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Article

Neuroprotective Effect of *Hericium erinaceum* against Oxidative Stress on PC12 Cells

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The free radical scavenging activity of various enzymatic extracts prepared from *Hericium erinaceum* was evaluated by using an ESR spectrometer. For this study, *H. erinaceum* was enzymatically hydrolyzed by eight carbohydrases and eight proteases. All of the hydrolyzed extracts scavenged DPPH, hydroxyl, superoxide, and alkyl radicals. Especially, the the pepsin-treated extracts (PTE) exhibited the highest scavenging activity on hydroxyl radical, which is deemed of the strongest radical. Also, the neuroprotective effects of PTE against hydrogen peroxide–induced oxidative stress in PC12 cells were investigated. PTE stimulated the expression of Bcl-2, on the contrary, the extracts suppressed the expression of Bax. These results indicate that PTE possess antioxidative activity on PC12 cells.

Key words: antioxidative activity, enzymatic hydrolysates, free radical, *Hericium erinaceum*, neuroprotective effect

Free radicals are known to be a major cause of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus, cancer, and neurological disorders like Alzheimer's disease [Slater, 1984; Cheng *et al*., 2003]. Reactive oxygen species (ROS) include free radicals such as $\cdot O2^-$ (superoxide anion), ·OH (hydroxyl radical), H_2O_2 (hydrogen peroxide), and $^1O_2$ (singlet oxygen), which can cause cellular injury and initiate the peroxidation of polyunsaturated fatty acids in biological membranes [Compori, 1985; Halliwell, 1997]. Therefore, research for identifying antioxidative compounds is an important issue.

Hericium genus (Heciciaceae) is white, fleshy fungus that primarily grows on dead or dying wood. All of the species of *Hericium* genus produce basidiomata as a mass of fragile, icicle-like spines that are suspended from either a branched supporting framework or from a tough, unbranched cushion of tissue; the basidiomata are considered to be edible and delicious mushrooms. *H. erinaceum,* which is a traditional edible mushroom in Asia, is composed of numerous constituents, such as polysaccharides, proteins, lectins, hericenone, erinacol, erinacine, and terpenoids, and the biological activities of many of these components have been studied [Lee *et al*., 2000; Kenmoku *et al*., 2002]. This mushroom has been used in several Asian countries as an edible folk medicine to treat various human diseases, and thus it has attracted considerable attention for its various bioactive properties [Mizuno *et al*., 1992; Chang, 1996; Kenmoku *et al*., 2002; Jia *et al*., 2004; Choi *et al*., 2005].

However, while literatures have reported on the chemistry of *H. erinaceum*, the antioxidative effects of *H. erinaceum* are not known so far. Thus, the present study aimed to investigate the free radical scavenging activities of enzymatic extracts from *H. erinaceum* by electron spin resonance (ESR) spectroscopy and their possible protective effects on PC12 cells against oxidative stress.

Materials and Methods

Materials. *H. erinaceum* was obtained in a local market (Chungju, Korea). The following substances were obtained from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark): 5,5-dimethyl-1-pyrroline N-oxide (DMPO),

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2,2-azobis(2-amidinopropane) hydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN), Dextrozyme, Amyloglucosidase (AMG), Promozyme, Maltogenase, Termamyl, Viscozyme, Celluclast, BAN, Flavourzyme, Neutrase, Protamex, and Alcalase. Four enzymes such as papain, pepsin, trypsin, and α-chymotrypsin were obtained from Sigma Chemical Co. (St. Louis, MO). Bcl-2 and Bax were purchased from Santa Cruz Biotechnology (Delaware, CA). Protein marker was obtained Fermentas (Brulington, Ontario, Canada). All other reagents were of the highest grade available commercially.

Preparation of enzymatic hydrolysates from *H. erinaceum*. The mushroom was pulverized into powder by 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°C; AMG, pH 4.5, 60°C; Promozyme, pH 5.0, 60°C; Maltogenase, pH 5.0, 60°C; Termamyl, pH 6.0, 60°C; Viscozyme, pH 4.5, 50°C; Celluclast, pH 4.5, 60°C; BAN, pH 7.0, 70°C; Flavourzyme, pH 7.0, 50°C; Neutrase, pH 6.0, 50°C; Protamex, pH 6.0, 40°C; Alcalase, pH 8.0, 50°C; trypsin, pH 7.0, 37°C; papain, pH 7.0, 37°C; pepsin, pH 7.0, 37°C; and α-chymotrypsin, pH 7.0, 37°C. Briefly, 100 mL of buffer solution was added to 2 g of each powder sample, and then 40 μL (or mg) of each enzyme was 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°C 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°C before use.

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 s, 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 (JEOL Ltd., Tokyo, Japan). 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 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5; and temperature, 298 K.

Hydroxyl radical scavenging activity. Hydroxyl radicals were generated by an iron-catalyzed Haber-Weiss reaction (i.e., a Fenton-driven Haber-Weiss reaction), and the generated hydroxyl radicals rapidly reacted with nitrone spin-trap DMPO. The resultant DMPO-OH adduct was detectable with an ESR spectrometer. Briefly, 0.2 mL of each enzymatic extracts at various concentrations was mixed with 0.2 mL of DMPO (0.3 M), 0.2 mL of $FeSO_4$ (10 mM), and 0.2 mL of H_2O_2 (10 mM) in a phosphate buffer solution (pH 7.2), and the resulting solution was then transferred into a 100-μL Teflon capillary tube. After 2.5 min, an ESR spectrum was recorded with the use of a JES-FA ESR spectrometer. Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5; and temperature, 298 K.

Superoxide radical scavenging activity. Superoxide radicals were generated as a result of the ultraviolet irradiation of a riboflavin/ethylenediaminetetraacetic acid solution. The reaction mixtures that contained 0.1 mL of 0.8 mM riboflavin, 0.1 mL of 1.6 mM EDTA, 0.1 mL of 800 mM DMPO, and 0.1 mL of various concentrations of extracts were irradiated for 1 min under an ultraviolet lamp at 365 nm. The mixtures were transferred to the 100 μL quartz capillary tubes of the ESR spectrometer for measurement. Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 10 mW; gain, 6.3×10^5; and temperature, 298 K.

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°C 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 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5; and temperature, 298 K.

Apoptosis analysis. The percentage of cells undergoing apoptosis was also determined by Annexin V- (FITC). 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°C, 990 μL of the enzymatic hydrolysate solution in DMEM was transferred to the well to give a final concentration of 1 mg/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 then double-stained with Annexin V-fluorescein-5-isothiocyanate (FITC) (10 μg/mL) and PI (20 μg/mL). The FITC and PI fluorescence were measured through an

FL-1 filter (530 nm) and an FL-2 filter (585 nm), respectively, on a FACS with the use of Cell Quest software (BD, Franklin Lakes, NJ); 10,000 events were acquired. The proportions of viable, necrotic, early apoptotic and late stage of apoptotic cells were determined by flow cytometer.

Western blot analysis. Total protein extracts were prepared with the use of a protein-extraction kit, and insoluble protein was removed by centrifugation at 13,000 rpm for 15 min. The protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The total cell lysate (40 to 50 μg) was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an immobile polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membrane was blocked with 5% skim milk for 1 h, washed, and then incubated overnight at 4°C with the primary antibodies. All antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). After the membranes were washed in TBS-T, secondary antibody reactions were performed with an appropriate source of antibodies labeled with horseradish peroxidase for 90 min at room temperature. The signals were detected with an enhanced-chemiluminescence advanced detection kit (Amersham Bioscience, Uppsala, Sweden) with the use of the LAS-3000 detector (Fujifilm, Tokyo, Japan). Immunoblotting for actin was performed as an internal control.

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

Results and Discussion

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 *H. erinaceum* to scavenge DPPH was measured by ESR spectrometry, and the results are shown in Table 1. PTE and Celluclast 1.5-treated extracts (CTE) exhibited the strongest scavenging activity among the various proteases and carbohydrases with the IC_{50} values of 205.1 and 81.2 μg/mL, respectively. Especially, CTE showed stronger scavenging activity on DPPH radical than water and methanolic extracts. It is suggested that the strongest DPPH scavenging activity of CTE is due to polysaccharides from *H. erinaceum* [Li and He, 1999]. And the further study is required for identification of the antioxidative compound from CTE. In previous studies, the DPPH radical scavenging activity of water and methanolic extracts from *Volvariella volvacea* showed 38.1% and 46.0% at 6 mg/mL, respectively. Also, the DPPH radical scavenging activity of water and methanolic extracts from *Lentinus edodes* exhibited 25.8% and 55.4% at 6 g/mL, respectively [Cheng *et al*., 2003]. These results indicate that CTE of *H. erinaceum* possessed more potent DPPH radical scavenging activity than water or methanolic extracts of *H. erinaceum* and other mushrooms.

Among the ROS, the hydroxyl radical showed the strongest chemical activity; it easily reacts with amino acids, DNA, and membrane components. Hydroxyl radicals generated in an Fe^{2+}/H_2O_2 system were trapped by a DMPO-forming spin adduct, which could be detected by an ESR spectrometer; the typical 1:2:2:1 ESR signal of the DMPO-OH adduct was observed. The decrease of the amount of DMPO-OH adduct was expressed by ESR signals, and the radical scavenging activity was expressed in a concentration-dependent manner. As shown in Table 2, it was observed that PTE and AMG 300L-treated extracts (ATE) exhibited the strongest scavenging activity among the various proteases and carbohydrases with the 50% inhibitory concentration (IC_{50}) values of 0.366 and 0.369 mg/mL, respectively. Moreover, the extracts showed stronger scavenging activity on hydroxyl radical than water and

Table 1. DPPH radical scavenging activity of various enzymatic hydrolysates from *H. erinaceum*

	Enzyme	IC_{50} (mg/mL)		Enzyme	IC_{50} (mg/mL)
Proteases	α-Chymotrypsin	0.383±0.018	Carbohydrases	Viscozyme	0.165±0.016
	Alcalase	0.289±0.022		Celluclast 1.5	0.081±0.016
	Flavozyme	0.355±0.007		Dextrozyme E	0.643±0.011
	Neutrase	0.263±0.011		AMG 300L	0.302±0.005
	Papain	0.365±0.009		Promozyme	0.484±0.019
	Pepsin	0.205±0.015		Maltogenase L	0.442±0.021
	Protamax	0.376±0.010		Termamyl SC	0.136±0.023
	Trypsin	0.222±0.013		BAN 480L	0.497±0.006
water		0.938±0.017	Methanol		0.130±0.021

Table 2. Hydroxyl radical scavenging activity of various enzymatic hydrolysates from *H. erinaceum*

	Enzyme	IC_{50} (mg/mL)		Enzyme	IC_{50} (mg/mL)
Proteases	α-Chymotrypsin	0.945±0.023	Carbohydrases	Viscozyme	0.581±0.014
	Alcalase	0.866±0.025		Celluclast 1.5	>1.0
	Flavozyme	0.781±0.014		Dextrozyme E	>1.0
	Neutrase	>1.0		AMG 300L	0.369±0.010
	Papain	0.806±0.017		Promozyme	>1.0
	Pepsin	0.366±0.009		Maltogenase L	>1.0
	Protamax	0.813±0.018		Termamyl SC	0.519±0.013
	Trypsin	0.449±0.014		BAN 480L	>1.0
water		0.921±0.022	Methanol		>1.0

Table 3. Alkyl radical scavenging activity of various enzymatic hydrolysates from *H. erinaceum*

	Enzyme	IC_{50} (mg/mL)		Enzyme	IC_{50} (mg/mL)
Proteases	α-Chymotrypsin	0.678±0.017	Carbohydrases	Viscozyme	0.236±0.003
	Alcalase	0.495±0.013		Celluclast 1.5	0.317±0.012
	Flavozyme	0.452±0.006		Dextrozyme E	0.373±0.015
	Neutrase	0.645±0.011		AMG 300L	0.538±0.014
	Papain	0.476±0.012		Promozyme	0.452±0.008
	Pepsin	0.419±0.013		Maltogenase L	0.507±0.018
	Protamax	0.428±0.008		Termamyl SC	0.609±0.017
	Trypsin	0.569±0.018		BAN 480L	0.570±0.027
water		0.096±0.011	Methanol		0.214±0.017

methanolic extracts. In previous research, at 5-20 mg/mL, the scavenging abilities of cold water and hot water extracts from *Hypsizigus marmoreus* on hydroxyl radicals slowly increased from 36.1 to 65.5% and 25.9 to 51.8%, respectively whereas ethanolic extracts showed no abilities [Lee *et al*., 2007]. These results suggest that PTE, CTE and ATE of *H. erinaceum* possess more effective antioxidative activity than water or organic solvent extracts of *H. erinaceum* and other mushrooms.

The alkyl radical spin adduct of 4-POBN/free radicals was generated from AAPH at 37°C for 30 min, and the decrease in ESR signals was observed with the dose increment of all enzymatic extracts. All enzymatic extracts of *H. 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 mg/mL, respectively (Table 3).

Superoxide anions are the most common free radicals *in vivo* and are generated in a variety of biological systems and the concentration of superoxide anions increases under conditions of oxidative stress [Lee *et al*., 2002]. The superoxide radical scavenging activities of various enzymatic extracts from *H. erinaceum* are shown in Table 4. It was observed that pepsin and Viscozyme extracts showed the strongest scavenging activity among the various proteases and carbohydrases with the IC_{50} values of 0.209 and 0.089 mg/mL, respectively. In addition, the extracts showed stronger scavenging activity on superoxide radical than water and methanolic extracts. These results suggest that enzymatic extracts of *H. erinaceum* possess more effective antioxidative activity than water or organic solvent extracts of *H. erinaceum*.

There are some previous researches neurodegenerative disorder such as, Alzheimer's, Parkinson's and Huntington's Disease caused by oxidative stress [Kalonia *et al*., 2009; 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 [Halliwell, 1999]. In the present study, the pepsin extracts were selected to investigate neuroprotective effects on H_2O_2-induced damage; this is because the extracts had the highest hydroxyl radical scavenging activities among various preteases and carbohyrases extracts. The neuroprotective effect of the pepsin extracts was determined with apoptosis analysis by a flow cytometer membrane events were analyzed by measuring the binding of the FITC-Annexin V protein to the phospholipid phosphatidylserine present on the external surface of the

Table 4. Superoxide radical scavenging activity of various enzymatic hydrolysates from *H. erinaceum*

	Enzyme	IC_{50} (mg/mL)		Enzyme	IC_{50} (mg/mL)
Proteases	α-Chymotrypsin	0.945±0.017	Carbohydrases	Viscozyme	0.089±0.003
	Alcalase	0.866±0.012		Celluclast 1.5	>1.0
	Flavozyme	0.857±0.013		Dextrozyme E	>1.0
	Neutrase	0.764±0.007		AMG 300L	>1.0
	Papain	0.807±0.009		Promozyme	>1.0
	Pepsin	0.209±0.004		Maltogenase L	>1.0
	Protamax	0.314±0.018		Termamyl SC	0.765±0.014
	Trypsin	0.842±0.022		BAN 480L	0.781±0.012
water		>1.0	Methanol		>1.0

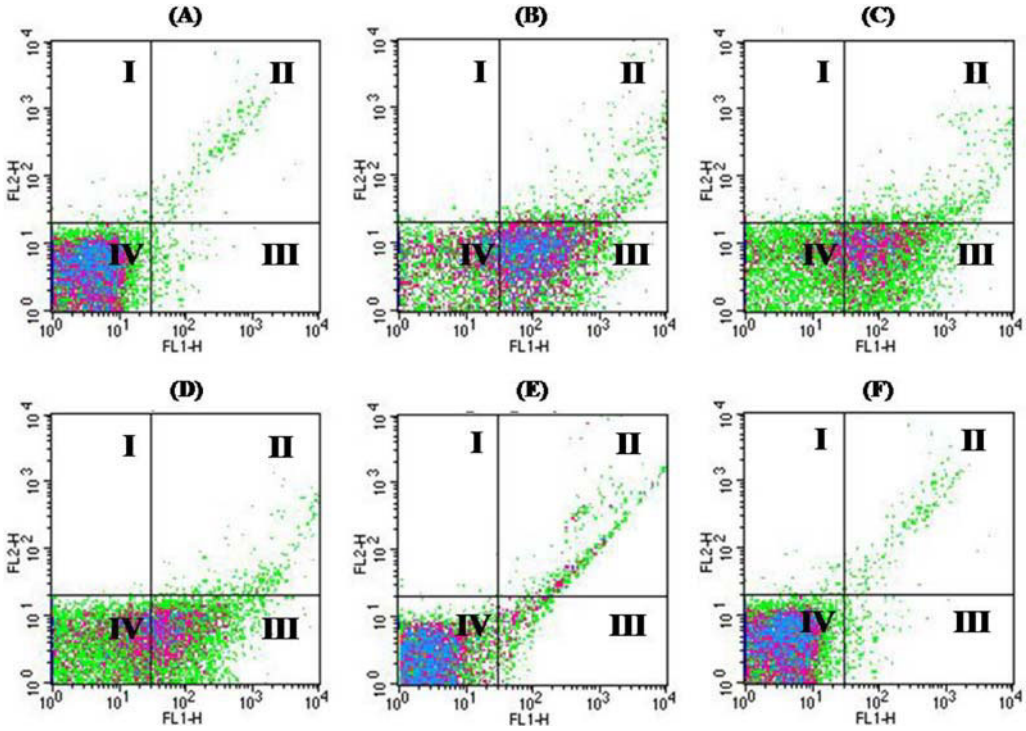

Fig. 1. Identification of the cell death by Annexin-V-PI staining. I, necrotic region; II, secondary apoptotic region; III, primary apoptotic region; IV, live region. (A) control; (B) 1.0 mM H_2O_2 treated alone; (C) 1.0 mM H_2O_2+0.125 mg/mL pepsin extracts; (D) 1.0 mM H_2O_2+0.25 mg/mL pepsin extracts; (E) 1.0 mM H_2O_2+0.5 mg/mL pepsin extracts; and (F) 1.0 mM H_2O_2+1.0 mg/mL pepsin extracts.

apoptotic cell membrane. The assay was performed with a two-color analysis of FITC-labeled Annexin V binding and PI uptake with the use of the Annexin V-FITC apoptosis detection kit. The positioning of quadrants on Annexin V/PI dot plots was performed, and live cells (IV, Annexin V/PI), primary apoptotic cells (III, Annexin V+/PI), secondary apoptotic cells (II, Annexin V+/PI+), and necrotic cells (I, Annexin V/PI+) were distinguished (Fig. 1) [Vermes *et al*., 1995]. Therefore, the total apoptotic proportion included the percentage of cells with fluorescent Annexin V+/PI and Annexin V+/PI+. The neuroprotective effect of the pepsin extracts was confirmed by measuring the presence of phosphatidylserine on the outer cell membrane and in the nucleus of a cell. Fig. 1 shows the results of Annexin-V and PI assay measured the presence of phospha-tidylserine on the nucleus on the outer cell membrane. These results revealed that 52.94% of cells demonstrated apoptosis after H_2O_2 treatment (Fig. 1B), but the proportions decreased to 48.50, 39.46, 7.97, and 2.97%, respectively, after treatment with 0.125, 0.25, 0.5, and 1.0 mg/mL of the pepsin extracts. These results suggest that the pepsin extracts protect neuronal cells against oxidative stress.

Bcl-2 and Bax are well-known and potent anti- and pro-

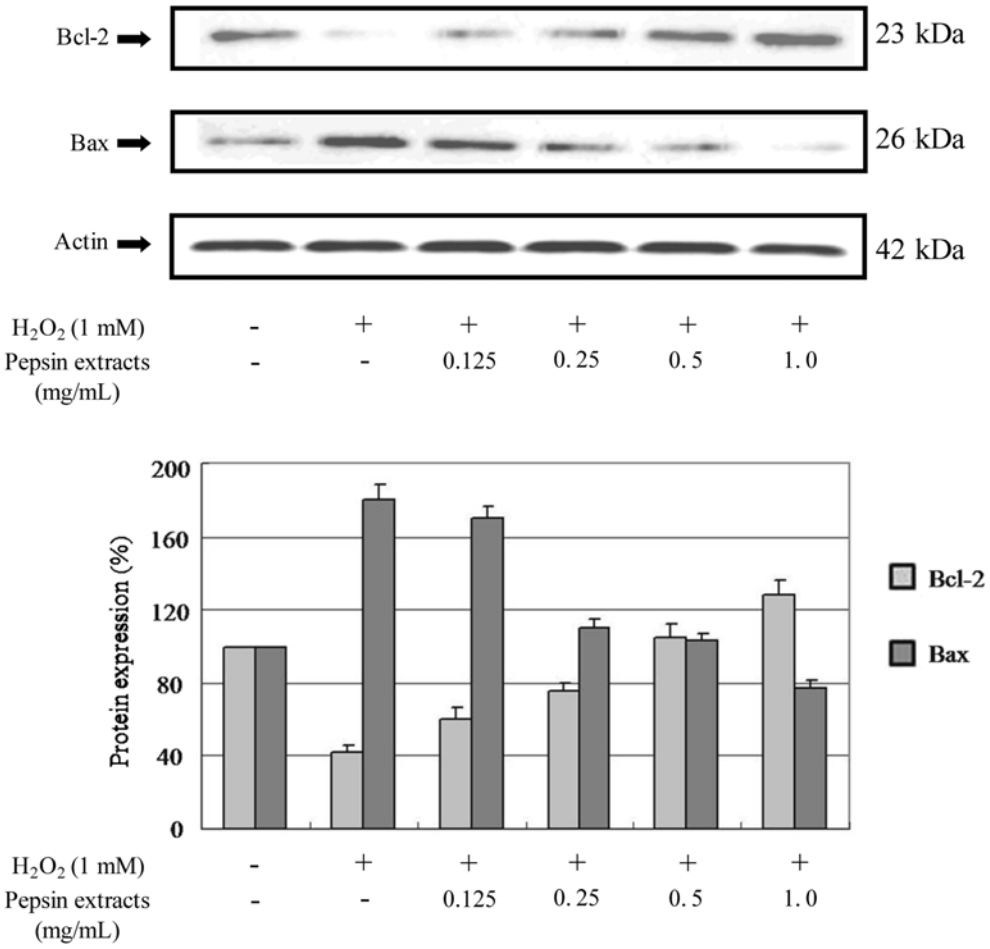

Fig. 2. Change of apoptosis-related proteins on PC-12 cells. Equal amounts of whole cell lysates (30 μg) were subjected to electrophoresis and analyzed for Bcl-2 and Bax by Western blotting. Data are given as the mean±the standard deviation of three replicate values in four separate experiments.

apoptotic proteins, respectively, that are involved in the mitochondrial apoptotic pathway. An increased level of Bax expression or a lowered level of Bcl-2 expression has been shown to reduce mitochondrial membrane potential and to increase the production of reactive oxygen species in neurons [Pastorino *et al*., 1998]. In this study, the expression of Bax on cytosolic fraction tends to increase H_2O_2-treated group compared with control group. Contrary to the oxidative stress group, H_2O_2-treated with the pepsin extracts group showed lower expression of Bax compared with H_2O_2-treated group. In addition, expression of Bcl-2 tends to decrease on H_2O_2-treated group compared with control group. On the other hand, H_2O_2-treated with the pepsin extracts group showed higher expression of Bcl-2 than H_2O_2-treated group (Fig. 2). These results strongly suggest that the pepsin extracts protect PC12 cells against H_2O_2-induced apoptosis through regulating of Bcl-2 family. However, further detailed studies on the pepsin extracts in regard to antioxidative activity *in vivo* are needed.

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