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Full Length Research Paper

Black soybean peptide mixture purified from *Rhynchosia volubilis* exerts antioxidant activity against H_2O_2-induced cytotoxicity and improves thrombosis

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Oxidative stress has been reported to be associated with cellular injury. Here, we investigated the antioxidant ability of a novel peptide mixture purified from black soybean (*Rhynchosia volubilis*) that exhibited significant antioxidant ability (IC_{50} = 119.8 ± 1.2 µg/ml) under *in vitro* ABTS assay conditions. To confirm this result, we next examined the antioxidant ability of the peptide mixture using cell culture systems. Black soybean peptide (BSP) significantly reduced the H_2O_2-induced cell death in C2C12 myocyte. Moreover, extracellular signal-regulated kinase (ERK) signaling was activated by BSP treatment. Under the same conditions, inhibition of the ERK signaling pathway with PD98059 significantly reduced the antioxidant ability of BSP in C2C12 cells. Finally, we also assessed the antithrombotic ability of BSP using a rat model; we found that the BSP also exerted antithrombotic effects in rat blood. Collectively, these results indicate that BSP purified from black soybean exerts an inhibitory effect on both oxidative stress and thrombosis.

Key words: Black soybean peptide (BSP), anti-oxidant, thrombosis, extra cellular signal regulated protein kinase, oxidative stress.

INTRODUCTION

Oxidative stress has been established to be a serious factor of physiological and pathological injury such as neurodegenerative disease and mitochondrial diseases (Jung et al., 2010; Wang et al., 2006). Therefore, the prevention of degenerative diseases that are often associated with oxidative stress generation poses a great challenge for maintaining a healthy life (Kang, 2005). Therefore, intracellular oxidative stress scavenging capabilities of natural sources have been suggested by many investigators. In particular, bioactive peptides widely found in many beverages and food products have been proposed to be potent agents in the prevention of oxidative stress-related diseases; these peptides are believed to function by increasing the rate of expressions of cellular antioxidative proteins (Ros, 2009; Landis and Tower, 2005; Ben-shaul et al., 2001). Naturally occurring products, rather than chemically synthesized compounds, attract the attention of researchers with regard to the prevention of degenerative diseases (Surh, 2003).

The novel black soybean peptide (BSP) is obtained from a purified novel peptide mixture of soybeans and has been implicated as a preventive agent against the development of various degenerative diseases. The basic properties of BSP, such as the antioxidative and antithrombotic activities, were tested by examining its preventive effects against cell injury involving the oxidative stress or oxidative stress-induced thrombosis, while focusing on the extracellular signal-regulated kinase (ERK) signaling pathways. One of the several possibilities that have been proposed is that proteins that control cell signaling, including those activating cell

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proliferation enzymes such as the extracellular signal regulated protein kinase (ERK) and Akt, effectively reduce cell cytotoxicity and cell death induced by the oxidative stress (Wu et al., 2006; Lind et al., 2006). ERK is the most distinctive protein kinase responsible for cellular proliferation and plays an essential role in cell survival under stress conditions (Ostrakhovitch and Cherian, 2005). Among several kinases, ERK is known to play a major protective role under various stress conditions in cells. On activation, ERK upregulates several cell proliferation enzymes through specially characterized cascade pathways and inhibits cell death (Wu et al., 2004). Several investigators have observed the antioxidant potential of ERK exerted by activating anti-oxidative enzymes such as superoxide dismutase under intracellular toxic conditions (Wu et al., 2006; Xu et al., 2006).

In this study, we investigated the anti-oxidant ability and protective role of novel peptides in reducing cell death induced by H_2O_2. We observed that novel peptides from black soybeans exhibited antioxidant properties and reduced cell death via ERK activation. Their efficacy in preventing oxidative stress-generated thrombosis was also examined using a rat model.

MATERIALS AND METHODS

Reagent

Black soybean peptide (BSP) was produced by Nong Shim Co. Ltd (Seoul, South korea) and the composition of amino acids was well described in the previous report comparing with casein (Roh et al., 2007). Specific antibodies that recognize the phosphorylated forms of ERK, and ERK were from Cell signaling technology (Danvers, MA). Pro-caspase-3 antibody was purchased from Santacruz biotechnology (Santacruz, CA). PD98059 and hydrogen peroxide were purchased from Sigma (St. Louis, MO).

ABTS assay

To generate the radical cation ($ABTS^{\bullet+}$) needed for the determination of antioxidant activity, 7 mM ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] diammonium salt dissolved in distilled water was allowed to react with 2.45 mM potassium persulfate. The reaction solution was left in the dark at room temperature for 16 h after which was diluted with 5 mM Na-phosphate buffer (pH 7.4) to an absorbance of 1.00 ± 0.02 at 734 nm. 10 µl of the methanol extract were added to 1 ml of the diluted free radical solution ($ABTS^{\bullet+}$) and the reaction mixture was incubated at 30°C for 7 min. After incubation, the absorbance of the reaction mixture was spectrophotometrically measured at 734 nm (Re et al., 1999; Moure et al., 2006). The standard curve was prepared using Trolox and the free radical scavenging activity (%) was reported as µg Trolox equivalent antioxidant capacity (TEAC)/g DW of sample.

Cell culture

C2C12 myocyte were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a CO_2 incubator.

Inhibition of platelet aggregation in rat (anti-thrombosis assay)

Rat blood was drawn into a syringe in 1/6 volume of ACD-C (130 mM citric acid,124 mM trisodium citrate and 110 mM glucose) and platelet-rich plasma (PRP) was obtained by centrifugation at 120 g, 15 min at room temperature. Platelets were washed from plasmatic contaminants (Drouet, 1990; Fiat et al., 1993) and resuspended in a buffer (11.9 mM NaHCO3, 0.33 mM NaH_2PO_4, 16.3 mM NaCl, 2.8 mM KCl,1.1 mM $MgCl_2$, 11.1 mM glucose and pH 7.4) to adjust the platelet counts to 5×10^8 /ml with a hemacytometer (Superior, Germany).

Platelet aggregation was performed in a Chrono-Log aggregometer (Havertown, PA). A 470 µl volume of PRP was placed in the cuvette of the aggregometer and this was incubated at 37°C for 5 min. To this, 10 µl $CaCl_2$ solution (final concentration of 1.0 mM) and 10 µl of test solution were added successively with 2 min incubation after each addition. Platelet aggregation was induced by the addition of 10 µl of ADP solution (final concentration of 10 µl). Results were given in terms of change in light transmission 5 min after the addition of the inducer and expressed as percent inhibition of maximal intensity of control, and are expressed as the mean of two measurements.

Cell proliferation assay

Cell proliferation was measured by the MTT method. Cells were seeded in 24-well plates (2×10^5 cells) containing the test compounds for indicated dose or time dependently, and then incubated with 30 µl MTT solution (5 mg/ml in PBS) for 2 h at 37°C. Optical densities of the solutions were determined by an ELISA reader.

Western blotting

Cells were lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% MP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NAF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin), solubilized proteins were centrifuged at 14,000 × g in a microfuge (4°C) for 5 min, and supernatant protein was collected. The protein concentration was determined by Bio-Rad Bradford assay reagent (Hercules, CA). Proteins (30 µg) were separated on a 10% SDS-PAGE, transferred to a nitrocellulose membrane. The western blot was performed with specific antibodies (ERK 1:2000, p-ERK 1:2000 β-actin 1:4000 dilutions), and probed using horseradish-peroxidase conjugated secondary antibody. The immunoreactive protein bands were detected by enhanced chemiluminescence (ECL) solution of Amersham Bioscience (Piscataway, NJ).

Statistical analysis

Data are presented as mean ± S.E.M of at least three independent experiments performed in triplicate. P values<0.05 were considered to be significant using SPSS 9.0 (SPSS Inc., Chicago, IL).

RESULTS

The novel peptide, BSP, has antioxidant ability

We investigated whether the BSP possesses free radical

Table 1. Anti-oxidant ability of Black soybean peptides.

Anti-oxidant assay ($ABTS^{\bullet+}$ assay)
BSP
IC_{50}=119.8 µg/ml ±1.2

The absorbance of the reaction mixture was measured at 734 nm. The free radical scavenging activity (%) was reported.

scavenging capacity using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) assay, we found that the free radical scavenging capacity (IC_{50}) of the BSP mixture was 119.8 µg (Table 1).

BSP prevents H_2O_2-induced cellular injury

Oxidative stresses are reported to be strongly associated with many diseases (Jung et al., 2010; Wang et al., 2006; Kang and Hamasaki, 2005). Thus, we next examined whether BSP exert radical scavenging activity in a myoblast system under oxidative stress. The cells were pretreated with 40 or 80 µg/ml of BSP for 1 h and then exposed to 1 mM H_2O_2 for the indicated time periods. Cells death was determined by the MTT assay (Figure 1A), Cell morphology (Figure 1B), and pro caspase-3 expression (Figure 1C). As shown in Figure 1A and B, BSP effectively protected the cells from H_2O_2-induced cell death. In addition, cleaved caspase-3 by H_2O_2 was restored in the treatment of BSP. These results suggest that the novel peptide (BSP) has an antioxidant potential and the ability to protect cells from ROS-induced cell injury.

Anti-oxidant ability of BSP is associated with ERK phosphorylation

Previous studies indicated that several active components in beans exhibited antioxidant ability as well as regulated intracellular signaling pathway as ERK signaling pathway (Su et al., 2006; Hwang et al., 2005; Bendia et al., 2005). Therefore, we next examined whether the ERK signaling pathway is involved in the protective effect of BSP against cell death. The C2C12 cells were treated with BSP for 1 h in a dose-dependent manner, and the phosphorylation levels of ERK was then examined using the western blot analysis. As shown in Figure 2A and B, BSP significantly activated the ERK signaling pathway. To confirm the mode of ERK activation, the cells were pretreated with an ERK inhibitor, PD98059 (20 µM for 1 h) and exposed to H_2O_2 (1 mM for 24 h); cell death was then measured using the MTT assay. As shown in Figure 3, the protective ability of BSP against the oxidative stress was abolished by treatment of the cells with the ERK inhibitor. These results suggest that BSP exerts beneficial effects on cells by protecting them from oxidative stress by activating the intracellular ERK signaling pathway.

BSP has antithrombotic ability *in vitro*

There is strong evidence about the role of intracellular oxidative stress in the pathogenesis of various conditions, including cardiovascular disease, cell senescence, and thrombosis (Szotowski et al., 2007). We tested whether BSP possesses an antithrombotic ability by using a rat thrombosis model. Rat blood was drawn into a syringe in 1/6 volume of ACD-C, and platelet aggregation was performed in a Chrono-Log aggregometer. As shown in Table 2, BSP significantly reduced thrombosis in rat blood since it inhibited ADP-induced platelet aggregation in a dose-dependent manner. These results suggest that BSP, a novel peptide, also has an antithrombotic ability, which might be related to their anti-oxidant abilities.

DISCUSSION

In this study, we investigated whether BSP has scavenging ability on oxidative stress in C2C12 myocyte. We show that BSP, a novel peptide, has the ability to reduce oxidative stress in an *in vitro* cell culture system. Moreover, BSP also possesses the ability of blocking oxidative stress-induced thrombosis. Several previous studies have shown that active peptide mixtures derived from natural sources can be used to reduce oxidative stress, which is implicated in causing various diseases such as cardiovascular diseases, cell senescence, cancer, and thrombosis (Szotowski et al., 2007; Loscalzo, 2003). In particular, soybean peptides have been shown to exert a beneficial effect on human health via modulation of various intracellular signaling pathways (Cigremis et al., 2006). For example, soybean-derived genistein inhibited cancer cell proliferation and also exhibited a neuro-protective effect through the control of a signaling pathway (Torres et al., 2006; Sarkar and Li, 2003; Sawada and Shimohama, 2003). The purpose of the present study was to evaluate the antioxidant and antithrombotic potential of the soybean-derived BSP and to determine the basis of anti-oxidant signaling in cells under oxidative stress.

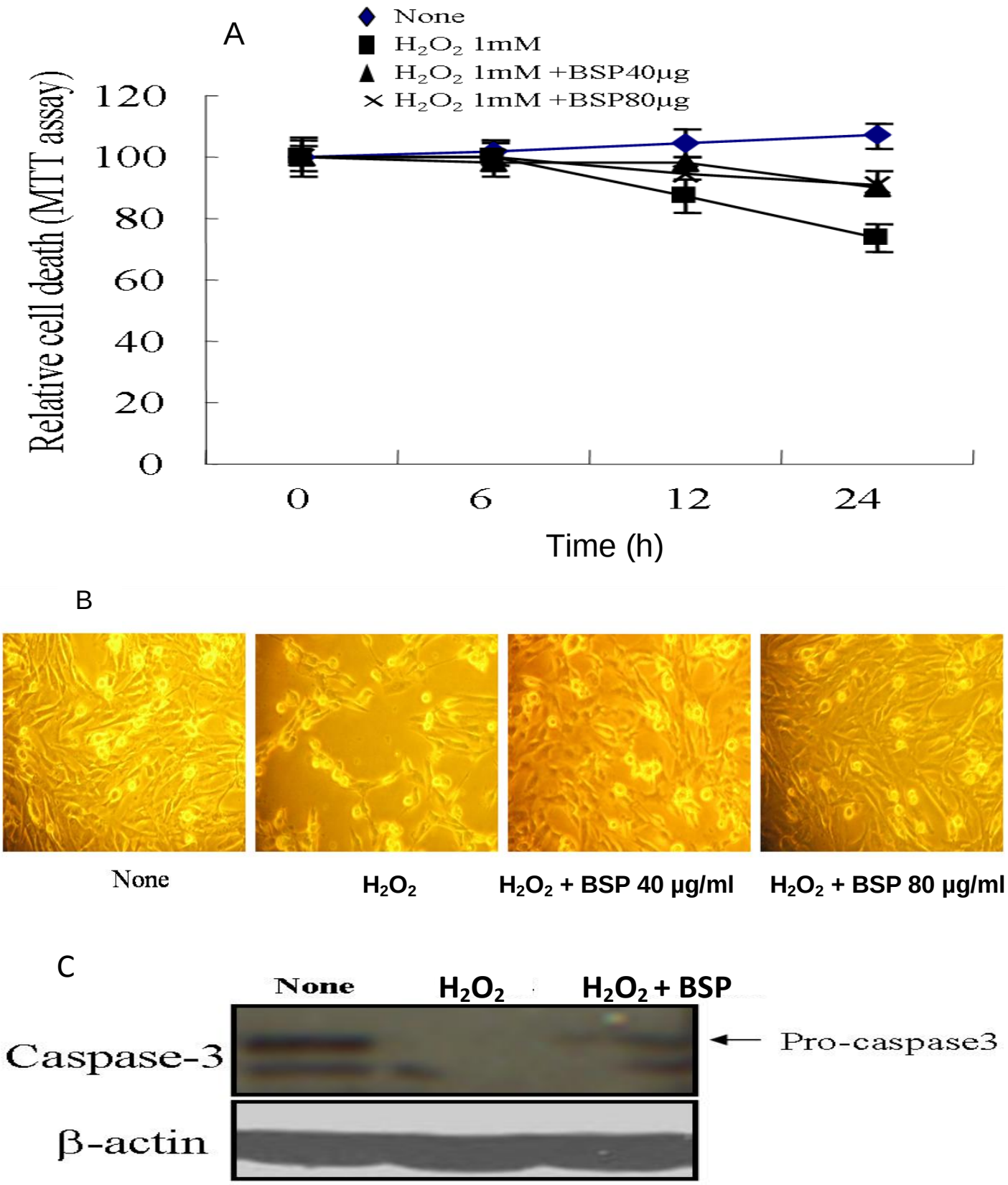

Figure 1. Cell protection effects of BSP in H_2O_2-treated C2C12 cells. Cells were cultured in 24 well plate (2×10^5 / well) for 24 h, and pre-treated with BSP (40, 80μg/ml) for 1h, exposed to H_2O_2, 1 mM for the indicated time periods, and then cell viability were measured by MTT assay (A), Cell morphology (B), and Caspase-3 expression (C).

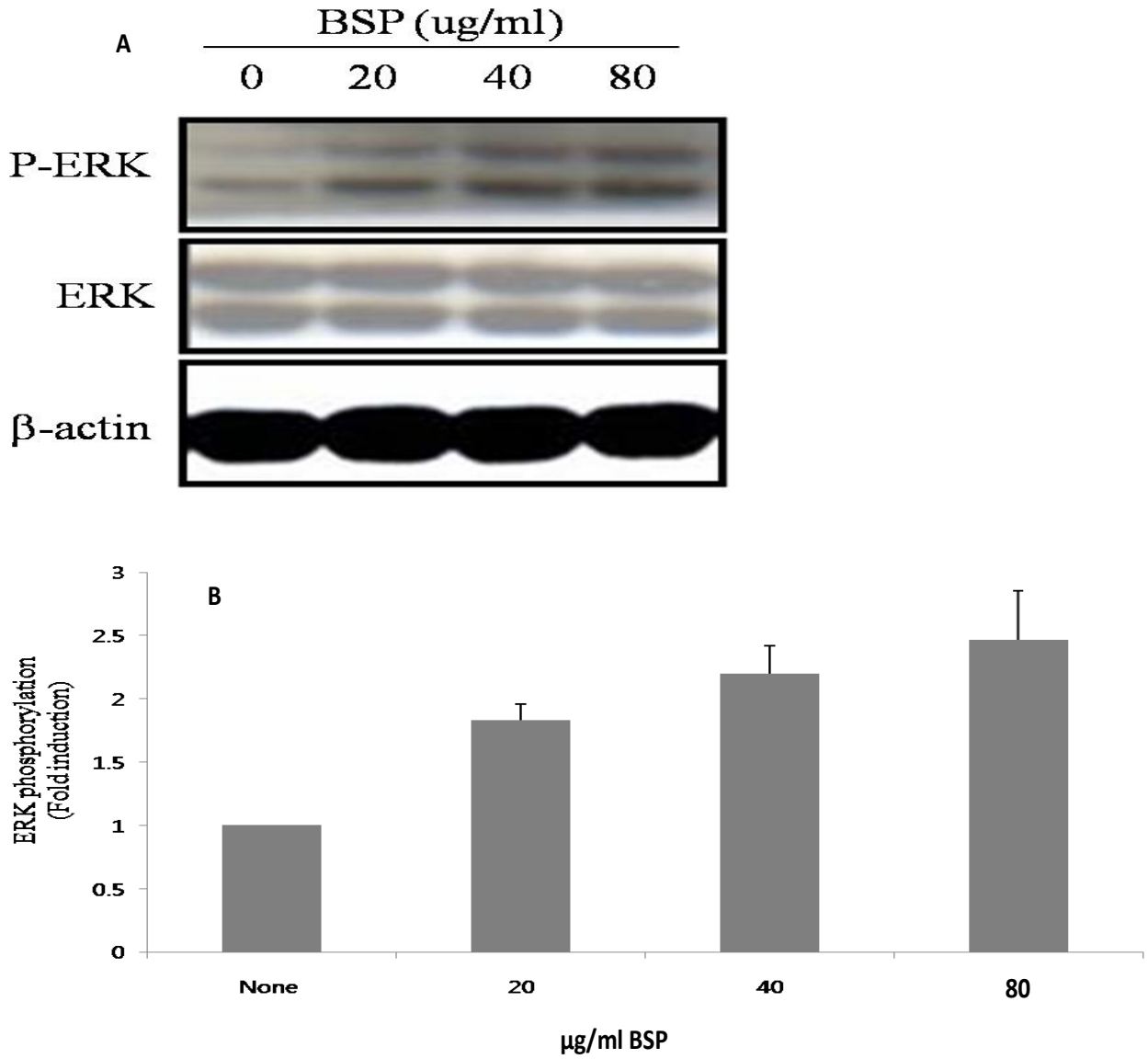

Figure 2. Effects of BSP on ERK phosphorylation in C2C12 cells. Cells were cultured in 12 well plate (4×10^5 / well) for 24 h, and treated with either BSP for 1 h in a dose dependently, and were analyzed by western blot. All experiment was replicated at least 3 times (n=3) and graph bar indicate significant difference (*P < 0.05; mean ± SD; n = 3) versus none sample.

It was reported that soy peptides have the potential to reduce cell death under oxidative stress based on the fact that many soybean sources exert their antioxidative effects (Torres et al., 2006; Sarkar and Li, 2003; Sawada and Shimohama, 2003). However, the mechanisms of these effects are poorly understood because the involvement of anti-oxidative signaling pathways appears very complex. In this study, we investigated the molecular basis of the anti-oxidant activity of BSP using the C2C12 myocytes. We found that BSP inhibited the H_2O_2-induced cell death; this ROS-scavenging effect was accompanied by ERK phosphorylation. Recently published data indicated that modulation of cellular survival or apoptotic proteins was significant in the inhibition of oxidative stress-induced cell death and the prevention of oxidative stress-induced events such as thrombosis, because several proliferative proteins were required for cell survival after the induction of cell death often by oxidative stress. Thus, the modulation of these survival proteins by newly discovered antioxidative components is also important in studying the basis of the anti-oxidant effects and associated diseases such as thrombosis (Lissin et al., 2004).

The ERK signaling pathway is a well-known mitogen-induced protein pathway involved in the process of cellular survival. Currently, the mitogen-activated protein kinase (MAPK) signaling pathway might be considered to play an important role in preventing apoptotic cell death

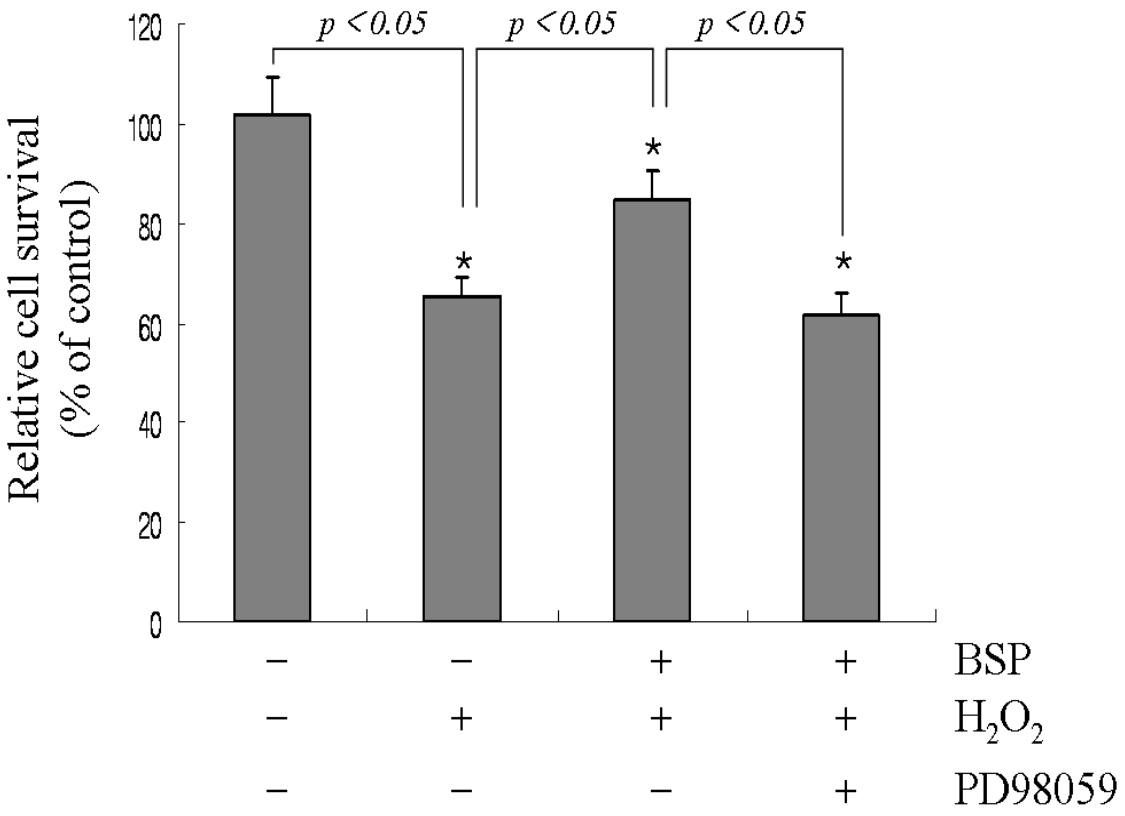

Figure 3. Effects of ERK inhibitor on BSP treatment in H_2O_2-induced cell death. Cells were treated with BSP (40 µg/ml) in the presence or absence of 20 µM PD 98059 for 1 h, exposed to H_2O_2 1 mM for 24 h, and then cell death were measured by MTT assay. $p<0.05$ was considered to statistically significant.

Table 2. Effects of BSP on ADP-induced platelet aggregation.

Concentration (µg/mL)	Inhibitory effect of platelet aggregation (%)
	BSP
200	37.5±1.6
70	12.5±0.5
20	4.0±0.2

Results were given in terms of change in light transmission 5 min after the addition of the inducer and expressed as percent inhibition of maximal intensity of control, and are expressed as the mean±S.D of three measurements. Tukey's test was used to determine the difference of means, and $p<0.05$ was considered to statistically significant.

and reducing the cytotoxic effects of death stimuli (Muro and Muzykantov, 2005). In particular, one of the well-known MAP-kinases, ERK, is representatively involved in the prevention of diseases by exerting antioxidative, antithrombotic, antiinflammatory, and anticancer effects through the modulation of other proteins. For example, recent papers have suggested that some natural compounds exhibit their anti-oxidant ability through ERK signaling modulation (Owuor and Kong, 2002).

In this study, we examined whether ERK activation induced by BSP in the C2C12 cells is important in the prevention of cell death as well as oxidative stress-induced thrombosis. Under the applied conditions, ERK activation was significantly increased by treatment with BSP and inhibition of ERK signaling by PD98059 reduced the cell protective ability of BSP in the H_2O_2-treated C2C12 cells. It appears that BSP exerts both anti-oxidative and cell protective effects through the modulation of the ERK signaling pathway. However, the precise molecular signaling pathways explaining the relation between BSP-induced cell survival and ERK remain to be elucidated. Furthermore, signal networks of

ERK and other regulatory kinases have to be defined.

In addition, we examined whether the other survival factors, Akt is also involved in the process of cellular protection under various stress conditions. Previous studies have reported that the PI3 kinase/Akt signaling pathway is critical for stress-induced cellular homeostasis (Kang and Hamasaki, 2005; Su et al., 2006; Hwang et al., 2005).

However, in the present study, no significant Akt activation was observed in the C2C12 cells in response to the treatment with BSP. Finally, we investigated whether BSP could be useful in the prevention of thrombosis. Several published papers have indicated that oxidative stress is an important causal factor for thrombosis, which is associated with intracellular oxidative or antioxidative signal molecules (Bonomini et al., 2008; Lentz, 2005). Thus, we investigated the antithrombotic potential of BSP; our data showed that BSP effectively blocked the process of thrombosis in rat blood. These results indicated that BSP also possesses the antithrombotic potential.

In summary, our results strongly suggest that BSP purified from soybean can be used as an antioxidant for protecting cells from oxidative stress, and that BSP exhibits this effect through the ERK signaling pathway. Furthermore, BSP could also prevent thrombosis in rat blood.

ACKNOWLEDGEMENT

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