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Full Length Research Paper

An antioxidant peptide produced by autolysis reactions from wheat germ

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Wheat germ was incubated in a buffer solution of sodium citrate and dibasic sodium phosphate and its proteins were hydrolyzed by endogenous proteases to produce peptides. A peptide with high antioxidant activity was purified using ultrafiltration, Sephadex G-25 gel filtration column and consecutive chromatographic methods. The purified peptide was identified as Val-His-His-His (520.66 m/z) using reversed-phase high performance liquid chromatography (RP-HPLC) connected online to an electrospray ionization mass spectrometry. This antioxidant peptide showed high reducing power per unit, hydroxyl radical scavenging ratio and Cu^{2+} chelating ability. This study provides an economical and convenient method to isolate bioactive peptides from wheat germ.

Key words: Antioxidant peptide, wheat germ, autolysis.

INTRODUCTION

Wheat is one of the world's major grains with several million tons of annual production. As a byproduct of flour milling industry, wheat germ has not been fully utilized, except for being used to feed animals. In fact, Wheat germ is a valuable source of protein for humans because of its high content and essential amino acids. Defatted wheat germ (DWG) has relatively high protein content (27.8 to 30.0 g/100 g) (Arshad et al., 2007). Most essential amino acids are present at higher concentrations in wheat germ proteins than in the reference egg protein (Ge et al., 2001). Bioactive peptides have possible regulatory functions such as antioxidant (Zhu et al., 2006), antihypertension (Qu et al., 2010), immune defense (Tsuruki et al., 2003) and anticancer (Blanca et al., 2009). Several studies have recently reported preparation of bioactive peptides from wheat germ hydrolyzed with exogenous enzymes such as alcalase (Jia et al., 2010), flavourzyme and papain (Claver et al., 2005). Generally, wheat germ protein was first obtained through alkaline extraction and acid precipitation and then hydrolyzed. However, the active endogenous proteases in fresh wheat germ are lack of attention. Firstly, endogenous proteases have more sites of action as a system than as a protease; so it is possible to find new peptides. Secondly, the operation is simple without extraction of isolated proteins. The objective of this study is to look for an economical method of preparation of antioxidant peptides from wheat germ.

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Abbreviation: RT-HPLC, Reversed-phase high performance liquid chromatography.

MATERIALS AND METHODS

Fresh wheat germ was kindly donated by Xuzhou Xueyuan Flour Co. Lpd (Jiangsu, China). When peeled from wheat, it was immediately stored at 20°C until use. Wheat grains appeal white and hard skin, a technology named Middle Road Preparation of flour was used during the milling process. Sephadex G-25 was purchased from Pharmacia Co. Shanghai Agency (Shanghai, China). All other chemicals and reagents used were of the highest analytical grade commercially available.

Incubation of wheat germ

To activate endogenous proteases in wheat germ, four factors including pH, temperature, solid-solvent ratio (m/v) and time were optimized using Response Surface Method (RSM) during

incubation.

The best condition was done with pH 4.39 at 45°C for 35 and 270 min. After hydrolysis, the mixture was heated in boiling water for 10 min to inactivate enzymes, and centrifuged at 4000 g for 15 min. The supernatants were then collected for purification.

Purification of the antioxidant peptide

Ultra filtration was first applied for purification. The supernatant from wheat germ incubation was passed through a 0.45 µm filter membrane, and then fractionated through four different UF membrane bioreactor systems (Millipore Co. Lpd), with ranges of molecular weight cut-offs (MWCO) of 100, 50, 10 and 3 KDa, respectively. Five fractions were obtained, lyophilized in a freezed drier for the determination of peptide content and reducing power. The fraction with the strongest antioxidant activity was loaded onto a Sephadex G-25 gel chromatography column (2.5 × 90 cm) which was previously equilibrated with distilled water (Je et al., 2004). The column was eluted with distilled water at a flow rate of 1.0 ml per min; the effluent was continuously monitored at 280 nm and collected at 2 min intervals (4 ml par tube). Elutes were lyophilized and compared for antioxidant activity. The fraction with the highest antioxidant activity was injected into a Waters Bridge prep C18 (19 mm × 150 mm) preparative reverse phase HPLC column and eluted with a linear gradient of acetonitrile and 0.1% trifluoroacetic acid (TFA, 0 to 50% v/v, for 15 min) at 6.0 ml par min flow rate. Finally, the peak with the highest activities was purified on a Zorbax SB C18 (4.6 × 250 mm) reverse phase HPLC column (Agilent Technologies, USA) with a linear gradient of acetonitrile and 0.1% TFA (same as that given previously) at 0.5 ml per min rate. The activity of the top two peaks was compared, and the highest one was chosen for structure identification.

Identification of peptide by LC-ESI-MS/MS

The most potent peptide was dissolved in 50% methanol and 50% water of HPLC grade, infused into a Finnigan TSQ Quantum ultra AM mass spectrometer (Thermo, USA) at a flow rate of 30 µL per min, which was operated in the positive electro spray ionization (ESI + ve) mode, via the electro spray interface. High purity nitrogen was used for the drying (35 psi) and ESI nebulizing gas (45 psi). It was recorded over the mass/charge (m/z) range of 0 to 700. Amino acid composition was determined by L-8900 Automatic amino acid analyzer (Hitachi, Japan). The peptide sequencing was performed by manual calculation.

Peptide content assay

Peptide content was measured using the method described by Umemoto (1966). An aliquot of 3 ml sample was added to 3 ml 10% (w/v) TCA solution, mixed vigorously for 30 s and centrifuged at 4000 g for 15 min. A volume of 3 ml from the supernatant was then mixed thoroughly with 2 ml biuret reagent (sample solution: biuret reagent = 3:2, v/v), stood still for 10 min and centrifuged at 4000 g for 15 min again. Finally, the supernatant was monitored at 540 nm, and the content of the sample peptides was calculated in accordance to standard plot. Gluthatione-γ-L-glut amyl-L-cysteinylglycine was used as standard preparation.

Measurement of antioxidant activity

Reducing power

Reducing power was measured according to the method of Zhu et al. (2006) with slight modifications. Antioxidants transformed electronic Fe^{3+} to Fe^{2+} absorbance in 700 nm and revealed the number of Fe^{2+}. Reducing power reflecting electron donating ability was closely related to antioxidant activity. However, 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide (w/v) were added to an aliquot of 1 mL sample solution. The mixture was incubated at 50°C for 20 min, cooled down quickly, and then 2.5 ml of 10% TCA (w/w) was added to it. The mixture was then centrifuged at 10,000 g for 10 min. Finally, 2.5 ml supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride (w/v) in a test tube. After a 10 min reaction time, the absorbance of the solution was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Triplicate tests were conducted for each sample. The reducing power per unit was indicated with the absorbance and defined as follows:

$$\text{Reducing power per unit} = \frac{A_{700}}{peptide\ content}$$

Scavenging activity of DPPH radical

DPPH is a stable nitrogen centered free radical that changes the color of ethanol solution to purple with 517 nm maximum absorption. When DPPH solution was added to radical scavenger, its lone electron pair was saturated, which resulted in disappearance or weakness of absorption and lighter color. The degree of color change was linear with free radical scavenging. The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured according to the method of Xie et al. (2008) with some modifications. An aliquot of 2 ml of sample solution at different concentrations (0.0 to 1.0 mg/ml) was mixed with 2 ml of DPPH alcoholic solution (0.2 mM), shaken vigorously and then incubated for 30 min in darkness at room temperature. The resulting solution was measured at 517 nm with spectrophotometer. Alcohol was used as a control. The scavenging activity was calculated by the following equation:

$$\text{Scavenging ration (\%)} = (1 - A_{sample} / A_{control}) \times 100$$

Scavenging activity of hydroxyl radical

Hydroxyl radical scavenging and results calculation was used according to the method of Zhang et al. (2011). Concentration of H_2O_2 is proportional to the amount of hydroxyl radicals generated by Fenton reaction. When given electron acceptor, it formed a red substance whose color was proportional to the amount of hydroxyl radicals. The hydroxyl radical scavenging ability of samples is inverse ratio with light color of reaction solution. Briefly, $FeSO_4$ (0.75 mM) and 1.10-phenanthroline (0.75 mM) were dissolved in a phosphate buffer (pH 7.4), mixed thoroughly and 0.01% of H_2O_2 (w/v) was added to the sample. The mixture was then incubated at 37°C for 60 min, and the absorbance was measured at 536 nm.

Cu^{2+} chelating activity

Cu^{2+} chelating activity was measured according to the method described by Zhang et al. (2011). A mixture of catechol and copper ion resulted to a blue color with 632 nm absorbance. After dissociation of copper ions and catechol, no absorbance was shown at 632 nm; so this mixture resulted to a faded blue after adding a metal ion chelator. As such, the absorbance could reflect metal

Table 1. Proportion and reducing power per unit of different *M*w fractions.

Index	Fraction				
	U-5	U-4	U-3	U-2	U-1
Peptide proportion (%)	46.40 ± 0.07[a]	7.75 ± 0.03[d]	16.60 ± 0.05[b]	16.32 ± 0.03[b]	12.93 ± 0.04[c]
Reducing power per unit	0.24 ± 0.01[a]	0.22 ± 0.02[a]	0.16 ± 0.01[c]	0.15 ± 0.02[c]	0.19 ± 0.01[b]

The wheat germ incubation permeation solution was defined as follows: U-1, It did not permeate 100 KDa membrane; U-2, WGI permeated 100 KDa membrane but not 50 KDa; U-3, WGI permeated 50 KDa membrane but not 10 KDa; U-4, WGI permeated 10 KDa membrane but not 3 KDa; and U-5, permeated 3 KDa membrane.

chelation ability. However, an aliquot of 1 ml $CuSO_4$ (2 mM) was mixed with 1 ml of 10% pyridine (w/v) and 20 µl of 0.1% pyrocatechol violet (w/v). Then 1 ml sample was added to the mixture, stirred and it reacted for 5 min. The disappearance of the blue color was monitored by measuring the absorbance at 632 nm. Deionized water was used as control. The chelating activity was obtained by the following formula:

$$Cu^{2+}\text{ chelating activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100\%$$

In the equation, A_0 represented the sample absorbance, while A_1 represented the control absorbance.

Statistical analysis

Each data point represented the mean and standard deviation of three samples subjected to analysis of variance followed by multiple range tests. However, the significance level of $p < 0.05$ was employed.

RESULTS AND DISCUSSION

Isolation of the antioxidant peptide

After ultra filtration, each fraction was named U-1, U-2, U-3, U-4 and U-5. They were pooled, lyophilized and measured for reducing power per unit. The yield and reducing power per unit of different molecular weight (MW) fractions is shown in Table 1. It was observed that U-5 had the largest amount of peptide content (46.40%). However, both U-5 and U-4 showed significantly high activities in reducing power. Considering its highest production, U-5 was dissolved in distillated water at 50 mg/ml for further purification, loaded onto a Sephadex G-25 column and eluted with deionized water. The eluted solution was monitored at 280 nm, and the fraction was pooled into three portions (Figure 1). Each portion was dealt with as shown previously. The results showed that S-2 had the highest activity (Figure 1). Consequently, S-2 (30 mg/mL) was then subjected to a Waters Bridge prep C18 preparative reverse phase HPLC column and divided into three peaks: P- 1, P- 2 and P- 3 (Figure 2). After comparison, P- 3 (20 mg/mL) was chosen and further separated by RP-HPLC on a Zorbax SB C18 (Figure 3). RP-HPLC showed that there were two main components (A-1 and A-2) in P- 3. Reducing power per unit of A-1 (9.20 ± 0.81 mg^{-1}) which was close to GSH (9.80 ± 0.70 mg^{-1}) ($p > 0.05$) was significantly higher than that of A-2 (4.00 ± 0.16 mg^{-1}), and was collected for identification. It was reported that the lower the molecular weight, the higher the chance of crossing the intestinal barrier and exerting biological effects on it (NSS et al., 2010); therefore, low MW peptides showed more active weights (Suetsuna et al., 2000).

Identification of the antioxidant peptide

A-1 was subjected to LC-ESI-MS/MS analysis, and its MS spectrum is shown in Figure 4. The MS/MS spectrum of a single charged ion with m/z at 520.66 is illustrated in Figure 5. The fragmentation spectrum contained major ion at m/z 384.94 and 249.03. The results indicate the loss of 135.7 in MW from a fragment of 520.87 and 384.94 respectively which indicate histidine (His) presented in the peptide. Amino acid composition analysis reveals that there are 3.65% valine (Val) and 14.61% His in A-1fraction. Therefore, the peptide sequence as Val-His-His–His (VHHH) was determined. The sequence of the purified peptide showed the presence of 25% valine and 75% histidine, which are both essential amino acid for people. Hydrophobic amino acid valine located at N side was combined with three histidines. Although, the relation between the structure and biological activity of peptides was not fully clear, some had demonstrated that molecular weight, amino acid type and sequence of peptides played a key role on the activity (Xie et al., 2008; Zhang et al., 2011; NSS et al., 2010).

Antioxidant activities of the purified peptide

Different systems as direct free radical scavenging and metal ion chelating were used for antioxidant activity evaluation of VHHH. Firstly, hydroxyl and DPPH radical were employed. VHHH can quench hydroxyl radical effectively in a dose dependent manner (Figure 6). The scavenging ration was up to 76.43 ± 4.71% in the presence of 1.0 mg/mL concentration, but the scavenging

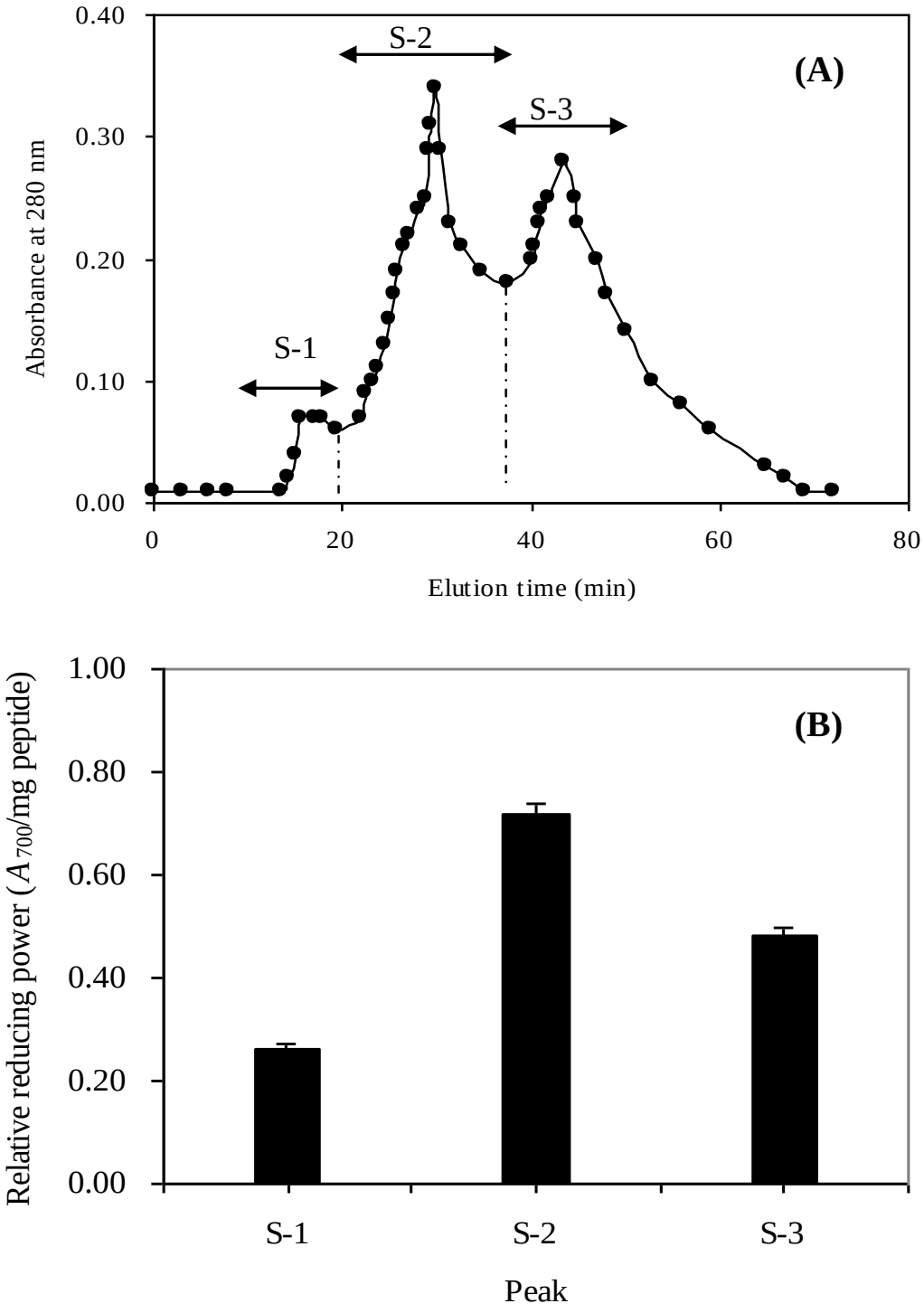

Figure 1. Antioxidant activity of S-1, S-2 and S- 3 after Sephadex G-25.

activity was not so excellent on DPPH (Figure 6), since it was related to the difference seen in their chemical activities. Hydroxyl radical had the strongest activity among all radicals, while DPPH was relatively stable. Hydroxyl radical can easily react with the bioactive molecule such as lipids, proteins, DNA etc., which will bring damage or disease to the living body (Suetsuna et al., 2000). So elimination may be the most effective way

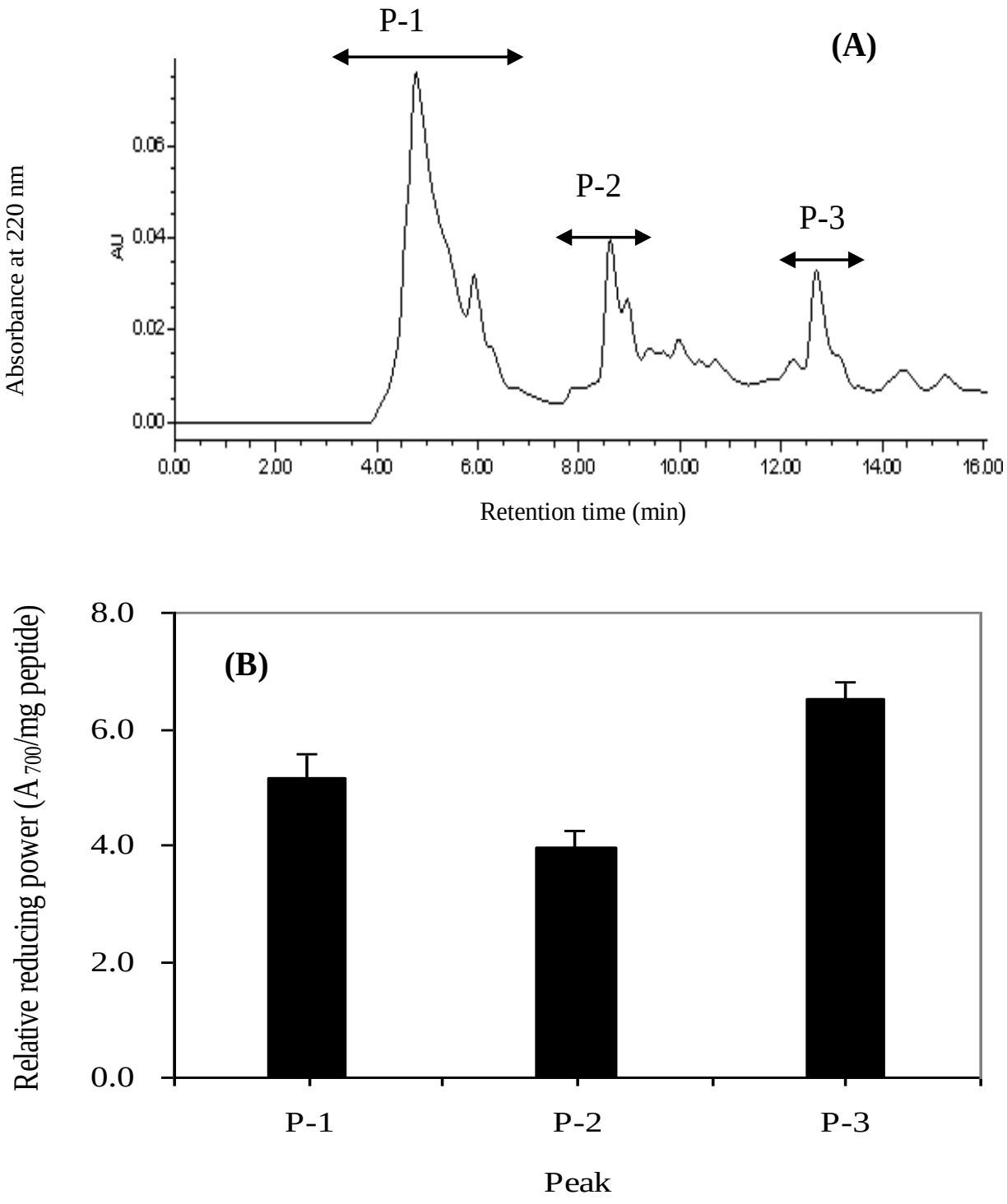

Figure 2. Antioxidant activity of P-1, P-2 and P-3.

against those diseases. Je et al. (2007) also showed that the peptide of APTBP had more active hydroxyl radical than other radicals. Peptide with hydrophobic amino acid valine or leucine in the N side showed more potent antioxidation; this may be due to better interaction of hydrophobic amino acids and fatty acids (Chen et al., 1966). Riisom et al. (1980) and Rajapakse et al. (2005) confirmed that peptide containing His appeared to have stronger antioxidant activity; whereas the imidazole ring could chelate metal ions and remove free radicals. Seo et

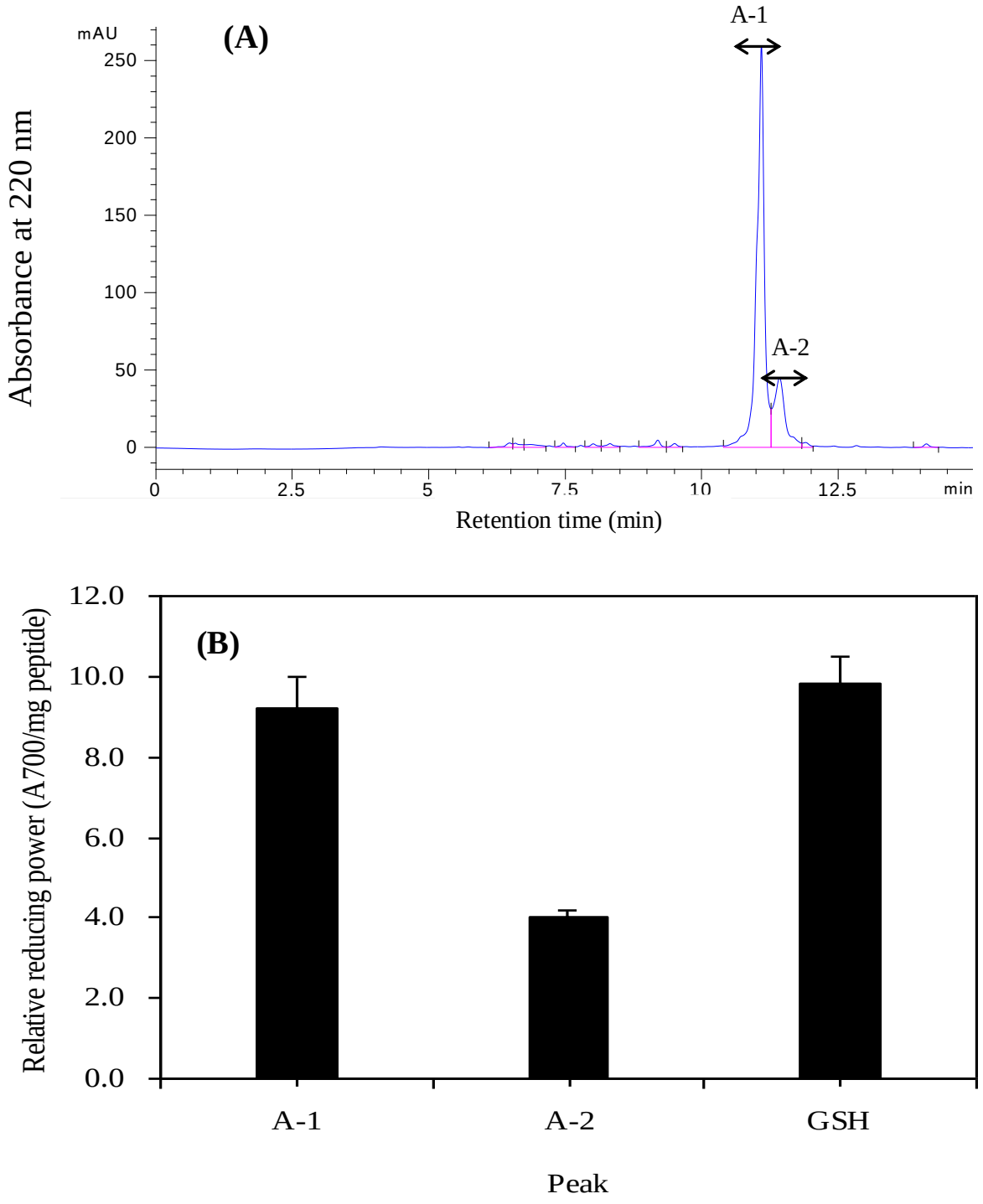

Figure 3. Antioxidant activity of A-1 and A-2.

al. (2010) even thought His-His in the C terminal could synergize with other antioxidants. Secondly, Cu2+ was tested for metal ion chelating ability. VHHH appeared to have a relatively high Cu2+ chelating ability in a dose dependent manner (Figure 6), due to the fact that it can chelate 65% Cu2+ at the concentration of 1.0 mg/ml. It is thought that acidic and basic amino acids play an important role in the chelation of metal ions by carboxyl and amino groups in their side chains (Suetsuna et al., 2000). In addition, histidine was frequently observed in

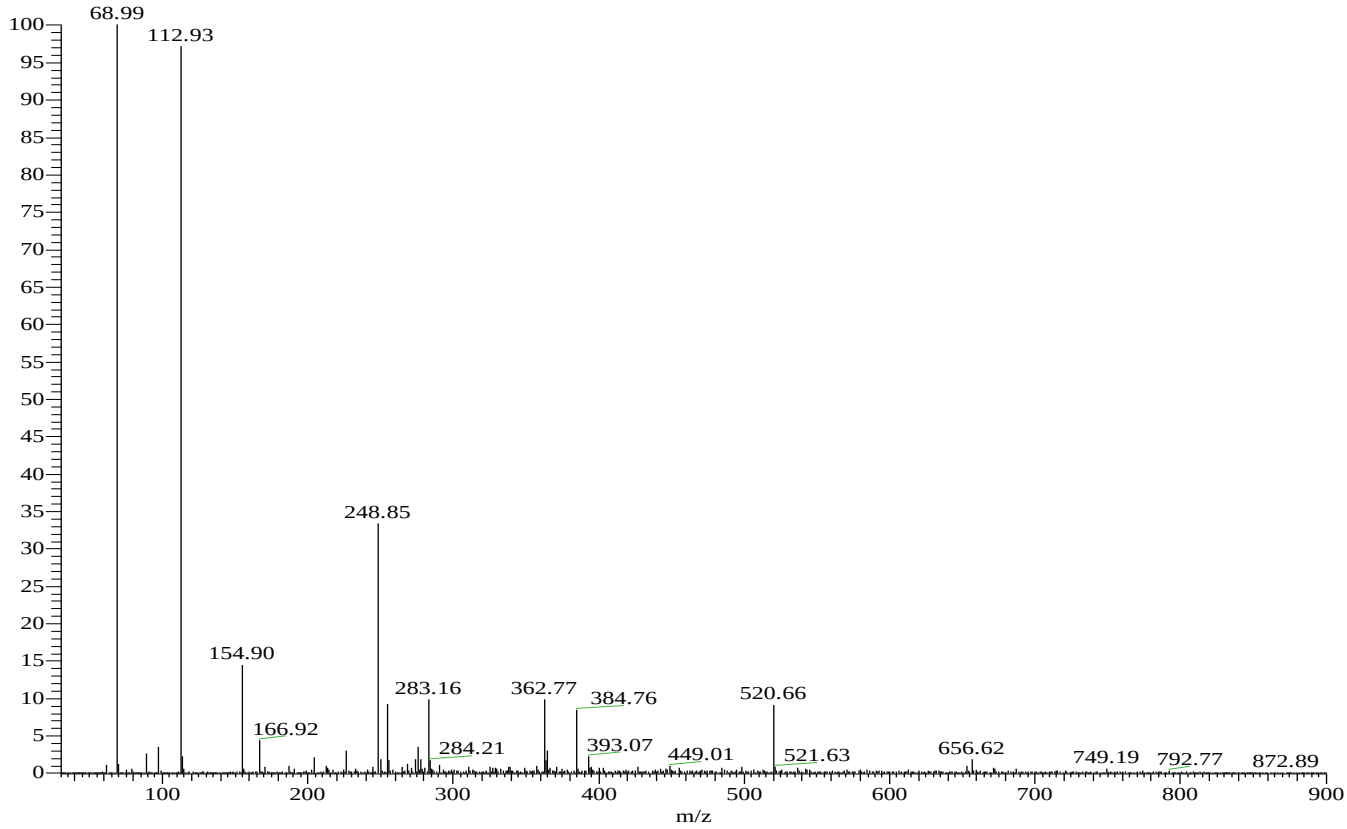

Figure 4. ESI mass spectra of A-1.

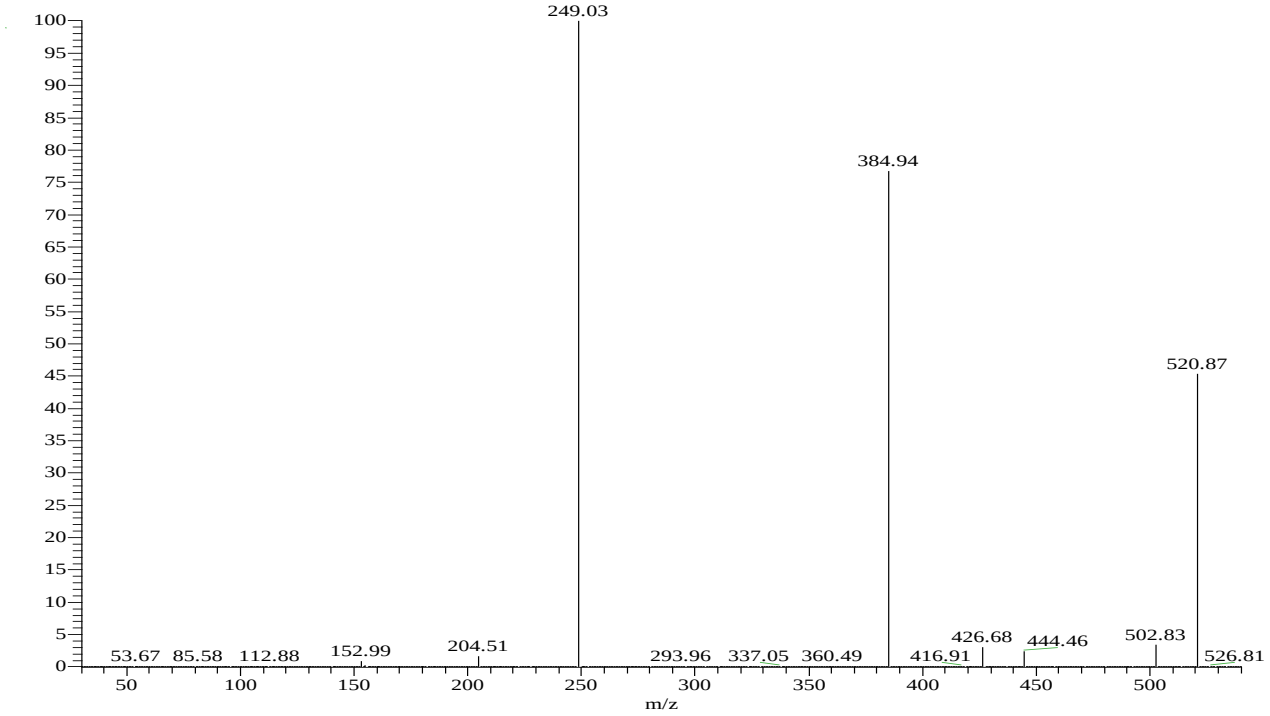

Figure 5. MS/MS spectrum of ion *m/z* 520.66.

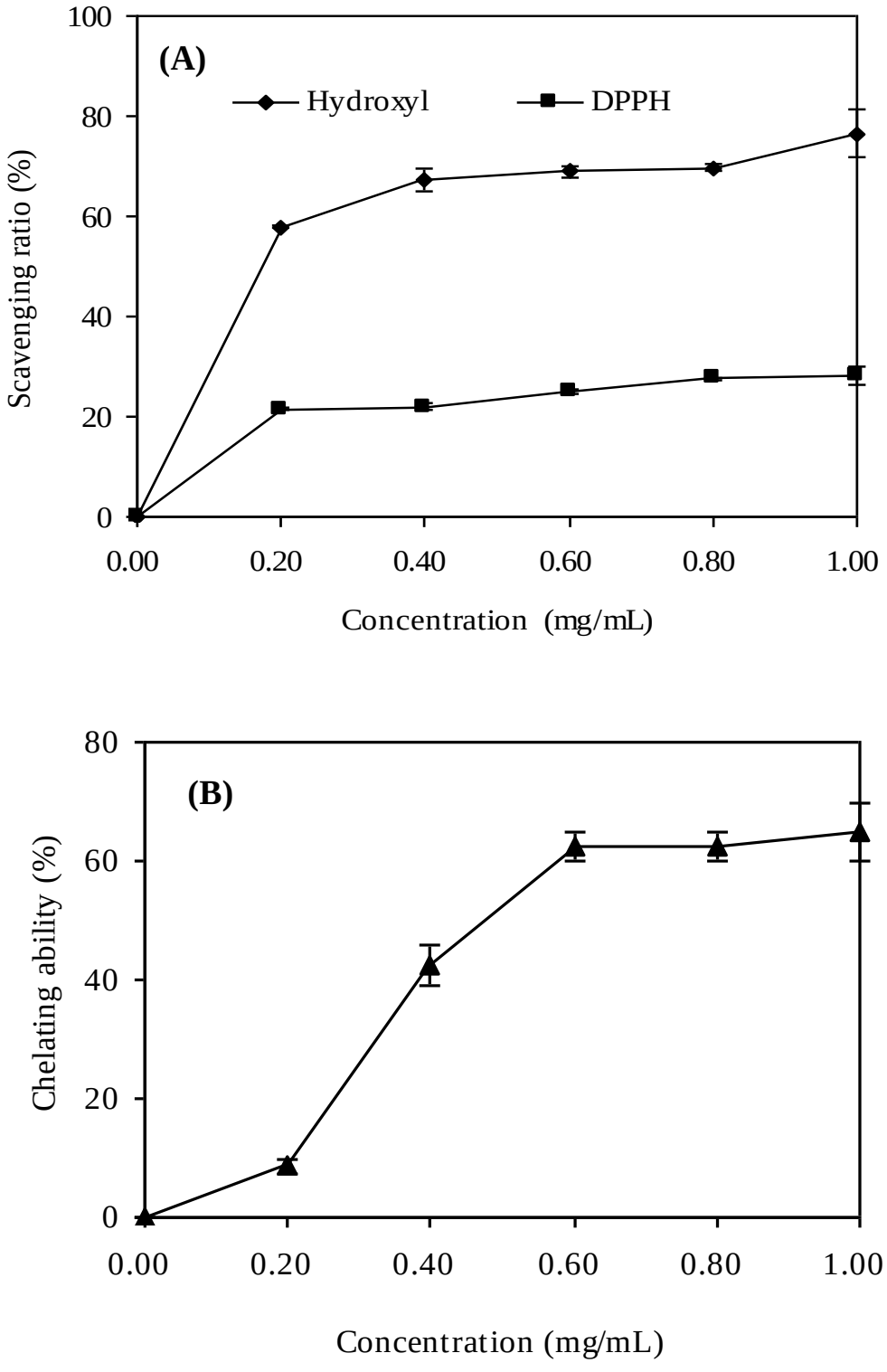

Figure 6. Antioxidant activities of the purified peptide.

sequences of peptide ion chelators; as such, the three histidine residues found in the tripeptide strengthened this ability.

Conclusions

Endogenous protease in wheat germ was activated for

hydrolysis of its own proteins. After consecutive purification, a peptide with good hydroxyl radical scavenging ability was obtained. Usage of endogenous protease provides a new way for designing functional peptide. However, further studies on antioxidant activities *in vivo* are necessary for the purified peptide.

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