Physicochemical and Biological Characteristics of Squid β-Chitosan Nanoparticle

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***Abstract*—A squid chitosan nanoparticle (SCN) manufactured using a dry milling method was used to evaluate the physicochemical and various biological properties; antioxidant, antibacterial and enzymatic inhibitory activities. The degrees of deacetylation and molecular weights (Mw) of squid chitosan (SC) and SCN were 91.5 and 90.4%, and 554.6 and 44.7 kDa, respectively. The average sizes of the dispersed SC and SCN in cetyltrimethylammonium chloride were 1198.1 and 543.7 nm, respectively, which were lower than 3945.3 and 2055.9 nm of the ordinary chitosan. SCN possessed moderate antioxidant and angiotensin-I converting enzyme inhibitory activity. Interestingly, SCN had strong α-glucosidase inhibitory activity, which was comparable to that of a commercial α-glucosidase inhibitor. The minimum inhibitory concentrations of SC and SCN ranged from 90 to 200 μg/ml against eight bacteria tested. Therefore, the squid chitosan nanoparticle could be used as a neutraceutical or preservatives.**

***Index Terms*—Antibacterial, Antidiabetes, Antioxidant, Byproduct, Nanoparticle, Squid chitosan**

I. Introduction

Chitosan, a nontoxic copolymer consisting of β-(1,4)-2-amino-2-deoxy-D-glucose units derived by deacetylation of chitin, is the principal component of protective cuticles of crustaceans including crabs, shrimps, prawns, lobsters and cell wall of some fungi [1]. There are two kinds of chitin, α and β-form in nature. α-Chitin extracted from shrimp or crab shells has a structure of antiparallel chains, whereas β-chitin found in squid pen has parallel chains joined through intrasheet hydrogen bonding [2]. β-Chitin is characterized by weak intermolecular forces and has been confirmed to exhibit higher reactivity under various modification conditions as well as higher affinity for solvents than α-chitin [3]. Hence, chitosan has been applied in various fields including waste water treatment, agriculture, fabric and textiles, cosmetics, food processing, etc. due to the its diverse biological activities such as immuno-enhancing effects, antitumor, antifungal, and antibacterial activities [1]. Due to the lack of toxicity and allergenicity, chitosan is a very attractive substance for diverse applications as a biomaterial in the pharmaceutical and medical fields [4]. However, because of its high molecular weight and water insolubility, the applications of chitosan are severely limited. Compared with ordinary chitosan, low molecular weight chitosan has improved water-solubility and some special biological functions such as immuno-enhancing effects, antitumor, antioxidant, and antifungal activities [5, 6]. Furthermore, chitosan nanoparticles have been found to possess antioxidant [7], antibacterial [8] and angiotensin-I converting enzyme (ACE) activity [9]. The objectives of this study were to investigate the physicochemical and biological activities of squid β-chitosan nanoparticle including average molecular weight, average size, solubility, antioxidant, antidiabetic, antiaging, antibacterial, and antihypertension activities.

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II. Material and Methods

A. Materials

A squid pen chitosan (Arabio, Seoul, Korea) was ground to nanoparticles by dry milling method in Nanotechworld Co. (Pohang, Korea). 2,2'-Azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), α-tocopherol, p-nitrophenyl α-D-glucopyranoside (pNPG), S. cerevisiae α-glucosidase, rabbit lung angiotensin I-converting enzyme and hippuryl-l-histidyl-l-leucine (HHL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Four gram-negative bacteria (*Escherichia coli, Salmonella typhimurium, Vibrio parahaemolyticus, Vibrio vulnificus*) and four gram-positive bacteria (*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Staphylo coccusmutans*) were obtained from Korean Culture Center of Microorganisms (Seoul, Korea). All other chemicals were used of analytical grade.

B. Characterization

The size distribution and mean diameter of squid chitosan nanoparticle were determined according to the method of Nguyen et al. [10]. In brief, squid chitosan samples were dissolved either in phosphate buffered saline (PBS, 30 mM, pH 7.4) or in cetyltrimethylammonium chloride (CTAC) solution (0.1 % w/v) in PBS so that

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the intensity of light scattering was 90~100 kcps. CTAC was used as a dispersant for the de-agglomeration of chitosan nanoparticle.

The weight average molecular weight of squid chitosan was determined according to the method of Nguyen et al. [10]. Squid chitosan powder (20 mg) was dissolved in 1% acetic acid (1 ml) with mechanical stirring at room temperature. The solution was centrifuged at 12,869×g for 10s, and then the supernatant was injected into the high performance size exclusion chromatography coupled to a multiangle light scattering (HPSEC-MALLS-RI) system consisted of a pump (Model 321, Gilson, Middleton, WI, USA) and a multiangle laser light refractive index detector (RI-150, Thermo Electron Corp., Yokohama, Japan). The aqueous solution of 0.2 M CH_3COOH/0.1 M CH_3COONa was used as a mobile phase at a flow rate of 0.4 ml/min. The dn/dc value was set to 0.162 for chitosan polymers [11]. The Mw of chitosan polymers was calculated from the data collected from MALLS and RI detector using ASTR 5.3 software (Wyatt Technology Corp., Goleta, CA, USA).

The deacetylation degree of squid chitosan samples was determined according to the method of Nguyen et al. [10]. Chitosan sample (10 mg) was dried overnight at 60 ℃ under reduced pressure. The dried chitosan sample was mechanically blended with 100 mg of KBr. The mixture was compacted using an IR hydraulic press at a pressure of 8 tons for 60 s, and then dried at 60 ℃ for 24 h under reduced pressure before measuring. The spectra of chitosan sample in the form of KBr disk was determined using a Perkin Elmer Spectrum FTIR spectrometer (Perkin-Elmer, Notasulga, AL, USA) with a frequency range of 4000~400 cm^{-1}. The deacetylation degree of the chitosan sample was calculated using the method of Baxter et al. [12].

Water solubility of squid chitosan at different pHs was determined according to the method of Kubota et al. [13]. Squid chitosan sample (50 mg) was dissolved in deionized water (5 ml), and then the transmittance of the solution was determined using a spectrophotometer (Jasco Co., Tokyo, Japan) at 600 nm. The water solubility of chitosan at different pHs was also estimated from the transmittance of the solution previously prepared with aqueous acetic acid (10%) by the stepwise addition of concentrated NaOH. The sample concentration was 0.5%.

DPPH radical scavenging activity of squid chitosan nanoparticle was determined according to the method of Blois [14]. Briefly, 1 ml of the sample (2 mg/ml) in 0.1% acetic acid was mixed with 4 ml of DPPH solution (0.15 mM) in ethanol. The mixture was then vortexed vigorously and incubated at room temperature for 30 min in the dark. The absorbance was measured at 517 nm using a spectrophotometer (Jasco Co.).

Hydroxyl radical scavenging activity of chitosan nanoparticle was determined according to the method of Chung et al. [15]. A reaction mixture containing each 0.2 ml of 10 mM $FeSO_4 \cdot 7H_2O$, 10 mM EDTA and 10 mM 2-deoxyribose was mixed with 0.2 ml of the sample (2 mg/ml) in 0.1% acetic acid. And then 0.1 M phosphate buffer (pH 7.4) was added into the reaction mixture until the total volume reached to 1.8 ml. 0.2 ml of 10 mM H_2O_2 was finally added to the reaction mixture and then incubated at 37 ℃ for 4 h. After incubation, each 1 ml of 2.8% trichloroacetic acid (TCA) and 1.0% thiobarbituric acid (TBA) was added. Then, the mixture was placed in a boiling water bath for 10 min. The absorbance at 532 nm was measured.

Hydrogen peroxide scavenging activity of squid chitosan nanoparticle was determined according to the method of Müller [16]. The chitosan sample (2 mg/ml) in 0.1% acetic acid was mixed with 100 μL of phosphate buffer (0.1 M, pH 5) and 20 μL of hydrogen peroxide (10 mM) in a 96-well microplate, and then incubated at 37 ℃ for 5 min. ABTS (30 μL, 1.25 mM) and peroxidase (30 μL, 1 Unit/ml) were added to the mixture and then incubated at 37 ℃ for 10 min. The absorbance at 405 nm was read with a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

α-Glucosidase inhibitory activity was determined according to the modified method of Kurihara et al. [17]. A reaction mixture containing 2.2 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml of α-glucosidase (0.5 U/ml) in 0.01 M phosphate buffer (pH 7.0), and 0.1 ml of sample at different concentrations in 0.1% acetic acid was incubated at 37 ℃ for 5 min, and then 0.1 ml of 3 mM pNPG as a substrate in the same buffer was added to the mixture. The reaction was stopped by adding 1.5 ml of 0.1 M Na_2CO_3 after further incubated at 37 ℃ for 30 min. α-Glucosidase activity was quantified by measuring the absorbance at 405 nm.

ACE-inhibitory activity was determined according to the modified method of Cushman and Cheung [18]. One hundred μL of the sample (2 mg/ml) was incubated with 100 μl of borate buffer (0.1 M, pH 8.3) containing 0.3 M NaCl and 5 mM hippuryl–histidyl–leucine. Twenty μl of ACE (2 mU) was added, and then incubated at 37 ℃ for 30 min. The reaction was stopped by adding 150μl of 1M HCl. The hippuric acid formed was extracted with ethyl acetate (1000 μl). After removal of ethyl acetate by heat evaporation, hippuric acid was dissolved in distilled water (800 μl). The absorbance was determined at 228 nm using a spectrophotometer (Jasco Co.).

Antibacterial activity of squid chitosan was assayed according to the method of No et al. [19]. One ml of chitosan solutions at different concentrations in 1% acetic acid was added to 9 ml of Muller Hinton broth (MHB, Merck, Germany). The pH of the broth was adjusted to 5.9 with 0.1 N NaOH or 0.1 N HCl. 0.05 ml of each bacterium (1.1×10^2 CFU/ml) subcultured in tryptic soy broth (TSB, Difco, Sparks, MD, USA) at 37 ℃ for 24 h were inoculated into 10 ml of MHB and then incubated at 37 ℃ for 24 h by shaking at 100 rpm. Viable cells (log CFU/ml) were enumerated on tryptic soy agar (Difco) by pour plating 1 ml of serial dilutions of MBH followed by incubation at 37 ℃ for 48 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of chitosan required to completely inhibit bacterial growth after incubated at 37 ℃ for 72 h.

Data were analyzed with Duncan's multiple comparison test ($P \leq 0.05$) using the SPSS software package (Version 11.0; SPSS Inc., Chicago, IL, USA).

III. Results and Discussion

Average sizes of SC and SCN dispersed in cetyltrimethylammonium chloride were 1198.1 and 543.7 nm (Fig. 1 and Table Ⅰ), respectively, which were lower than 3945.3 and 2055.9 nm of the ordinary chitosan. Various particles with different sizes are existed in SC (Fig. 1), which significantly affected the average particle sizes, whereas very few in SCN. Molecular weight (Mw) and the deacetylation degree (DD) of SC and SCN are presented in Table 1. The Mw of SCN was 44.7 kDa, which was lower than 554.6 kDa of SC. The DDs of SC and SCN were 91.5 and 90.4%, respectively, in which no significant difference between chitosan and its nanoparticle. Thus, dry milling method affected molecular weight and average size, but not deacetylation degree of chitosan nanoparticle, which was the same as the result of crab chitosan nanoparticle [10]. The molecular weight of squid chitosan determined by viscosity method was 650 [5], which was similar to in this study, but much lower than 1,024 kDa of Chandumpai et al. [20], in which Mw decreased as the deacetylation period increased.

TABLE I. Average Sizes, Molecular Weight and Deacetylation Degree of Squid Chitosan and Squid Chitosan Nanoparticle

Sample	Average size (nm)	Molecular weight (kDa)	Deacetylation degree (%)
SC	1198.1	554.6±12.3[a]	91.5[a]
SCN	543.7	44.7± 1.7[b]	90.4[a]

*Means(three replicate), ±Standard Deviation, same small letters in the columns indicate non significant differences at 5%. Where: SC=squid chitosan; SCN=squid chitosan nanoparticle.

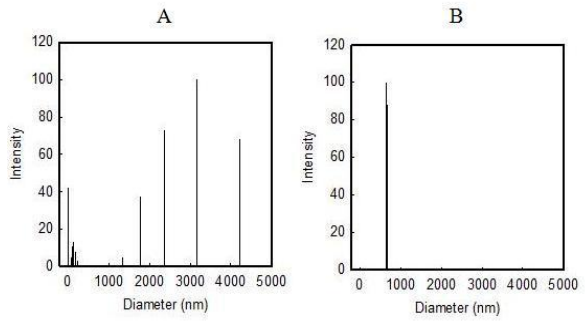

Figure 1. Size determination of squid chitosan dispersed in cetyltrimethylammonium chloride. A, squid chitosan; B, squid chitosan nanoparticle.

Overall, there was no significant difference in solubility between SC and SCN (Fig. 2). The solubility of SC and SCN was almost 100% at pH 4~6 and was much higher than 10% at pH 8~11, which was the same as in crab chitosan nanoparticle [10]. When chitosan was dissolved in aqueous acetic acid, its solubility at neutral pH appeared to be higher than that in pure water [11]. Qin et al. [21] reported that the solubility of degraded shrimp shell chitosan depended on their molecular weight, which was different from this study.

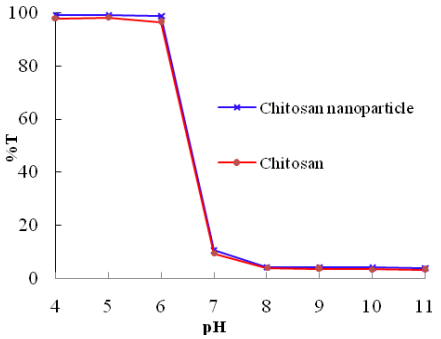

Figure 2. The solubility of squid chitosan and chitosan nanoparticle at different pHs.

The DPPH method is known as a simple, rapid, convenient and independent on sample polarity for screening quickly many antioxidants with radical scavenging activity [22]. At 2 mg/ml, the scavenging abilities of SC and SCN on DPPH radicals were 18.02 and 25.16%, respectively (Table Ⅱ), which was higher than 15.63 and 16.19% of crab α-chitosan nanoparticles [10]. DPPH radical scavenging activity of SCN was significantly ($P<0.05$) higher than that of SC, indicating that molecular weight and/or size of chitosan nanoparticle may affect DPPH radical scavenging ability. This result is in good agreement with the results of Huang et al. [5], Nguyen et al. [10] and Cho et al. [23], in which the DPPH scavenging activity of squid chitosan generally increased with decreasing Mw. This could be explained by the fact that the higher molecular weight chitosan would have lower mobility than the lower Mw chitosan. Consequently, this would increase the possibility of inter- and intramolecular bonding of the high Mw chitosan molecules, and thus the chance of exposure of their amine groups might be restricted [23]. Furthermore, the interaction of hydrophilic carboxyl groups on β-chitin decreased the intramolecular hydrogen bonds resulting in the exposure of more hydroxyl groups, thus led to greater availability of the hydroxyl groups in the β-chitin molecular [24]. However, the mechanism of squid chitosan nanoparticle against DPPH radical scavenging ability needs to be further studied.

Hydroxyl radical (HO ·), a most reactive oxygen species, can easily penetrate through cell membranes and react readily with other groups or substances in the body, resulting in cell damaging and human disease [25]. Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of a living body against various diseases. The scavenging abilities of SC and SCN at 2 mg/ml on hydroxyl radicals were 13.50 and 16.42%, respectively, which was however much lower than 92.41% of α-tocopherol at the same concentration (Table Ⅱ). The hydroxyl radical scavenging ability of SCN was a little higher than that of SC, but moderately low compared with α-tocopherol. The hydroxyl radical

scavenging ability of squid β-chitin might be due to scavenging free radicals by electron transfer to form a stable free radical ion [26]. Furthermore, low molecular weight (LMW) chitosan has short chains and thus their ability to form intramolecular hydroxyl bonds declines sharply, that is, the hydroxyl and amino groups remain activated and this enhance to scavenge the radicals [5].

The hydrogen peroxide scavenging activities of SC and SCN at 2 mg/ml were 35.14 and 41.61%, respectively in which the lower Mw, the higher activity (Table Ⅱ), which was similar to the results of crab α-chitosan nanoparticles [10]. Park et al. [27] reported that free radical scavenging activities of three kinds of crab shell chitosans with different deacetylation degrees were mainly dependent on the degree of deacetylation. In fact, although there was no significant difference in DD between SC and SCN, the hydrogen peroxide scavenging activity of SCN was significantly ($P<0.05$) higher than that of SC (Table Ⅱ). Hence, molecular weight is also a very important factor affecting the hydrogen peroxide scavenging activity of SCN.

TABLE II. ANTIOXIDANT ACTIVITIES OF SQUID CHITOSAN AND SQUID CHITOSAN NANOPARTICLE

Sample	Antioxidant activity[1,2] (%)		
	DPPH	HO ·	H_2O_2
SC	18.02 ± 0.40^{c}	13.15 ± 0.12^{c}	35.14 ± 0.74^{c}
SCN	25.16 ± 0.05^{b}	16.42 ± 0.74^{b}	41.61 ± 0.42^{b}
α-Tocopherol	97.89 ± 0.41^{a}	92.41 ± 0.73^{a}	96.01 ± 0.73^{a}

*Means(three replicate)±Standard Deviation with different supercript in the same columns are significantly different ($P<0.05$). Where: SC, squid chitosan; SCN, squid chitosan nanoparticle.

α-Glucosidase is a key enzyme in the digestive process of carbohydrates. Hence, α-glucosidase inhibitors can retard the liberation of D-glucose of oligosaccharides, delay glucose absorption, and suppress postprandial hyperglycaemia, a characterization of type 2 diabetes [28]. SC and SCN inhibited *S. cerevisie* α-glucosidase in a dose-dependent manner (Fig. 3).

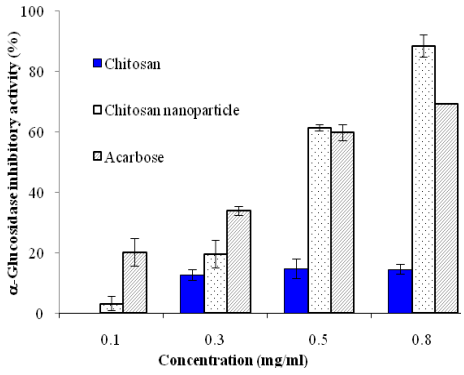

Figure 3. α-Glucosidase inhibitory activity of squid chitosan and squid chitosan nanoparticles.

The IC_{50} value (0.46 mg/ml) of SCN was slightly higher than 0.42 mg/ml of acarbose, a commercial drug, which was similar to the results of crab α-chitosan nanoparticles [10]. At 0.8 mg/ml, the α-glucosidase inhibitory activity of SCN was 85.60% (Table Ⅲ), which was significantly ($P<0.05$) higher than 15.60 and 65.30% of SC and acarbose, respectively (Table Ⅲ).

TABLE III. α-GLUCOSIDASE AND ACE INHIBITORY ACTIVITIES OF SQUID CHITOSAN AND SQUID CHITOSAN NANOPARTICLE

Sample	Enzymatic inhibitory activity (%)	
	α-Glucosidase	ACE
SC	15.60 ± 0.28^{c} (>0.8)[1]	7.58 ± 0.85^{b}
SCN	85.60 ± 1.20^{a} (0.46)[1]	13.48 ± 0.99^{a}
Acarbose	65.30 ± 0.52^{b} (0.42)[1]	–

*Means(three replicate), ±Standard Deviation, different small letters in the same columns are significantly different ($P< 0.05$). [1]means the IC_{50} value (mg/mL) is defined as the inhibitor concentration to inhibit 50% of its activity under assayed conditions. Where: SC=squid chitosan; SCN=squid chitosan nanoparticle.

Hence, the size and/or molecular weight of squid chitosan might affect α-glucosidase inhibitory activity. Up to now, few reports are available on α-glucosidase inhibitory effect of polysaccharides [29]. The interactions between proteins and polysaccharides on the inhibition of enzyme have been suggested [30]. The amino groups of chitosan nanoparticle may, therefore, have an important role in promoting inhibitory activity. Based on above results, the findings of antidiabetic agents from polysaccharides could be possible and developed. The strong enzymatic inhibitory activity of SCN against yeast α-glucosidases comparable to that of a commercial α-glucosidase inhibitor will decrease the blood glucose level with no adverse gastrointestinal effects and abdominal discomfort caused by acarbose [31]. In our previous study, crab α-nanochitosan decreased total cholesterol and low density cholesterol in rat by 46.6 and 55.7%, respectively, whereas increased high density cholesterol by 16.5% [32]. In addition, it also decreased total serum cholesterol and insulin by 31.6 and 92.9% higher than 18.2 and 51.7% of the control chitosan in db/db mice, respectively [33]. Therefore, it was considered that squid β-chitosan nanoparticle might have the antidiabetic effect by retarding the liberation of D-glucose of oligosaccharides and disaccharides, and delaying glucose absorption through the inhibition of intestinal α-glucosidase.

Angiotensin-I converting enzyme (ACE) plays an important physiological role in regulating blood pressure. Hence, ACE inhibitors are widely used as a drug in cardiac conditions [34]. The ACE inhibitory activity of SC and SCN is shown in Table Ⅲ. At 2 mg/ml, ACE inhibitory of SCN was 13.48%, which was higher than 7.58% of SC and 11.42% of crab α-nanochitosan [10]. Most studies on ACE inhibitors were focused on peptides derived from food proteins. Hence, there are very few reports on the ACE inhibitory activity of carbohydrate. IC_{50} values of hetero-chitooligosaccharides with different deacetylated degrees ranged from 1.5 to >3.2 mg/ml [6], whereas those of differently carboxylated COSs by carboxyl modifica-

tions were 10~26% at 1.5 mg/ml [35]. On the other hand, crab chitin derivatives synthesized by grafting amino functionality at the C-6 position showed the strong ACE inhibitory potential with IC_{50} of less than 0.1 μM [36]. Therefore, squid chitosan can be developed as an ingredient for ACE inhibitor. Not mentioned above, squid chitosan and its nanoparticle exhibited weak inhibitory activities against tyrosinase, collagenase and elastase, which was similar to the results of crab α-chitosan and its nanoparticles [10].

The minimum inhibitory concentration (MIC) of squid β-chitosan and its nanoparticle ranged from 90 to >200 μg/ml. Both squid chiotsan and nanoparticle resulted in the highest antibacterial activity against *S. mutans* followed by *L. monocytogenes* with MIC values of 90 and 110 μg/ml, respectively, while exhibited the weak antibacterial activity against *B. cereus*, *S. typhimurum*, and *V. parahaemolyticus* with MIC value of >200 μg/ml. This finding is similar to the results of crab α-nanochitosan, in which its MIC values were generally a little lower than those of squid β-nanochitosan [10]. Antibacterial activities of squid chitosan nanoparticle were higher than those of the control chitosan against *S. aureus* and *S. mutans*, whereas the reversed was true in *E. coli* and *V. vulnificus* (Table IV).

TABLE IV. ANTIBACTERIAL ACTIVITIES OF SQUID CHITOSAN AND SQUID CHITOSAN NANOPARTICLE

Microorganism	MIC (μg/ml)	
	SC	SCN
B. cereus	190	180
L. monocytogenes	100	110
S. aureus	190	150
S. mutans	110	90
S. typhimurium	>200	>200
E. coli	160	200
V. parahaemolyticus	>200	>200
V. vulnificus	160	200

* Where: MIC=Minimum inhibitory concentration.

Therefore, the antibacterial effects of squid chitosan differed with the Mw of chitosan and the bacterial species. Antibacterial activity of chitosan oligomers increased with increasing Mw [19], which was different from in this study. [19] also found that chitosan generally showed stronger bactericidal effects on gram-positive bacteria than gram-negative bacteria, which was similar to the results of this study. On the other hand, [37] found that for chitosan with Mw below 300 kDa, the antimicrobial activity against *S. aureus* increased as the Mw increased, while the effect on *E. coli* was weakened. [38] reported that the antimicrobial activity of partially hydrolyzed chitosan oligomers was much higher than that of chitosan, which was well agreed to in this study. They also found that the antimicrobial activity of chitooligosaccharide was correlated with the content of protonated amino groups and relative molecular weights. Although there are many reports discussing chitosan's antimicrobial activities at different conditions with conflicting results, they all confirmed that chitosan and its oligomers present a profitable potential for developing an antimicrobial agent or food preservative in food or pharmaceutical industry.

IV. CONCLUSION

The physicochemical and biological activities of squid β-chitosan nanoparticle manufactured by dry milling method were investigated for the development as a nutraceutical. There were no significant differences in the deacetylation degree and the solubility between chitosan and its nanoparticle. Squid β-chitosan nanoparticle exhibited strong antidiabetic and antibacterial activities. Furthermore, both squid chitosan and its nanoparticle exhibited moderate antioxidant and ACE inhibitory activities. Because squid chitosan nanoparticle possessed many biofunctional activities, it could be used as a neutraceutical, cosmeceutical and pharmaceutical in food, medical and cosmetic industries.

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