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Free Radical Scavenging Activity of Enzymatic Hydrolyzates of Hot Water Extract from the Shell of Reeve's Turtle (*Chinemys reevesii*)

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The shell of Reeve's turtle has been used as a traditional folk medicine in Korea. We produced a hot water extract from Reeve's turtle shell according to the traditional medical practice. To release bioactive peptides, the hot water extract was enzymatically hydrolyzed with various proteases, and the free radical scavenging activity of the hydrolysate was investigated against 1,1-diphenyl-2-picryl-hydrazyl (DPPH), hydroxyl and peroxyl radicals. The free radical scavenging activity of the enzymatic hydrolysates varied from 1 to 79% depending on the enzymes, free radical species, and concentration. The EC_{50} values demonstrated that the enzymatic hydrolysates of hot water extract from the shell of Reeve's turtle are potential antioxidants.

Key words: Antioxidant activity, Reeve's turtle shell, Free radical, Enzymatic hydrolysis

Introduction

Reactive oxygen species (ROS) and free radicals such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) are an unavoidable consequence in aerobic organisms during respiration. Under normal conditions, ROS and free radicals are effectively eliminated by the antioxidant defense system such as antioxidant enzymes and non-enzymatic factors. However, under pathological conditions, the balance between the generation and elimination of ROS is broken, as a result of these events, biomacromolecules including DNA, membrane lipids and proteins are damaged by ROS-mediated oxida-tive stress. Uncontrolled generation of free radicals that attack membrane lipids, proteins and DNA is believed to be involved in many health disorders such as diabetes mellitus, cancer, neurodegenerative and inflammatory diseases (Pryor and Ann, 1982; Butterfield et al., 2002). Free radical and ROS scavengers are used as preventive antioxidants. They exert their abilities by scavenging free radical and ROS, preventing the generation of free radical and ROS, or activating a battery of detoxifying proteins. Therefore, an antioxidant supplement is important for the prevention and/or reduction of oxidative stress.

The shell of Reeve's turtle has been used as a traditional folk medicine in Korea, however, its functional components and biological activities have been unclear until now. Therefore, in this study, our aim was to examine the antioxidant activity of enzymatic hydrolysates of hot water extract from the shell of Reeve's turtle, prepared according to the traditional medical practice.

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Materials and Methods

Reagents

All chemicals including DPPH, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2,2-azobis-(2-amidinopropane)-hydrochloride (AAPH) and α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (4-POBN) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals and reagents used were of analytical grade commercially available.

Sample preparation

Reeve's turtle shell was donated by Shin Hung

Pharm. Co. (Yeosu). Hot water extract was prepared at 120°C for 2 hr (shell:water=1:2.5, w/v) and then hydrolyzed using various proteases such as alcalase, flavourzyme, neutrase, protamex and trypsin. One hundred milliliters of 0.1 M sodium phosphate buffer solution and then 10 μL (or mg) of each enzyme were added to 1 g of the dried sample. The reaction was mixed after pre-incubation for 30 min. The enzymatic hydrolysis reactions were performed for 8 hr to achieve an optimum hydrolytic level and were then immediately heated at 100°C for 10 min. Finally, the supernatants were filtered, lyophilized and stored at -20°C until use. The optimum hydrolysis conditions for each enzyme were as follows: alcalase, pH 7.0, 50°C; flavourzyme, pH 7.0, 50°C; neutrase, pH 7.0, 50°C; protamex, pH 7.0, 50°C; and trypsin, pH 7.0, 37°C.

Amino acid analysis

An aliquot of hot water extract (50 mg) was hydrolyzed with 6.0 N HCl in a vacuum-sealed ampoule at 110°C for 24 hr for amino acid analysis. The HCl was removed on a rotary evaporator, and the sample was brought to a volume of 10 mL with 0.2 M sodium citrate buffer (pH 2.2). Amino acids were determined with a Biochrom 20 amino acid analyzer (Biochrom Ltd., Cambridge, UK) using a single ion-exchange resin column (4.0×150 mm) with ninhydrin as the color reactant.

DPPH radical scavenging activity

DPPH radical scavenging activity was measured as described by Nanjo et al. (1996) A 60 μL of each hydrolysate (or ethanol itself as control) was added to 60 μL of DPPH (60 μM) in ethanol solution. After mixing vigorously for 10 s, the solution was transferred into a 100 μL quartz capillary tube, and the scavenging activity of the hydrolysates on DPPH radical was measured using a JES-FA electron spin resonance (ESR) spectrometer (JEOL Ltd., Tokyo, Japan). The spin adduct was measured on an ESR spectrometer exactly 2 min later. Experimental conditions were as follows: magnetic field, 336.5±5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000; sweep time, 30 sec.

Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by iron-catalyzed Haber-Weiss reaction (Fenton driven Haber-Weiss reaction) and the generated hydroxyl radicals were rapidly reacted with nitrone spin trap DMPO (Rosen and Rauckman, 1984). The resultant DMPO-OH adduct was detectable with an ESR spectrometer. Various concentrations of the hydrolysate was mixed with DMPO (0.3 M), $FeSO_4$ (10 mM) and H_2O_2 (10 mM) in a phosphate buffer solution (pH 7.2), and the mixture was transferred into a 100 μL quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. Experimental conditions were as follows: magnetic field, 336.5±5 mT; power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1×200; sweep time, 4 min.

Peroxyl radical scavenging activity

Peroxyl radicals were generated by AAPH. A phosphate buffered saline (PBS) reaction mixture containing 10 mM AAPH, 10 mM 4-POBN and the hydrolysate at various concentrations was incubated for 30 min at 37°C in a water bath (Hiramoto et al., 1993), and then transferred to 100 μL quartz capillary tube. The spin adduct was recorded using an ESR spectrometer. Experimental conditions were as follows: magnetic field, 336.5±5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000; sweep time, 1 min.

Statistical analysis

The data are presented as mean ± S.E. of three determinations. The significance of differences was determined by Student's *t*-test. Values with $p<0.05$ were considered significant.

Results and Discussion

The shell of Reeve's turtle has been used as a traditional folk medicine in Korea, but the pharmaceutical characterization of this material has not been undertaken until now. We extracted the functional components using traditional medical practice

Table 1. Amino acids contents of hot water extract

Amino acids	Contents (g/100 g)
Aspartic acid	4.33
Threonine	2.22
Serine	1.56
Glutamic acid	4.89
Proline	1.62
Glycine	1.92
Alanine	1.45
Cysteine	0.07
Valine	2.11
Methionine	0.96
Isoleucine	1.01
Leucine	1.14
Tyrosine	0.89
Phenylalanine	1.52
Histidine	1.47
Lysine	1.43
Arginine	0.87
Total	29.46

and then hydrolyzed the hot water extract using various proteases. The amino acid analysis showed that the extract is rich in aspartic acid, glumatic acid, valine, threonine, proline and histidine (Table 1). Several reports have suggested that phenolic hydroxyl groups present in aromatic amino acids contribute substantially to the scavenging of radicals by acting as potent electron donors (Suetsuna et al., 2000). Other amino acids such as histidine, proline, alanine and leucine have been reported to contribute to free radical scavenging as well (Kim et al., 2001). Hernández-Ledesma et al. (2005) have reported that the high antioxidant activity of tryptophan and tyrosine may be explained by the capacity of the indolic and phenolic groups, respectively, to serve as hydrogen donors, resulting in the formation of more stable indoyl and phenoxyl radicals. Therefore, we could expect small peptides generated by enzymatic hydrolysis to show antioxidant activity by scavenging free radicals. DPPH is a stable free radical and accepts an electron or hydrogen to become a stable diamagnetic molecule. Therefore, DPPH is often used as a substrate to evaluate antioxidant activity. As shown in Fig. 1A, the ESR spectrum with neutrase hydrolysate decreased with increasing concentrations of hydrolysate. All of the enzymatic hydrolysates exhibited dose-dependen t DPPH radical scavenging, with enzyme-dependent activities ranging from 22.36%

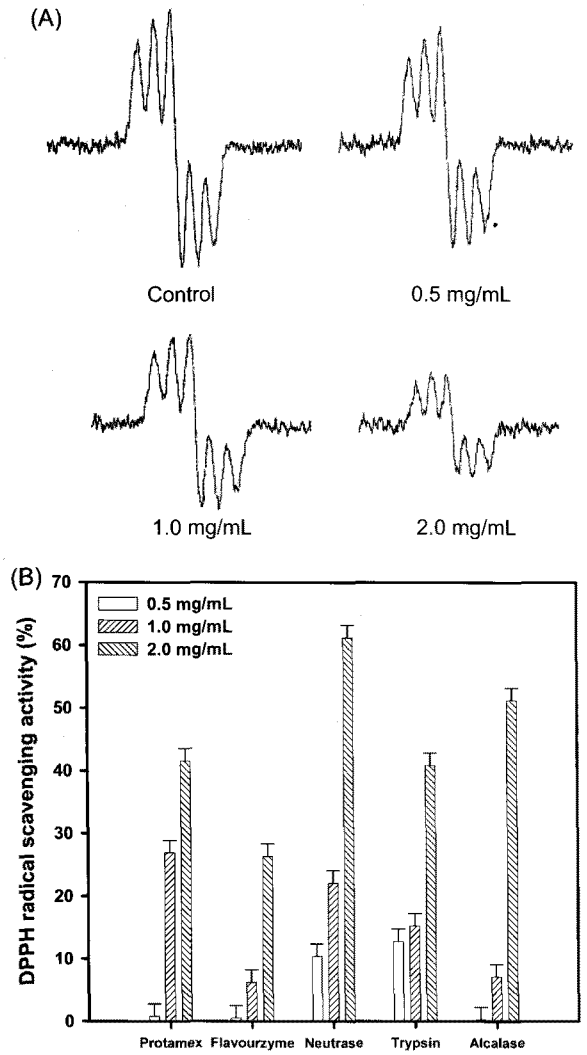

Fig. 1. (A) DPPH radical ESR spectrum with neutrase hydrolysate. (B) DPPH radical scavenging activity of the enzymatic hydrolysates from Reeve's turtle shell hot water extract measured by ESR spectroscopy. Values represent mean ± SD (n=3).

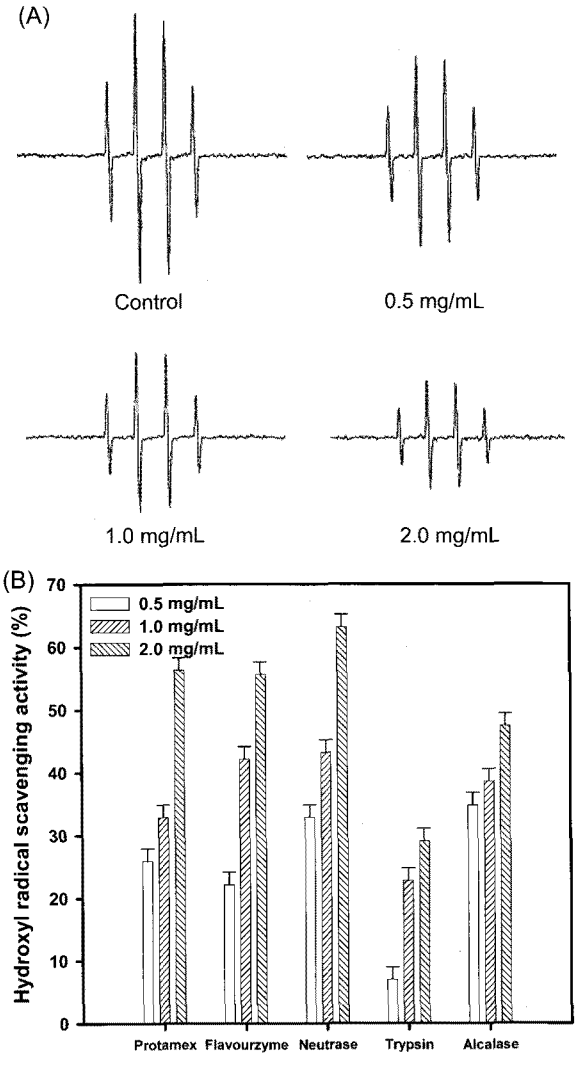

Fig. 2. (A) Hydroxyl radical ESR spectrum with neutrase hydrolysate. (B) Hydroxyl radical scavenging activity of the enzymatic hydrolysates from Reeve's turtle shell hot water extract measured by ESR spectroscopy. Values represent mean ± SD (n=3).

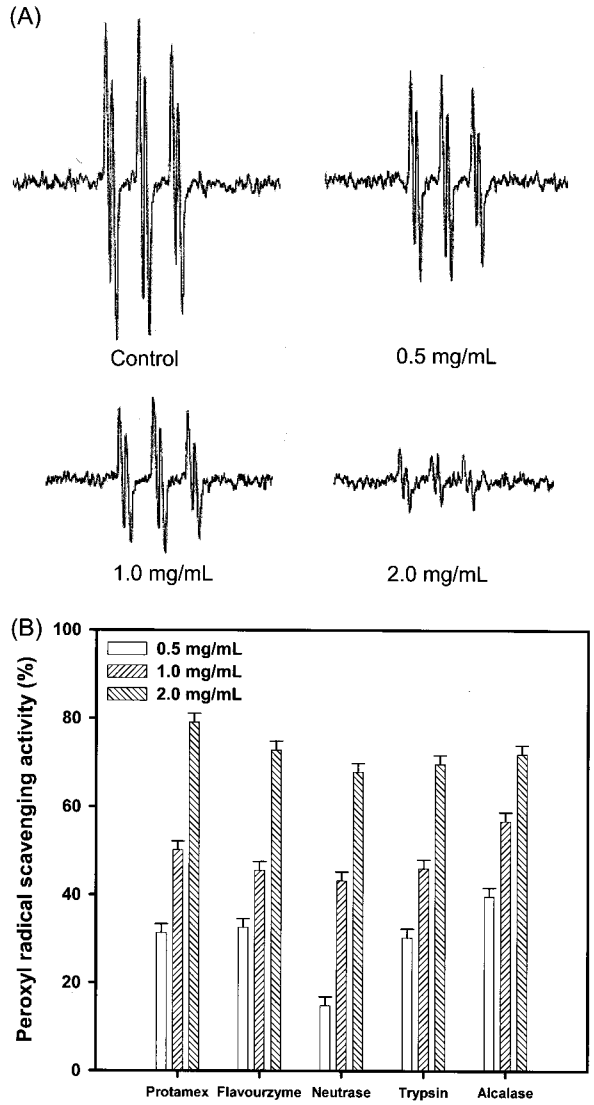

Fig. 3. (A) Peroxyl radical ESR spectrum with protamex hydrolysate. (B) Peroxyl radical scavenging activity of the enzymatic hydrolysates from Reeve's turtle shell hot water extract measured by ESR spectroscopy. Values represent mean ± SD (n=3).

for the flavourzyme hydrolysate to 61.24% for the neutrase hydrolysate, at the concentration of 2.0 mg/mL (Fig. 1B). Hydroxyl radicals were generated in a Fenton reaction and visualized by ESR spectrometry. The ESR signal is inhibited by the presence of ·OH scavengers, which compete with DMPO for ·OH. As shown in Fig. 2A, the neutrase hydrolysate ESR spectrum was decreased with increasing concentrations of hydrolysate. All of the enzymatic hydrolysates effectively and dose-dependently suppressed hydroxyl radicals although to different extents (Fig. 2B). At a concentration of 2.0 mg/mL, the neutrase hydrolsate showen the greastest hydroxyl radical scavenging activity at 63.21%, followed by the protamex (56.33%), flavourzyme (55.70%), trypsin (29.11%) and alcalase (47.47%) hydrolysates. Among the ROS, the hydroxyl radical has the strongest chemical activity and readily reacts with biomolecules such as amino acids, proteins, and DNA (Cacciuttolo et al., 1993). Therefore, the removal of the hydroxyl radical is probably one of the most effective defenses against various diseases.

AAPH can decompose to form carbon-centered radicals that can react swiftly with O_2 to yield peroxyl radicals, which stimulate lipid peroxidation (Mossman, 1983). As shown in Fig. 3A, the protamex hydrolysate ESR spectrum decreased in a dose-dependent manner. In decreasing order of strength, the peroxyl radical scavenging activities of the protamex, flavourzyme, alcalase, trypsin, and neutrase hydrolysates were 79.14, 72.87, 71.97, 69.73 and 67.88%, respectively, at a concentration of 2.0 mg/mL. The protamex hydrolysate showed strong peroxyl radical scavenging activity, in contrast to its weaker DPPH and hydroxyl radical scavenging activities. The differrences in the relative scavenging activities of the hydrolysates may be attributed to differences in the sizes and amino acid sequences of the peptides released by the various proteases, owing to their different specific activities on the hot water extract of Reeve's turtle shell. The EC_{50} values for the scavenging activities of the enzymatic hydrolysates were calculated by the non-linear regression method and are summarized in Table 2. These results indicate that some of the enzymatic hydrolysates are potent antioxidants.

This study demonstrates that enzymatic hydrolysates of Reeve's turtle shell hot water extract

Table 2. EC_{50} values of the enzymatic hydrolysates and vitamin C as positive control. [a]EC_{50} value was defined as the concentration of elimination required to eliminate 50% of free radical. Different superscript letters indicate statistically significant difference ($p<0.05$)

Radical species	[a]EC_{50} values of hydrolysates and vitamin C (mg/mL)					
	Protamex	Flavourzyme	Neutrase	Trypsin	Alcalase	Vitamin C
DPPH	>2.0[b]	>2.0[b]	1.71±0.12[a]	>2.0[b]	1.93±0.09[a]	0.030±0.005
Hydroxyl	1.62±0.05[b]	1.73±0.07[b]	1.36±0.10[a]	>2.0[c]	>2.0[c]	0.032±0.004
Peroxyl	0.98±0.04[b]	1.17±0.08[c]	1.26±0.11[c]	1.17±0.12[c]	0.79±0.05[a]	0.023±0.002

possess strong free radical scavenging activity and might provide as a good source of safe natural antioxidants. Further studies are required to identify and characterize of the antioxidant compounds. This work is in progress.

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