, $CuSO_4$, Phen, Vc, and other chemicals and reagents were also of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

Extraction of SEP. Raw ESM pieces used in the experiment were from the same eggshell membrane. These pieces were suspended in 1.25 M aqueous 3-mercaptopropionic acid in the presence of 10% acetic acid at 90 °C for 6 h (*13*). The undissolved ESM was extracted further by pepsin in a new method. The undissolved ESM was mixed with water at a ratio of 1:30 (w/v). After adjustment of the pH to 2.0 with 0.5 M acetic acid, the above mixture was digested with pepsin at an enzyme to substrate ratio of 2% (w/w) and at 37 °C for 4 h. The supernatant, collected after the mixture had been centrifuged at 5000 rpm for 10 min, was mixed with the supernatant of the first reaction. The mixed supernatant was neutralized to pH 5 and allowed to stand for 30 min. SEP was obtained by centrifugating the solution at 5000 rpm for 10 min and by freeze-drying the sediment. The SEP extraction rate could rise to 90% by this new method, basically achieving the purpose of using the whole membrane.

Protease Hydrolysis of SEP. The optimum pH, temperature, and characterization of various enzymes are summarized in **Table 1**. Briefly, 100 mL of 0.1 M buffer solution was added to 1 g of SEP and then was digested with different commercial proteases (alcalase, trypsin, Neutrase, caroid, and pepsin) at an enzyme to substrate ratio of 2% (w/w). The enzymatic hydrolysis reactions were performed for 2 h and immediately heated to inactivate enzyme at 90 °C for 10 min. The mixture was then rapidly cooled to 20−25 °C in an ice bath. Degree of hydrolysis (DH) of every hydrolysate was determined using a pH-stat method. It was found that the DH of the hydrolysate obtained through hydrolysis by alcalase was higher than those obtained by other enzymes. Therefore, the antioxidant activity was investigated on SEP obtained from AH.

The pH of the 5% (w/v) SEP suspension was adjusted to 9.0 with 0.1 M NaOH. The suspension was incubated at 55 °C for 4 h (pH of the system was kept stable with pH-stat method). After inactivation by maintenance at 90 °C for 10 min, the liquid was centrifuged at 5000 rpm for 10 min. The supernatant was desalted by using a macroporous resin and then concentrated under vacuum. Finally, SEP hydrolysate was obtained by freeze-drying and then stored at −20 °C until use.

Purification of the Antioxidative Peptide. At a concentration of 10 mg/mL, the SEP hydrolysate was fractionated through a Sephadex G-25 (2.6 × 65 cm) column with distilled water at a flow rate of 0.5 mL/min. A fixed amount of sample (1.5 mL) was applied to the column, and 3.0 mL fractions were collected. The absorbance of the effluent was measured at 280 nm. Each fraction with a bioactive peak was collected and then lyophilized, respectively.

Determination of Superoxide Anion Scavenging Ability. The superoxide anion scavenging ability of sample was determined by a CL method in the pyrogallol−luminol system. Ten microliters of sample and 50 μL of pyrogallol (6.25×10^{-4} M) were mixed. The light emission from the reactive vial was recorded soon after 0.94 mL of a mixture containing luminol (0.05 M) and carbonate buffer (pH 10.2) was added and mixed. The test was accomplished at the spectral scan range of 180−800 nm, with high voltage of 800 V and temperature of 30 °C. The emission light intensity was recorded every 2 s, and the total integral of the light intensity of 180 s was determined. The control was performed in the same manner in the mixture without the sample solution, and the background was detected without pyrogallol addition (*18*).

Determination of Scavenging Ability on Hydroxide Radicals. The scavenging ability of sample on hydroxide radical was measured by using a $FeSO_4$−luminol−H_2O_2 CL system in a BPCL-4 Ultraweak Luminescence Analyzer (Institute of Biophysics, Academia Sinica, China). It was composed of 50 μL of a 0.4 mM $FeSO_4$, 50 μL of a 1.5% H_2O_2 solution, and 50 μL of sample solution. The light emission from the reactive vial was recorded immediately after 0.6 mL of a mixture containing luminol (0.1 mM) and phosphate buffer (0.05 M, pH 7.4) was added to the glass vial. The test was accomplished at the spectral scan range of 180−800 nm, with high voltage of 800 V and temperature of 30 °C. The emission light intensity was recorded every 1 s, and the total integral of the light intensity of 60 s was determined. The control was performed in the same manner in the mixture without the sample solution, and the background was detected without H_2O_2 addition.

Determination of Preventing DNA Damage Effect. Preventing the DNA damage effect of sample was determined by a $CuSO_4$−Phen−Vc−H_2O_2−DNA CL system. Copper and 1,10-phenanthroline were premixed in 0.1 M NaOAc/HOAc (pH 5.5) buffer, and 3 μg/mL DNA was incubated with a phen−Cu solution. Then, 800 μL of phen−Cu/DNA solution, 100 μL of 4.2×10^{-3} M ascorbate, and 200 μL of 6% H_2O_2 were added without interval to a 100 μL sample solution to give a final volume of 1.2 mL. The kinetic curve of CL produced in the phen−Cu−H_2O_2−ascorbate system was immediately recorded (*19*). The control was performed in the same manner in the mixture without the sample solution, and the background was detected without H_2O_2 addition.

Determination of Amino Acid and Molecular Weight Distribution. Amino acids contained in SEP peptides were determined with an automatic amino acid analyzer (Hitachi L-8800, Japan). The molecular weight distribution of SEP peptide was measured by Sephadex G-25 standard curve method. Vitamin B_{12} (1.355 kDa), cytochrome *c* (12 kDa), and reduced glutathione (0.307 kDa) as molecular weight standards were chromatographed, separately. The standard curve $y = -39.902x + 223.30$ ($R^2 = 0.9994$) was obtained in this study and could be used to measure the molecular weight distribution of SEP peptide.

Data Analysis. The integrated area of the curve expressed the relative luminescent intensity. The scavenging activity was represented by the equation

$$\text{scavenging activity} = \frac{(CL_{control} - CL_0) - (CL_{sample} - CL_0)}{CL_{control} - CL_0}$$

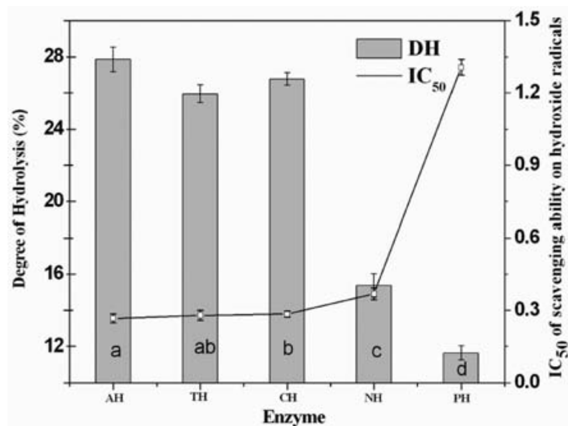

Figure 1. Degree of hydrolysis of SEP treated with different proteases and its IC_{50} of scavenging effect on hydroxide free radical. Means with different letters (a−d) differ significantly ($p < 0.05$).

where $CL_{control}$ is the relative luminescent intensity of the control group, CL_0 is the relative luminescent intensity of the background group, and CL_{sample} is the relative luminescent intensity of the experimental group.

The SAS system (SAS for Windows 6.12, SAS Institute Inc., Cary, NC) was used for statistical analysis. Data were expressed as mean ± standard deviation ($n = 3$). Student's t test was used to determine the level of significance at $p < 0.05$.

RESULTS AND DISCUSSION

Preparation of Protein Hydrolysates. EP was digested with different commercial proteases. The result is shown in **Figure 1**. It was found that the DH of the hydrolysate obtained through hydrolysis by alcalase was higher than those obtained by trypsin, Neutrase, caroid, and pepsin, respectively. The DH of AH samples ascended obviously with hydrolysis time increasing in the first 4 h ($p < 0.05$), and then the enzymatic reaction reached the steady-state phase when no apparent hydrolysis took place (figure not shown). Moreover, the antioxidative activity of the hydrolysates was evaluated using a hydroxyl radical, the strongest free radical, scavenging activity by CL. As shown in **Figure 1**, among various hydrolysates, AH appeared to show the highest antioxidative activity. Therefore, the 4 h AH was selected for further study.

It has been demonstrated that the antioxidant activities of proteins can be increased through hydrolysis with certain enzymes, and some peptides or fractions possess stronger antioxidant potential than others. Furthermore, the antioxidant activity of protein hydrolysates depends on the protein substrate, the specificity of the enzyme, the conditions used during proteolysis, and the degree of hydrolysis (*20*). Because enzymes have specific cleavage positions on polypeptide chains, protein hydrolysates were prepared from SEP by treatment with different enzymes to obtain peptides with different amino acid sequences and peptide lengths.

Alcalase is an alkaline protease industrially used and produced from *Bacillus licheniformis*, in which one of the amino acids at the active site is serine. It is capable of producing bioactive peptides when it is used to hydrolyze natural proteins (*21*), with a rather broad range of specificity for peptide bonds, leading to the production of antioxidant peptides (*22*, *23*). Bioactive peptides produced by alcalase are resistant to digestive enzymes such as pepsin, trypsin, and Pronase E, which would be absorbed when present in this sort of hydrolysate (*3*, *24*). Moreover, related studies have clearly shown that alcalase produces shorter peptide sequences as well as terminal amino acid sequences responsible for various bioactives, including antioxidant activity (*25*, *26*).

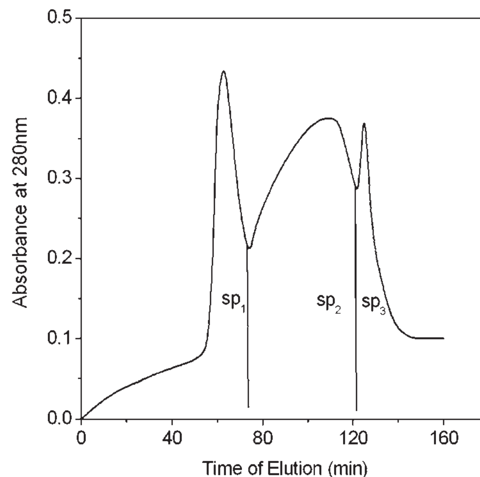

Figure 2. Gel filtration pattern of SEP at 4 h AH.

Purification of Antioxidative Peptide. To understand the possible effect of peptide composition on the antioxidant activity of SEP hydrolysates, the 4 h AH samples were subjected to fractionation with a Sephadex G-25 gel filtration column. As displayed in **Figure 2**, the column successfully separated the 4 h hydrolysate into three fractions (SP_1, SP_2, and SP_3), corresponding to average molecular weights (MW) of 10522.14, 618.86, and 300.83 Da. SP_2 was observed with stronger antioxidant activity by the CL method (data not shown), accounting for 68.15% of total peptides.

Superoxide Anion ($O_2^{\bullet -}$) Scavenging Ability of Purified Peptide. The superoxide anion radical is normally formed first in cellular oxidation reactions. It can produce hydrogen peroxide and hydroxyl radical through dismutation and other types of reaction and is the source of free radicals formed in vivo. Not only superoxide anion radical but also its derivatives are cell-damaging, which can cause damage to DNA and membrane of cell. Therefore, it is of great importance to scavenge superoxide anion radical (*27*, *28*).

The scavenging activity of SP_2 on superoxide anion induced by CL of pyrogallol−luminol system is shown in **Figure 3A**. The results showed that light emission in the pyrogallol−luminol system increased for the first 6 s after the luminescent measurement described above was initiated and then declined. All kinetic curves of CL were superposed at around 180 s. According to the detection principle, there is a negative correlation between the peak values of CL and the superoxide anion scavenging activity of SP_2.

Figure 3B shows the percentage inhibition of superoxide anion radicals by SP_2 at different concentrations (0.0−0.5 mg/mL). The superoxide anion scavenging activities and concentrations were almost linearly correlated for SP_2 ($p < 0.01$). The IC_{50} (concentration of hydrolysate to scavenge 50% superoxide anion activity) value of SP_2 on scavenging superoxide anion was 0.10 mg/mL, which was similar to that of glutathione (*28*).

Hydroxide Radical Scavenging Ability of Purified Peptide. Among the oxygen radicals, hydroxyl radicals are the most reactive and can react with almost all of the substances in the cell and induce severe damage to cells (*29*). Hydrogen peroxide can also change to hydroxyl radicals in vivo. Hydroxyl radical generation systems include the Fenton reaction system, $CuSO_4$−Phen−Vc−H_2O_2 CL system, $FeSO_4$−luminol−H_2O_2 CL system, $FeSO_4$−luminol CL system, $CuCl_2$−H_2O_2−Phen−carbonate buffer CL system, and $CuSO_4$−yeast−Vc−H_2O_2 CL system, etc. (*16*). In this study, the $FeSO_4$−luminol−H_2O_2 CL system was used to determine the hydroxyl radical scavenging

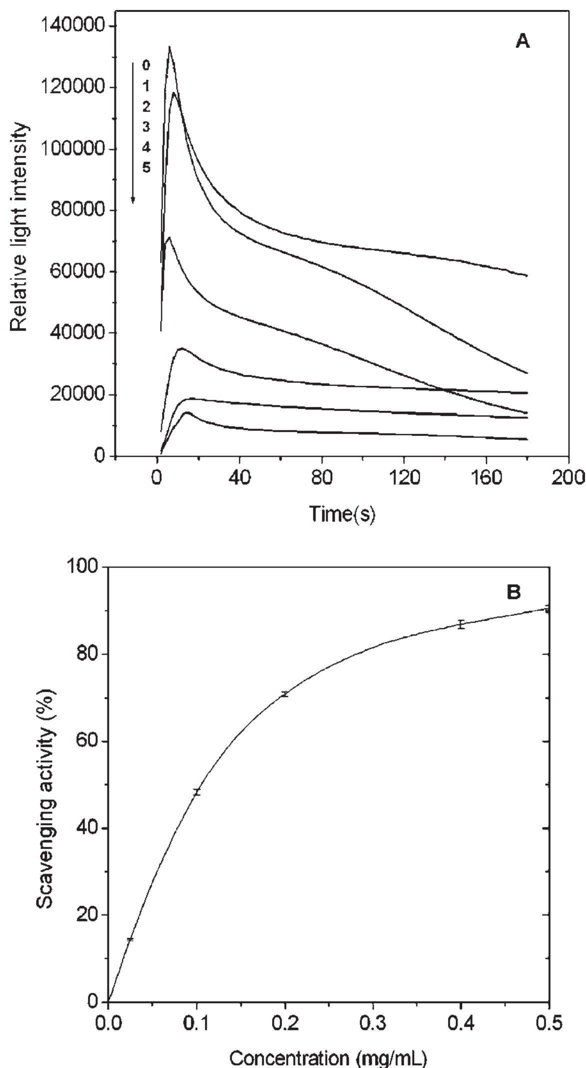

Figure 3. Scavenging activity of SP_2 on superoxide anion radical induced by CL of the pyrogallol–luminol system: (**A**) CL curves; (**B**) scavenging activity of SP_2 on superoxide anion at different concentrations. Each value is the mean ± SD of triplicate measurements.

capacity of SP_2. This CL system was also a rapid-response CL system. The kinetics of the reaction showed maximal luminescence rapidly in the first 2 s after the reactants were mixed and then declined. All kinetic curves of CL were superposed at around 16 s (figure not shown).

The hydroxyl radical scavenging activity of SP_2 at different concentrations is shown in **Figure 4**. SP_2 scavenged hydroxyl radicals in a concentration-dependent way. The IC_{50} value of SP_2 on scavenging hydroxyl radical was 0.18 mg/mL.

Hydroxyl radical scavenging capacity has been confirmed for peptides from other sources. Je, Park, and Kim (*30*) reported that the purified peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate scavenged 35% on hydroxyl radical at 53.6 M using electron spin resonance (ESR) spectroscopy. Soy protein hydrolysates from the fraction with molecular weight between 30 and 50 kDa showed the highest hydroxyl radical scavenging capacity (69.75%) (*31*); alfalfa leaf protein hydrolysate exhibited 80% of scavenging activity on hydroxyl radicals at a concentration of 1.2 mg/mL(*28*).

The chemical activity of hydroxyl radical is very strong, and it easily reacts with biomolecules such as amino acids, proteins, and DNA. Therefore, the removal of the hydroxyl radical is probably

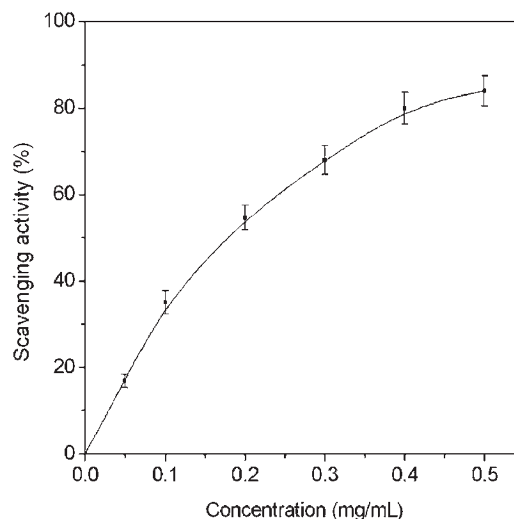

Figure 4. Scavenging activity of SP_2 on superoxide anion radical induced by CL of the pyrogallol–luminol system. Each value is the mean ± SD of triplicate measurements.

one of the most effective defenses of a living body against various diseases. The hydroxyl radical is also effective at initiating lipid peroxidation reactions. Determination of hydroxyl radical scavenging activity provides useful information on antioxidant activities (*28*, *32*) The high hydroxyl radical scavenging activity shown by SP_2 strengthened its antioxidant activity and the ability to protect hydroxyl radical induced damage.

DNA Damage Prevention Effect of Purified Peptide. Hydroxyl radicals generated by the Fenton reaction are known to cause oxidative breaks in DNA strands to yield its open circular or relaxed forms. There is a considerable amount of evidence revealing an association between DNA damage and some human problems, such as cancer and aging. It is meaningful to study the DNA protection activity of SP_2.

The protective effect of different concentrations of SP_2 samples on damaged DNA in the CL system is shown in **Figure 5**. In this system, an initial small peak was found within 12 s, and a second peak appeared around 2.5 min after the addition of H_2O_2. $^{\bullet}OH$ produced in the Fenton reaction attacked Phen to generate the initial peak. The second was a lag peak that had DNA damage by hydroxide radical (*33*). In this system, the reduction of the two peak values and integral areas was observed with SP_2. Results also showed that its protective effect varies with the concentration of the purified peptide; therefore, SP_2 possesses DNA protection activity in a dose- and effect-dependent manner, and its IC_{50} was 0.95 mg/mL. The results of this study clearly explain that peptide can prevent oxidative damage to DNA when DNA is exposed to $^{\bullet}OH$.

In addition, according to the differences in affecting the CL, antioxidants were sorted into three types, namely, inhibiting, delaying, and mixed types, respectively. The CL yield was decreased with the increase of antioxidant concentration, which was called the inhibiting type. The luminescence peak was delayed, and the interval became longer and longer with increasing antioxidant concentration. This was called the delaying type. The mixed type showed both inhibiting and delaying activities (*33*). Thus, SP_2 belonged to the inhibiting type.

Antioxidant activity present in alcalase-hydrolyzed SEP has been investigated by using a CL method. A short peptide (SP_2) with a low molecular weight of 618.86 Da had the strongest radical quenching effect and therefore seemed to play a major role in the overall antioxidant effect of SEP hydrolysates.

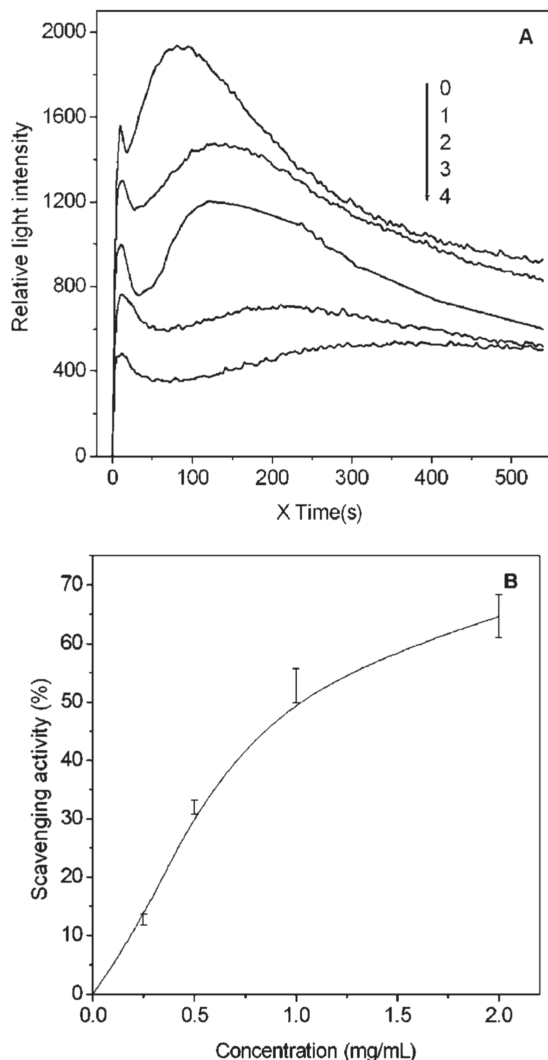

Figure 5. Protective effect of SP_2 on damaged DNA in $CuSO_4$−Phen−Vc−H_2O_2−DNA CL system: (**A**) CL curves; (**B**) protective effect of SP_2 on damaged DNA at different concentrations. Each value is the mean ± SD of triplicate measurements.

Table 2. Amino Acid Composition (Grams per 100 g) of SP_2

amino acid	amount	amino acid	amount
aspartic acid	3.37	methionine[a,b,c]	5.82
threonine[a]	2.76	isoleucine[a,c]	0.82
serine	2.85	leucine[a,c]	2.29
glutamic acid	9.27	tyrosine[b,c]	5.67
proline[c]	10.83	phenylalanine[a,b,c]	2.83
glycine	5.28	lysine[a]	1.16
alanine[c]	1.55	histidine[b]	7.62
cysteine[b]	3.58	tryptophan[a,b,c]	2.48
valine[a],[c]	4.87	arginine	4.27

[a] Essential amino acid. [b] Antioxidant amino acid. [c] Hydrophobic amino acid.

Amino Acid Composition Analysis. Table 2 presents the amino acid profiles of SP_2. The total amount of amino acid was 77.32 g/100 g of sample. SP_2 contained some amino acids, such as His, Tyr, Met, Cys, Val, Pro, Phe Trp, Leu, Ile, Ala, Asp, and Glu, which had been reported to show antioxidant activity. Histidine exhibited a strong radical scavenging activity due to the decomposition of its imidazole ring (*34*). The Cys residue of the peptide was expected to protect lipid and other biomolecules by donating protons to peroxyl radicals and other free radicals in the cell. The aromatic amino acids (Tyr, Phe) could make active oxygen stable through direct electron transfer (*35*). Some residues (Val, Pro, Phe Trp, Leu, Ile, Met, Tyr, and Ala) of SP_2 were hydrophobic amino acids, and hydrophobic amino acids might increase the affinity and reactivity to the cell membrane in the living cells (*36*). Therefore, the antioxidant activities of SP_2 seemed to be caused by these amino acids in the peptides. Moreover, the antioxidant activity of the SP_2 depended upon the amino acid sequence of the peptides. Furthermore, the ratio of amino acids in the SP_2 was reasonable, with almost all kinds of amino acids, especially the essential amino acids, present. Therefore, SP_2 possessed not only an antioxidant property but also a high nutritive value.

The antioxidative properties of peptides were highly influenced by molecular mass and molecular structure properties (*37*). Most of the reported peptides exhibiting antioxidative activity were those with low molecular weights (*38*, *39*). The peptide in the study had a low molecular weight of 618.86 Da. On the other hand, the characteristic amino acid composition of the peptide was also important for the antioxidative activity. The oxidative susceptibility of a given amino acid in the peptide to free radical attack was dictated in large part by its R groups. The most reactive amino acids tend to be those containing nucleophilic sulfur-containing side chains, aromatic side chains, or imidazole-containing side chains. Furthermore, the antioxidative activity was also limited by the structure of the peptide, and the solvent accessibility of the amino acids in the peptide was also important (*8*, *40*).

On the basis of the above research, peptides purified from SEP hydrolysates can be exploited as antioxidants in cosmetic or food additives and a potential nutraceutical material. Also, our results raised a number of issues for further investigation: the isolation and characterization of protein present in ESM, the determination of the mechanisms involved in the antioxidant abilities of SEP peptides, the isolation of the individual antioxidative peptides in SEP hydrolysates, and the identification of their amino acid sequences for a better understanding of the peptide structure−functionality relationship. Further in vivo studies on the antioxidant activity of SEP and its peptides are still underway.

ABBREVIATIONS USED

ESM, eggshell membrane; SEP, soluble eggshell membrane protein; AH, Alcalase hydrolysate; TH, trypsin hydrolysate; NH, Neutrase hydrolysate; CH, caroid hydrolysate; PH, pepsin hydrolysate; CL, chemiluminescence.

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