Food Sci. Biotechnol. 21(4): 1149-1155 (2012)
DOI 10.1007/s10068-012-0150-x

RESEARCH ARTICLE

Bioactive Compounds Extracted from *Gamtae* (*Ecklonia cava*) by Using Enzymatic Hydrolysis, a Potent α-Glucosidase and α-Amylase Inhibitor, Alleviates Postprandial Hyperglycemia in Diabetic Mice

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Received: 20 February 2012 / Revised: 9 April 2012 / Accepted: 10 April 2012 / Published Online: 31 August 2012

Abstract This study was designed to investigate whether the brown alga *gamtae* (*Ecklonia cava*) may inhibit α-glucosidase and α-amylase activities, and alleviate postprandial hyperglycemia in streptozotocin-induced diabetic mice. For that purpose, we prepared an enzymatic hydrolysate from *gamtae* (EHG) by using the carbohydrase, Celluclast. EHG evidenced prominent inhibitory effect against α-glucosidase and α-amylase. The IC_{50} values of EHG against α-glucosidase and α-amylase were 0.62 and 0.59 mg/mL, respectively, which evidenced the higher activities than that of acarbose. EHG did not exert any cytotoxic effect in human umbilical vein endothelial cells (HUVECs) at various concentrations (from 0.25 to 2 mg/mL). The increase of postprandial blood glucose levels were significantly suppressed in the EHG administered group than those in the streptozotocin-induced diabetic or normal mice. Moreover, the area under curve (AUC) was significantly reduced via EHG administration (6,102 vs. 10,425 mg·min/dL) in the diabetic mice as well as it delays absorption of dietary carbohydrates. These result indicated that EHG might be a potent inhibitor for α-glucosidase and α-amylase.

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Keywords: *gamtae*, celluclast, α-glucosidase, α-amylase, hyperglycemia

Introduction

Diabetes mellitus is a chronic disease representing one of the world's most serious health concerns, developing increasingly with the increasing obesity and advancing age in the general global population. Diabetes mellitus is a complex disorder characterized by hyperglycemia. The disease is primarily classified into insulin-dependent diabetes mellitus (type 1 diabetes) and non-insulin-dependent diabetes mellitus (type 2 diabetes). The prevalence of type 2 diabetes is increasing globally (1). Postprandial hyperglycemia plays an important role in the development of type 2 diabetes, as well as in complications associated with the condition, including micro-vascular and macro-vascular diseases (2). Therefore, the control of postprandial hyperglycemia has been shown to be important in the treatment of diabetes and the prevention of cardiovascular complications.

One of the therapeutic approaches adopted thus far to ameliorate postprandial hyperglycemia involves the retardation of glucose absorption via the inhibition of carbohydrate-hydrolyzing enzymes including α-glucosidase and α-amylase, in the digestive organs (3). The powerful synthetic α-glucosidase and α-amylase inhibitors, such as acarbose, miglitol, and voglibose, function directly in reducing the sharp increases in glucose levels that occur immediately after food uptake (4-6). However, the continuous use of those synthetic agents should be limited because those agents may induce side effects such as flatulence, abdominal cramps, vomiting, and diarrhea (7). Additionally, there have been some reports describing an increased

incidence of renal tumors, serious hepatic injury, and acute hepatitis (8,9). Therefore, a number of studies have been conducted in the search for naturally derived α-glucosidase and α-amylase inhibitors that induce no deleterious side effects (10-12).

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 (13). 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, *gamtae* by using enzymatic hydrolysis technique. The enzymatic hydrolysate from *gamtae* has numerous advantages such as water solubility, high extraction efficiency, non toxicity, great variation of constituents, and multiple biological activities including antioxidant, immunomodulatory effect, anticoagulant, and anticancer (14-18). These enzymes work principally by macerating the tissues and breaking down the cell walls and complex interior storage materials.

The aim of this research is to investigate an enzymatic hydrolysate from *gamtae* (EHG) as a potential source of natural anti-hyperglycemic agent. We demonstrated previously that the EHG is a potential therapeutic agent that may ameliorate the damage induced by diabetes-associated hyperglycemia-induced oxidative stress (19). Therefore, in this study, we attempted to determine whether EHG may inhibit α-glucosidase and α-amylase activities, and also whether EHG alleviates postprandial hyperglycemia in streptozotocin-induced diabetic mice.

Materials and Methods

Materials The brown alga *gamtae* (*Ecklonia cava*) was collected from the coast of Jeju Island, Korea. Salt, sand, and epiphytes were removed using tap water. Then, the samples were rinsed carefully with fresh water and freeze-dried. Dried alga sample was ground and sifted through a 50-mesh standard testing sieve. All chemicals and reagents used were of analytical and obtained from commercial sources.

Preparation of *gamtae* enzymatic hydrolysate (EHG) The preparation of enzymatic hydrolysate followed the method previously reported (14,19). One g of the ground dried *gamtae* powder 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 (3,000×*g* for 20 min) to remove any unhydrolyzed residue. Finally, the enzymatic hydrolysate, EHG, obtained after filtration of the supernatant, was adjusted to pH 7.0 and stored for use in experiments. The EHG contained rich polyphenols and sulfated polysaccharides with high contents of fucose and sulfate group (19) (Table 1).

Inhibitory effect of EHG on α-glucosidase and α-amylase *in vitro* The α-glucosidase inhibitory assay was done by the chromogenic method described by Watanabe *et al.* (20) using a readily available yeast enzyme. Briefly, yeast α-glucosidase (0.7 U, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 100 mM phosphate buffer (pH 7.0) containing 2 g/L bovine serum albumin and 0.2 g/L NaN_3 and used as an enzyme solution. Five mM *p*-nitrophenyl-α-D-glucopyranoside in the same buffer (pH 7.0) was used as a substrate solution. The 50 μL of enzyme solution and 10 μL of sample were mixed in a microtiter plate and measured absorbance at 405 nm at zero time. After incubation for 5 min, substrate solution (50 μL) was added and incubated for another 5 min at room temperature. The increase in the absorbance from zero time was measured. The α-amylase inhibitory activity was assayed in the same way as described for α-glucosidase inhibitory assay except the using of porcine pancreatic amylase (100 U, Sigma-Aldrich) and blocked *p*-nitrophenyl-α-D-maltopentoglycoside (Sigma-Aldrich) as enzyme and substrate, respectively. Percent inhibitory activity was expressed as 100 minus relative absorbance difference (%) of test compounds to absorbance change of the control where test solution was replaced by carrier solvent.

Measurement of cytotoxicity Cell viability was assessed by measuring the uptake of the supravital dye neutral red (21). Human umbilical vein endothelial cells (HUVECs) were seeded at the concentration of 2×10^4 cells/mL in 96-well plate and pre-incubated in humidified atmosphere containing 5% CO_2 at 37°C for 24 h. After that, the cells were treated with various concentrations (0.25, 0.5, 1, and 2 mg/mL) of EHG, and further incubated for 24 h. Thereafter, the medium was carefully removed from each well, and replaced with 0.5 mL of fresh medium containing 1.14 mM neutral red. After 3 h of incubation, the medium was removed and the cells were washed twice with phosphate buffered saline (PBS, pH 7.4). The incorporated neutral red was released from the cells by incubation in the presence

of 1 mL of the cell lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM dithiothreitol (DTT), and 1% Triton X-100] containing 1% acetic acid and 50% ethanol at room temperature for 15 min. The cell lysis products were centrifuged to measure the dye taken up and absorbance of supernatant was measured spectrophotometrically at 540 nm.

Experimental animals Male ICR mice (4 weeks of age; purchased from Joong Ang Lab Animal Co., Seoul, Korea) were used. All animals were housed individually in a light (12 h on/12 h off) and temperature-controlled room with food and water available *ad libitum*. The animals were maintained with pelleted food, while tap water was available *ad libitum*. After an adjustment period of approximately 2 weeks, diabetes was induced by intraperitoneal injection of streptozotocin (60 mg/kg i.p.) dissolved in a freshly prepared citrate buffer (0.1 M, pH 4.5). After 7 days, tail bleeds were performed and animals with a blood glucose concentration above 250 mg/dL were considered to be diabetic.

Measurement of blood glucose level Normal mice and streptozotocin-induced diabetic mice fasted overnight were randomly divided into 2 groups. Fasted animals were deprived of food for at least 12 h but allowed free access to water. After overnight fasting, the mice were administrated orally soluble starch (2 g/kg BW) alone or with EHG (250 mg/kg BW). Blood samples were taken from the tail vein at 0, 30, 60, and 120 min (22). Blood glucose was measured using a glucometer (Roche Diagnostics GmbH, Mannheim, Germany). Areas under the curve (AUC) were calculated using the trapezoidal rule.

Data and statistical analysis The data are represented as mean±standard error (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 and Discussion

A sudden increase in blood glucose levels, which causes hyperglycemia in type 2 diabetes patients, occurs as the result of the hydrolysis of starch by pancreatic α-amylase and glucose uptake due to intestinal α-glucosidases (23). An effective strategy for the management of type 2 diabetes patients involved the profound inhibition of intestinal α-glucosidases and the mild inhibition of pancreatic α-amylase (24). Several algal species have been evaluated for their ability to suppress the production of glucose from carbohydrates in the gut or glucose absorption from the intestine (11,12).

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 into water-soluble materials, also this method do 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 (14). Previously, we reported that Celluclast hydrolysate of *gamtae* contains a high amount of sulfated polysaccharide and polyphenolic compounds (19). 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 *gamtae* displayed high extraction yield (40.66%) and highest antioxidant activity among the tested hydrolysates (14,15). In addition, enzymatic hydrolysate of *gamtae* (EHG) by using the carbohydrase, Celluclast displayed a potential therapeutic agent that may ameliorate the damage induced by diabetes-associated hyperglycemia-induced oxidative stress (19). Presently, we attempted to determine whether EHG may inhibit α-glucosidase and α-amylase activities, and also whether EHG alleviates postprandial hyperglycemia in streptozotocin-induced diabetic mice.

Chemical composition of EHG The chemical composition of EHG is shown in Table 1 (19). The moisture content was 6.8%, and the ash, protein, and lipid contents for EHG were 17.6, 9.3, and 4.1%, respectively. The EHG contained significantly high portions of polyphenols and crude sulfated polysaccharide (about 60.11%). In addition, the fucose and sulfate groups constituted about 60.7 and 4.3%, respectively, of the crude sulfated polysaccharides. Fucoidan usually contains large amounts of fucose and sulfates. So, polyphenols and fucoidan were the most major component in the EHG.

Inhibitory effect of EHG on α-glucosidase and α-amylase *in vitro* α-Glucosidase is one of the glucosidases located within the brush-border surface membranes of intestinal cells, and is a key enzyme in carbohydrate digestion (25). Similarly, α-amylase catalyzes the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen, and a variety of oligosaccharides, and α-glucosidase further degrades the

Table 1. Chemical composition of *gamte* enzymatic hydrolysate (EHG)[1)]

Compounds[2)]	Contents (%)
Moisture	6.82
Ash	17.64
Protein	9.39
Lipid	4.18
Polyphenols	20.09
Crude sulfated polysaccharides (CSP)	40.02
Fucose	60.71
Galactose	19.04
Xylose	20.25
Sulfate/total CSP	4.3[2)]

[1)]Information from Lee *et al.* (19)
[2)]Contents of sugar and sulfate group are expressed as a percentage of total crude sulfated polysaccharides.

disaccharides into simpler sugars, which are readily available for intestinal absorption. The inhibition of their activity in the human digestive tract is regarded as an effective method for the control of diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes (26). Therefore, effective and nontoxic inhibitors of α-glucosidase and α-amylase have long been sought. The inhibitory effect of EHG against α-glucosidase was determined using *p*-nitrophenyl-α-glucopyranoside (*p*NPG) as a substrate. EHG inhibited α-glucosidase activity in a dose-dependent manner as 40.32, 47.26, 58.66, and 63.19% at the concentrations of 0.25, 0.5, 0.75, and 1 mg/mL ($p<0.05$), respectively (Fig. 1A). Moreover, the EHG evidenced more effective than that of acarbose even at the low concentration (0.5 mg/mL). The α-amylase inhibitory effect of EHG was also illustrated using *p*-nitrophenyl-α-maltopenotoglycoside (*p*NPM) as a substrate. Both activities were compared with a commercial inhibitor, acarbose. The inhibitory effect of EHG against α-amylase was dose-dependent and the increment was 25.45, 44.24, 58.18, and 63.64% at the concentrations of 0.25, 0.5, 0.75, and 1 mg/mL ($p<0.05$), respectively (Fig. 1B). IC_{50} values of EHG against α-glucosidase and α-amylase were 0.62 and 0.59 mg/mL ($p<0.05$), respectively, which were evidenced stronger inhibitory effect than was observed with acarbose (Table 2). In the present study, EHG exhibited stronger inhibitory activity against both α-glucosidase and α-amylase than that of the commercial carbohydrate digestive enzyme inhibitor, acarbose. These results indicate that EHG may prove useful as a natural anti-hyperglycemic compound by inhibitory effects against α-glucosidase and α-amylase.

Cytotoxic effect of EHG in HUVECs To evaluate whether EHG has toxic effect to the cells, HUVECs were treated with EHG for 24 h, and cell viability was measured via neutral red assay. EHG was not exerting any cytotoxic effect at the various concentrations (0.25, 0.5, 1, and 2 mg/mL) in HUVECs (Fig. 2).

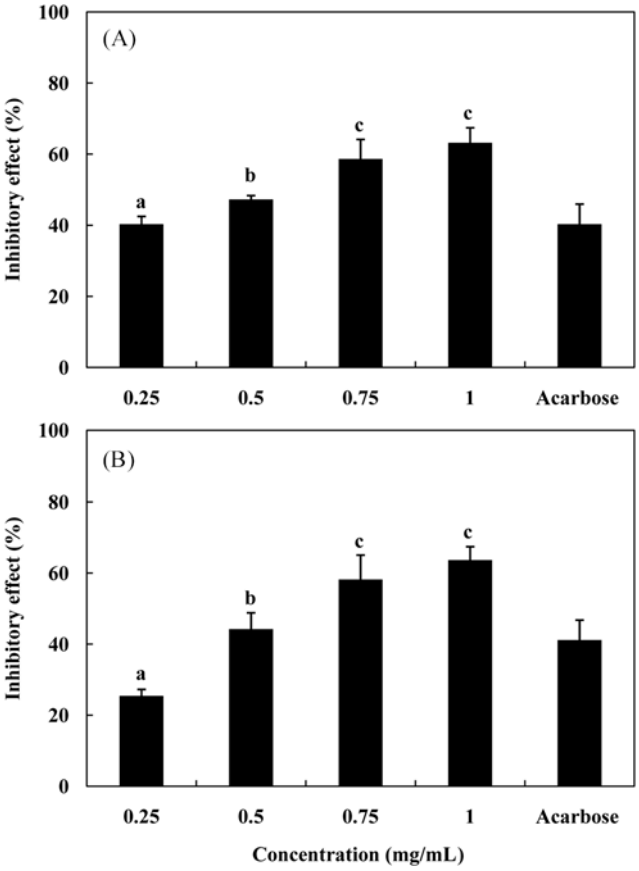

Fig. 1. Inhibitiory effect of EHG on α-glucosidase (A) and α-amylase (B). Inhibition effect was determined using *p*NPG and *p*NPM as a substrate, respectively and acarbose was used as positive control. Each value is expressed as mean±SE ($n=3$); Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test. Final concentration of acarbose is 0.5 mg/mL.

Table 2. IC_{50} values of inhibitory of EHG α-glucosidase and α-amylase

Sample	IC_{50} (mg/mL)[1)]	
	α-Glucosidase	α-Amylase
Acarbose	0.68±0.03[2)]	0.71±0.07
EHG	0.62±0.10*	0.59±0.10*

[1)]IC_{50} value is the concentration of sample required for 50% inhibition.
[2)]Significantly different from control at *$p<0.05$; Each value is expressed as mean±SE ($n=3$).

Effect of EHG on blood glucose level *in vivo* It has been previously established that postprandial hyperglycemia performs a critically important function in the development of type 2 diabetes and complications associated with cardiovascular diseases (2). Therefore, the control of

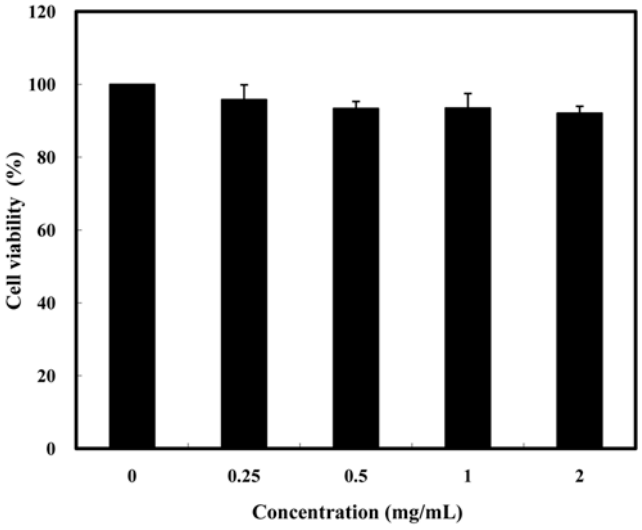

Fig. 2. Effect of EHG on cytotoxicity in HUVECs. Cells in well of 24 well plates (4×10^4) were preincubated for 48 h, and then incubated without or with indicated concentrations (0.25, 0.5, 1, and 2 mg/mL) of EHG for 24 h. Each value is expressed as mean ±SE (n=3).

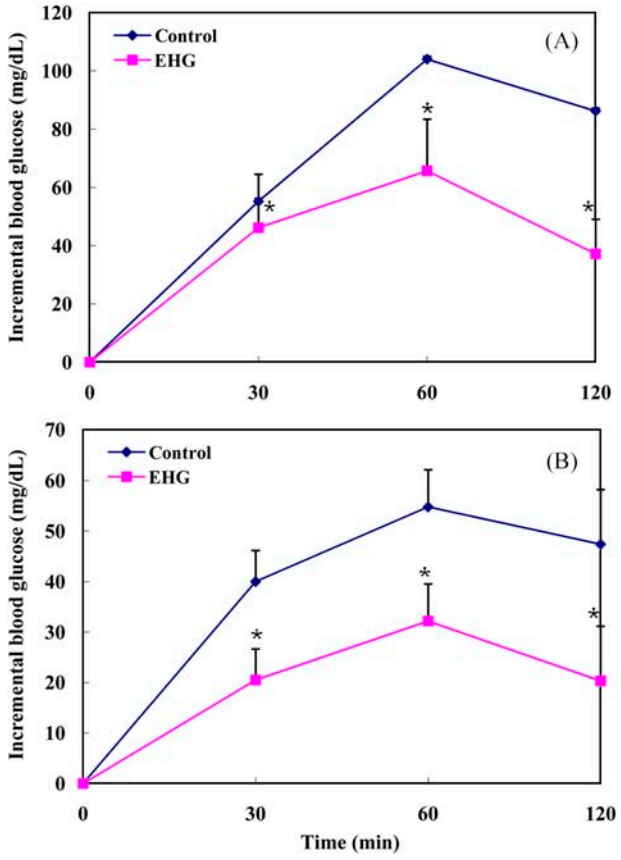

Fig. 3. Blood glucose levels after administration of EHG in streptozotocin induced diabetic mice (A) and normal mice (B). Control (distilled water) and EHG (250 mg/kg) were co-administered orally with starch (2 g/kg). Each value is expressed as mean±SE of 7 mice (n=14); Significantly different from control at *p<0.05 as analyzed by Duncan's multiple range test.

Table 3. Area under curve (AUC) of postprandial glucose responses of normal and streptozotocin-induced diabetic mice

Group[1)]	AUC (mg·min/dL)[2)]	
	Normal mice	Diabetic mice
Control	6,142±101.3	10,425±108.4
EHG	4,099±121.3**	6,102±102.3**

[1)]Control (distilled water) and EHG (250 mg/kg) were co-administered orally with starch (2 g/kg).
[2)]Each value is expressed as mean±SD of 7 mice (n=28); Significantly different from control at **p<0.01.

postprandial hyperglycemia has been regarded as important in the treatment of diabetes and the prevention of cardiovascular complications. In this study, we determined the anti-hyperglycemic effect of EHG in streptozotocin-induced diabetic and normal mice after consuming of starch. Postprandial blood glucose level of the administered EHG was lower than those of the control in diabetic mice (Fig. 3A). The blood glucose level increased until 104 mg/dL at 60 min after a meal, and decreased thereafter. However, the increase in postprandial blood glucose level was significantly suppressed (p<0.05) when the mice was fed after the administration of EHG as 46.17, 65.67, and 37.17 mg/dL at 30, 60, and 120 min, respectively. The postprandial blood glucose level was also significantly decreased when the normal mice were orally administered with starch together with EHG (Fig. 3B). In normal mice, EHG significantly suppressed (p<0.05) the postprandial hyperglycemia caused by starch. Collectively, the increase in postprandial blood glucose levels was suppressed significantly in both streptozotocin-induced diabetic and normal mice which were treated with EHG. These results suggest that EHG may delay the absorption of dietary carbohydrates, resulting in the suppression of an increase in postprandial blood glucose level.

Inoue *et al.* (27) reported that the medication, which flattens peak postprandial blood glucose, reduces the AUC of the blood glucose response curve. The AUC for glucose response of EHG administered group (6,102±102.3 mg·min/dL) was significantly lower (p<0.01) than that of the control group (10,425±108.4 mg·min/dL) in the diabetic mice (Table 3). The AUC in normal mice corroborated the hyperglycemia effect of EHG. The results indicate that EHG was shown to reduce both the blood glucose level at the peak time point and the AUC. Postprandial hyperglycemia is also involved in a variety of metabolic disorders and other diseases, including virus-based diseases and cancer (28,29). Glycosidase inhibitors are important tools for studying the mechanisms of actions on glycosidases, and are also prospective therapeutic agents for certain degenerative diseases (30). Many synthetic compounds have already been employed in efforts to develop a treatment for

diabetes. However, they have, generally, been associated with marked toxic or undesirable side effects (7,31). Therefore, marine algae are currently recognized as good candidate sources for naturally-derived anti-diabetic compounds. Previously, we evaluated the effects of *Ishige okamurae,* a brown alga, on postprandial hyperglycemia in an *in vivo* test, and also assessed the prominent effects of *I. okamurae* in both streptozotocin-induced diabetic mice and normal mice (11). Cherng and Shih (32) and Kang *et al.* (33) also noted that *Chlorella* and *Petalonia* exerted anti-diabetic effects via both insulin-like and insulin-sensitizing activities on *in vivo* tests. We presently observed that as EHG rich in polyphenols and sulfated polysaccharides showed anti-diabetic effects, it is regarded as having great potential against postprandial hyperglycemia. The findings suggest that EHG may prove useful as an effective natural anti-diabetic compound.

In conclusion, EHG exerts a profound inhibitory effect against α-glucosidase and α-amylase, which may eventually provide a method for generating a carbon source, such as starch, in the fermentation process. Further, EHG may delay the absorption of dietary carbohydrates in the intestine, resulting in the suppression of increased blood glucose levels after a meal. Thus, we suggested that EHG might be developed into medicinal preparations, nutraceuticals, or functional foods for diabetes, and may also be applied in other therapeutic fields.

Acknowledgments This research was supported by a grant from Marine Bioprocess Research Center of the Marine Biotechnology Program funded by the Ministry of Land, Transport and Maritime, Republic of Korea.

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