THREE COMPOUNDS WITH POTENT α-GLUCOSIDASE INHIBITORY ACTIVITY PURIFIED FROM SEA CUCUMBER *STICHOPUS JAPONICUS*

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Abstract

Diabetes mellitus is a worldwide health problem which is increasing every year. One therapeutic approach to decrease postprandial hyperglycemia is to slow down the absorption of glucose through inhibition of α-glucosidase. Here we explore the potential α-glucosidase inhibitory activity of compounds extracted from sea cucumber, *Stichopus japonicus.* We found that the aqueous methanol extract of sea cucumber, *Stichopus japonicus* inhibited yeast α-glucosidase activity by 68% at 0.5 mg/ml. The following hexane fraction was the most potent (≈98% inhibition at 10 μg/ml). Three compounds with potent α-glucosidase inhibitory activities were purified from *S. japonicus*. IC_{50} values of compound 1, 2 and 3 were 1.22, 0.17 and 0.36 μg/ml against *Saccharomyces cerevisiae* α-glucosidase, and 2.49, 0.24 and 0.21 μg/ml against *Bacillus stearothermophilus* α-glucosidase, respectively. Both compound **1** and 2 inhibited yeast α-glucosidase activity non-competitively (K_i value of 0.98 and 0.06 μg/ml), while compound 3 showed a mixed type inhibition (K_i value of 0.61 μg/ml). In addition, compound 1, 2 and 3 were very stable under thermal and acidic conditions up to 30 and 60 min. We conclude that compounds of *S. japonicus* have potential as natural nutraceuticals and that these compounds could be use to prevent diabetes mellitus because of their high α-glucosidase inhibitory activity.

Keywords: α-Glucosidase, α-Glucosidase inhibitors, *Stichopus japonicus*, Diabetes mellitus.

1. INTRODUCTION

Diabetes has become an alarming global problem in recent years. According to the International Diabetes Foundation (IDF), the number of people diagnosed with diabetes in the last twenty years has risen from 30 million to over 246 million. It is also a lifestyle-related disease known to trigger many complications, nephropathy, retinopathy, neuropathy, cardiovascular diseases, and so on (Saijyo *et al*., 2008). Type-2 diabetes is the most common form of diabetes, accounting for 90% of cases, and it is usually characterised by an abnormal rise in blood sugar right after a meal, called postprandial hyperglycemia (Apostolidis, Kwon, & Shetty, 2006). Mammalian α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) located in the brush-border surface membrane of intestinal cells, is the key of enzyme which catalyses the final step in the digestive process of carbohydrates. Hence, α-glucosidase inhibitors can retard the liberation of D-glucose of oligosaccharides and disaccharides from dietary complex carbohydrates, delay glucose absorption, and, therefore suppress postprandial

hyperglycaemia (Lebovitz, 1997). Such inhibitors, including acarbose and voglibose, are currently used clinically in combination with either diet or other anti-diabetic agents to control blood glucose levels of patients (Van de Laar *et al*., 2005). A main drawback of the current α-glucosidase inhibitors (such as acarbose) is the presence of side effects such as abdominal bacterial fermentation of undigested carbohydrates in the colon (Bischoff *et al*., 1985). To either avoid or decrease the adverse effects of current agents and also to provide more candidates of drug choices, it is still necessary to search for new α-glucosidase inhibitors for further drug development (Lam *et al*., 2008).

The sea cucumber (a cylindrical marine invertebrate) has long been used as a tonic food in the Ido-pacific region as well as a traditional medicine in East Asia due to its high nutraceutical value (Zhong, Khan, & Shahidi, 2007). Several papers published in the last two decades support these medical claims and document multiple biological activities of sea cucumber extracts such as wound healing promoter and exhibiting antimicrobial, anticancer and immunomodulatory properties (Mamelona *et al*., 2007).

The sea cucumber, *Stichopus japonicus*—a widespread species in East Asia, including Korea, China, Japan and Far Eastern Russia (Kanno, Li, & Kijima, 2005)—contains antifungal triterpene glycosides, holotoxins A, B, and C (Kitagawa, Sugawara, & Yosioka, 1976). However, its α-glucosidase inhibitory activities have not been reported. The objective of this study was to investigate the inhibitory effects of sea cucumber on α-glucosidase activity.

2.. MATERIALS AND METHODS

2.1 Materials

Live specimens of sea cucumbers *Stichopus japonicus* were purchased from a fishery market in Kangnung, South Korea. *B. stearothermophilus* and *S. cerevisiae* α-glucosidase, rat intestinal acetone powder, p-Nitrophenyl-α-D-glucopyranodase (pNPG), and glucose assay kit (GAGO-20) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sephadex LH-20 and Sephacryl HR-100 columns were purchased from Pharmacia Biotech Ltd. (Uppsala, Sweden). The other chemicals used in this study were of analytical grade.

2.2 Sample preparation

Fresh sea cucumber specimens were washed with tap water. The body wall was cut into small species. All samples were frozen at -80 ^{0}C for 24 h, and vacuum dried at 30 ^{0}C for 72 h. The dried samples were ground to fine powder and sieved with a ≤ 600 μm sieve and kept frozen at -20 ^{0}C until used.

2.3 Extraction and purification of α-glucosidase inhibitors

The powder of sea cucumber (200 g) was extracted with refluxing 80% methanol (2 × 5 l, each for 2 h). After the solvent was removed under reduced pressure at <40^{0}C, the residue (70.5 g) was suspended in water and then successively partitioned with n-hexane, $CHCl_3$, EtOAc, and n-BuOH. The n-hexane fraction (17.6 g) was chromatographed over silica gel (2.0

× 15.0 cm) eluting with n-hexane, hexane–$CHCl_3$, and $CHCl_3$–MeOH (in order of increasing polarity) to give nine main fractions (F1-F9). Fraction F1 and F4 were further purified by Sephadex LH-20 (3.0 × 30.0 cm) to obtain three fractions (compound 1, 102 mg; compound 2, 67 mg; compound 3, 82 mg).

2.4 Assay for α-glucosidase inhibitory activity

α-Glucosidase inhibitory activity was performed following the modified method of Kim, Nam, Kurihara, and Kim (2008). A reaction mixture containing 2.2 ml of 0.01 M phosphate buffer (pH 7), 0.1 ml of 0.25 U/ml α-glucosidase in 0.01 M phosphate buffer (pH 7), and 0.1 ml of sample was pre-incubated for 5 min at 37 ^{0}C, and then 0.1 ml of 3 mM pNPG as a substrate in the same buffer was added to the mixture. After further incubation at 37 ^{0}C for 30 min, the reaction was stopped by adding 1.5 ml of 0.1 M Na_2CO_3. Enzymatic activity was quantified by measuring the absorbance at 405 nm. The percentage of α-glucosidase enzyme inhibition by the sample was calculated by the following formula: % inhibition = [AC – AS]/AC×100, where AC is the absorbance of the control and AS is the absorbance of the tested sample. The concentration of an inhibitor in the reaction mixture required to inhibit 50% of enzyme activity under the foregoing assay conditions is defined as the IC_{50} value.

2.5 Purification of rat intestinal α-glucosidase

Rat intestinal α-glucosidase was purified according to the method of Kim, Nam, Kurihara, and Kim (2008). Commercial rat intestine acetone powder (10 g) was dissolved in buffer A (100 ml) (0.1 M potassium phosphate buffer containing 5 mM EDTA, pH 7.0), sonicated at 4 °C for 15 s, and then centrifuged at 27,000*g* at 4 °C for 60 min to obtain supernatant A. The precipitate was dissolved in buffer A (100 ml), sonicated, and then centrifuged (32,000*g*, 60 min, 4 °C) to obtain supernatant B. The combined supernatant was dialysed against buffer B (0.05 M potassium phosphate buffer containing 0.4 mM EDTA, pH 7.0) for 48 h. This was concentrated to 20 ml by ultrafiltration (cut off membrane 10 kDa) and then loaded onto a Sephacryl HR-100 column (2.6 × 60.0 cm) equilibrated with buffer B in advance. Rat intestinal α-glucosidase was eluted with buffer B at a flow rate of 0.2 ml/min.

2.6 Inhibitory assay for rat intestinal α-glucosidase (sucrase and maltase) activity

Rat intestinal α-glucosidase inhibitory activity was determined according to the modified method of Kurihara, Mitani, Tawabata, and Takahashi (1999). Sucrase activity was determined in a mixture of 500 mM sucrose (0.1 ml), the isolated compound in MeOH (0.05 ml), and 0.1 M maleate buffer (pH 6.0, 0.75 ml). The mixture was preincubated at 37 C for 5 min, and reaction was initiated by adding rat intestinal α-glucosidase (0.1 ml) to the reaction mixture. The mixture was incubated at 37 C for 60 min. The reaction was terminated by adding 2.0 M maleate–Tris–NaOH buffer (pH 7.4, 1.0 ml). To measure maltase activity, maltose (500 mM) was used instead of sucrose. The glucose release in the solution was determined using a glucose assay kit based on the glucose oxidase/peroxidase method. One

unit of α-glucosidase activity was defined as the amount of enzyme that liberated 1.0 μM of substrate per min. One unit of α-glucosidase inhibitory activity was defined as a 1 unit decrease in α-glucosidase activity.

2.7 Stability under thermal and acidic conditions

The stability of purified compounds under thermal and acidic conditions assays were performed following the method of Kim *et al*. (2005). Compounds 1, 2 and 3 were treated at 100 ^{0}C for 30 min or 37 ^{0}C for 40 min and 60 min at pH 2. Each compound was then used for inhibition assay against *B. stearothermophilus* and *S. cerevisiae* α-glucosidases. The relative inhibitory activity of bromophenol at pH 2 was calculated based on the inhibitory activity of the control.

2.8. Kinetics of α-glucosidase inhibitor

For kinetic analyses of *S.cerevisiae* α-glucosidase by compounds 1, 2 and 3, the enzyme and test compounds were incubated with increasing concentration of pNPG. Inhibitory kinetics of compounds 1, 2 and 3 for yeast α-glucosidase was determined by Lineweaaver-Burk plot analysis of the data (Lineweaver & Burk, 1934), which were calculated from the result according to Michaelis-Menten kinetics.

2.9 Statistical analysis

SPSS for windows (version 10.0, SPSS Inc., Chicago, IL) was used for statistical analysis. Each value is expressed as the mean ± standard deviation (SD). Differences among groups at various times of the experiment were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's multiple-range *t*-test. A value of $p < .05$ is considered to indicate a statistically significant effect.

3. RESULTS AND DISCUSSION

3.1 Extraction and isolation of α-glucosidase inhibitors

The powder of *S. japonicus* (200 g) was reflux extracted using 80% MeOH. The aqueous MeOH extract inhibited yeast α-glucosidase by 68% at 0.5 mg/ml. In order to isolate α-glucosidase inhibitors from sea cucumber, the 80% MeOH extract was fractionated by monitoring *S.cerevisiae* α-glucosidase inhibitory activity. Firstly, the concentrated extract was suspended in water and successively partitioned with n-hexane, $CHCl_3$, EtOAc, and n-BuOH. The n-hexane fraction showed the most potent inhibitory activity effects on yeast α-glucosidase (approximately inhibition 98% at 10 μg/ml), followed by EtOAc (20.7%), even though the H_2O fraction resulted in the highest solute yield (68.1%), followed by the n-hexane (23.2%), BuOH (4.0%), , $CHCl_3$ (3.3%), and EtOAc (1.4%) fractions (Table 1). A large amount of the solute in the aqueous MeOH (80%) extract was shifted over to H_2O fraction, but this fraction showed no α-glucosidase inhibitory activity at 0.5 mg/ml. Lipids, chlorophyll, refined oil, and sterols were dissolved and fractioned in n-hexane; , $CHCl_3$ is a excellent solvent for extracting resin. In addition, polyphenols such as flavonoids and tannins are typically extracted in EtOAc and

water-soluble components are shifted over to BuOH fraction (Suffness, Newman, & Snader, 1989). In a previous study, we found that the highest α-glucosidase inhibitory activity was observed in the EtOAc fraction of the red alga, *Grateloupia elliptica*, followed by BuOH, and H_2O fractions, while n-hexane and , $CHCl_3$ fractions did not show any α-glucosidase inhibitory activity at 0.1 mg/ml (Kim, Nam, Kurihara, & Kim, 2008). Because the n-hexane fraction appeared to be the most potent α-glucosidase inhibitory activity, the main α-glucosidase inhibitor of *S. japonicus* was provisionally considered to be a fatty acid compound.

The hexane fraction in *S. japonicus* was further purified to isolate the compounds with α-glucosidase inhibitory activity using silica gel, Sephadex LH-20 chromatographic separations, respectively. Two fractions among nine fractions eluted from silica gel column showed α-glucosidase inhibitory activity. Finally, three fractions from Sephadex LH-20 chromatographic separation were pooled, and then confirmed and classified as compound 1 (102 mg), 2 (67 mg) and 3 (82 mg). We are currently analysing the structure of these compounds.

Table 1. α-Glucosidase inhibitory activities of the solvent-partitioned fraction of sea cucumber, *S. japonicus* at different concentrations.

Fractions	Yield (%)	α-Glucosidase inhibitory activity (%)		
		0.01 mg/ml	0.1 mg/ml	0.5 mg/ml
n-Hexane	23.2	98.2 ± 0.2[a]	98.3 ± 0.1[a]	98.8 ± 0.8[a]
$CHCl_3$	3.3	NI[3)]	NI	8.0 ± 2.3[c]
EtOAc	1.4	20.7 ± 4.5[b]	92.5 ± 4.7[b]	98.5 ± 0.1[a]
n-BuOH	4.0	17.9 ± 5.3[b]	18.5 ± 3.6[c]	42.9 ± 1.4[b]
Water	68.1	NI	NI	NI

[1] Values are expressed as mean ± SD. Mean in the same column with different superscripts are significantly different ($p < 0.05$). [2] The final concentration in the reaction mixture. [3] No inhibition.

3.2 Assay for α-glucosidase inhibitory activity

Isolated compounds inhibited *Saccharomyces cerevisiae* and *Bacillus stearothermophilus* α-glucosidase activity in a dose-dependent manner (Figure 1). The IC_{50} values for compounds 1, 2 and 3 were determined to be 1.22, 0.17 and 0.36 μg/ml against *S. cerevisiae* α-glucosidase, and 2.49, 0.24 and 0.21 μg/ml against *B. stearothermophilus* α-glucosidase, respectively (Table 2). Compound 2 had strongest yeast α-glucosidase inhibitory activity among these compounds, and IC_{50} value (0.17 μg/ml) of 2 was about seven and two-fold lower than those of compounds 1 and 3, respectively. Compounds 2 and 3 showed more potent inhibitory activity against both yeast and bacterial α-glucosidases than compound 1. Nacao *et al*., (2004) reported that two sulphated fatty acids from a marine sponge *Penares sp*., sulphates A1 and B1, inhibited yeast α-glucosidase inhibitory activity (IC_{50} values of 1.2 and 1.5 μg/ml, respectively). It can be seen that the IC_{50} of compounds 2 and 3 were lower than those of

sulphates fatty acids, A1 and B1. In contrast, the commercial inhibitor, acarbose exhibited no α-glucosidase inhibitory activities against *S. cerevisiae* and *B. stearothermophilus* α-glucosidases (Table 2). This is in agreement with other reports that either described a very weak inhibitory activity of acarbose (Oki, Matsui, & Osajima, 1999) or no inhibition against *S. cerevisiae* α-glucosidase (Kim *et al*., 2004; Kim, Nam, Kurihara, & Kim, 2008). A similar result was observed for voglibose and glucono-1,5-lactone, which strongly inhibited mammalian α-glucosidases, whereas no inhibition was observed in yeast and bacterial α-glucosidases (Oki, Matsui, & Osajima, 1999; Kim, Nam, Kurihara, & Kim, 2008). In contrast, Oki, Matsui, & Osajima (1999) showed that (+)-catechin, an inhibitor of *S. cerevisiae* α-glucosidase, had no inhibitory effect on enzymes from mammalian species.

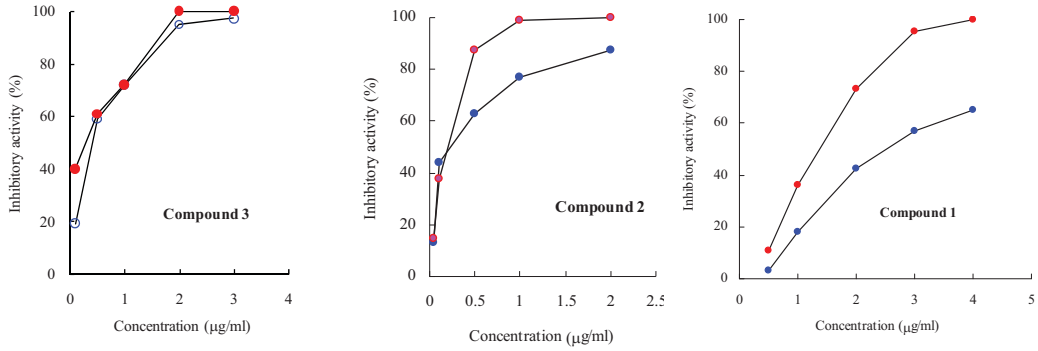

Figure 1. Dose-dependent inhibition of *S.cerevisiae* (●) and *B. Stearothermophilus* (●) α-glucosidases by sea cucumber compounds.

Table 2. IC_{50} values of isolated compounds from *S. japonicus* against α-glucosidases

Inhibitors	*IC_{50}*[1,2]			
	S. cerevisiae α-glucosidase	*B. Stearothermophilus* α-glucosidase	Sucrase (mg/ml)	Maltase (mg/ml)
Compound 1	1.22 ± 0.02^{a}	2.49 ± 0.07^{a}	3.52 ± 0.15^{a}	4.21 ± 0.31^{a}
Compound 2	0.17 ± 0.01^{c}	0.24 ± 0.02^{b}	2.30 ± 0.11^{b}	3.47 ± 0.07^{b}
Compound 3	0.36 ± 0.01^{b}	0.21 ± 0.01^{c}	0.08 ± 0.02^{c}	0.10 ± 0.03^{c}
Acarbose	NI[3]	NI	0.02 ± 0.01^{d}	0.05 ± 0.01^{d}

[1] Values are expressed as mean ± SD. Means in the same column with different superscripts are significantly different ($p < .05$). [2] The IC_{50} value is defined as the inhibitor concentration to inhibit 50% of its activity under assayed conditions. [3] NI: no inhibition.

3.3 Inhibitory activity against rat-intestinal sucrase and maltase

The inhibitory activities of *S. japonicus* compounds against rat-intestinal sucrase and maltase were also compared with those of acarbose (Table 2). The IC_{50} values of compounds 1, 2 and 3 were 3.52, 2.30 and 0.08 mg/ml against rat-intestinal sucrase, and 4.21, 3.47 and 0.10

against maltase, respectively. The IC_{50} value of acarbose was 0.02 mg/ml against sucrase and 0.05 mg/ml against maltase, respectively. Hence, acarbose was a better inhibitor against mammalian α-glucosidase than the *S. japonicus* compounds. In previous studies, most mammalian α-glucosidase inhibitors did not effectively inhibit microbial α-glucosidases, whereas catechin, an inhibitor of *S. cerevisiae* α-glucosidase, did not inhibit mammalian α-glucosidases (Oki, Matsui, & Osajima, 1999). Therefore, the higher mammalian α-glucosidase inhibitory activity of acarbose compared to sea cucumber compounds is likely due to the substrate specificities that depend on the source of α-glucosidases. This suggests that the binding of compounds is less specific to the enzyme because these compounds can bind to various proteins included in the crude enzyme solution. Bacterial, yeast, and insect enzymes, called α-glucosidase I, show higher activity toward heterogeneous substrates such as sucrose and *p*NPG, and either less or no activity toward homogeneous substrates such as maltooligosaccharides; this implies that α-glucosidase I recognises the "glucosyl structure" in the substrate (Kimura, 2000). The mould, plant, and mammalian enzymes, called α-glucosidase II, hydrolyze homogeneous substrates more rapidly than heterogeneous substrates, indicating that this class of α-glucosidases recognises the "maltostructure" (Kimura *et al*., 2004). The hydrolysis of *p*-nitrophenol 2-deoxy-α-D-arabino-hexopyranoside was catalysed by α-glucosidase II (Nishio *et al*., 2002), but no such reaction was observed with α-glucosidase I, suggesting that the 2-OH groups in the glucose moiety are essential for α-glucosidase I (Kimura *et al*., 2004). The α-glucosidase II catalysed the hydration of D-glucal to produce 2-deoxy-α-D-arabino-hexose, but α-glucosidase I yielded no detectable hydration product (Chiba *et al.,* 1988). The strong enzymatic inhibitory activity against microbial α-glucosidases shown by sea cucumber compounds is clearly better than the activity of commercial inhibitors such as acarbose and voglibose at low concentration. It is likely that sea cucumber compounds can decrease blood glucose level but with fewer, if any, adverse gastrointestinal effects, and abdominal discomfort than acarbose and voglibose (Tewari *et al*., 2003; Iwai, 2008).

3.4 Kinetics of enzyme inhibition

The inhibition mode of isolated compounds against S. *cerevisiae* α-glucosidase was analysed from the data derived from enzyme assays containing difference concentrations of pNPG, ranging from 2 to 10 mM at each different of the compounds. The data indicate that both compounds 1 and 2 showed non-competitive inhibition against S. *cerevisiae* α-glucosidase, while compound 3 displayed a mixed type inhibition mode (Figure 2). The different inhibition kinetics of these compounds seemed to be due to structural differences formed by the origins of the enzymes (Kim *et al*., 2005). The K_i (inhibition constant) values of compounds 1, 2, and 3 were 0.98, 0.06, and 0.61 μg/ml, respectively (Table 3), which shows that compound 2 was a more effective inhibitor than compounds 1 and 3.

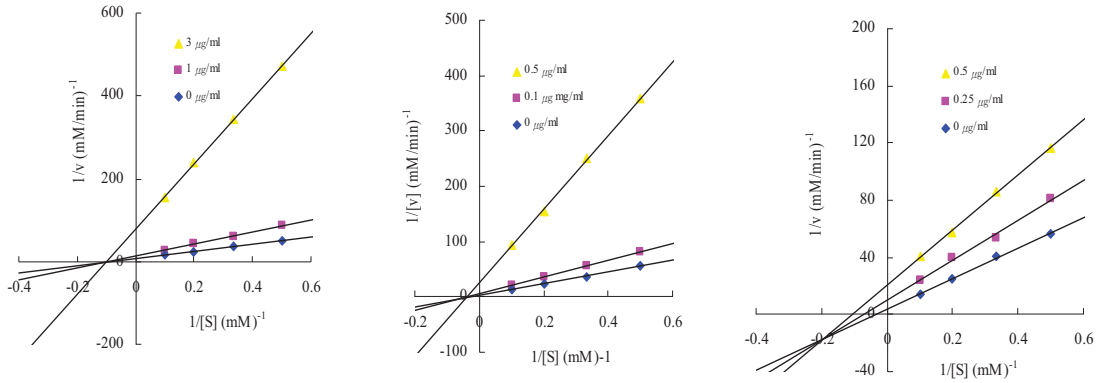

Figure 2. Lineweaver-Burk plot of *S. cerevisiae* α-glucosidase inhibition of isolated compounds from *S. japonicus* at different concentrations of pNPG.

Table 3. K_i values and inhibition mode of isolated compounds against yeast α-glucosidase.([1] K_i value was determined by Dixon plot analysis.[2] Inhibition mode was determined by Lineweaver–Burk plot).

Compounds	*K_i (μg/ml)*[1]	*Inhibition mode*[2]
1	0.98	Non-competitive
2	0.10	Non-competitive
3	0.61	Mixed

3.5 Stability of purified compounds under thermal and acidic conditions

To determine the potential for industrial usage and stability in digestive organs of the sea cucumber compounds, we investigated the stability of extracts at high temperature and low pH by measuring inhibitory activity on bacteria *B. stearothermophilus* and yeast *S. cerevisiae* α-glucosidases of sea cucumber compounds. Table 4 shows that the inhibition activity of all compounds retain more than 99% of its value against both bacteria and yeast α-glucosidases after standing for up to 30 min at 37 ^{0}C. The acidic condition (pH 2) was used to mimic the pH encountered by the compound in the stomach. The inhibitory activity of sea cucumber compounds against *S. cerevisiae* α-glucosidase was very stable at pH 2 for 40 min and remained from 80% (compound 1) to 88% (compound 3) for 60 min, respectively, while these compounds remained moderately against *B. stearothermophilus* α-glucosidase, the inhibitory activities were remained from 75 to 83% and 67 to 69% for 40 and 60 min, respectively. This finding was similar to the result of pine bark extract, where its inhibitory activity against yeast *S.cerevisiae* α-glucosidase was very stable under thermal condition and mildly stable under acidic condition (Kim *et al*., 2005). This result suggests that the purified compounds from *S. japonicus* are stable under thermal and acidic conditions and can be a candidate for development of a α-glucosidase inhibitor. Moreover, *S. japonicus* may be useful for potential usage of industrial use or as food additive.

Table 4. Effects of thermal and acidic conditions on inhibitory activities of *B. sterothermophilus* and *S. cerevisiae* α-glucosidase.Results are expressed as means ± SD, $n = 5$

Conditions	Compounds	Relative inhibition[a] (%)	
		B. sterothermophilus α-glucosidase	*S. cerevisiae* α-glucosidase
100 ^{0}C for 30 min	1	99.6 ± 0.4	99.4 ± 0.2
	2	99.8 ± 0.1	99.8 ± 0.2
	3	99.7 ± 0.4	99.8 ± 0.2
pH 2 for 40 min	1	78.4 ± 2.4	92.8 ± 1.2
	2	75.3 ± 2.5	90.5 ± 2.1
	3	82.7 ± 3.3	99.3 ± 0.1
pH 2 for 60 min	1	69.5 ± 2.5	85.2 ± 3.2
	2	67.5 ± 1.7	80.6 ± 1.4
	3	67.0 ± 5.7	87.8 ± 0.2

4. Conclusion

One of the therapeutic approaches for preventing diabetes mellitus is to retard absorption of glucose via inhibition of α-glucosidase. Hence, the search for α-glucosidase inhibitors in marine organisms is important because these inhibitors could control the postprandial hyperglycemia of diabetic patients. In this study, three compounds with strong α-glucosidase inhibitory activity were purified from sea cucumber *S. japonicus* collected from the eastern coastal area of the Korean peninsula. Therefore, compounds of *S. japonicus* can potentially be developed as a novel natural nutraceutical to prevent diabetes mellitus because of their strong α-glucosidase inhibitory activity.

The search for α-glucosidase inhibitors in marine organisms is important because these inhibitors can help control the postprandial hyperglycemia of diabetic patients. Although it is still not clear whether sea cucumber α-glucosidase inhibitors can suppress hyperglycemia, we found that sea cucumber compounds greatly inhibited yeast and bacterial α-glucosidase

Acknowledgements. *This research was supported by the Regional Research Centers Program of the Ministry of Education, Republic of Korea. T.H Nguyen is the recipient of a graduate fellowship provided by the Brain Korea (BK21) program sponsored by the Ministry of Education, Science and Technology, Republic of Korea.*

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