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Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry

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Abstract

The porcine skin collagen was hydrolyzed by different protease treatments to obtain antioxidative peptides. The hydrolysate of collagen by cocktail mixture of protease bovine pancreas, protease *Streptomyces* and protease *Bacillus* spp. exhibited the highest antioxidant activities on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, metal chelating and in a linoleic acid peroxidation system induced by Fe^{2+}. And degree of hydrolysis highly affected the antioxidant properties of the hydrolysates. Four different peptides showing strong antioxidant activity were isolated from the hydrolysate using consecutive chromatographic methods including gel filtration chromatography, ion-exchange chromatography and high-performance liquid chromatography. The molecular masses and amino acid sequences of the purified antioxidant peptides were determined using electrospray ionization (ESI) mass spectrometry. One of the antioxidative peptides, Gln-Gly-Ala-Arg, was then synthesized and the antioxidant activities measured using the aforementioned methods. The results confirmed the antioxidant activity of this peptide, and adds further support to its feasibility as a provider of natural antioxidants from porcine skin collagen protein.

Keywords: Antioxidant activity; Radical scavenging activity; Lipid peroxidation; Antioxidative peptide; Porcine collagen; Mass spectrometry

1. Introduction

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been commonly used to preserve food products by delaying discoloration and deterioration due to oxidation. However, these synthetic antioxidants have been limited in their applications as food additives because of potential health hazards (Branen, 1975; Becker, 1993). The past two decades have witnessed a growing interest in finding safe and natural antioxidants that enhance the body's antioxidant defenses through dietary supplementation, and inhibit lipid oxidation in foods (Chow, 1988; Finkel & Holbrook, 2000).

Several natural proteins have been reported as water soluble antioxidants due to their chelating effect on metal ions (see Cervato, Cazzola, & Cestaro, 1999; Lu & Baker, 1986; Tong, Sasaki, McClements, & Decker, 2000; Wang, Fujimoto, Miyazawa, & Endo, 1991). Antioxidant activity has also been identified in several protein hydrolysates such as those in soybean (Pena-Ramos & Xiong, 2002), myofibrillar (Saiga, Tanabe, & Nishimura, 2003), and milk proteins (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; Sakanaka, Tachibana, Ishihara, & Juneja, 2005; Suetsuna, Ukeda, & Ochi, 2000), egg-white albumin (Tsuge, Eikawa, Nomura, Yamamoto, & Sugisawa, 1991),

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and soluble elastin (Hattori, Yamaji-Tsukamoto, Kumagai, Feng, & Takahashi, 1998). Further, a number of antioxidative peptides, usually composed of 3 to 16 amino acid residues, have been isolated from these hydrolysates. Among these peptides, some contained hydrophobic amino acids (Val or Leu) at the N-terminus, Pro, His, or Tyr in sequences (Chen, Muramoto, & Yamauchi, 1995), and some containing mainly acidic acid residues (Glu, Asp) (Saiga et al., 2003). However, little is known about the structure of antioxidative peptides in the various proteins.

Collagen is a cheap and resourceful meat byproduct whose main product is a gelatin that is used extensively as a food additive to increase the texture, water-holding capacity and stability of several food products. Although several studies have been performed to identify potential antioxidative peptides in fish skin gelatin (Kim et al., 2001a; Mendis, Rajapakse, & Kim, 2005), there has yet to be any research on antioxidative peptide activity in porcine skin collagen.

The functional properties of peptides are highly influenced by their molecular structure and weight, which are greatly affected by processing conditions. Enzymatic hydrolysis has become a valuable tool for modifying the functionality of proteins (Korhonen, Pihlanto-Leppala, & Tupasela, 1998). While the antioxidant activity of protein hydrolysates has been examined using a single enzyme, little is known about the antioxidative character of protein hydrolysates stemming from a cocktail mixture of proteases.

Analysis of the amino acid sequences of peptides and proteins is one of the most important issues in peptide identification. Peptide sequencing was initially achieved using the Edman degradation method (Edman, 1950; Heinrikson, 1984), which was time consuming, had low throughput, and poor sensitivity in comparison with the mass spectrometric approach. Tandem mass spectrometry, where the peptide sequencing is achieved by software using iterative calculations, is now a widely used approach in peptide sequencing (Siuzdak, 1996; Lin & Glish, 1998). The software looks for continuous sequences at the N- and/or C-terminal fragments that differ by just one amino acid and allowing the sequence to be obtained from a complete series of fragments (Hernández-Ledesma et al., 2005). Unfortunately, the peptides, and especially small peptides, do not usually break at each amino acid conjunction, making the problem very difficult for existing peptide sequencing methods. In this research, we developed an example for determining peptide amino acid sequences by processing mass spectrometry data. Our objective was to determine whether porcine collagen may represent a source of antioxidative peptides upon hydrolysis. The antioxidant activities of hydrolysates by single protease and cocktail enzymes were evaluated in three oxidation systems. Further, the purification and identification of several of the antioxidative peptides was determined using consecutive chromatography and tandem mass spectrometry.

2. Materials and methods

2.1. Materials

Porcine skin collagen powder was obtained from Proliant Co., USA. Enzymes, pepsin, papain, protease from bovine pancreas (PP), protease from *Streptomyces* (PS), and protease from *Bacillus polymyxa* (PB) were purchased from Sigma Chemical Co. (St. Louis, MO). Chemicals required for the assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-trinitrobenzenesulfonic acid (TNBS), butylated hydroxytoluene (BHT), and ethylenediaminetetraacetic acid (EDTA) were also purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals and reagents used were of analytical grade and commercially available.

2.2. Preparation of collagen hydrolysates

Porcine skin collagen in deionized water (5% w/w) was preheated to 90 °C for 5 min, and then hydrolyzed for 24 h with pepsin. The resulting supernatant was further hydrolyzed for 24 h by papain, protease from bovine pancreas (PP), and the cocktail mixture of three enzymes: PP, protease from Streptomyces (PS), and protease from *Bacillus polymyxa* (PB), respectively. The hydrolysis temperature was 37 °C for the pepsin and cocktail mixture of PP, PS and PB, and 25 °C for papain.

Considering the enzymatic assay and activity of the different proteases (μ/mg powder: pepsin, 800–2500; papain, 1.5–3.5; PP, 5; PS, 4; PB, 1.0) which were offered by Sigma Chemical Co., we used the different dilution factors. The ratio of the enzyme/collagen substrate was 1/2500 for pepsin, 1/50 for papain, and 1/125 for PP, PS, PB, respectively.

Pepsin was dissolved in a 10 mM HCl solution (pH 2.0), papain dissolved in deionized water, and the PP and cocktail mixture of PP, PS and PB dissolved in 10 mM sodium acetate buffer (pH 7.5). The pH of substrate solution was adjusted to the optimal value for the specific proteases (pH 2.0 for pepsin, pH 6.2 for papain and pH 7.5 for PP, PS and PB) before initiating the hydrolysis. Inactivation of the enzymes was accomplished by heating for 3 min in boiling water and then centrifuged at $20{,}000 \times g$ for 10 min. The supernatant was stored at 0 °C for future use.

2.3. DPPH radical scavenging assay

DPPH radical scavenging activity was measured based on methods described in Bersuder, Hole, and Smith (1998). A 500 μl test sample was mixed with 500 μl of 99.5% ethanol and 125 μl of 99.5% ethanol containing 0.02% DPPH. This mixture was kept in the dark at room temperature for 60 min before measuring for absorbance at 517 nm. Radical scavenging activity was calculated as follows:

$$\text{Radical scavenging activity}(\%) = \frac{\text{Control} + \text{Blank-sample}}{\text{Control}} \times 100\%$$

where the control was the absorbance value of 500 μl of distilled water + 125 μl of ethanol including 0.02% DPPH + 500 μl of ethanol, the sample that for 500 μl of sample solution + 125 μl of ethanol including 0.02% DPPH + 500 μl of ethanol, and the blank that for 500 μl of sample solution + 125 μl of ethanol + 500 μl of ethanol.

2.4. Metal chelating assay

The metal chelating effect of the sample was determined using a ferrous ion chelating assay (Chung, Chang, Chao, Lin, & Chou, 2002). A test sample of 800 μl was mixed with 10 μl of 2 mM $FeCl_2$ and 20 μl of 5 mM ferrozine, the mixture vortexed and kept at room temperature for 10 min prior to measuring for absorbance at 562 nm. EDTA was used as a standard metal chelating agent.

$$\text{Chelating effect}(\%) = \left(1 - \frac{\text{Absorbance of sample at 562 nm}}{\text{Absorbance of control at 562 nm}}\right) \times 100$$

2.5. Assay in a linoleic acid oxidation system

Linoleic acid was oxidized in a linoleic acid model system to measure the antioxidant activity of hydrolysates following a modified version of the Mendis et al. (2005) methodology. Briefly, 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.0) and 1.5 ml of 50 mM linoleic acid in ethanol (99.5%) were mixed in a glass test tube and 2.0 ml of the test sample (pH 7.0) added. BHT (2.0 mM and 20 mM in methanol) was used instead of test sample as positive control. The mixed solution in a lightly sealed screw cap glass tube was incubated in the dark at 60 °C. The degree of linoleic acid oxidation was measured at 24-h intervals using the ferric thiocyanate method described in Osawa and Namiki (1981). An aliquot (50 or 100 μl) of the reaction mixture was mixed with 75% ethanol (4.55 ml or 4.5 ml) followed by the addition of 30% ammonium thiocyanate (100 μl), HCl of 1.0 N (200 μl) and 20 mM ferrous chloride solution in 3.5% HCl (100 μl). The reaction solution was 5 ml in total. After 5 min incubation, the color development, which represents the linoleic acid oxidation, was measured at 500 nm.

2.6. Degree of hydrolysis

Degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved, was calculated by the determination of free amino groups by reaction with TNBS according to methods described in Alder-Nissen (1979). The total number of amino groups was determined in a sample which was 100% hydrolyzed in 6 N HCl at 110 °C for 24 h (10 mg sample in 4 ml of HCl).

2.7. Amino acid analysis

Hydrolysates of collagen were hydrolyzed in 6 N HCl at 110 °C for 22 h. Amino acid analysis of the hydrolysates was performed with a Dionex D-300 amino acid analyzer (Dionex Co., USA) according to standard methods of ion exchange chromatography. The amino acid composition of the peptides was determined by subtracting the free amino acids from the total amino acids in the hydrolysate, and the hydrolysate concentration then calculated according to amino acid composition.

2.8. Purification of antioxidant peptide

Hydrolysates showing antioxidant activity were concentrated and loaded onto a Sephadex LH-20 gel filtration column (2.5 × 80 cm), which was previously equilibrated with distilled water. The column was eluted with distilled water and the eluted peptides detected at 254 nm. Each of the fractions showing antioxidant activity were pooled and concentrated using a roller-evaporator (38 °C). Each pooled fraction was loaded onto an ion-exchange column (2.5 × 18 cm) with a DEAE-Sephadex A-25 (chloride form, Sigma Chemical Co., St. Louis, MO) previously equilibrated with 0.5 M tris–HCl buffer (pH 8.3). The column was washed with the same buffer and eluted with a linear gradient of NaCl concentrations from 0 to 0.5 M. The fractions showing antioxidant activity were concentrated and the NaCl removed by 5 ml D-Salt Excellulose Plastic Desalting Column (Pierce Biotechnology co., USA). This fraction was further separated by reversed-phase high performance liquid chromatography (RP-HPLC, SHIMADZU Scientific Instruments, Japan) on an ODS column (YMC ODS-AQ S-5 120 Å, 4.6 × 250 mm, USA) using a linear gradient of acetonitrile containing 0.05% trifluoroacetic acid (TFA). The peptides were detected at 215 nm.

2.9. Identification of peptide by LC–MS and peptide sequencing

The molecular mass and De novo peptide sequencing of the peptide from cocktail protease digestions was done on positive ion mode on both the electrospray ionization–mass spectrometry (ESI–MS) and the tandem mass spectrometry (MS/MS) using the quadrapole time-of-flight micro mass spectrometer with capillary liquid chromatography capability and electrospray ion source (Waters Corp., Milford, MS). Each peptide sample was directly injected into the electrospray ion source and sampled for 10 min using a mobile phase of 98% water and 2% acetonitrile containing 0.1% formic acid at a flow rate of 1.5 μl/min. The mass scanning range was from 50 to 1500 msu at 1 second with a 0.1-s inter-scan delay in continuum mode. Glu-fibrinopeptide was used in MS and MS/MS mode and infused through the nano-Lockspray (Waters Corp., Milford, MS) for single point real time external mass calibration.

Raw spectra were processed using Masslynx software (V4.0, Waters Corp., Milford, MS). A precursor ion scan of purified antioxidant fraction was used to identify unique peptide mass, which was then fragmented using a low energy collision induced dissociation to reveal the peptide fragment for de novo sequencing.

2.10. Peptide syntheses

The antioxidant peptide was synthesized (New England Peptide, Inc.) using solid phase peptide synthesis methods. The synthesized peptide was purified by Gilson HPLC on an ODS column. The molecular masses of the isolated peptides were determined by mass spectrometry (PE Biosystems Voyager DE MALDI-TOF).

2.11. Statistical analysis

All assays were carried out in triplicate. Data were expressed as means with standard deviations. Statistical analysis was conducted on SAS (SAS institute, Inc. Cary, NC). All means were subjected to analysis of variance at $P < 0.05$ to determine statistical significance.

3. Results and discussion

3.1. Antioxidant activities of collagen hydrolysates

Porcine skin collagen was first hydrolyzed by pepsin for 24 h to produce gelatin, which showed some antioxidant activity (Table 1). Because the accessibility to the oxidant–antioxidant test systems has been shown greater for small peptides than for large peptides and protein (Moosman & Behl, 2002), we further hydrolyzed the gelatin (pepsin hydrolysate) using papain, PP, and a cocktail mixture of PP, PS and PB.

Table 1 shows the DPPH radical scavenging and metal chelating activities of the above gelatin and the gelatin hydrolysates. Among the four hydrolysates, the cocktail hydrolysate exhibited the highest radical scavenging activity (87.18 ± 1.84%), which was as strong as that of 2 mM of BHT. However, its metal chelating activity (37.4 ± 1.5%) was significantly lower than that of 1.0 mM of EDTA.

All the hydrolysates were tested for their antioxidant activity against the peroxidation of linoleic acid in a lipid peroxidation model system (Fig. 1). The cocktail hydrolysate exhibited a stronger peroxidation inhibition activity compared to the other three hydrolysates, which suggests that the antioxidative property of peptides is highly influenced by the processing conditions. In addition, the cocktail protease treatment was the most effective means of obtaining the antioxidant hydrolysate from porcine collagen. This may be due to the fact that the cocktail protease treatment is capable of increasing the DH of the collagen hydrolysate.

It was speculated that DH may influence the antioxidant activities of the hydrolysates except the certain types of enzymes. In this research, the cocktail hydrolysate had the highest DH (Table 1). To further examine the effect DH may have on antioxidant activity, the cocktail hydrolysate (24 h) was continuously hydrolyzed for 48 and 72 h (Fig. 2). The results indicated that the DH of the hydrolysates increased with time. The metal chelating activity also increased with increases in DH during the test. The radical scavenging activity of the cocktail hydrolysate also increased with DH at first (below 85%), but then decreased because most of collagen is already hydrolyzed into free amino acids when the DH of the hydrolysate is greater than 85%. Based on these findings, we believe that DH can highly affect the antioxidant properties of peptides, and that smaller peptides have a higher level of radical scavenging and metal chelating activity than larger peptides; a finding consistent with that of Moosman and Behl (2002).

The hydrolysate for 48 h had the highest radical scavenging activity (89.12 ± 2.1%) while that for 72 h had the highest metal chelating activity (85.5 ± 3.8%). The cocktail hydrolysate from 24 h had the highest peroxidation inhibition activity (Fig. 1).

Table 1
Antioxidant activities and degree of hydrolysis of the hydrolysates by different protease treatment

Sample[A]	Enzyme	Antioxidant activities[C] (%)		Degree of hydrolysis[C](%)
		DPPH radical	Iron chelating	
A	Pepsin	13.44 ± 3.22e	9.5 ± 1.2d	8.39 ± 1.04c
B	Papain	20.45 ± 4.84d	32.9 ± 2.3b	20.43 ± 3.12b
C	PP	27.01 ± 7.35c	16.5 ± 0.7c	21.55 ± 1.67b
D	Cocktail[B]	87.18 ± 1.84a	37.4 ± 1.5b	55.32 ± 4.08a
Standard		85.31 ± 0.63a,b	93.5 ± 3.3a	

a–e, Antioxidant activities/Degree of hydrolysis are significantly different at $p < 0.05$ with Student's t-test. Means followed by the same letter are not significantly different.

[A] Porcine skin collagen was hydrolyzed with pepsin (A), then A was continuously hydrolyzed with papain (B), PP (C) and cocktail (D), respectively. The concentration of sample A was 1.38 ± 0.02%, and the concentration of sample B, C and D was 1.11 ± 0.04% respectively. BHT (2.0 mM in methanol) was used as standard for DPPH radical scavenging activity of the hydrolysates and EDTA (1.0 mM in distilled water) for iron chelating activity.

[B] Cocktail mixture of PP, PS and PB (D).

[C] Results are presented as the means ($n = 3$) ±SE.

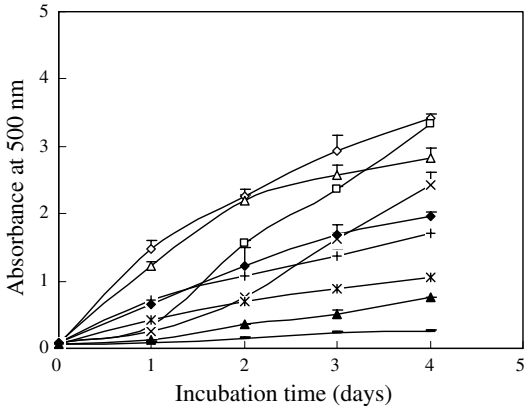

Fig. 1. Antioxidant activities of collagen hydrolysates by different protease treatment in linoleic acid oxidation system. The degree of linoleic acid oxidation was assessed by measuring optical density at 500 nm at every 24 h interval. Butylated hydroxytoluene (BHT) was used as standard. Key: (–◇–) Control; (–□–) porcine skin collagen was hydrolyzed with pepsin (A); sample A was continuously hydrolyzed for 24 h with papain (–△–), PP (–×–) and cocktail (–⁎–) respectively; (–+–) the cocktail hydrolysates of 48 h; (–♦–) the cocktail hydrolysates of 72 h; (–▲–) 2 mM BHT; (—), 20 mM BHT.

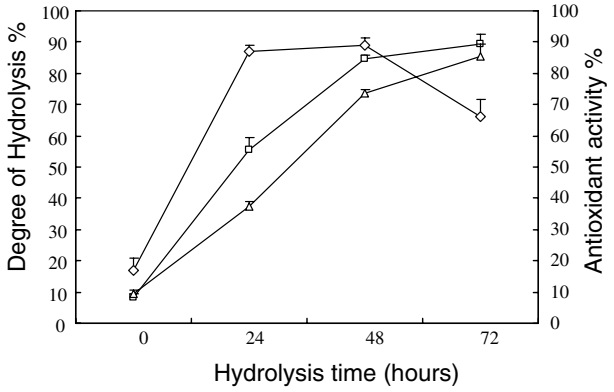

Fig. 2. Degree of hydrolysis and antioxidant activities of collagen hydrolysates by cocktail of three crude enzymes, PP, PS and PB at different hydrolysis time. Key: (–□–) DH; (–◇–) DPPH radical scavenging activity; (–△–) metal chelating activity.

When the amino acid composition of the cocktail gelatin hydrolysate was analyzed, it was rich in Hyp (19.0%), Gly (15.0%), Pro (12.6%), Glu (10.6%), Ala (8.0%) and Arg (5.2%) (data not shown). The amino acid composition of the porcine skin cocktail hydrolysate was different from that of the fish skin gelatin hydrolysate, although the major amino acid components of the two gelatin hydrolysates was same.

3.2. Purification and characterization of radical scavenging peptide (RSP)

Fig. 3 illustrates the column chromatographic profiles of the cocktail hydrolysates and their radical scavenging effects. The cocktail hydrolysate was initially separated into five fractions (P1-P5) on a Sephadex LH-20 gel filtration column as shown in Fig. 3A. Fraction P2, P3, P4 showed stronger radical scavenging activity compared with a control sample (no peptides), and fraction P4 showed the strongest activity ($61.08 \pm 1.24\%$) among all fractions. Fraction P4 was pooled according to its peak value and concentrated in vacuo prior to loading onto a DEAE-Sephadex A-25 ion exchange column. The peptides in frac-

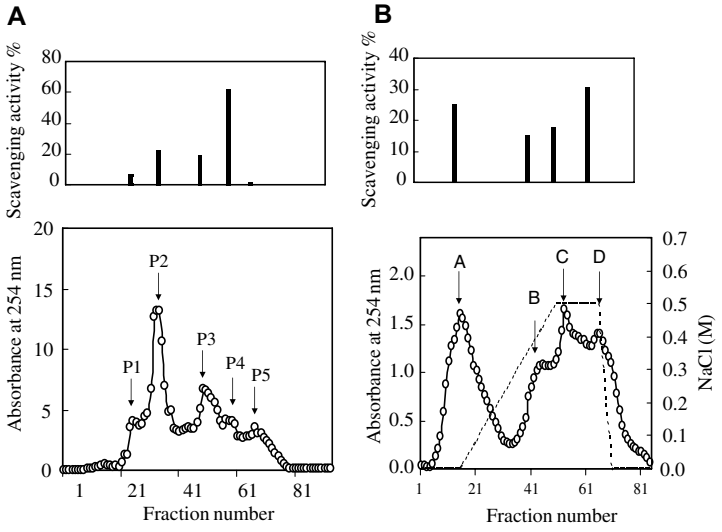

Fig. 3. Separation scheme of radical-scavenging peptide obtained from cocktail hydrolysate of porcine skin collagen (48 h). (A) gel filtration chromatography on a Sephadex LH-20 column. The hydrolysate solution was concentrated and filtered (0.45 μm), then loaded onto the gel filtration column. The sample was separated into five fractions (P1–P5) and tested for radical-scavenging activities of DPPH and illustrated as a percentage in the upper panel. (B) ion-exchange chromatography on a DEAE-Sephadex A-25. Potent fraction P4 obtained from gel filtration chromatography was separated into four fractions and tested for DPPH free radical scavenging activities.

tion P4 were then further separated into four fractions (A–D) as shown in Fig. 3B, with fraction A indicating the unbound fraction and B–D representing the bound fractions. That means one neutral or basic peptide fraction (A) and three weak acidic peptide fractions (B-D) were obtained. Among the bound fractions, fraction D had the highest radical scavenging activity ($30.33 \pm 0.44\%$). The fractions A and D were further separated by RP-HPLC on an ODS column and the fraction A yielded seven additional fractions (Fig. 4). Fraction RSP, which was fully identified, was responsible for the highest radical scavenging activity.

For peptide identification, the RSP fraction was subjected to nano-ESI + mass spectrometry. The precursor ion scan mass spectrum is shown in Fig. 5A. The MS/MS spectrum of a single charged ion with m/z at 430.91 (Da) is shown in Fig. 5B. Following sequence interpretation and a database search, the RSP with m/z 430.91 (Da) was determined to be the peptide Gln-Gly-Ala-Arg, which matched 72–75 and 180–183 residues of the α1 chain in rat (residues 1–402) skin collagen (Hulmes, Milier, Parry, Piez, & Woodhead-Galloway, 1973). However, the MS/MS spectrum for Fig. 5B did not contain the complete series of fragments; which is often the case because as the collision energy increases, the peptide may break at locations that are not an amino acid conjunction and making peptide sequencing very difficult. According to Bruni, Gianfranceschi, and Koch (2005), each peak in the spectrum can be due to the presence of any one of the various classical types of fragments (a-ion, b-ion, c-ion, x-ion, y-ion, z-ion,). As such, each ion has a unique weight depending on its components, which are determined according to the following criteria: a-ions, molecular mass of amino acid residue plus -27; b-ions, plus 1; c-ions, plus 18; x-ions, plus 45; y-ions, plus 19; z-ions, plus 3, y-NH_3-ions, plus 37; y-H_2O-ions, plus 37; etc.

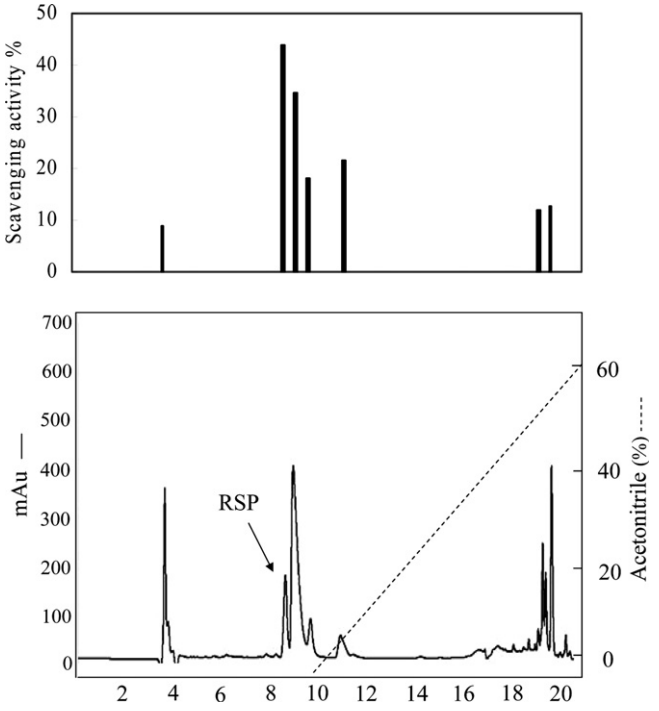

Fig. 4. The potent fraction A obtained from ion-exchange chromatography was further purified into a RSP by a C_{18} reverse phase HPLC column chromatography. The peaks from HPLC were tested for the radical scavenging activities and presented in relative activity (percentage) in the upper panel.

Although the Biolynx peptide sequencer failed to obtain an amino acid sequence from the MS/MS spectrum (Fig. 5B), each mass signal and corresponding fragmentation spectra could be matched to a single peptide fragment by manual calculation. In fact, the manual analysis gave three sequences, Gln-Gly-Ala-Arg, Lys-Gly-Ala-Arg, and Gly-Ala-Gly-Ala-Arg. This is because the fragmentation is not complete and the molecular weight of the amino acids inside the peptide chain may represent the sum of two amino acids:

$$\mathrm{Gln}(128) \quad \text{or} \quad \mathrm{Lys}(128) = \mathrm{Gly}(57) + \mathrm{Ala}(71).$$

Notice that a non-unique or a partial peptide sequence may still be considered as satisfactory if the emphasis is on identification of the protein from database blasting (Bruni et al., 2005). In our case, the selected amino acid sequence Gln-Gly-Ala-Arg agrees with the 72–75 and 180–183 residues of the α1 chain from rat skin collagen (Hulmes et al., 1973). Although it was found from the database of rat skin collagen, according to Xu, Olivry, and Chan (2004), the domain amino acid sequence identity between porcine and that of canine and mouse was calculated to be 55% and 57%, respectively.

The other three peptides derived from Fraction D were also identified (Table 2). With regard to the conversion of proline to hydroxyproline and conversion of lysine to hydroxylysine, sequences No. 2, No. 3 and No. 4 matched well with the NCBI (National Center for Biotechnology Information) database. The fragments contained between 2 and 5 residues. The sequence for No. 3 was found to be the same as that for No. 2, except for one additional amino acid residue, Hyp, at the C-terminus of No. 2. Three of the four antioxidant peptides identified had the Gln-Gly sequence. In the peptides No. 2, No. 3 and No. 4, Met and Cys may be important to the radical scavenging activity of the identified peptides because Met is prone to oxidation of the Met sulfoxide and Cys donates the sulfur hydrogen (Hernández-Ledesma et al., 2005).

Da'valos, Miguel, Bartolome', and Lo'pez-Fandiño (2004) reported that among the amino acids, Trp, Tyr, Met showed the highest antioxidant activity, followed by Cys, His and Phe. The rest of the amino acids (Arg, Asn, Gln, Asp, Pro, Ala, Val, Lys, Ile, Tre, Leu, Glu and Gly) did not exhibit antioxidant activity. Therefore, the radical scavenging activity of the peptides obtained from the β-Lactoglobulin hydrolysate by Corolase PP could be attributed to the presence of Trp, Tyr, Met and His in the identified peptides (Hernández-Ledesma et al., 2005). The antioxidative peptides isolated from soybean hydrolysate

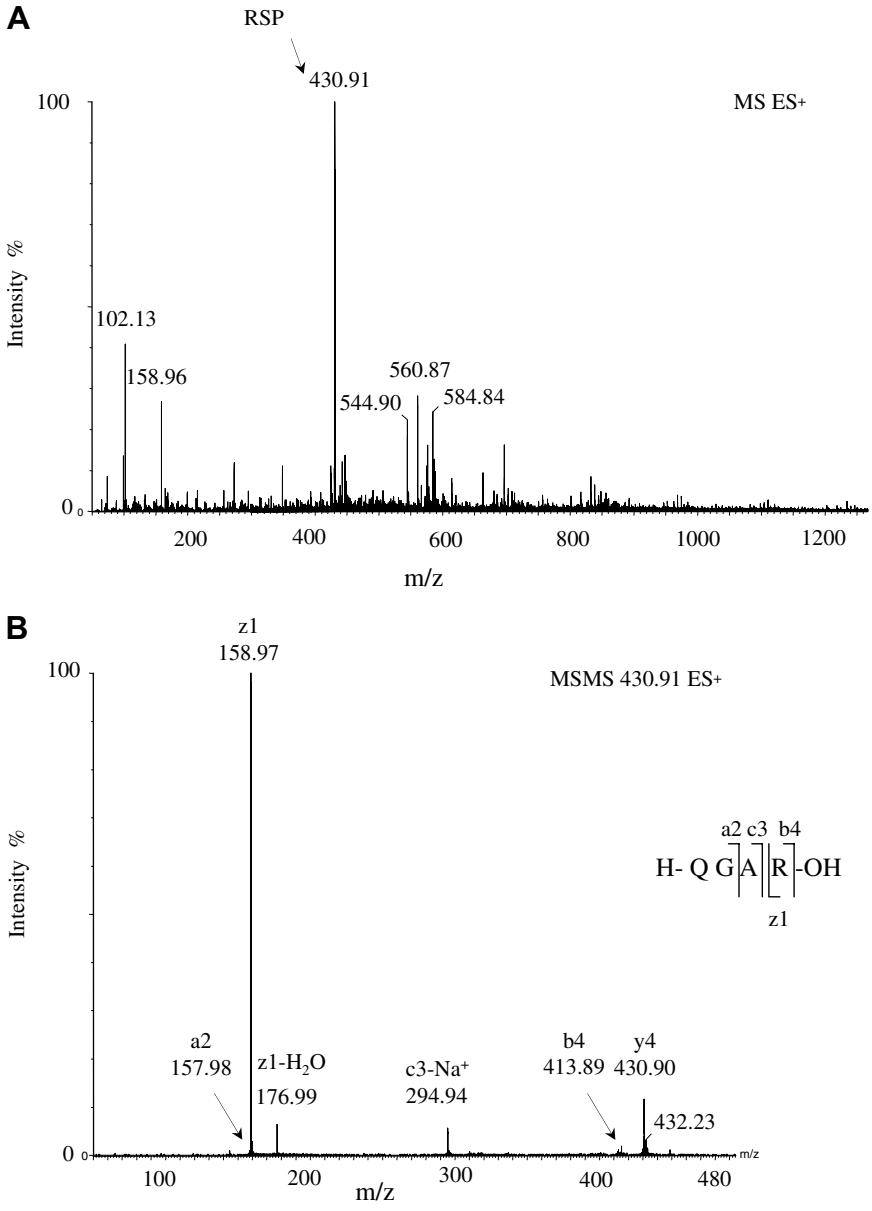

Fig. 5. Identification of the antioxidant peptide. The mass spectrum of the highest active peak (RSP) was acquired (A). The accurate molecular weight of RSP (M + H) was determined to 430.91 Da. The collision induced fragmentation of 430.9 was illustrated (B). Following sequence interpretation and database searching, the MS/MS spectrum was matched to the a1 chain of collagen (residues 72–75, 180–183).

Table 2
Sequences of antioxidant peptides identified by MS/MS in fractions separated by RP-HPLC of the cocktail hydrolysate from porcine skin collagen

Fraction	No.	MW	Sequence	Fragment
A	1	430.2	Gln-Gly-Ala-Arg	72–75[a], 180–183[a]
D	2	447.2	Leu-Gln-Gly-Met	123–126[b]
D	3	560.3	Leu-Gln-Gly-Met-Hyp	123–127[b]
D	4	265.1	Hyl-Cys	37–38[b]

[a] The amino acid sequence of the α1chain from rat (residues 1–402) skin collagen (26).
[b] The amino acid sequences are found from porcine collagen databases on NCBI (<http://www.ncbi.nlm.nih.gov/>).

(Chen et al., 1995) and egg white albumin hydrolysate (Tsuge et al., 1991) have also been attributed to His due to the proton-donation ability of the His imidazole group. It has also reported that His and Pro play an important role in the antioxidant activity of peptides designed peptides test, among which Pro-His-His was the most antioxidative (Chen, Muramoto, Yamauchi, & Nokihara, 1996).

Generally, the quenching of free radical has been attributed to the donation of hydrogen. Further, some amino acids such as His, Leu, Tyr, Met enhance the scavenging activities of peptides. However, the purified peptide RSP (Gln-Gly-Ala-Arg) in this study did not contain any of the strong proton-donating amino acid residues in the

sequence. To find out whether RSP has strong antioxidant activities, RSP was further synthesized and the antioxidant activities tested and compared to the artificial antioxidant BHT (Fig. 6A). Even though the antioxidant activity was very weak at low concentrations (less than 2 mM), RSP is a potential antioxidative peptide at high concentrations. Here, the radical scavenging activity of RSP was 40% (37.27 ± 2.66%) of BHT at 20 mM and 51% (38.48 ± 6.49%) of BHT activity at 20 mM in the lipid peroxidation system (Fig. 6B). However, the metal ion chelating activity (Fe^{2+}) was poor at 20 mM (21.4 ± 3.8%). Compared to reported antioxidative peptides (Chen et al., 1995; Saiga et al., 2003), this is a hydrophilic basic peptide.

In fact, collagen has a specifically arranged amino acid sequence where glycine strictly represents every third amino acid residue. Kim et al. (2001a, 2001b) were able to isolate gelatin-derived antioxidative peptides containing the **Gly-Pro**-Hyp sequence from marine fish and bovine skin (Kim et al., 2001b). The strong radical scavenging peptide from Hoki fish skin (His-**Gly-Pro**-Leu-**Gly-Pro**-Leu) also produced **Gly-Pro** amino acid residues with the exception of His (Mendis et al., 2005). In this study, we speculate that the **Gln-Gly** sequence may play an important role in RSP as a potent radical scavenger.

In this paper, we have investigated the antioxidant properties of peptide obtained from collagen by enzymatic hydrolysis, with the final aim of using these as antioxidant ingredients. For this purpose, collagen was hydrolyzed by different protease treatments. The results indicated that the cocktail protease treatment was the most effective to obtain the antioxidant hydrolysate in the utilization of collagen, because the cocktail protease treatment is more advantageous to the yield of antioxidative peptides than single protease treatment.

In conclusion, this research reports the results obtained from porcine skin collagen by cocktail-enzymatic hydrolysis relative to the antioxidant properties of peptides. The cocktail-derived hydrolysate exhibited higher radical scavenging and metal chelating activity than the others. Four new peptides were identified from the porcine gelatin hydrolysate by cocktail proteases treatment using tandem mass spectrometry. Of special interest is the peptide Gln-Gly-Ala-Arg, which has high antioxidant activity without strong proton-donating amino acid residues in the sequence. Results from this study indicated that it is feasible to produce natural antioxidants from porcine collagen by enzymatic hydrolysis.

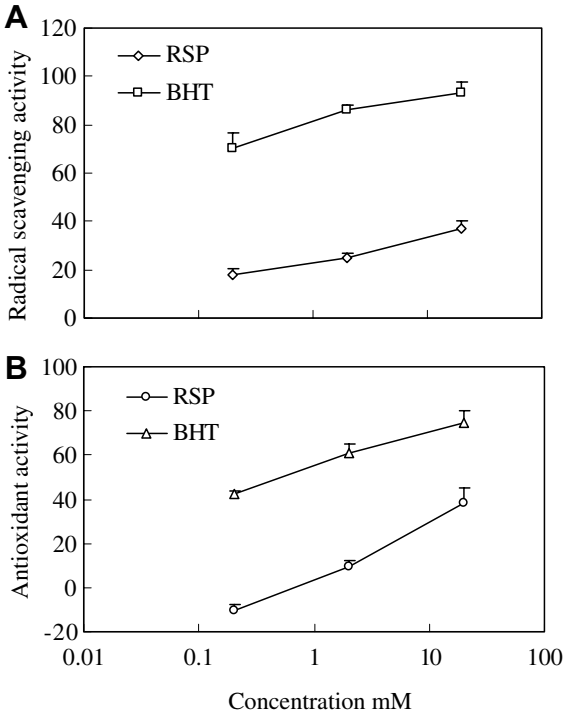

Fig. 6. Effects of RSP on antioxidant activities using DPPH radical scavenging activity (A) and linoleic acid oxidation system (B). The antioxidant activity was estimated as the rate of inhibition of hydroperoxide production at 48 h. Butylated hydroxytoluene (BHT) was used as positive controls. Values were means ± SE of triplicate experiments. Data were significantly different from the control at $P < 0.05$ with Student's *t*-test.

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