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Antimicrobial Activity of Hetero-Chitosans and Their Oligosaccharides with Different Molecular Weights

PARK, PYO-JAM, JAE-YOUNG JE, HEE-GUK BYUN, SUNG-HOON MOON, AND SE-KWON KIM*

Department of Chemistry, Pukyong National University, Busan 608-737, Korea

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Abstract This study was performed to investigate the antimicrobial effects of hetero-chitosans and their oligosaccharides against three Gram-negative bacteria and five Gram-positive bacteria. Nine classes of hetero-chitosan oligosaccharides consisted of partially deacetylated chitosans; 90%, 75%, and 50% deacetylated chitosans. Based on molecular weight, they were prepared using an ultrafiltration membrane reactor system. Seventy-five percent deacetylated chitosan showed the highest antimicrobial acitivity as compared with the 90% and 50% deacetylated chitosan, and the activity was dependent on their molecular weights. It was apparent that the growth of Gram-negative bacteria is less inhibited in the presence of the heterochitosans and their oligosaccharides than Gram-positive bacteria. These results revealed that the antimicrobial effects of hetero-chitosans and their oligosaccharides depend on the degree of deacetylation, and their molecular weights.

Key words: Hetero-chitosan, chitooligosaccharide, antimicrobial activity, minimum inhibitory concentration

Chitosan is a partially deacetylated polymer of *N*-acetyl glucosamine, which is obtained after alkaline deacetylation of the chitin derived from the exoskeletons of crustaceans and arthropods. It has received considerable attention for its commercial applications in biomedical, food, and chemical industries [13, 14]. In addition, chitosan and chitosan oligosaccharides (COSs) have attracted considerable interest due to their biological activities; that is, antitumor activity [8, 23, 24, 30], immuno-enhancing effects [7, 25, 27], enhancing protective effects against infection with some pathogens in mice [26, 35], antifungal activity [6, 11, 16], and antimicrobial activity [1, 5, 6, 9, 10, 12, 17, 31, 32].

*Corresponding author
Phone: 82-51-620-6375; Fax: 82-51-628-8147;
E-mail: sknkim@mail.pknu.ac.kr

Recent studies on the antimicrobial activity of chitosan and COSs have revealed that chitosan is more effective in inhibiting the growth of bacteria than COSs [1, 5, 6, 9, 10, 12, 17, 31, 32]. Furthermore, the antimicrobial activity of chitosan and COSs was dependent on its molecular weight [10, 31]. However, most studies were performed with only one or a few different molecular weights of chitosans or COSs [28, 31, 33, 34]. Therefore, there is little information available on the antimicrobial activity of hetero-chitosans with different degrees of deacetylation and their COSs with widely different molecular weights.

In this study, the antimicrobial activity of hetero-chitosans and hetero-COSs with different degrees of deacetylation and molecular weights was investigated against three Gram-negative and five Gram-positive bacteria.

MATERIALS AND METHODS

Materials

Chitin prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). The chitosanase (35,000 units/g protein) derived from *Bacillus* sp. was purchased from Amicogen Co. (Jinju, Korea) and cellulase was donated by Pacific Chemical Co. Ltd. (Seoul, Korea). An ultrafiltration (UF) membrane reactor system used for the preparation and the fractionation of hetero-COSs based on their molecular weights was purchased from Millipore Co. (Bedford, MA, U.S.A.). The microorganisms tested for antibacterial activity were obtained from KCTC (Korean Collection of Type Cultures) and ATCC (American Type Culture Collection). All other reagents were of the highest grade available commercially.

Preparation of Hetero-chitosan and Their Oligosaccharides

Three kinds of partially deacetylated chitosans, designated as 90%, 75%, and 50% deacetylated chitosan, were prepared from crab chitin, according to our previous method [18]. Hetero-COSs were prepared from partially deacetylated hetero-

Table 1. Bacterial strains used for antibacterial activity.

Bacteria		Sources
Gram (-) bacterium	*Escherichia coli*	KCTC 1682
	Salmonella typhimurium	KCTC 2424
	Pseudomonas aeruginosa	KCTC 1750
Gram (+) bacterium	*Micrococcus luteus*	KCTC 10240
	Staphylococcus epidermidis	KCTC 1917
	Staphylococcus aureus	ATCC 65389
	Bacillus subtilis	KCTC 1028
	Bacillus cereus	KCTC 1012

chitosans by two enzymatic reactions using chitosanase and cellulase in an UF membrane reactor system according to a previously reported method [17]. Three different UF membranes with molecular weight cutoffs (MWCO) of 1, 5, and 10 kDa were used, and fractionated into nine kinds of COSs with relatively higher molecular weights (MW= 10,000- 5,000 Da; 90-, 75-, and 50-HMWCOSs), medium molecular weights (MW= 5,000- 1,000 Da; 90-, 75-, and 50-MMWCOSs), and lower molecular weights (MW<1,000 Da; 90-, 75-, and 50-LMWCOSs). The nine kinds of COSs recovered were lyophilized on a freezing-drier for 5 days.

Assays for Antimicrobial Activity

The antimicrobial activity of hetero-chitosans and their oligosaccharides was investigated against three Gram-negative bacteria (*Escherichia coli, Salmonella typhimurium,* and *Pseudomonas aeruginosa*) and five Gram-positive bacteria (*Micrococcus luteus, Staphylococcus epidermidis, Staphylococcus aureus, Bacillus subtilis,* and *Bacillus cereus*). The assays were carried out by colony count on incubated agar plates. The mixture of 0.5 ml of cultured bacteria, 0.5 ml of sample solution and 4 ml of 0.05 M acetate buffer (pH 5.5) was incubated with shaking at 37°C for 1 h. In control experiments, 4.5 ml of acetate buffer was used. The mixture solution (1 ml) was diluted 10-fold, added to Tryptic soy agar (TSA, Difco Lab., Detroit, MI, U.S.A.) medium, plated on a plastic petri-dish, and then incubated at 37°C for 24 h. After incubation, colony forming units (CFUs) were counted to indicate the level of bactericidal activity. The minimum inhibitory concentration (MIC) was tested by two-fold serial dilution in broth. To determine the MIC, bacteria culture (10^6- 10^7 colonies/ml) grown in 5 ml Tryptic soy broth (TSB), containing 1 ml of the test sample, was incubated at 37°C for 18 h. MIC was defined as the lowest concentration of the tested sample at which cell growth was not visible with naked eye.

Results and Disscussion

Antimicrobial Activity of Hetero-Chitosans and Their Oligosacharides

As shown in Fig. 1, hetero-chitosans markedly inhibited the growth of Gram-negative bacteria such as *E. coli, S.*

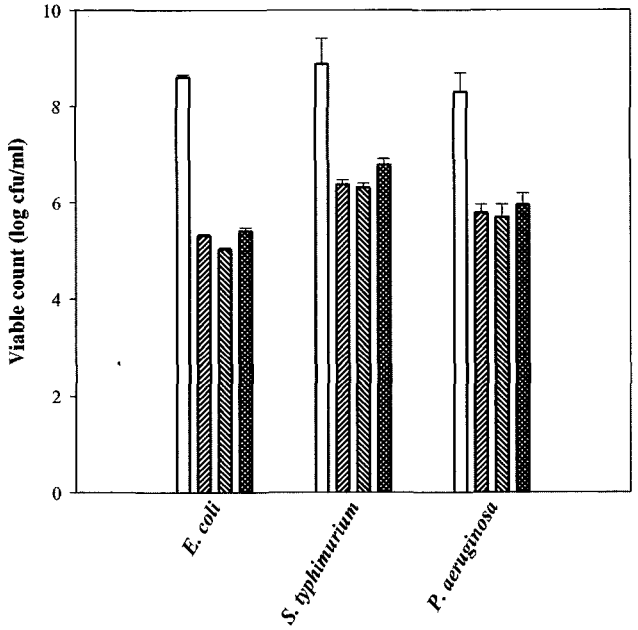

Fig. 1. Growth inhibition of Gram-negative bactera in the absence and presence of 0.1% hetero-chitosans.
Results are represented as means±SD of three different experiments. □, control; ▨, 90% deacetylated chitosan; ▧, 75% deacetylated chitosan; ▩, 50% deacetylated chitosan.

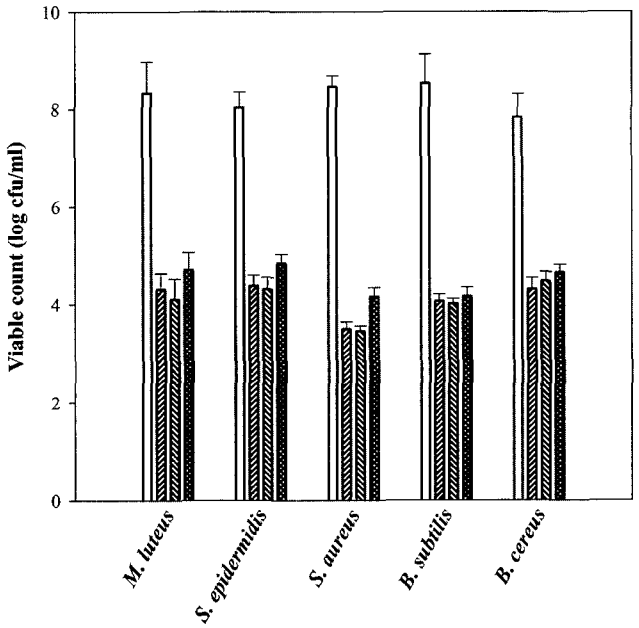

Fig. 2. Growth inhibition of Gram-positive bacteria in the absence and presence of 0.1% hetero-chitosans.
Results are represented as means±SD of three different experiments. □, control; ▨, 90% deacetylated chitosan; ▧, 75% deacetylated chitosan; ▩, 50% deacetylated chitosan.

typhimurium and *P. aeruginosa*. However, the inhibitory activities were different from the factors, the degree of polymerization, and the type of bacterium. According to the result of the present study, hetero-chitosans possessed the most antibacterial activity against *E. coli.* among the Gram-negative bacteria tested, and the 75% deacetylated chitosan revealed effective antimicrobial activity against the Gram-negative bacteria tested. In addition, the growth of Gram-positive bacteria (*M. luteus*, *S. epidermidis*, *S. aureus*, *B. subtilis* and *B. cereus*) was inhibited in the presence of hetero-chitosans (Fig. 2).

Jeon *et al.* [10] reported that 89% deacetylated chitosan and its oligosaccharides have more effective activity against pathogens than against nonpathogens, except in the case of lactic acid bacteria. In addition, in this study, hetero-chitosans showed stronger bactericidal effects on Gram-positive bacteria than on Gram-negative bacteria in the presence of 0.1% hetero-chitosans. These results agree with those of No *et al.* [15].

The relationship between the molecular weights of COSs and their antibacterial activities has been reported by several investigators. However, information concerning the relationship between their antimicrobial activities and hetero-COSs with different degrees of deacetylation and molecular weights has been greatly lacking until now. Therefore, nine kinds of hetero-COSs as previously described were prepared from partially deacetylated hetero-chitosans, and their antibacterial activity was investigated against 3 Gram-negative bacteria and 5 Gram-positive bacteria.

As shown in Fig. 3A-3C, hetero-COSs inhibited the growth of 3 Gram-negative bacteria (*E. coli*, *S. typhimurium* and *P. aeruginosa*), and the antibacterial activity depended on their molecular weights. In particular, the growth of *E. coli.* was markedly inhibited with the

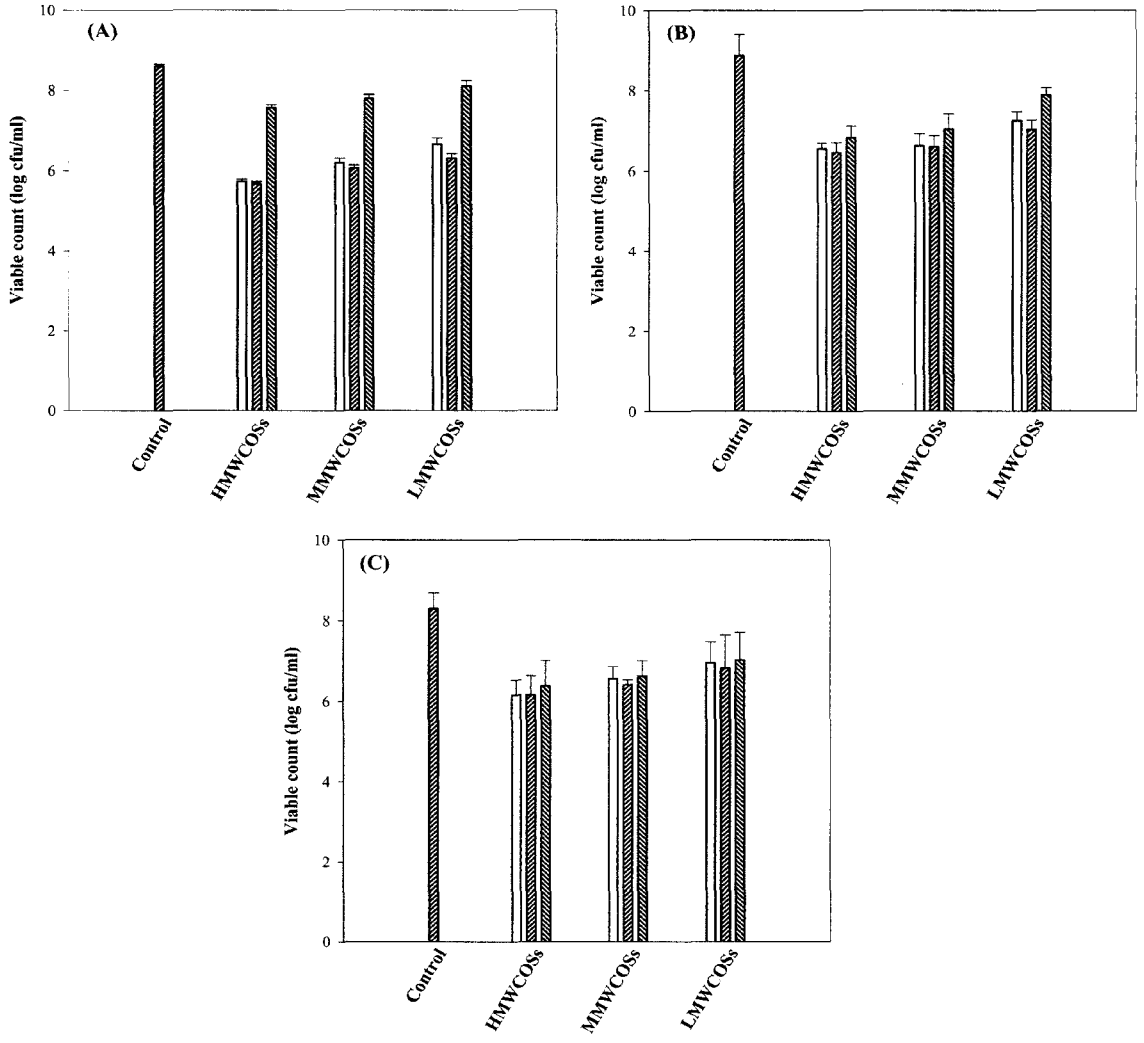

Fig. 3. Growth inhibition of Gram-negative bacteria [*E. coli.* (A), *S. typhimurium* (B), and *P. aeruginosa* (C)] in the absence and presence of 0.1% hetero-COSs.
Results are represented as means±SD of three different experiments. □, 90% deacetylated COSs; ▨, 75% deacetylated COSs; ▩, 50% deacetylated COSs.

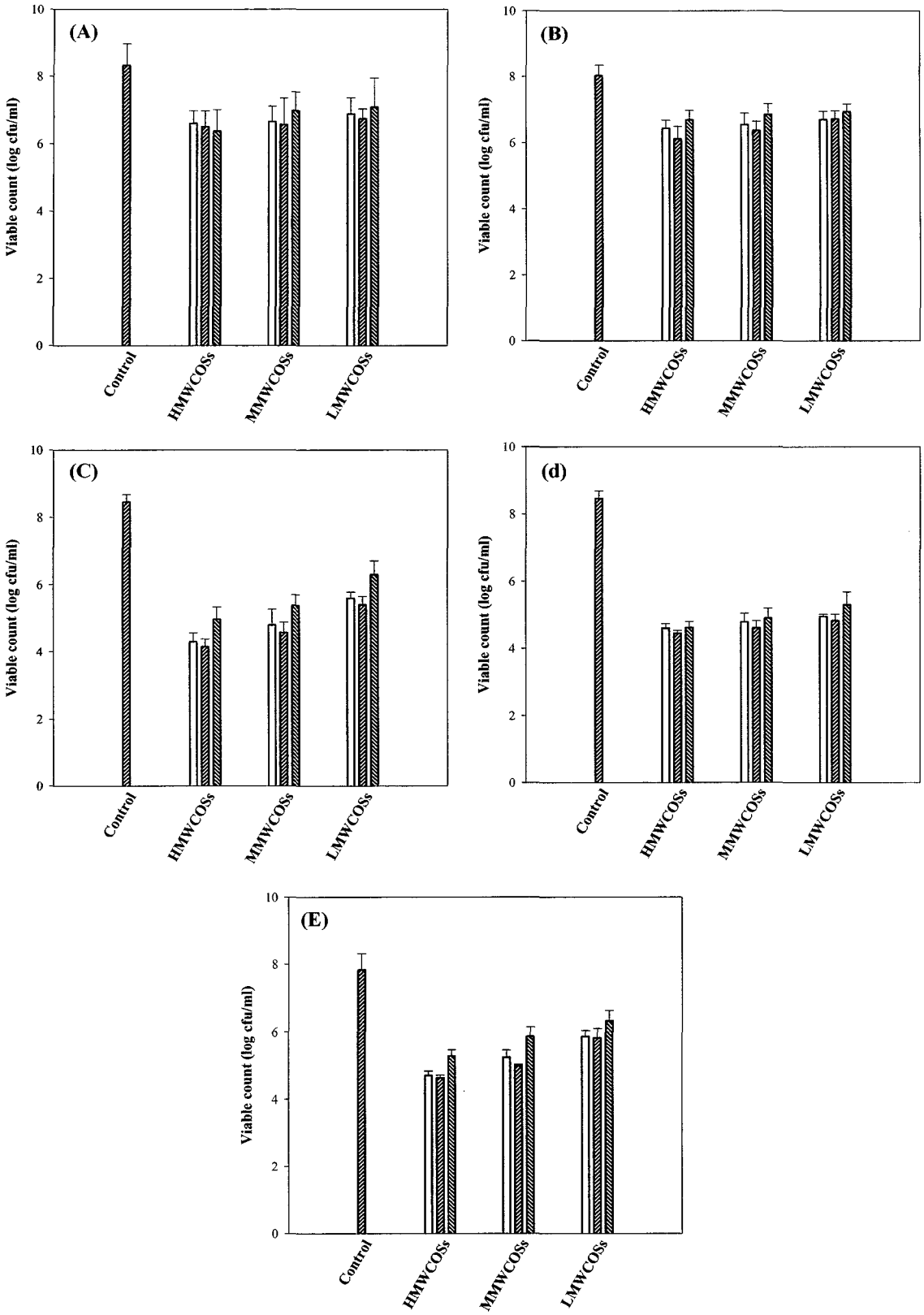

Fig. 4. Growth inhibition of Gram-positive bacteria [*M. luteus* (A), *S. epidermidis* (B), *S. aureus* (C), *B. subtilis* (D) and *B. cereus* (E)] in the absence and presence of 0.1% hetero-COSs.

Results are represented as means±SD of three different experiments. □, 90% deacetylated COSs; ▨, 75% deacetylated COSs; ▧, 50% deacetylated COSs.

increment of the molecular weights of hetero-COSs. Antibacterial activity against 5 Gram-positive bacteria (*M. luteus*, *S. epidermidis*, *S. aureus*, *B. subtilis*, and *B. cereus*) also decreased in the treatment with all hetero-COSs (Fig. 4A-4E). In particular, hetero-COSs possessed strong antibacterial activity against *S. aureus* and *B. subtilis* among the 5 Gram-positive bacteria tested. In addition, the antibacterial activity of hetero-COSs depended on their molecular weights against *S. aureus* and *B. cereus*.

Uchida *et al.* [31] further found that chitosan hydrolyzate, slightly hydrolyzed with chitosanase, was more active as an antibacterial agent than native forms of chitosan and COSs. Cho *et al.* [2] reported that the antibacterial activity of chitosan against *E. coli* and *Bacillus* sp. increased with decreasing viscosity from 1,000 to 10 cp. In addition, Jeon *et al.* [10] reported that molecular weights (1 to 10 kDa) of COSs were critical for the inhibition of microorganism growth and that efficacy increased in proportion to the increase in molecular weight. Sekiguchi *et al.* [21] investigated antibacterial activities of COSs (molecular weight ranging from 2,350 to 21,600 Da) against various bacteria. The growth of *Bacillus cereus* on agar culture was suppressed by 0.2%-0.3% chitosan oligosaccharide with molecular weight of 11,000 Da. Yalpani *et al.* [34] found that COSs with various degrees of polymerization reduced the viability of *E. coli* by 2.47-2.84 log cycles at a concentration of 1,000 ppm. In this study, the growth of *E. coli* was reduced by 0.4 to 3.5 log cycles at a 0.1% concentration of chitosan and COSs.

MIC Values of Hetero-Chitosans and Their Oligosacharides

MICs of hetero-chitosans and their oligosaccharides against Gram-negative bacteria and Gram-positive bacteria were investigated. Hetero-chitosans markedly inhibited the growth of most Gram-negative bacteria tested such as *E. coli*, *S. typhimurium* and *P. aeruginosa* in low concentrations, and their MIC values were less than 1.25 mg/ml against most Gram-positive bacteria. In the antimicrobial effect of hetero-chitosans against Gram-positive bacteria, MIC values against *M. luteus* were lower than those against other tested Gram-positive bacteria, and the MIC values were less than 0.15625 mg/ml (Table 2). Hetero-COSs also effectively blocked the growth of most Gram-negative and Gram-positive bacteria, although their effects were lower than that of hetero-chitosans (Table 3). In addition, the MIC values of the bacteria tested varied according to the molecular weights of hetero-COSs used.

Table 2. Minimum inhibitory concentration (MIC) of hetero-chitosans against eight different bacteria.

Bacteria		MIC (mg/ml)		
		90%	75%	50%
Gram (-)	*Escherichia coli*	0.625	0.625	1.25
	Salmonella typhimurium	0.625	0.3125	0.625
	Pseudomonas aeruginosa	0.625	0.625	1.25
Gram (+)	*Micrococcus luteus*	0.15625	0.15625	0.15625
	Staphylococcus epidermidis	0.3125	0.3125	0.625
	Staphylococcus aureus	0.625	0.625	0.625
	Bacillus subtilis	0.625	0.625	0.625
	Bacillus cereus	0.625	0.625	0.625

Seo *et al.* [22] tested the effect of chitosan on the growth of 11 different bacteria and found that the MIC values of chitosans ranged from 10 to 1,000 ppm. Among the microorganisms tested, the growth of *E. coli*, *P. fluorescens*, *B. cereus*, and *S. sureus* were inhibited by chitosan concentrations of 20, 500 and 1,000 μg/ml (that is, 0.002%, 0.05% and 0.1% respectively), respectively. Uchida *et al.* [31] also reported that the MIC values of chitosans for *E. coli* and *S. aureus* were 0.025% and 0.5%, respectively. In addition, Yun *et al.* [35] found that the differences in MIC values correlated with those in molecular weights of chitosan, ranging from 0.05% to >0.2% for *E. coli* and 0.04% to 0.1% for *S. aureus*. In our previous study [17], we investigated the antimicrobial effects of hetero-chitosans and their oligosaccharides on thirty two strains of V. parahaemolyticus isolated from various organisms

Table 3. Minimum inhibitory concentration (MIC) of hetero-chitooligosaccharides against eight different bacteria.

Bacteria		MIC (mg/ml)								
		90-HMWCOS	90-MMWCOS	90-LMWCOS	75-HMWCOS	75-MMWCOS	75-LMWCOS	50-HMWCOS	50-MMWCOS	50-LMWCOS
Gram (-)	*Escherichia coli*	1.25	1.25	2.5	1.25	1.25	2.5	2.5	2.5	5.0
	Salmonella typhimurium	1.25	1.25	2.5	1.25	1.25	2.5	2.5	2.5	5.0
	Pseudomonas aeruginosa	2.5	5.0	5.0	2.5	5.0	5.0	2.5	5.0	5.0
Gram (+)	*Micrococcus luteus*	0.3125	0.625	1.25	0.3125	0.625	1.25	0.625	1.25	2.5
	Staphylococcus epidermidis	0.625	1.25	2.5	0.625	1.25	2.5	1.25	1.25	5.0
	Staphylococcus aureus	1.25	1.25	2.5	1.25	1.25	2.5	1.25	1.25	2.5
	Bacillus subtilis	1.25	2.5	5.0	1.25	2.5	5.0	2.5	5.0	5.0
	Bacillus cereus	1.25	12.5	2.5	1.25	12.5	2.5	2.5	2.5	5.0

such as shellfish, shrimps, octopus, seabirds and so on. Seventy-five percent deacetylated chitosan showed the highest antimicrobial activity, whereas the MIC was 0.5 mg/ml on 14 strains and MIC of the rest of the strains (18 strains) was 1.0 mg/ml.

It has been postulated that the antimicrobial action of chitosan occurs as a result of several mechanisms [20, 29]. It seems that chelating is one of the most important actions of chitosan. Deprivation of metals, trace elements, or essential nutrients by chelation limits the growth of microorganisms. It has also been suggested that chitosan acts even more aggressively. Fang *et al.* [4] showed that 0.2-0.5% chitosan caused the leakage of proteinaceous and UV-absorbing material from *Aspergillus niger*. Chitosan is also able to interact with flocculate proteins, but this action is highly pH-dependent [20]. The highly reactive amino groups in chitosan have the ability to interact with anionic groups on the cell surface and to form polyelectrolyte complexes with bacterial surface compounds, thereby forming an impermeable layer around the cell, which prevents the transport of essential solutes into the cell. In addition, Choi *et al.* [3] reported that exposure to COSs either rapidly killed the cells of *Actinobacillus actinomycetemcomitans* or rendered them non-culturable by directly attacking their membranes. Generally, the degree of chitosan polymerization is known to affect its antimicrobial activity and the effectiveness of different chain length chitosan hydrolysates against microorganisms has been described. In the present study, the 75% deacetylated chitosan showed more effective antimicrobial activity compared with that of 90% deacetylated chitosan and 50% deacetylated chitosan. In addition, the chitosan with high molecular weights had the higher inhibitory activity. Rhoades and Roller [19] reported that mild hydrolysis of chitosan enhanced its inhibitory activity against some species of spoilage yeasts grown in complex media, whereas highly degraded forms showed no antimicrobial activity. The antimicrobial activity of chitosan varies among species [31]. In conclusion, the antimicrobial activity of different deacetylated chitosans and nine kinds of their oligomers was investigated against three Gram-negative bacteria and five Gram-positive bacteria. Although the antimicrobial activity of hetero-chitosans and their COSs was different against the tested bacteria, 75% deacetylated chitosan had the highest inhibitory activity as compared with the activity of 90% and 50% deacetylated chitosan, and the activity was dependent on molecular weights in most of the bacteria tested.

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References

1. Allan, G. R. and L. A. Hadwiger. 1979. The fungicidal effect of chitosan on fungi of varying cell wall composition. *Exp. Mycol.* **3:** 285-287.
2. Cho, H. R., S. S. Chang, W. D. Lee, E. T. Jeong, and E. W. Lee. 1998. Utilization of chitosan hydrolysate as a natural food preservative for fish meat paste products. *Korean J. Food Sci. Technol.* **30:** 817-822.
3. Choi, B. K., K. Y. Kim, Y. J. Yoo, S. J. Oh, J. H. Choi, and C. Y. Kim. 2001. *In vitro* antimicrobial activity of a chitooligosaccharide mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*. *Int. J. Antimicrobial Agents* **18:** 553-557.
4. Fang, S. W., C. F. Li, and D. Y. C. Shih. 1994. Antifungal activity of chitosan and its preservative effect on low-sugar candied kumquat. *J. Food Prot.* **56:** 136-140.
5. Hadwiger, L. A. and J. M. Beckman. 1980. Chitosan as a component of pea-*Fusarium solani* interactions. *Plant Physiol.* **66:** 205-211.
6. Hirano, S. and N. Nagao. 1989. Effects of chitosan, pectic acid, lysozyme, and chitinase on the growth of several phytopathogens. *Agric. Biol. Chem.* **53:** 3065-3066.
7. Jeon, Y. J. and S. K. Kim. 2001. Potential immunostimulating effect of antitumoral fraction of chitosan oligosaccharides. *J. Chitin Chitosan* **6:** 163-167.
8. Jeon, Y. J. and S. K. Kim. 2002. Antitumor activity of chitosan oligosaccharides produced in ultrafiltration membrane reactor system. *J. Microbiol. Biotechnol.* **12:** 503-507.
9. Jeon, Y. J. and S. K. Kim 2000. Production of chitooligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity. *Carbohyd. Polym.* **41:** 133-141.
10. Jeon, Y. J., P. J. Park, and S. K. Kim. 2001. Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohyd. Polym.* **44:** 71-76.
11. Kendra, D. F., D. Christian, and L. A. Hadwiger 1989. Chitosan oligomers from *Fusarium solani*/pea interactions, chitinase/β-glucanase digestion of sporelings and from fungal wall chitin actively inhibit fungal growth and enhance disease resistance. *Physiol. Mol. Plant. Pathol.* **35:** 215-230.
12. Kim, S. K., Y. J. Jeon, and H. C. Zan. 2000. Antibacterial effect of chitooligosaccharides with defferent molecular weights prepared using membrane bioreactor. *J. Chitin Chitosan* **5:** 1-8.
13. Knorr, D. 1984. Use of chitinous polymers in food a challenge for food research and development. *Food Technol.* **5:** 1-8.
14. Muzzarelli, R. A. A. 1977. Chitin, Pergamon Press, Oxford.
15. No, H. K., N. Y. Park, S. H. Lee, and S. P. Meyers. 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *Int. J. Food Microbiol.* **74:** 65-72.
16. Park, N. D., K. J. Jo, Y. Y. Jo, Y. L. Jin, K. Y. Kim, J. H. Shim, and Y. W. Kim. 2002. Variation of antifungal activities of chitosans on plant pathogens. *J. Microbiol. Biotechnol.* **12:** 84-88.

17. Park, P. J., H. K. Lee, and S. K. Kim. 2004. Preparation of hetero-chitooligosaccharides and their antimicrobial activity on *Vibrio parahaemolyticus*. *J. Microbiol. Biotechnol.* **14:** 41-47.
18. Park, P. J., J. Y. Je, and S. K. Kim. 2004. Free radical scavenging activities of differently deacetylated chitosan using an ESR spectrometer. *Carbohyd. Polym.* **55:** 17-22.
19. Rhoades, J. and S. Roller. 2000. Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. *Appl. Environ. Microbiol.* **66:** 80-86.
20. Roller, S. and N. Covill. 1999. The antifungal properties of chitosan in laboratory media and apple juice. *Int. J. Food Microbiol.* **47:** 67-77.
21. Sekiguchi, S., Y., H. Miura, S. Kaneko, I. Nishimura, N. Nishi, M. Iwase, and S. Tokura. 1994. Molecular weight dependency of antimicrobial activity by chitosan oligomers, pp. 71-76. *In* K. Nishinari and E. Doi (eds.), *Food Hydrocolloids: Structures, Properties, and Functions*, Plenum Press, New York, U.S.A.
22. Seo, H., K. Mitsuhashi, and H. Tanibe. 1992. Antibacterial and antifungal fiber blended by chitosan, pp. 34-40. *In* C. Brine, P. Sanford, and J. Zikakis, (eds.), *Advances in Chitin and Chitosan*. Elsevier, London, U.K.
23. Suzuki, K., T. Mikami, Y. Okawa, A. Tokoro, S. Suzuki, and M. Suzuki. 1986. Antitumor effect of hexa-*N*-acetylchitohexaose and chitohexaose. *Carbohydr. Res.* **151:** 403-408.
24. Suzuki, S. 1996. Studies on biological effects of water soluble lower homologous oligosaccharides of chitin and chitosan. *Fragrance J.* **15:** 61-68.
25. Suzuki, S., T. Watanabe, T. Mikami, T. Matsumoto, and M. Suzuki. 1992. Immuno-enhancing effects of *N*-acetylchitohexaose, pp. 96-105. *In* C. J. Brine, P. A. Sanford, and J. P. Zikakis (eds.), *Advances in Chitin and Chitosan*, Elsevier Applied Science, Barking, U.K.
26. Tokoro, A., M. Kobayashi, N. Tatekawa, S. Suzuki, and M. Suzuki. 1989. Protective effect of *N*-acetyl chitohexaose on *Listeria monocytogens* infection in mice. *Microbiol. Immunol.* **33:** 357-367.
27. Tokoro, A., N. Tatewaki, K. Suzuki, T. Mikami, S. Suzuki, and M. Suzuki. 1988. Growth-inhibitory effect of hexa-*N*-acetylchitohexaose and chitohexaose against Meth-A solid tumor. *Chem. Pharm. Bull.* **36:** 784-790.
28. Tsai, G. J. and W. H. Su. 1999. Antibacterial activity of shrimp chitosan against *Escherichia coli*. *J. Food Prot.* **62:** 239-243.
29. Tsai, G. J., Z. Y. Wu, and W. H. Su. 2000. Antibacterial activity of a chitooligosaccharide mixture prepared by cellulase digestion of shrimp chitosan and its spplication to milk preservation. *J. Food Prot.* **63:** 747-752.
30. Tsukada, K., T. Matsumoto, K. Aizawa, A. Tokoro, R. Naruse, S. Suzuki, and M. Suzuki, 1990. Antimetastatic and growth-inhibitory effects of *N*-acetylchitohexaose in mice bearing Lewis lung carcinoma. *Jpn. J. Cancer Res.* **81:** 259-265.
31. Uchida, Y., M. Izume, and A. O htakara. 1989. Preparation of chitosan oligomers with purified chitosanase and its application, pp. 373-382. *In* G. Skjak-Braek, T. Anthonsen, and P. Sandford (eds.), *Chitin and Chtosan*, Elsevier Applied Science, London/New York.
32. Walker-Simmons, M., L. Hadwiger, and C. A. Ryanee. 1983. Chitosans and pectic polysaccharides both induce the accumulation of the antifungal phytoalexin pisatin in pea pods and antinutrient proteinase inhibitors in tomato leaves. *Biochem. Biophys. Res. Commun.* **110:** 194-199.
33. Wang, G. H. 1992. Inhibition and inactivation of five species of foodborne pathogens by chitosan. *J. Food Protect.* **55:** 916-919.
34. Yalpani, M., F. Johnson, and L. E. Robinson. 1992. Antimicrobial activity of some chitosan derivatives. pp. 543-548. *In* C. J. Brine, P. A. Sandford, and J. P. Zikakis (eds.), *Advances in Chitin and Chitosan*, Elsevier Applied Science, London, U.K.
35. Yamada, A., N. Shibuya, O. Kodama, and T. Akatsuka. 1993. Induction of phytoalexin formation in suspension-cultured rice cells by *N*-acetyl chitooligosaccharides. *Biosci. Biotechnol. Biochem.* **57:** 405-409.
36. Yun, Y. S., K. S. Kim, and Y. N. Lee. 1999. Antibacterial and antifungal effect of chitosan. *J. Chitin Chitosan* **4:** 8-14.