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Original Article

Effect of deacetylation conditions on antimicrobial activity of chitosans prepared from carapace of black tiger shrimp (*Penaeus monodon*)

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Abstract

Chitosan was prepared from black tiger shrimp carapace by deacetylation process performed in 50% NaOH at 100°C under vacuum, nitrogen and regular atmospheres. Each condition was maintained for 0.5, 1.0 and 2.0 h. Chitosan obtained from 1.0 h of deacetylation under regular atmosphere showed the lowest minimum inhibitory concentration (MIC) value of 625 ppm against *Escherichia coli* and *Staphylococcus aureus*, while *Candida albicans* was inhibited at MIC value of 312.5 ppm due to its higher degree of deacetylation (% DD) and lower molecular weight (MW) compared to chitosan deacetylated under vacuum and nitrogen atmospheres. Chitosan hydrolysates obtained from both chemical (H_2O_2 in the presence of ferric ions) and enzymatic (lysozyme) hydrolysis were not as inhibitory as the native one, except that from chemical hydrolysis, which showed the elevation of the antifungal activity against *C. albicans* when longer hydrolysis was performed. However, antibacterial activity against *E. coli* decreased when the MW of hydrolyzed chitosan decreased.

Keywords: chitosan, antimicrobials, deacetylation, black tiger shrimp, *Penaeus monodon*

1. Introduction

Chitosan, the deacetylated derivative of chitin, is a copolymer made up of 2-amino-2-deoxy-D-glucose (GlcN) and 2-acetamido-2-deoxy-D-glucose units. It is a versatile biopolymer exhibiting various unique biological properties hence its wide application in food, biomedical and chemical industries (Shahidi *et al*., 1999). Suzuki *et al*. (1986 cited by No *et al*., 2002) and Tokoro *et al*. (1988 cited by No *et al*., 2002) reported antitumor activity of hexa-*N*-acetylchitohexaose and chitohexaose. Hypocholesterolemic activity in rat of chitosan hydrolysates has also been studied (Sugano *et al*., 1992 cited by No *et al*., 2002). Recently most studies on chitosan have focused on its strong antimicrobial activity and use of chitosan as a food preservative. Antifungal activity of chitosan was reported against growth of *Aspergillus niger* and aflatoxin production of *Aspergillus parasiticus* (Fang *et al*., 1994). Spoilage yeast such as *Candida* sp., *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Saccharomycodes ludwigii* and *Rhodococcus* sp. were inactivated by chitosan hydrochloride and its hydrolysates (Rhoades and Roller, 2000). Additionally, chitosan has shown antibacterial activity against many Gram-positive and Gram-negative bacteria including food-borne pathogens (Helander *et al*., 2001; Knowles and Roller, 2001; No *et al*., 2002; Tsai and Hwang, 2004; Vishu Kumar *et al*., 2004). Antibacterial activity of chitosan was shown against *Streptococcus mutans* and *Micrococcus luteus* at minimum inhibitory concentrations (MIC) of 0.8 % v/v, as well as *Staphylococcus aureus*, *Staphylococcus epidermis* and *Bacillus subtilis* at MIC of 0.6% v/v (Jeon *et al*., 2001). Lactic acid bacteria, including *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus fermentum* and *Streptococcus faecalis* were completely

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inhibited by chitosan at MIC of 0.03 % v/v. Chitosan exhibited stronger inhibition against Gram-positive bacteria than Gram-negative bacteria such as *Escherichia coli*, *Escherichia coli* O157:H7, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Vibrio* sp., *Shigella dysenteriae* and *Salmonella typhimurium*, which were inhibited at MICs 100-10,000 ppm (Chen *et al*., 1998; Tsai and Su, 1999; Rhoades and Roller, 2000; Tsai *et al*., 2000; Jeon *et al*., 2001). However, growth of certain Gram-negative bacteria such as *Erwinia* sp., *Klebsiella pneumoniae* and *S. enteritidis* PT4 could not be inhibited by chitosan at concentration as high as 5000 ppm (Chen *et al*., 1998; Roller and Covill, 2000).

Apart from microbial strains, antimicrobial activity of chitosan varies widely, depending on % DD, molecular weight (MW), pH, temperature and the presence of interfering substances such as proteins, fats and other antimicrobials (Rhoades and Roller, 2000; Tsai *et al*., 2000; Jeon *et al*., 2001; Knowles and Roller, 2001; No *et al*., 2002; Zheng and Zhu, 2003). This study has investigated antimicrobial activity of native chitosan prepared from black tiger shrimp carapace and chemically, and enzymatically-hydrolyzed chitosan.

2. Materials and Methods

2.1 Materials

Frozen shrimp heads were supplied by Piti Seafood Co., Ltd., Songkhla (Thailand). Muller Hinton Broth (for bacteria) and Potato Dextrose Broth (for yeast) were obtained from Difco (Detroit, MI, USA). A sterile 96 well microplate was purchased from Nunc™ Brand Products (Denmark). An Ubbelohde capillary viscometer was purchased from Canon®, No.200 M548 (USA). Commercial grade 70%, 80% and 90% DD chitosan were obtained from Katokichi (Japan). Lysozyme (EC3.2.1.17) from chicken egg white (catalog no. 6876) was supplied by Sigma (St. Louis, MO, USA). Standard solution of n/400 potassium polyvinylsulfate (PVSK) was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). All chemicals (reagent grade) were supplied by Lab-Scan Ltd. (Ireland).

A microplate reader was obtained from EL_x808 Bio-TEX instrument INC. (Belgium). High performance liquid chromatography (HPLC) system including column, detector, recorder and other accessories were manufactured by Waters Co. (Massachusetts, USA). Pullulans standard was supplied by Polymer Laboratories Ltd. (Shropshire, UK).

2.2 Microorganisms

Pathogenic *Escherichia coli* isolated from a patient was obtained from culture collection of Prince of Songkla Hospital, Prince of Songkla University, Hat Yai campus. *Staphylococcus aureus*, and *Candida albicans* were from the culture collection of the Microbiology Laboratory, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai campus.

2.3 Preparation of chitin and chitosan

As shown in Figure 1, black tiger shrimp heads were cleaned and washed with water several times. They were then dried at temperature of 65°C for 6 hours, and ground into 2-4 mm in size. The dried shrimp head powder was kept in brown bottles and stored at room temperature.

Preparation of chitin began with deproteination process performed by alkaline treatments with 5 % (w/v) sodium hydroxide solution and heating at 100°C for 1 hour. The alkaline-treated materials were then washed with water to completely remove the alkali. Demineralization process was carried out thereafter by soaking the alkaline- treated materials in 2.0 N of hydrochloric acid for 1 hour at room temperature, and then washed with water to reach neutral pH. The final product called "chitin" was obtained and was kept in a brown bottle, which was stored at room temperature (Benjakul and Sophanodora, 1990).

Chitosan was prepared under various conditions of deacetylation processes, which were performed in 50% (w/v) NaOH (1 g chitin/15 ml NaOH) at 100°C under normal, vacuum and nitrogen atmospheres, each of which was conducted for 0.5, 1.0 and 2.0 hours. Chitosan obtained from each condition was further investigated for its antimicrobial activity, molecular weight and degree of deacetylation.

2.4 Antimicrobial Activity assay

Pathogenic *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* were used as the test microorganisms. The bacteria were cultured in Muller Hinton Broth (MHB), while yeast was cultured in Potato Dextrose Broth (PDB) and incubated at 37°C for 24 hours. Then, the active cultures were inoculated into 10 ml of MHB for bacteria and PDB for yeast and incubated at 37°C for 15 hours. Microorganisms were diluted with MHB/PDB to obtain bacterial/yeast count of $5\text{-}10 \times 10^5$ CFU/ml.

Antimicrobial activity of prepared chitosan was assayed by microdilution method, performed using a sterile 96 well-microplate (Rhoades and Roller, 2000). Chitosan solutions was prepared in 1% acetic acid at a concentration of 20 mg/ml (20,000 ppm) before being applied to broth and each solution was diluted (serial two-fold dilutions) with MHB or PDB to give final chitosan concentrations of 2500, 1250, 625, 312.5, 156.25 and 78.125 ppm (2-fold dilutions). One hundred microliters of each chitosan concentration and 100 ml of microbial suspension (prepared above) were added to obtain final chitosan concentrations of 1250, 625, 312.5, 156.25, 78.125 and 39.06 ppm. The plate was then incubated at 37°C for 48 h. Growth of microorganisms was determined by measuring the absorption at 595 nm at 0, 24 and 48 h using the microplate reader. Antimicrobial activity of chitosan was recorded in terms of MIC, which was defined as the lowest concentration of chitosan required to completely inhibit microbial growth after incubation at 37°C for 24 hours.

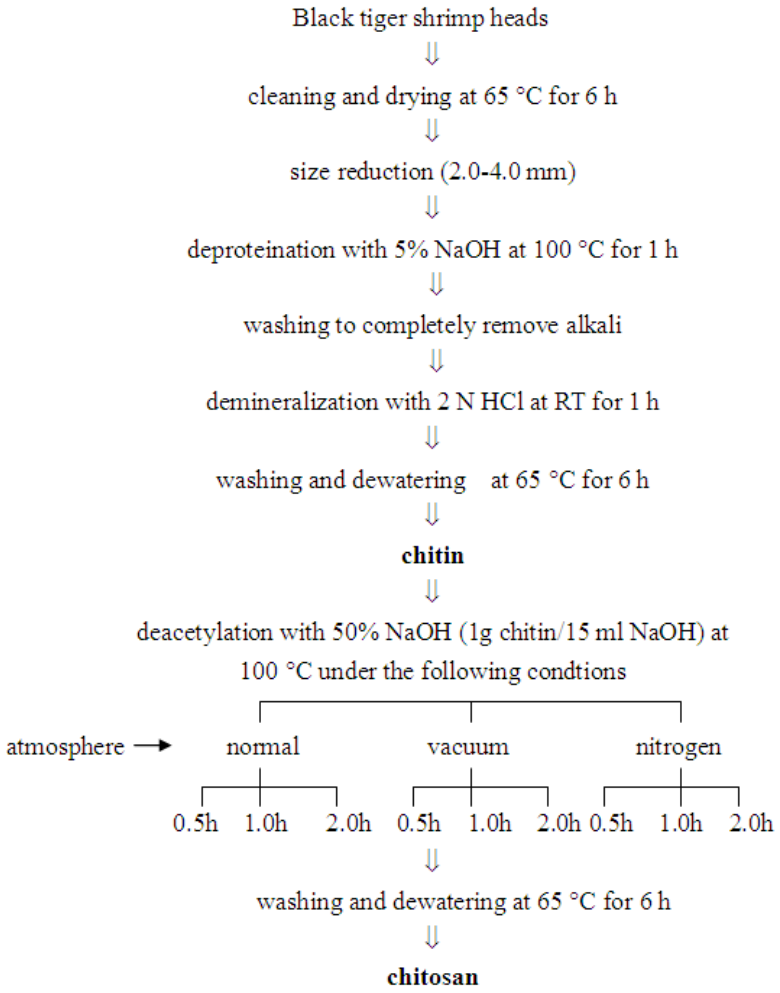

Figure 1. Preparation of chitin and chitosan

2.5 Molecular weight determination

1) Viscosity molecular weight determination

Five chitosan concentrations of 0.20, 0.10, 0.05, 0.025 and 0.0125% (w/v) were prepared in 1% acetic acid. The relative viscosity measurement was performed by using a Ubbelohde capillary viscometer at 25±0.1°C and intrinsic viscosity is defined as

$$[\eta] = (\eta_{red})c \rightarrow 0$$

It was obtained by extrapolating the reduced viscosity *vs* concentration data to zero concentration. The intercept on the abscissa is the intrinsic viscosity. Viscosity molecular weight was calculated based on Mark Houwink equation

$$[\eta] = KM^a \text{ or } \log [\eta] = \log K + a \log M$$

where, $[\eta]$ is intrinsic viscosity; M is viscosity average-molecular weight; $K = 8.93 \times 10^{-4}$ and a = 0.71 (Chen and Hwa, 1996).

2) Molecular weight determination by gel permeation chromatography (GPC)

Arcidiacono and Kaplan's (1992 cited by Chen and Hwa, 1996) method was modified using PL-GPC 110 column packed with Ultrahydrogel linear 1 loaded with 20 ml of 0.1 % (w/v) samples/standard (pullulans, polysaccharide standard kits, MW 5,800-788,000) were eluted with 0.5 M acetic buffer (0.5 M acetic acid and 0.5 M sodium acetate, pH 3.0) at flow rate of 0.6 ml/min and detected by refractive index detector. The number average molecular weight was determined from K_{av} *vs* log MW (logarithm molecular weight) standard curve. K_{av} was obtained by the following equation:

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

Here, V_e is the elution volume; V_o is the void volume; V_t is the total volume.

2.6 Degree of deacetylation determination

The determination of % DD was measured by the

colloid titration procedure using toluidine blue as an indicator reported by Toei and Kohara (1976) and Chen and Hwa (1996). Chitosan 0.5 g was dissolved in 100 ml of 5% acetic acid. One gram of chitosan/ acetic acid solution was mixed with 30 ml distilled water. After adding 2 to 3 drops of 0.1% toluidine blue (indicator), the solution was titrated with n/400 potassium polyvinylsulfate (PVSK) which had been calibrated with cetylpyridium chloride monohydrate. The degree of deacetylation was calculated as following:

$$\% \text{ DD} = [X/161/(X/161+Y/203)] \times 100$$

Here, $X = 1/400 \times 1/1000 \times f \times 161 \times V$

$$Y = 0.5 \times 1/100 - X$$

V: Titrated volume (ml) of n/400 PVSK; *f*: Factor of n/400 PVSK solution (0.995).

2.7 Preparation of hydrolyzed chitosan

The selected chitosans were degraded *via* oxidative-reductive reaction and hydrolysis with lysozyme from chicken egg white with an activity of 58,100 units/mg of protein. (Rhoades and Roller, 2000)

In the oxidative-reductive reaction, twenty gram of chitosan was dissolved in one liter of 1% acetic acid. Aliquots (8.5 ml) of chitosan solution were mixed with 1.0 ml of 10 mM aqueous $FeCl_3$. Hydrogen peroxide (1 M) was added to obtain final concentrations of 0.0, 5.0, 10.0 and 25.0 mM, and the volume of each reaction mixture was adjusted to 10.0 ml with distilled water. After 18 hours of incubation at room temperature, the viscosity and molecular weight of each mixture was measured by using a Ubbelohde capillary viscometer at 25°C. Degraded chitosan solutions were stored at 4°C for no more than 24 hours before antimicrobial activity was tested.

Lysozyme from chicken egg white was dissolved in 0.1 M potassium chloride and added to a solution containing 5.0 g of chitosan per liter in 1% acetic acid/0.2 M acetic acid - sodium acetate buffer (pH 4.9) so that the final enzyme concentration was 0.05% (wt/v). The reaction mixture was stirred at room temperature, and samples taken periodically for 0, 5, 10, 30 min and 24 hours to determine viscosity molecular weight and antimicrobial activity. Samples taken at 0, 5, 10, 30 min and 24 hours were boiled for 30 min to inactivate the enzyme and were store at 4°C for no more than 24 hours for antimicrobial activity was tested.

3. Results and Discussion

3.1 Effects of deacetylation conditions on % yield, characteristics and antimicrobial activities of chitosans prepared from black tiger shrimp

Production yield of chitin and chitosan obtained from black tiger shrimp head were only 0.91% and 0.67% based on initial fresh weight of raw material (Table 1). The major component of shrimp head was lost as shrimp gut, which was accounted to 58.4%, and only 13.3% of dried shrimp carapace was obtained from the initial fresh weight. After grinding and sizing, only 34.4% of ground carapace was obtained due to the low efficiency of grinding machine and sizing through 2.0-4.0 mm sieves. Only 19.9% of chitin was obtained from ground carapace, which was higher than that obtained from crab shell (10 % yield of chitin), but lower than that from squid and crayfish, which yielded 40 and 32%, respectively (Tolaimate *et al*., 2000).

Chitosan prepared by deacetylation processes, in which shrimp chitin was heated in the presence of alkaline under normal atmosphere, vacuum and nitrogen flux for 0.5, 1 and 2 hours, exhibited different characteristics in term of molecular weight and degree of deacetylation (% DD). Chitosan obtained from deacetylation under normal atmostphere condition for 1 and 2 hours exhibited % DD of 74.80% and 74.19%, respectively, whereas chitosan obtained from deacetylation under vacuum condition for 1 and 2 hours exhibited % DD of 65.41% and 68.28%, respectively. Deacetylation under nitrogen flux was not successful, because major component remained as chitin with % DD of 14.72, 13.03, and 13.88 at 0.5, 1.0 and 2 hours of deacetylation and the chitosan product was partially insoluble in 1% acetic acid. Deacetylation under normal atmosphere was low in an early stage of the process due to the presence of oxygen. However, the oxygen effect was minimized due to a formation of water vapor in the headspace of the closed system. The replacement of water vapor led to a reduction in oxygen content, and therefore facilitated the process of deacetylation under normal atmospheric condition. Degree of deacetylation of 35.67% was accomplished after 30 min, whereas that of chitosan prepared under vacuum condition was 63.91% (Table 2), indicating the presence of oxygen in

Table 1. Yield percentage of chitin and chitosan obtained from black tiger shrimp head

Preparation steps	Wet weight (kg)	% Yield
Fresh shrimp head	100	100[a]
Shrimp carapace (after gut removal)	41.6±1.77	41.6[a]
Drying	13.3±0.92	13.3[a]
Grinding and sizing	4.58±0.23	4.58[a]
Demineralization and washing (chitin)	0.91±0.04	0.91[a]
		19.9[b]
Deacetylation (chitosan)	0.67±0.07	0.67[a]
		14.6[b]
		73.6[c]

[a] % yield based on initial raw materials
[b] % yield based on dried and ground shrimp carapace
[c] % yield based on chitin weight before deacetylation

Table 2. Effect of deacetylation process and reaction time on molecular weight and % DD of chitosan from black tiger shrimp carapace

Atmosphere conditions	Time (hours)	M_v	M_n	% DD
Atmosphere	0.5	345±67.85^{c}	63,173	35.67±0.44^{d}
	1.0	1500±269.1^{b}	34,756	74.80±1.03^{a}
	2.0	2470±611.2^{b}	56,579	74.19±0.53^{a}
Vacuum	0.5	4200±513.1^{a}	84,555	63.91±0.83^{c}
	1.0	4950±1189^{a}	114,050	65.41±0.62^{c}
	2.0	5260±1490^{a}	106,496	68.28±0.24^{b}
Nitrogen	0.5	ND	ND	14.72±0.24^{e}
	1.0	ND	ND	13.03±0.24^{e}
	2.0	ND	ND	13.88±0.24^{e}

$^{a\text{-}e}$ means with different superscripts with a column indicate significant difference (P<0.05)
% DD = % Degree of deacetylation.
M_v = viscosity average molecular weight.
M_n = number average molecular weight.
ND = cannot be detected due to insolubility of chitosan.

Table 3. Minimum inhibitory concentration (MIC) values of chitosans prepared under various deacetylation processes against *E. coli, S. aureus* and *C. albicans*

Types of chitosan		MIC (ppm)		
Conditions	Reaction times (h)	*E. coli*	*S. aureus*	*C. albicans*
atmosphere	0.5	625^{B,a}	625^{B,a}	625^{C,a}
	1.0	625^{B,a}	625^{B,a}	312.5^{D,b}
	2.0	1250^{A,a}	625^{B,b}	312.5^{D,c}
vacuum	0.5	1250^{A,a}	1250^{A,a}	625^{C,b}
	1.0	1250^{A,a}	1250^{A,a}	625^{C,b}
	2.0	625^{B,b}	625^{B,b}	>1250^{A,a}
nitrogen	0.5	1250^{A,b}	1250^{A,b}	>1250^{A,a}
	1.0	1250^{A,b}	1250^{A,b}	>1250^{A,a}
	2.0	1250^{A,a}	1250^{A,a}	1250^{B,a}

$^{A\text{-}D}$ means with different superscripts with a column indicate significant difference (P< 0.05)
$^{a\text{-}c}$ means with different superscripts with a row indicate significant difference (P< 0.05)

the system when deacetylation was conducted under normal atmospheric condition. In addition, deacetylation under normal atmospheric condition yielded chitosan with lower molecular weight than the one obtained from deacetylation under vacuum atmosphere, indicating that better hydrolysis had occurred. This finding shows that vacuum atmosphere is not the only option for deacetylating chitin to chitosan, regular heating in the closed system, which can prevent the entry of air, makes it possible to accomplish the deacetylation process.

The MIC values of chitosans were determined against pathogenic *E. coli, S. aureus* and *C. albicans.* As shown in Table 3, MIC values of chitosans varied depending on conditions of deacetylation processes, reaction times and test microorganisms. In general, chitosan prepared under atmosphere showed the lowest MIC value or highest inhibitory effect on test microorganisms, whereas that deacetylated under N_2 showed the least inhibitory effect. As shown in Table 2, deacetylation under normal atmosphere yielded chitosan with highest % DD, but % DD was lowest when chitosan was prepared under nitrogen atmosphere. Plus, deacetylation under normal atmosphere yielded lower MW chitosan compared to that obtained under vacuum atmosphere, indicating hydrolysis of chitosan had occurred. There-

Table 4. Antimicrobial activity of native and hydrolyzed chitosan prepared by chemical hydrolysis catalyzed with H_2O_2 at different concentrations against *E. coli*, *S. aureus* and *C. albicans* in 1% acetic acid

mM H_2O_2	MW (kDa)	MIC (ppm)		
		E. coli	*S. aureus*	*C. albicans*
0	3260^{A}	$625^{B,a}$	$625^{A,a}$	$312.5^{A,b}$
5	157	$625^{B,a}$	$625^{A,a}$	$156.25^{B,b}$
10	3.17^{C}	$625^{B,a}$	$625^{A,a}$	$78.13^{C,b}$
25	0.24^{D}	$1250^{A,a}$	$625^{A,b}$	$78.13^{C,c}$

$^{A-D}$means with different superscripts with a column indicate significant difference (P< 0.05)

$^{a-c}$means with different superscripts with a row indicate significant difference (P< 0.05)

Table 5. Antimicrobial activity of native and hydrolyzed chitosan prepared by enzymatic hydrolysis catalyzed with lysozyme against *E. coli*, *S. aureus* and *C. albicans* in 0.2 M acetate buffer

Time for hydrolysis	MW (kDa)	MIC (ppm)		
		E. coli	*S. aureus*	*C. albicans*
0 min	438^{A}	$625^{A,a}$	$>1250^{A,b}$	$>1250^{A,b}$
5 min	586^{A}	$625^{A,a}$	$>1250^{A,b}$	$>1250^{A,b}$
10 min	473^{A}	$625^{A,a}$	$>1250^{A,b}$	$>1250^{A,b}$
30 min	543^{A}	$625^{A,a}$	$>1250^{A,b}$	$>1250^{A,b}$
24 hours	501^{A}	$625^{A,a}$	$>1250^{A,b}$	$>1250^{A,b}$

^{A} means with different superscripts with a column indicate significant difference (P<0.05)

$^{a-b}$ means with different superscripts with a row indicate significant difference (P<0.05)

fore, antimicrobial activity of chitosan was dependent on the deacetylation methods used. However, there is not enough evidence to specify which parameter (between % DD and MW) has the major contribution. Variation of deacetylation process yielded chitosan with significant differences in % DD as well as variation of the molecular weight. As Tsigos *et al.* (2000) reported, chitosan with high the degree of deacetylation had more inhibitory activity against microorganisms than chitosan with low the degree of deacetylation. Similarly, Simpson *et al.* (1997) reported that chitosan with a degree of deacetylation of 92.5% was more effective than chitosan with a degree of deacetylation of 85%. On the contrary, Ikinci *et al.* (2002) reported that change in degree of deacetylation (73, 84 and 95%) did not have any effect on antimicrobial activity of chitosan (similar inhibition zone) against *Porphyromonas gingivalis*.

Chitosan obtained from 1 hour deacetylation under atmospheric condition exhibited highest antifungal activity, but inhibited pathogenic *E. coli* and *S. aureus* at the same level with the one from 0.5 h deacetylation. Under atmospheric condition, chitosan deacetylated for 1 hour demonstrated higher inhibitory activity against test microorganisms than those deacetylated for 0.5 and 2.0 hours. Among all test microorganisms, *C. albicans* appeared to be most susceptible to chitosan from black tiger shrimp carapace, compared to pathogenic *E. coli* and *S. aureus*. Generally, spoilage yeasts were more sensitive to chitosan than Gram-positive bacteria, which were in turn more sensitive than Gram-negative bacteria. (Sagoo *et al.*, 2002). Nikaido (1996) reported that as a polymeric macromolecule, chitosan is unable to pass the outer membrane of Gram-negative bacteria, since this membrane functions as an efficient outer permeability barrier against macromolecules. Moreover, the antimicrobial effect of chitosan is reported to be dependent on its MW (Zheng and Zhu, 2003). Various investigators have reported the close relationships between molecular weight and antimicrobial activity of chitosan. Jeon and Kim (1998) found that the highest MW oligomers (MW 500-10,000 Da) had the strongest bactericidal and fungicidal activities against most pathogens tested. Jeon *et al.* (2001) reported that MW (10-1 kDa) of chitosan oligomers was critical for microorganism inhibition and efficacy increased with MW increased although the native one (MW 685 kDa) was the most inhibitory. Zheng and Zhu (2003) reported that the effect of

chitosan with MW below 300 kDa on *Staphylococcus aureus* was strengthened as the MW increased, whereas the antimicrobial effect on *Escherichia coli* increased as the MW was decreased. Thus, two possible antimicrobial mechanisms were proposed that the chitosan of higher MW forms a film which may inhibit nutrient adsorptions, but that with a lower MW enters the microbial cell more easily to disturb the metabolism of the cell.

3.2 Antimicrobial activity of hydrolyzed chitosan

The hydrolyzed chitosan prepared by chemical reaction led to a significant reduction of molecular weight. The treatment of chitosan with H_2O_2 also resulted in a change in the chemical structure, as the degradation of chitosan by H_2O_2 is a random process and can be completely degraded to its constituent monomers. The changes, such as formation of carboxyl groups and deamination, increased with the decrease of molecular weight. Nordtveit *et al.* (1994) prepared chitosan hydrolysates using H_2O_2, in the presence of Fe (III) to generate hydroxyl radicals, which cleaved the chitosan molecule by nucleophilic attack. As shown in Table 4, the molecular weight of chitosan decreased as hydrogen peroxide concentration increased. The molecular weight of chitosan obtained at H_2O_2 concentration of 0, 5, 10 and 25 mM were 3260, 157, 3.17 and 0.24 kDa, respectively. Antimicrobial activities of chitosan hydrolysates (3.17-157 kDa) against both pathogenic *E. coli* and *S. aureus* were shown at MIC of 625, 625 ppm, respectively, which were not different from native chitosan (3260 kDa). However, the low molecular weight chitosan obtained at H_2O_2 concentration of 10 and 25 mM showed lowest MIC of 78.125 ppm against *C. albicans*. The results are in agreement with Hirano and Nagao (1989) which found that low molecular weight chitosan in agar system inhibited a range of phytopathogenic fungi more effectively than high molecular weight chitosan. However, hydrolyzed chitosan with lower MW had no inhibitory activity against pathogenic *E. coli* and *S. aureus*; particular, the one with MW of 0.24 kDa had less inhibitory effect against pathogenic *E. coli*. This phenomenon was different from the finding of Jeon *et al*. (2001), which indicated that native chitosan (685 kDa) was more effective on Gram-positive and Gram-negative bacteria than chitosan oligomers, whose MW was between 1-10 kDa. No *et al*. (2002) was also reported the same result that native chitosan showed higher antibacterial activities than chitosan oligomers.

For enzymatic degradation of chitosan, several researchers have studied the preparation and physiological activities of chitosan oligomers with the aid of lysozyme. Nordtveit *et al.* (1996) reported lysozyme from chicken egg used for chitosan degradation has been shown to be most efficacious when the chitosan is only partially deacetylated. Rhoades and Roller (2000) also found that a degraded form of chitosan was successfully produced by using lysozyme-overnight treatment of chitosan (viscosities >800 s) with lysozyme resulted in degraded forms of chitosan with viscosities of 140 s. However, the hydrolyzed chitosan prepared by using lysozyme in this study did not show significant difference in the molecular weight compared to the native one. As shown in Table 5, hydrolysis of native chitosan with lysozyme for 0, 5, 10, 30 minutes and 24 hours had the molecular weight 438, 586, 473, 543 and 501 kDa, respectively. These results disagree with the results of other authors, which may be due to differences of chitosan in terms of chemical structure and complexity. Antimicrobial activity of hydrolyzed chitosan (473-583 kDa) against pathogenic *E. coli* was not different from the native chitosan (438 kDa), whereas the inhibition against *S. aureus* and *C. albicans* was lower than the native one. The chitosan molecular weight and antimicrobial activity from the enzymatic study were different from the previous result due to the limitation of chitosan solubility in the buffer (instead of 1% acetic acid), which was used to facilitate the lysozyme activity.

4. Conclusions

Deacetylation of chitin obtained from black tiger shrimp carapace under atmosphere yielded chitosans exhibiting highest antimicrobial activity, due to higher % DD chitosan, whereas chitosans obtained under nitrogen atmosphere showed the least inhibition against all test microorganisms. Degree of deacetylation exhibited significant effect on the antimicrobial activity of chitosan as well as its MW. Chitosan hydrolyzed by chemical reaction can improve antifungal activity greatly against *C. albicans* as the MW decreases, but had antibacterial activity that decreased with the MW. However, hydrolyzed chitosan prepared by using lysozyme did not show siginificant effect on the molecular weight. The native chitosan and the hydrolyzed chitosan were equally inhibitory against pathogenic *E. coli*, but the degraded ones were not effective on *S. aureus* and *C. albicans* at a concentration of 1250 ppm, the highest concentration tested. This is maybe caused by performing the antimicrobial assay of the chitosan in a buffer system, which may limit the solubility of chitosan.

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