韓藥作誌(Korean J. Medicinal Crop Sci.) 19(2) : 77 – 82 (2011)

능이버섯 효소 추출물의 항산화 활성 및 H_2O_2로 유도된 스트레스에 대한 신경보호 효과

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Free Radical Scavenging Activity and Protective Effect against H_2O_2-Induced Stress in Neuronal Cells of Enzymatic Extracts from *Sarcodon aspratus*

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ABSTRACT : The antioxidative activity of various enzymatic extracts from *Sarcodon aspratus* (*S. aspratus*) was evaluated by measuring 1,1-diphenyl-2-picrylhydrazyl (DPPH), and alkyl radical scavenging activity using an electron spin resonance (ESR) spectrometer. For this study, the *S. aspratus* were enzymatically hydrolyzed by seven carbohydrases (Viscozyme, Celluclast, Dextrozyme, AMG, Promozyme, Maltogenase, and Termamyl) and eight proteases (α-chymotrypsin, Alcalase, Flavourzyme, Neutrase, papain, pepsin, Protamax, and trypsin). The DPPH radical scavenging activities of Viscozyme and pepsin extracts were the highest, and the half maximal inhibitory concentration (IC_{50}) values were 0.896 and 0.734 ㎎/mL, respectively. The Celluclast and trypsin extracts showed the highest scavenging activities on alkyl radical, and their IC_{50} values were 0.278 and 0.575 ㎎/mL, respectively. The Celluclast extracts was decreased cell apoptosis in PC-12 cells against H_2O_2-induced oxidative damage. The findings of the present study suggest that enzymatic extracts of *S. aspratus* exhibit antioxidative activity against oxidative stress on PC-12 cells.

Key Words **:** Antioxidative Activity, Enzymatic Hydrolysates, Free Radical, *S. aspratus*, Neuroprotective Effect

INTRODUCTION

Recently, many researches tried to find new physiologically effective materials from medicinal plants for prevention and/or remedy of diseases (Lee *et al*., 2004, 2008). The etiology of a range of diseases is associated with the generation of excess reactive oxygen species (ROS). Steady-state maintenance of ROS/antioxidant ratio is, however, essential for cell signaling. ROS generated in cells include the superoxide anion radical ($O2 \cdot^-$) (Boveris and Cadenas, 1997). ROS are generated by biochemical reactions in the cell. Indeed, leakage of electrons from the mitochondrial electron transport chain is a significant source of mitochondrial ROS (Cashman, 1997). H_2O_2 peroxide is produced by mitochondrial monoamine oxidase (Fridovich, 1995) and by the superoxide dismutase (MnSOD and Cu/ZnSOD)-catalyzed dismutation of $O2^{\cdot-}$ (Reubsaet *et al*., 1988). In addition, peroxisomal acyl-CoA oxidases also generate H_2O_2 (Sheu *et al*., 2006). ROS generation may also be associated with external stimuli. UV and high-energy radiation, the metabolism of some xenobiotics, air pollutants and the redox cycling of quinones and nitroaromatics are all associated with ROS generation (Boveris and Cadenas, 1997). Currently, mushrooms are drawing attention as beneficial foods for human health, and have been valued as edible and medical resources for some time (Jeoung *et al*., 2009).

In an early work, Kaneda and Tokuda reported the hypocholesterolemic action of edible mushrooms (Kaneda and Tokuda, 1966) from cholesterol lowering properties of *Lentinus*

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Received 2010 December 7 / 1st Revised 2011 February 24 / 2nd Revised 2011 February 28 / Accepted 2011 March 4

edodes, Auricularria polytricha, Flammulina velutipes and *Agaricus bisporus* (Bobek *et al*., 1991, 1994; Bobek and Galba, 1999). Besides, some mushrooms was found hypotensive effect when blood pressure is already high (Kabir *et al*., 1987, 1988; Kabir and Kimura, 1989; Miyazawa *et al*., 2008). As well as, many species of mushrooms were turns out to include the compounds of antioxidant (Cheung and Cheung, 2003; Wong and Chye, 2009) and anti-inflammatory (Jose *et al*., 2004; Kohno *et al*., 2008). In addition, Eva *et al*. (2010) predicted these effects of antioxidant and anti-inflammatory involved with the management of heart and circulation health complications. Yoon *et al*. (2006) reported the antioxidative activities and antimicrobial effects of water and ethanol extracts from the *S. aspratus*. The enzymes high specificity, nontoxicity, water solubility, biodegradability, and mild operational conditions of pH, temperature, and pressure, are major advantages over inorganic catalysts (Taylor, 1991). However, while literature on the chemistry of *S. aspratus* and clinical trials reporting its effects are vast, research into the antioxidative effect of enzymatic extracts from *S. aspratus* is scarce, or even non-existent. Thus, the present study aimed to investigate the free radical scavenging activities of enzymatic extracts from *S. aspratus* by ESR spectroscopy and their possible protective effects on PC-12 cells against oxidative stress.

MATERIALS AND METHODS

1. Materials

PC-12 (ATCC CRL-1721), the standard model for neuronal function studies, was obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Invitrogen Corporation (Carlsbad, CA, USA). The following substances were obtained from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark): Dextrozyme, AMG, Promozyme, Maltogenase, Termamyl, Viscozyme, Celluclast, Flavourzyme, Neutrase, Protamex, and Alcalase. 5,5-dimethyl-1-pyrroline N-oxide (DMPO), 2,2-azobis (2-amidinopropane) hydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN), papain, pepsin, trypsin, α-chymotrypsin, propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tween-20 was supplied by USB (Cleveland, OH, USA). All other reagents were of the highest grade available commercially.

2. Preparation of enzymatic hydrolysates from *S. aspratus*

The mushroom was pulverized into powder with the use of a grinder (Hanil, Seoul, Korea), and the enzymatic hydrolysates were obtained according to the methods described by Park *et al*. (2005) and Cumby *et al*. (2008). The optimum hydrolysis conditions of particular enzymes are as follows: Dextrozyme, pH 4.5, 60℃; AMG, pH 4.5, 60℃; Promozyme, pH 5.0, 60℃; Maltogenase, pH 5.0, 60℃; Termamyl, pH 6.0, 60℃; Viscozyme, pH 4.5, 50℃; Celluclast, pH 4.5, 60℃; BAN, pH 7.0, 70℃; Flavourzyme, pH 7.0, 50℃; Neutrase, pH 6.0, 50℃; Protamex, pH 6.0, 40℃; Alcalase, pH 8.0, 50℃; trypsin, pH 7.0, 37℃; papain, pH 7.0, 37℃; pepsin, pH 7.0, 37℃; and α-chymotrypsin, pH 7.0, 37℃. Briefly, 100 mL of buffer solution were added to 2 g of each powder sample, and then 40 μL (or ㎎) of each enzyme were added after a pre-incubation period of 30 min. The enzymatic hydrolysis reactions were performed for 8 h to achieve an optimum hydrolytic level, and the samples were then immediately heated to 100℃ for 10 min to inactivate the enzyme. Finally, the supernatant was filtered to obtain the enzymatic extracts, which were then lyophilized and stored at –20℃ until use.

3. DPPH radical scavenging activity

DPPH radical scavenging activity was measured with the use of the method described by Nanjo *et al*. (1996). Briefly, 60 μL of each enzymatic extracts at various concentrations were added to 60 μL of DPPH (60 μM) in a methanol solution. After the solution was mixed vigorously for 10 sec, it was then transferred into a 100 μL Teflon capillary tube, and the scavenging activity of each enzymatic extracts with regard to DPPH radicals was measured with the use of an ESR spectrometer. A spin adduct was measured on an ESR spectrometer exactly 2 min later. Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 ㎑; modulation amplitude, 2 G; microwave power, 5 ㎽; gain, 6.3×10^5; and temperature, 298 K.

4. Alkyl radical scavenging activity

Alkyl radicals were generated by AAPH. The PBS (pH 7.4) reaction mixtures that contained 0.1 mL of 10 mM AAPH, 0.1 mL of 10 mM 4-POBN, and 0.1 mL of the indicated concentrations of the tested samples were incubated at 37℃ in a water bath for 30 min (Hiramoto *et al*., 1993), and they were then transferred to 100-μL Teflon capillary tubes. The spin adduct was recorded on an ESR spectrometer. Measurement conditions were as follows: central field, 3475 G; modulation frequency, 100 ㎑; modulation amplitude, 2 G; microwave power, 1 ㎽; gain, 6.3 ×

10^5; and temperature, 298 K.

5. Cell Culture

PC-12 cells were cultured and maintained in DMEM supplemented with 100 U/mL penicillin, 100 ㎍/mL streptomycin and 10% FBS and maintained at 37℃ under a humidified atmosphere with 5% CO_2. All the treatments were performed at 30% confluence.

6. Apoptosis analysis

The PC-12 cells were seeded at 2.1×10^5 cells/well in 6-well plates in a complete medium, DMEM with 10% FBS. After 24 h incubation in a humidified 5% (v/v) CO_2/air environment at 37℃, 990 μL of the enzymatic hydrolysate solution in DMEM was transferred to the well to give a final concentration of 1 ㎎/mL. Following 1 h incubation with the extracts, 10 μL of 100 mM H_2O_2 was added the medium. After 24 h the cell was harvested, and the harvested cells were suspended in ethanol with 0.5% Tween-20 and left for 24 h at 4℃. The cells were then harvested by centrifugation and re-suspended in 1.0 mL of PBS with 0.05 ㎎/mL of propidium iodide and 10 ㎍/mL of RNase A and incubated at 37℃ for 30 min. The analysis of apoptotic cell death was performed by measuring the hypodiploid DNA contents using a flow cytometer (FACS-caliber, Becton Dickinson, NJ). The cells in the sub-G1 population were considered as apoptotic cells, and the percentage of each phase of cell cycle was determined.

7. Statistical analysis

The experimental results were recorded as the mean ± the standard deviation of three parallel measurements

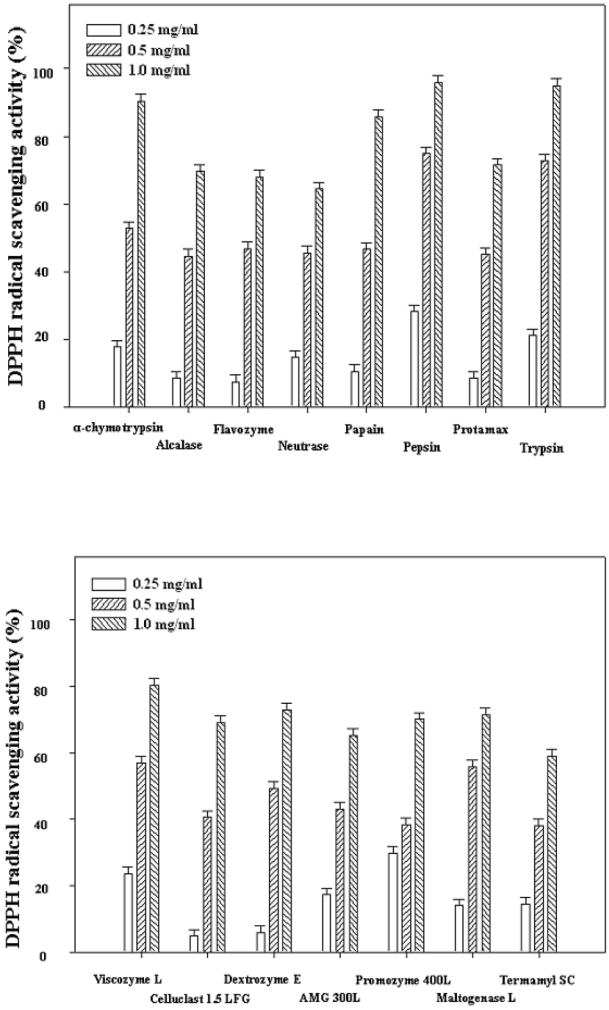

Fig. 1. DPPH radical scavenging activity of various enzymatic extracts by proteolytic (Upper) and carbohydratic (Lower) hydrolysis from *S. aspratus*. Values are given as the means ± SD of determinations were made in triplicate experiments.

RESULTS AND DISCUSSION

1. DPPH scavenging activity

DPPH is a stable free radical that has been used to evaluate the free-radical scavenging activity of natural antioxidants. The capacity of the enzymatic extracts of *S. aspratus* to scavenge DPPH was measured by ESR spectrometry, and the results are shown in Fig. 1. It was observed that carbohydratic extracts by Promozyme, Dextrozyme, Maltogenase, AMG, Celluclast, Termamyl, and Viscozyme had scavenging activity 70.17, 72.94, 71.36, 65.30, 69.14, 59.09, and 80.44% at 1.0 ㎎/mL on DPPH radical. The proteolytic extracts by Alcalase, Protamex, α-chymotrypsin, trypsin, papain, Neutrase, Flavourzyme, and pepsin from the leaves scavenged 69.68, 71.39, 90.37, 95.01, 85.82, 64.47, 67.88, and 95.84% at 1.0 ㎎/mL on DPPH radical. The radical scavenging activity was concentration-dependent manners. In addition, Viscozyme and pepsin extracts exhibited the strongest scavenging activity among the various carbohydrases and proteases with IC_{50} values of 0.896 and 0.734 ㎎/mL, respectively.

In the earlier reports, *S. aspratus* have been known for several biological activities (Kalonia *et al*., 2009; Halliwell, 1999; Je *et al*., 2007). However, to date, there have been no studies on radical scavenging activity by using ESR spectroscopy and protective effects of neuronal cells of enzymatic extracts from *S. aspratus*. Therefore, we prepared enzymatic extracts from *S.*

Table 1. Optimum hydrolysis conditions of various enzymes.

Enzyme	Optimum conditions		Buffer used[a]	Enzyme composition
	pH	Temperature		
Protamex	7.0	50	0.1 M PB[b]	Hydrolysis of food proteins
Flavourzyme	7.0	50	0.1 M PB	Containing both endoprotease and exopeptidase activities
Neutrase	7.0	50	0.1 M PB	An endoprotease
trypsin	7.0	37	0.1 M PB	A serine protease
papain	7.0	37	0.1 M PB	A digestive enzyme in the juice of papaya fruits and leaves, used for tenderizing meat
pepsin	2.2	37	0.1 N GH[c]	A digestive enzyme produced by the gastric glands that catalyses the partial breakdown of dietary protein
α-chymotrysin	7.0	37	0.1 M PB	A serine protease
Alcalase	7.0	50	0.1 M PB	A endo protease
Promozyme	5.0	60	0.1 M SB[d]	Debranching enzymes known as pullulanases
Celluclast	4.5	50	0.1 M SB	Catalyzing the breakdown of cellulose into glucose, cellobiose and higher glucose polymer
Maltogenase	5.0	60	0.1 N SB	An alpha-amylase
Viscozyme	4.5	50	0.1 M SB	Arabanase, celluiase, β-glucanase, hemi-cellulase and xyianase
Termamyl	6.0	60	0.1 M PB	A heat-stable α-amylase
Dextrozyme	4.5	60	0.1 M SB	A glucoamylase and pullulanase
AMG	4.5	60	0.1 M SB	An exo-1,4-α-d-glucosidase

[a] In Enzymatic hydrolysis, [b] Phosphate buffer, [c] Glycine-Hcl, [d] Sodium acetate - acetic acid buffer

aspratus using various enzymes to take water soluble extracts. The antioxidative activities of the enzymatic extracts were investigated using radical scavenging activities on DPPH radical. The earlier results were DPPH radical scavenging activity of another positive control of vitamin C, and the IC_{50} values was under the 10 ㎍/mL. Although DPPH radical scavenging activity of *S. aspratus* was lower than vitamin C, these results indicate that enzymatic extracts from *S. aspratus* appear to be good potential candidates for DPPH radical scavenger. Further studies are required for identification of the antioxidative compound from Viscozyme and pepsin extracts.

2. Alkyl radical scavenging activity

The alkyl radical spin adduct of 4-POBN/free radicals was generated from AAPH at 37℃ for 30 min, and the decrease in ESR signals was observed with the dose increment of all enzymatic extracts. All enzymatic extracts of *S. aspratus* scavenged alkyl radical in a dose-dependent manner. The extracts from *S. aspratus* exhibited the alkyl radical scavenging activities, and the scavenging activities of Viscozyme, Celluclast, Dextrozyme, AMG, Maltogenase, Promozyme, and Termamyl were 68.21, 78.56, 74.33, 75.87, 71.85, 74.16, and 66.61% at 1.0 ㎎/mL. In addition, same concentration of the extracts hydrolyzed from *S. aspratus* by the 8 types of proteases such as α-chymotrypsin, Flavourzyme, Neutrase, Protamex, pepsin, Alcalase, trypsin, and papain also scavenged 64.16, 76.63, 66.49, 69.52, 42.55, 63.02, 78.54, and 62.99% at 1.0 ㎎/mL. It was observed that Celluclast and trypsin extracts exhibited the strongest scavenging activities among the various carbohydrases and proteases with IC_{50} values of 0.278 and 0.575 ㎎/mL, respectively (Fig. 2).

Lee *et al.* (2010) reported that the free radical scavenging activity of various enzymatic extracts prepared from *Hericium erinaceum* was evaluated by using an ESR spectrometer, and all enzymatic extracts of *Hericium erinaceum* scavenged alkyl radical with dose-dependent manners. It was observed that pepsin and Viscozyme extracts exhibited the strongest scavenging activity among the various proteases and carbohydrases with the IC_{50} values of 0.419 and 0.236 ㎎/mL, respectively. These facts suggest that enzymatic extracts of *S. aspratus* might be potential source of alkyl radical scavenger with another mushroom.

3. Cell cycle and apoptosis analysis by flow cytometer

Some previous research has identified a link between neurodegenerative disorders such as, Alzheimer's, Parkinson's and Huntington's disease and oxidative stress (Nakajima *et al*., 2007; Fischer and Glass, 2010). Due to strong reactivity with biomolecules, ·OH is probably capable of doing more damage to biological systems than any other ROS (Kalonia *et al*., 2009). In the present study, the Celluclast extracts were selected to

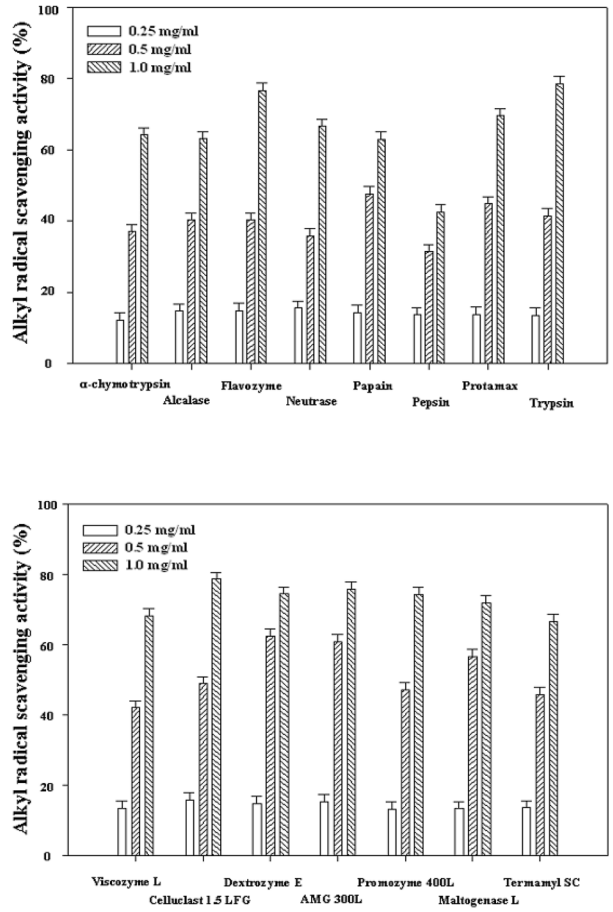

Fig. 2. Alkyl radical scavenging activity of various enzymatic extracts by proteolytic (Upper) and carbohydratic (Lower) hydrolysis from *S. aspratus*. Values are given as the means ± SD of determinations were made in triplicate experiments.

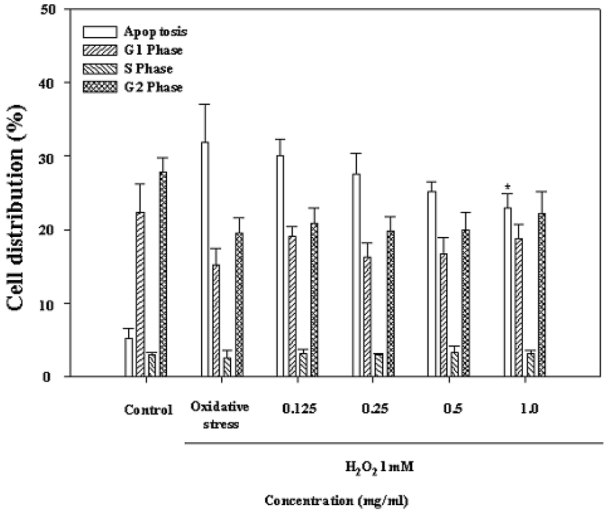

Fig. 3. PC-12 cells were treated with various concentrations of Maltogenase extract from *S. aspratus*. Values are given as the mean ± SD of determinations were made in triplicate experiments. * ($p < 0.05$) are significantly different as analyzed by paired t-test compared oxidative stress group with the Maltogenase extracts group, respectively.

investigate neuroprotective effects on H_2O_2-induced damage, as the extracts had the highest alkyl radical scavenging activities among the various carbohydrases and proteases extracts. The neuroprotective effect of the Celluclast extracts was determined by apoptosis analysis using a flow cytometer. The cells were treated with the extracts prior 1.0 mM H_2O_2. In the Celluclast extracts, the percentage of apoptotic cells was observed 32.62% at 1.0 mM H_2O_2, while the percentages of the Celluclast extracts treated cells were 24.23 and 21.52% at 0.5 and 1.0 ㎎/mL, respectively (Fig. 3). Therefore, the Celluclast extracts protect neuronal cells against H_2O_2-induced oxidative damage. In the present study, we focused on natural water-soluble antioxidants from *S. aspratus*, which prepared by enzymatic hydrolysis using different carbohydrate degrading enzymes and proteases. Their antioxidative effects were evaluated in two different reactive oxygen species assays including DPPH radical and Alkyl radical scavenging assays by an ESR spectrophotometer.

ACKNOWLEDGMENTS

This research was financially supported by the Ministry of Education, Science Technology (MEST) and Korea Industrial Technology Foundation (KOTEF) through the Human Resource Training Project for Regional Innovation.

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