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Bioactive Compounds Extracted from *Ecklonia cava* by Using Enzymatic Hydrolysis Protects High Glucose-Induced Damage in INS-1 Pancreatic β-Cells

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Abstract Pancreatic β-cells are very sensitive to oxidative stress and this might play an important role in β-cell death in diabetes. In the present study, we investigated whether the brown alga *Ecklonia cava* has protective effects against high glucose-induced damage in INS-1 pancreatic β-cells. For that purpose, we prepared an enzymatic hydrolysate from *E. cava* (EHE) by using the carbohydrase, Celluclast. High-glucose (30 mM) treatment induced glucotoxicity, whereas EHE prevented cells from high glucose-induced damage then

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restoring cell viability was significantly increased. Furthermore, lipid peroxidation, intracellular reactive oxygen species (ROS) and nitric oxide (NO) were overproduced as the result of the treatment by high glucose; however, these lipid peroxidation, ROS and NO generations were effectively inhibited by addition of EHE in a dose-dependent manner. Moreover, EHE treatment increased activities of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-px) in high glucose pretreated INS-1 pancreatic β-cells. EHE slightly reduced the expression of pro-apoptotic protein Bax induced by high glucose but increased the expression of Bcl-2, an anti-apoptotic protein. These findings indicate that EHE might be used as potential nutraceutical agent which will protect the glucotoxicity caused by hyperglycemia-induced oxidative stress associated with diabetes.

Keywords *Ecklonia cava* · Enzymatic hydrolysate · Pancreatic β-cells · Protective effects · High glucose · Oxidative stress

Introduction

Dysfunction of pancreatic β-cells performs a crucial function in the pathogenesis of type 2 diabetes. Although the exact mechanism underlying β-cell destruction remains unknown, it has been suggested that the oxidative stress induced by high glucose is one of the major factors contributing to the destruction of pancreatic β-cells [1]. Several studies have demonstrated that chronic exposure of β-cells to high glucose results in β-cell dysfunction and apoptosis [1, 2]. Under high glucose levels, mitochondria produce excessive amounts of reactive oxygen species (ROS) as they utilize alternative glucose-metabolizing pathways prone to induction of oxidative stress [3]. In addition to increasing the production of ROS by mitochondria, glucose is known to induce an increase in ROS generated by NAPDH oxidase in the cell membrane. The results of several studies suggested that antioxidants can prevent the pathological damage induced by the hyperglycemia-induced oxidative stress associated with diabetes [4, 5].

Marine algae are known to generate an abundance of bioactive compounds with great potential in the pharmaceuticals, food, and biomedical industries. In particular, the brown algae harbor a variety of biological compounds, including pigments, fucoidans, phycocolloids, and polyphenolic compounds [6]. Traditional extraction techniques for bioactive compounds derived from marine algae generally use various organic solvents or water. However, some controversy surrounds this approach, largely with regard to the extremely low recovery and strict regulations for the use of organic solvents in the food industry and the limited recovery of water-soluble components in water extractions. Previously, we reported that bioactive compounds were obtained from brown algae, *Ecklonia cava* by using enzymatic hydrolysis technique. The enzymatic hydrolysate from *E. cava* (EHE) has numerous advantages such as water solubility, great variation of constituents, multiple biological activities, high extraction efficiency, and nontoxicity [7–11]. These enzymes work principally by macerating the tissues and breaking down the cell walls and complex interior storage materials.

In the previous studies [7, 8], we demonstrated the effects of EHE on extracellular and intracellular antioxidant activities. However, the direct effects of EHE on β-cell functions and diabetes-related survival have not yet to be determined. Therefore, in this study, we investigated the protective effects of an EHE against high glucose-induced damage in INS-1 pancreatic β-cells.

Materials and Methods

Materials

The brown alga *E. cava* was collected from the coast of Jeju Island (33°29 N, 126°31 E), South Korea. The sample was washed three times with tap water to remove the salt, sand, and epiphytes attached to its surface, then carefully rinsed with freshwater and freeze-dried. Dried alga sample was ground and sifted through a 50-mesh standard testing sieve. RPMI 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, and trypsine–ethylenediamminetetraacetate (EDTA) were purchased from GIBCO (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), dimethyl sulfoxide (DMSO), and 2 ,7 -dichlorodihydrofluorescein diacetate (DCF-DA) were purchased from Sigma (St. Louis, MO, USA). Antibodies against Bax, Bcl-2, and β-actin were purchased from Cell Signaling Technology (Bedford, MA, USA), and other chemicals and reagents used were 99 % or greater purity.

Preparation of EHE

The preparation of enzymatic hydrolysate followed the method previously reported [7, 12]. Ground, dried *E. cava* powder (1 g) was homogenized with 100 ml of distilled water (pH 4.5) and mixed 100 μl of Celluclast (Novo Nordisk, Bagsvaerd, Denmark). The reaction with this enzyme was conducted at 50 °C for 24 h. As soon as the enzymatic reaction is completed, the digest was boiled for 10 min at 100 °C to inactivate the enzyme. The product was clarified by centrifugation (3000×*g* for 20 min) to remove any unhydrolyzed residue. Finally, the EHE obtained after filtration of the supernatant was adjusted to pH 7.0 and stored for use in experiments. The EHE contained polyphenols and sulfated polysaccharides with high contents of fucose and sulfate group (Table 1) [12].

Cell Culture

INS-1 pancreatic β-cells (insulin-secreting rat insulinoma cell line) were cultured in RPMI 1640 medium, supplemented with 10 % FBS, streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37 °C in an humidified atmosphere containing 5 % CO_2.

Assay of Cell Viability

Cell viability was assessed by a colorimetric MTT assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes, as described previously [13]. Cells (2×10^4 cells/well) in wells of 96-well plates were preincubated with glucose (5.5 or 30 mM) for

Table 1 Polyphenols and sulfated polysaccharides contents of *Ecklonia cava* enzymatic hydrolysate[a]

Compounds	Contents (%)
Polyphenols	20.09
CSP	40.02
Fucose	60.71
Galactose	19.04
Xylose	20.25
Sulfate/total CSP	4.3[b]

CSP Crude sulfated polysaccharides[a]Information from Ref. [12][b]The contents of sugar and sulfate group are expressed as a percentage of total crude sulfated polysaccharides

48 h, and then incubated without or with the indicated concentrations of EHE for 24 h. Thereafter, a 100 μl of MTT solution (1 mg/ml) was added to each well of 96-well culture plate, incubated for 4 h at 37 °C, and the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100 μl DMSO and the absorbance at 540 nm of each well was read using a microplate reader (Sunrise, Tecan, Salzburg, Austria). In this study, the treatment concentrations of glucose in pancreatic β-cells were selected according to the previous study [14].

Assay of Lipid Peroxidation

Lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARSs) production [15]. Cells (2×10^4 cells/well) in wells of 96-well plates were preincubated with glucose (5.5 or 30 mM) for 48 h, and then incubated without or with the indicated concentrations of EHE for 24 h. A 200 μl of each medium supernatant was mixed with 400 μl of TBARS solution then boiled at 95 °C for 20 min. The absorbance at 532 nm was measured and TBARS concentrations were extrapolated from the 1,1,3,3-tetraethoxypropane serial dilution standard curve, TBARS values were then expressed as equivalent nmoles of malondialdehyde (MDA).

Assay of Intracellular ROS Levels

Intracellular ROS levels were measured by the dichlorofluorescein assay [16]. 2′,7′-DCF-DA can be deacetylated in cells, where it can react quantitatively with intracellular radicals (mainly hydrogen peroxide) to converted into its fluorescent product, DCF, which is retained within the cells. Therefore, DCF-DA was used to evaluate the generation of ROS in oxidative stress. Cells (2×10^4 cells/well) in well of 96-well plates were preincubated with glucose (5.5 or 30 mM) for 48 h, and then incubated without or with the indicated concentrations of EHE for 24 h, after that the cells were washed with PBS and incubated with 5 μM DCF-DA for 30 min at room temperature. 2 ,7 -Dichlorodihydrofluorescein fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Perkin-Elmer LS-5B spectrofluorometer (Norwich, CT, USA).

Assay of Nitric Oxide (NO) Levels

Cells (2×10^4 cells/well) in well of 96-well plates were preincubated with glucose (5.5 or 30 mM) for 48 h, and then incubated without or with the indicated concentrations of EHE for 24 h. The nitrite accumulation in the supernatant was assessed by Griess reaction [17]. Each 50 μl of culture supernatant was mixed with an equal volume of Griess reagent [0.1 %*N*-(1-naphthyl)-ethylenediamine, 1 % sulfanilamide in 5 % phosphoric acid] and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in a microplate absorbance reader, and a series of known concentrations of sodium nitrite was used as a standard.

Antioxidant Enzyme Assays

Cells (1×10^6) in 10 mm dishes were preincubated with glucose (5.5 or 30 mM) for 48 h, and then incubated without or with the indicated concentrations of EHE for 24 h. The medium was removed and the cells were washed twice with PBS. One milliliter of 50 mM potassium phosphate buffer with 1 mM EDTA (pH 7.0) was added and cells were scraped. Cell suspensions were sonicated three times for 5 s on ice each time, then cell sonicates were

centrifuged at 10,000×*g* for 20 min at 4 °C. Cell supernatants were used for antioxidant enzyme activities. The protein concentration was measured by using the method of Bradford with bovine serum albumin as the standard. SOD activity was determined by monitoring the auto-oxidation of pyrogallol [18]. A unit of SOD activity was defined as the amount of enzyme that inhibited the rate of oxidation of pyrogallol. Catalase (CAT) activity was measured according to the method of Aebi [19] by following the decreased absorbance of H_2O_2. The decrease of absorbance at 240 nm was measured for 2 min. Standards containing 0, 0.2, 0.5, 1, and 2 mM of H_2O_2 were used for the standard curve. GSH-px activity was measured by using the method of Lawrence and Burk [20]. GSH-px (1 unit) was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per consumed per minute.

Western Blot Analysis

Cells (2×10^5) in 10 mm dishes were preincubated with glucose (5.5 or 30 mM) for 48 h, and then incubated without or with the indicated concentrations of EHE for 24 h. The cell lysates were prepared with ice-cold lysis buffer containing 250 mM NaCl, 25 mM Tris–HCl (pH 7.5), 1 % (v/v) NP-40, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (10 μg/ml aprotinin, 1 μg/ml leupeptin). Cell lysates were washed via centrifugation, and the protein concentrations were determined using a BCA™ protein assay kit (Bio-Rad, CA, USA). The lysates containing 30 μg of protein were subjected to electrophoresis on 10 % sodium dodecyl sulfate-polyacrylamide gels, and the gels were transferred onto nitrocellulose membrane. The membranes were incubated with antibody against Bax, Bcl-2, and β-actin in TTBS (25 mM Tris–HCl, 137 mM NaCl, 0.1 % Tween 20, pH 7.4) containing 5 % skim milk at 2 h. The membranes were then washed with TTBS and incubated with secondary antibodies. Signals were developed using an ECL Western blotting detection kit (Bio-Rad) and exposed to X-ray films.

Flow Cytometric Assessment of Apoptosis

Cell death by apoptosis was quantified in 5.5 mM glucose and 30 mM glucose without or with EHE treated cells using annexin-V/PI staining. A 100 μl of cell suspension was incubated with 5 μl of annexin-V and 5 μl of PI. Cells were kept in the darkness for 15 min on an ice bath. Thereafter, 400 μl of binding buffer was added to the cell suspension and promptly read in the flow cytometry (FACScalibur and CellQuest software; Becton Dickinson, San Jose, CA, USA). Apoptotic cells were expressed as a percentage of the total number of cells.

Data and Statistical Analysis

The data are represented as mean±SE. The statistical analysis was performed using SAS software. The values were evaluated by one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range tests.

Results

Cell Viability

Figure 1 shows the effects of EHE on cell viability in INS-1 pancreatic β-cells treated with a high glucose concentration (30 mM) as determined via an MTT assay. When INS-1

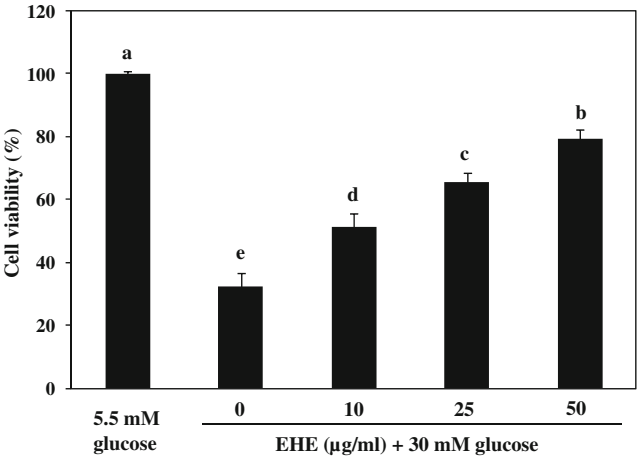

Fig. 1 Effect of EHE on cell viability in high glucose-treated INS-1 pancreatic β-cells. Cells in wells of 96-well plates (2×10^4 cells/well) were preincubated with 5.5 or 30 mM glucose for 48 h and, subsequently, incubated in the absence of EHE or 10, 25, or 50 μg/ml EHE for 24 h. The use of 5.5 mM glucose was representative of normal glucose conditions and the 30 mM glucose treatments represent high glucose conditions. Each value is expressed as mean ± SE (n=3). $^{a-e}$Values with different alphabets differ significantly at $p<0.05$ as analyzed via Duncan's multiple range test

pancreatic β-cells were treated with 30 mM glucose for 48 h, cell viability was reduced significantly. Cell viability was reduced to 32.22 % in 30 mM glucose-treated INS-1 pancreatic β-cells, but EHE protected against the cellular damage induced by 30 mM glucose in a dose-dependent manner. In particular, treatment with 50 μg/ml of EHE along with high glucose treatment resulted in a significant increase in cell viability to 79.41 %.

Lipid Peroxidation

As shown in Fig. 2, the effect of EHE on lipid peroxidation in high glucose-treated INS-1 pancreatic β-cells was determined by measuring TBARS, a lipid peroxidation product. When INS-1 pancreatic β-cells were incubated for 48 h with 30 mM glucose, TBARS was significantly increased relative to the cells treated with 5.5 mM glucose. Treatment with 50 μg/ml of EHE along with high glucose significantly inhibited TBARS formation, indicating protection against lipid peroxidation. When the cells were treated with 50 μg/ml of EHE, TBARS was reduced significantly by 0.175 nmol MDA.

Intracellular ROS Generation

As demonstrated in Fig. 3, the generation of intracellular ROS in INS-1 pancreatic β-cells increased significantly after treatment with 30 mM glucose as compared with those treated with 5.5 mM glucose. When INS-1 pancreatic β-cells were cultured with 30 mM glucose, intracellular ROS levels significantly increased to 215.81 %. However, EHE treatment dose-dependently reduced the levels of ROS in the cells induced by treatment with 30 mM glucose. In particular, treatment with 50 μg/ml of EHE resulted in a significant reduction in intracellular ROS to 144.37 %. EHE significantly reduced the elevated ROS levels induced by high levels of glucose.

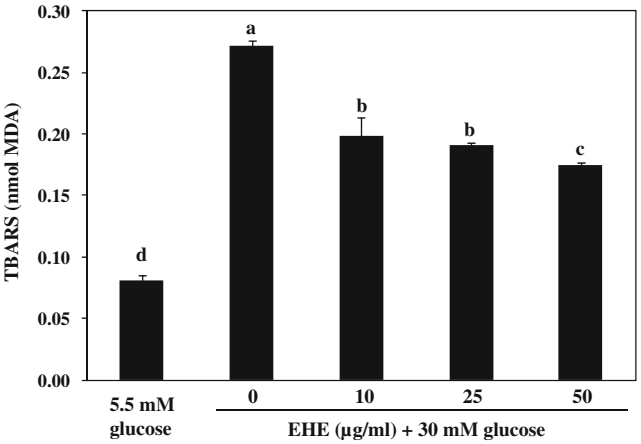

Fig. 2 Effect of EHE on TBARS generation in high glucose-treated INS-1 pancreatic β-cells. Cells in wells of 96-well plates (2×10^4 cells/well) were preincubated with glucose and incubated in the absence or presence of EHE as described in the legend to Fig. 1. Each value is expressed as mean ± SE (n=3). $^{a-d}$Values with different alphabets differ significantly at $p<0.05$, as shown by Duncan's multiple range test

NO Generation

As in shown in Fig. 4, the level of NO in INS-1 pancreatic β-cells was significantly elevated as the result of 30 mM glucose treatment as compared with the cells treated with 5.5 mM glucose. However, NO levels in the EHE-treated cells were reduced significantly, and this effect was concentration-dependent. The level of NO in the INS-1 pancreatic β-cells treated with 30 mM glucose was 216.22 %, but treatment with 50 µg/ml of EHE in conjunction with high-glucose exposure resulted in significant reductions in intracellular NO to 137.14 %. EHE scavenged the NO generated by high glucose-induced oxidative stress.

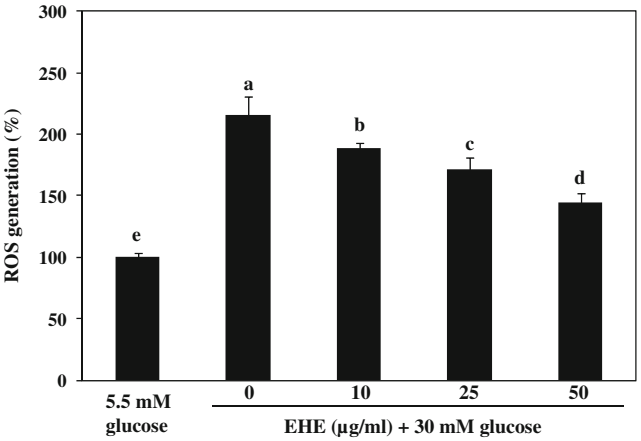

Fig. 3 Effect of EHE on intracellular ROS generation in high glucose-treated INS-1 pancreatic β-cells. Cells in 96-well plates (2×10^4 cells/well) were preincubated with glucose and incubated in the absence or presence of EHE as described in the legend to Fig. 1. Each value is expressed as mean ± SE (n=3). $^{a-e}$Values with different alphabets differ significantly at $p<0.05$ as analyzed via Duncan's multiple range test

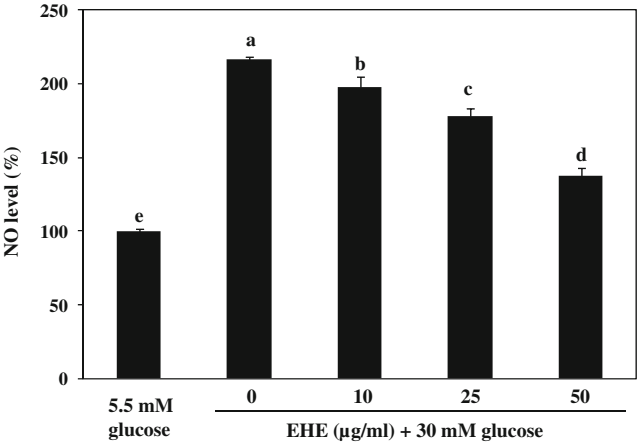

Fig. 4 Effects of EHE on NO levels in high glucose-treated INS-1 pancreatic β-cells. Cells in 96-well plates (2×10^4 cells/well) were preincubated with glucose and incubated in the absence or presence of EHE as described in the legend to Fig. 1. Each value is expressed as mean ± SE (n=3). $^{a-e}$Values with different alphabets differ significantly at $p<0.05$ as analyzed via Duncan's multiple range test

Antioxidant Enzyme Assays

Cells are protected from activated oxygen species by endogenous antioxidant enzymes including CAT, superoxide dismutase (SOD), and glutathione peroxidase (GSH-px). The effects of EHE on antioxidant enzyme activities in high glucose-treated INS-1 pancreatic β-cells are shown in Table 2. The 30 mM glucose treatment reduced CAT activity relative to that measured in the cells treated with 5.5 mM glucose. EHE treatment increased CAT activity in a dose-dependent manner. The treatment of INS-1 pancreatic β-cells with EHE increased CAT activity in the 30 mM glucose-pretreated cells. After the cells were treated with 50 μg/ml of EHE, CAT activity was increased significantly to 2.07 μmol/mg protein. Treatment for 48 h with 30 mM glucose significantly attenuated the SOD activity of INS-1 pancreatic β-cells relative to that seen in the cells pretreated with 5.5 mM glucose. The treatment of INS-1 pancreatic β-cells with EHE increased in SOD activity in the 30 mM glucose-pretreated cells. After the cells were treated with 50 μg/ml of EHE, SOD activity was significantly increased to 43.41 unit/mg protein. GSH-px activity in INS-1 pancreatic β-cells treated with 30 mM glucose was significantly reduced in

Table 2 Effects of EHE on antioxidant enzyme activities in high glucose-treated INS-1 pancreatic β-cells

	5.5 mM Glucose	EHE (μg/ml) + 30 mM glucose			
		0	10	25	50
SOD (unit/mg protein)	69.85±4.84^{a}	11.7±3.04^{d}	24.61±2.51^{c}	30.20±4.97^{c}	43.41±1.63^{b}
Catalase (μmol/mg protein/min)	2.52±0.30^{a}	1.45±0.01^{d}	1.68±0.02^{cd}	1.84±0.06^{c}	2.07±0.06^{b}
GSH-px (unit/mg protein)	5.46±0.03^{a}	0.39±0.07^{d}	1.50±0.14^{c}	2.15±0.07^{c}	3.98±0.09^{b}

Cells in 10 mm dishes (1×10^6 cells/dish) were preincubated with glucose and incubated in the absence or presence of EHE as described in the legend to Fig. 1

A Catalase (CAT) activity, *B* super oxide dismutase (SOD) activity, *C* glutathione peroxidase (GSH-px) activity, EHE *Ecklonia cava* enzymatic hydrolysate. Each value is expressed as mean ± SE (n=3)

$^{a-d}$ Values with different alphabets differ significantly at $p<0.05$ as analyzed via Duncan's multiple range test

comparison to the 5.5 mM glucose-treated cells. Treatment of 30 mM glucose-pretreated INS-1 pancreatic β-cells with EHE resulted in an increase of GSH-px activity, as shown by the measured GSH-px activity of 3.98 unit/mg protein at a dosage of 50 μg/ml.

Bax and Bcl-2 Expressions

To determine whether EHE induces expression of proteins related to high glucose-induced apoptosis, EHE concentrations of 25 and 50 μg/ml were added to INS-1 pancreatic β-cells. As shown in Fig. 5, the level of the Bax pro-apoptotic protein expression was clearly higher

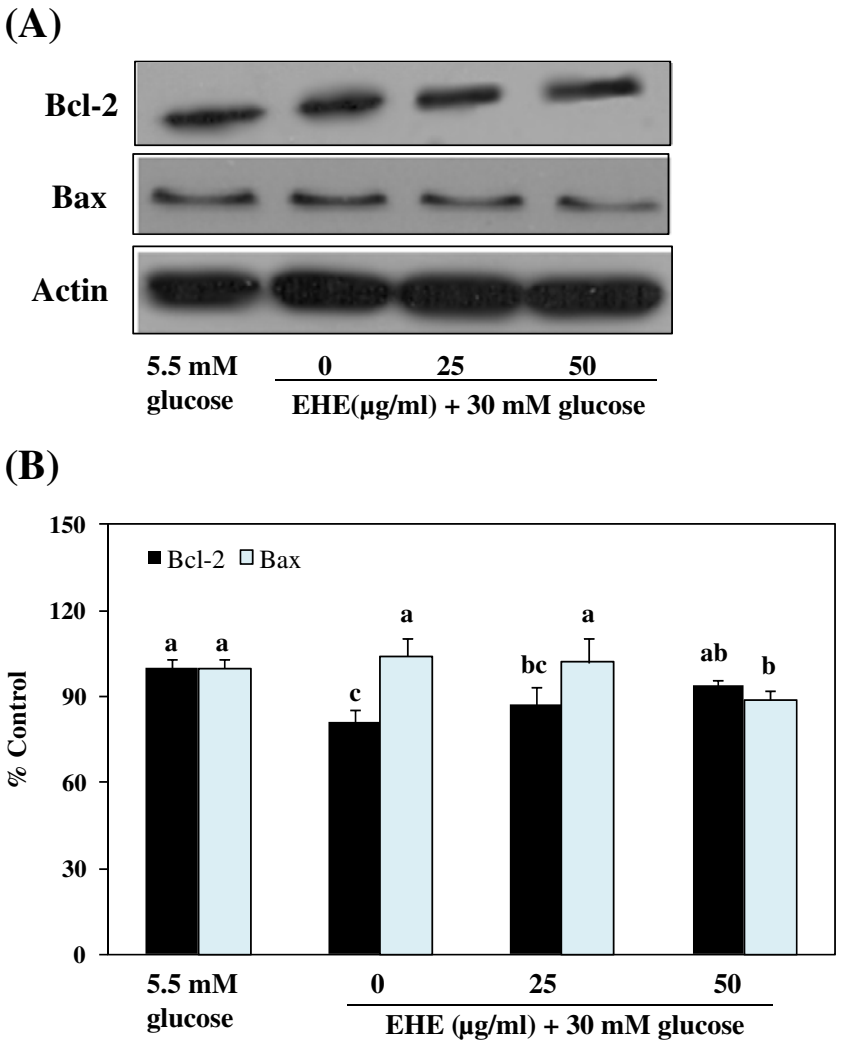

Fig. 5 Effects of EHE on Bcl-2 and Bax expressions. **A** Equal amounts of cell lysates (30 μg) were subjected to electrophoresis and analyzed for Bcl-2 and Bax expressions by Western blot. Actin was used as an internal control. **B** INS-1 pancreatic β-cells were preincubated with glucose and incubated in the absence or presence of EHE as described in the legend to Fig. 1. Each value is expressed as mean ± SD (n=3). $^{a-c}$Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test

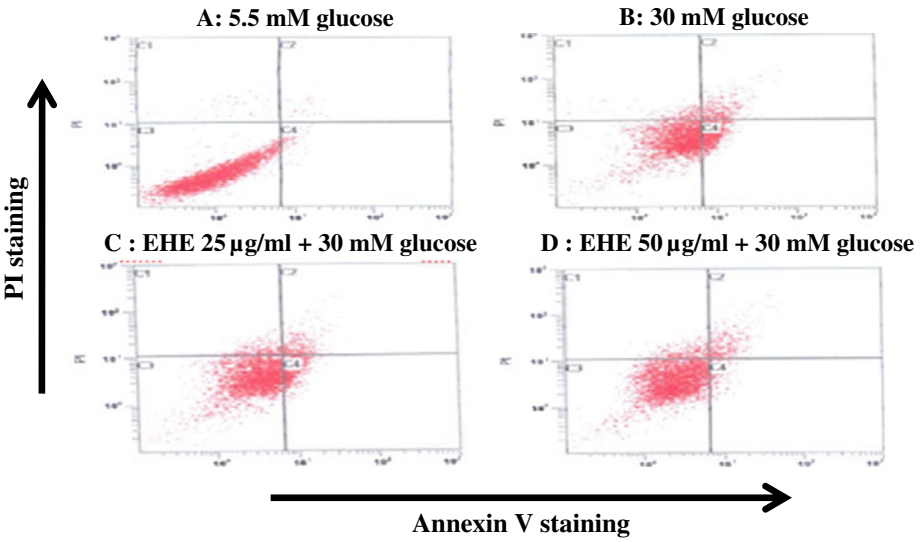

Fig. 6 Identification of type of the cell death by annexin-V/PI staining. The status of cell death was determined by counting the cells stained with annexin-V and propidium iodide using flow cytometer. Cells were preincubated with glucose and incubated in the absence or presence of EHE as described in the legend to Fig. 1. **A** 5.5 mM glucose, **B** 30 mM glucose, **C** EHE 25 µg/ml + 30 mM glucose, **D** EHE 25 µg/ml + 30 mM glucose

in 30 mM glucose-treated cells than in 5.5 mM glucose-treated cells. However, the expression level by the treatment of EHE was reduced markedly. In addition, expression of anti-apoptosis-related protein, such as Bcl-2, tends to decrease in 30 mM glucose-treated cells. On the other hand, the cells treated with EHE showed higher Bcl-2 expression than the 30 mM glucose-treated cells.

Protective Effects of Apoptosis

By flow cytometry analysis with annexin-V and PI double staining, Figure 6 shows, when INS-1 pancreatic β-cells were treated with 30 mM glucose, the number of apoptosis cells increased compared to the cells treated with 5.5 mM glucose (Fig. 6A and B). However, EHE treatment dose-dependently reduced the number of apoptosis cells in the cells induced by the treatment with 30 mM glucose (Fig. 6C and D). In particular, treatment with 50 µg/ml of EHE resulted in a significant reduction in number of apoptosis cells.

Discussion

Biochemical scientists have several techniques to extract bioactive compound from algal biomass. As one of the techniques, enzymatic hydrolysate of algal biomass gains more advantages over other conventional techniques. Enzymes can convert water-insoluble materials in to water-soluble materials; in addition, this method does not adapt any toxic chemicals. Interestingly, this technique gains high bioactive compound yield and shows enhanced biological activity in comparison with water and organic extract counterparts [7].

Previously, we reported that Celluclast hydrolysate of *E. cava* contains a high amount of sulfated polysaccharide and polyphenolic compounds [12]. Celluclast is used for catalyzing the breakdown of cellulose into glucose, cellobiose, and higher glucose polymer. Also, Celluclast can be used for the reduction of viscosity of soluble cellulosic substrates or the increase in yield of valuable products of plant origin. Accordingly, the Celluclast hydrolysis technique releases bioactive compounds by breaking down the cell walls of plant.

Several brown algal species were enzymatically hydrolysated with several carbohydrates and proteases to investigate their potential bioactivities. In that study, Celluclast hydrolysate of *E. cava* displayed high extraction yield (40.66 %) and highest radical scavenging activity among the tested hydrolysates [7]. In addition, EHE by using the carbohydrase, Celluclast displayed the highest protective effect on hydrogen peroxide-induced cell damage [8]. However, there have been no reports thus far assessing the effects of EHE on β-cell functions and survival-related oxidative stress; thus, we demonstrate herein that EHE prevents damage in INS-1 pancreatic β-cells under high glucose conditions.

To measure neutral red cell viability, MTT assays were conducted. The exposure of INS-1 pancreatic β-cells to high level of glucose resulted in significant reductions in cell viability (Fig. 1). However, EHE treatment was shown to inhibit cell death, thereby suggesting that EHE protects INS-1 pancreatic β-cells against high glucose-induced cytotoxicity.

Lipid peroxidation may be a form of cell damage mediated by free radicals [21]. Presently, high glucose treatment has been shown to induce lipid peroxidation in INS-1 pancreatic β-cells and EHE was shown to effectively inhibit TBARS formation (Fig. 2). One of the more serious consequences of lipid peroxidation is damage to biomembranes such as mitochondrial and plasma membranes. During lipid peroxidation, low molecular weight end products, most notably MDA, are formed via the oxidation of polyunsaturated fatty acids. These end products can react with two molecules of thiobarbituric acid to generate a pinkish red chromogen. The presently demonstrated protective action of EHE on TBARS formation can be attributed to its antiperoxidative effects.

High ROS levels induce oxidative stress, which can result in a variety of biochemical and physiological lesions. Such cellular damage frequently impairs metabolic function and results in cell death [22]. Our results demonstrated that the treatment of INS-1 pancreatic β-cells with 30 mM glucose significantly increased intracellular ROS levels. However, EHE inhibited high glucose-induced ROS generation (Fig. 3). Previously, we reported [8] that enzymatic hydrolysate of *E. cava* protected H_2O_2-induced cell damage in V79-4 cells. These results indicate that EHE alleviates oxidative stress via the inhibition of ROS generation induced not only by H_2O_2 but also by high glucose treatment.

High glucose treatment results in the overproduction of NO and superoxide anion (O_2^-) [23]. NO and O_2^- separately induce ischemic renal injury, and collectively, these radicals work together to inflict further damage. The toxicity and damage induced by NO and O_2^- are multiplied as they react to generate reactive ONOO-, which results in serious toxic reactions including SH-group oxidation, protein tyrosine nitration, lipid peroxidation, and DNA modifications [24]. In addition, NO and O_2^- induce highly reactive oxidative damage associated with diabetes [2]. In this study, we demonstrated that EHE scavenged the NO produced by high glucose-induced oxidative stress (Fig. 4). These findings indicate that EHE might confer important protection against the oxidative stress induced by hyperglycemia.

Cells are protected from activated oxygen species by endogenous antioxidant enzymes such as CAT, SOD, and GSH-px. We observed in this study that the application of high glucose pretreatment to INS-1 pancreatic β-cells with EHE resulted in increases in CAT activity, SOD activity, and GSH-px activity. SOD, the endogenous scavenger, catalyzes the dismutation of the highly reactive superoxide anion to H_2O_2 [25]. GSH-px catalyzes the reduction of H_2O_2 at the

expense of reduced GSH. H_2O_2 is also scavenged by CAT [26]. The reduced activities of both CAT and GSH-px in the INS-1 pancreatic β-cells treated with high glucose demonstrate a highly reduced capacity to scavenge H_2O_2 produced in the cells, with increases in ROS and oxidative stress occurring in response to high glucose treatment [27]. High superoxide anion radical production inhibits CAT activity [28]. The excess of the superoxide anion radical, as a consequence of a reduction in SOD activity, might prove responsible for the reduction in the activities of CAT in high glucose-treated INS-1 pancreatic β-cells.

ROS are by-products of normal cellular oxidative stress processes and are generated in the mitochondria and from other sources. They inflict serious damage to nucleic acids, protein, and membrane lipids, and they have been suggested to regulate the processes involved in the initiation of apoptotic signaling. Several recent studies have demonstrated that ROS generation performs a crucial function in the pro-apoptotic activities. Members of the Bcl-2 family (such as Bcl-xL) of proteins are critical regulators of the apoptotic pathway [29]. Bcl-2 and Bcl-xL are an upstream molecule in the apoptotic pathway and are identified as a potent suppressor of apoptosis [30]. Previous reports have demonstrated that Bcl-2 family-mediated caspase-3 activation is responsible for ROS-induced apoptosis [31]. In this study, we demonstrate that EHE protects damage in INS-1 pancreatic β-cells under high glucose conditions (Fig. 5). These effects were mediated by suppressing apoptosis and were associated with increasing in anti-apoptotic Bcl-2 expression; they reduce in pro-apoptotic Bax expression. In addition, flow cytometry measurements were performed using annexin-V as an apoptotic marker (Fig. 6). The exposure of INS-1 pancreatic β-cells to high levels of glucose resulted in increases in apoptosis cells rate. However, EHE treatment was shown to reduce the number of apoptosis cells. The results of two assays provide substantial evidence that EHE plays a protective role during high glucose-induced apoptosis.

Polyphenolic compounds and polysaccharides from marine algae have antioxidative activities on oxidative stress in vitro and in vivo [32, 33]. Also, marine algae exhibited hypoglycemic effect in diabetic mice [34]. Recently, several studies reported that bioactive compounds including high concentrations of polyphenols and crude sulfated polysaccharide obtained from *E. cava* enzymatic hydrolysis responsible for antioxidant activity [7] and protective effect against oxidative stress-induced cell damage [8, 12]. In the present study, we demonstrated that EHE possess a protective effect against high glucose-induced pancreatic β-cells damage through the antioxidant mechanisms as well as suppressing apoptosis.

In summary, β-cell damage occurred as a result of high glucose-induced overproduction of ROS, lipid peroxidation, and nitric oxide. Additionally, high glucose levels induced the increasing of apoptosis in β-cells. EHE protects cell damage in pancreatic β-cells under high glucose conditions via reducing ROS-induced oxidative stress and apoptosis. These findings demonstrate that that EHE might be developed into medicinal preparations, nutraceuticals, or functional foods for diabetes and may also be applied in other therapeutic fields.

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