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저분자량 수용성 키토산의 항균 활성에 관한 연구

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Antibacterial Activity of Low Molecular Weight Water-Soluble Chitosan

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초록: 항균제 대체제로 응용하기 위하여 다양한 저분자량 수용성 키토산(LMWSC; MW1, MW3, MW5, MW10)을 제조하였으며, 이들의 항균제 대체제 사용 가능여부와 그 작용 기작에 대하여 연구하였다. 먼저, 다양한 분자량 형태의 LMWSC를 이용하여 사람에게 유해한 각종 박테리아를 이용하여 항균효과를 확인하였고, 그 중 MW10의 항균효과가 가장 우수한 것으로 확인되었다. 그 반면 사람의 적혈구를 이용한 용혈활성 실험에서 독성을 나타내지 않았다. MW10의 항균 효과가 세균의 어느 부분에서 일어나는지 확인하기 위해 박테리아의 세포막 조건(PE/PG=7/3, w/w)으로 인공 리포좀을 만들었고, 여기에 MW10을 처리한 결과 세균 막에서 항균효과를 나타냄을 추론할 수 있었다.

Abstract: Chitosan is a natural polymer derived from chitin that has been widely used as a dietary supplement and in a variety of pharmacological and biomedical applications. In addition, water-soluble chitosan has been used to enhance the stability of chitosan in water and reduce cytotoxic activity induced by acetic acid. In this study, the antibiotic activity and mechanism of low molecular weight water-soluble chitosan (LMWSC; MW1, MW3, MW5, and MW10) were examined in pathogenic bacteria cells and vesicles containing bacterial membrane lipids. MW10 displayed potent antibacterial activity against pathogenic bacteria strains and no cytotoxicity against mammalian cells. In addition, the degree of calcein leakage was examined as a function of lipid composition (PE/PG=7/3 w/w). The results of these experiments demonstrated that MW10 promoted leakage in negatively-charged membranes. Furthermore, confocal microscopy revealed that MW10 was located in the plasma membrane.

Keywords: chitosan, antibacterial activity, pathogenic bacteria, lipid, water-soluble, negatively-charged membranes.

Introduction

Chitosan is formed by partial alkaline *N*-deacetylation of chitin, which is commercially developed from shrimp and crab shells. It is a high-molecular-weight linear polycationic heteropolysaccharide comprising copolymers of β-1,4-linked D-glucosamine and *N*-acetyl-D-glucosamine.[1] Chitosan has been shown to exhibit a broad range of biological activities including antitumor activities,[2] immunostimulating effects,[3] antibiotic effects,[4] free radical scavenging activities,[5] wound healing effects[6] and cholesterol-reducing effects.[7] Due to these beneficial biological activities, a significant effort has been dedicated to examining the potential of using chitosan for therapeutic purposes;[8,9] however, such applications have been restricted because chitosan is insoluble. Due to these special features, chitosan has been evaluated for use in antibiotics, biomedical resources, food additives, cosmetics and farming materials. Chitosan has also been used as a dietary supplement to reduce weight because it binds to fats and cholesterol and prevents them from entering the digestive tract before being processed.[7] Although the mechanism by which chitosan exerts its antibiotic activity

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is not yet fully understood, it has been suggested that this process involves cell lysis, breakdown of the cytoplasmic membrane barrier and the chelation of trace metal cations.[10-12]

In this study, we examined the antimicrobial mechanism of low molecule weight water-soluble chitosan (LMWSC). We also examined the effect of molecular weight (MW1, MW3, MW5, and MW10) on its antimicrobial activity using lipid compositions and a membrane probe.

Experimental

Materials. PE (L-α-phosphatidylethanolamine) (Type V, from *E. coli*), SM (sphingomyelin, from bovine brain), CH (cholesterol) (Type V, from *E. coli*) and FITC (fluorescein isothiocyanate) were purchased from Sigma Chemical Co. (St. Louis, MO). PG (L-α-phosphatidyl-DL-glycerol) and PC (egg yolk L-α-phosphatidylcholine) were obtained from Avanti Polar Lipids (Alabaster, AL). Calcein was acquired from Molecular Probes (Eugene, OR). All other reagents were of analytical grade. Buffers were prepared in double-glass-distilled water.

Preparation of Low Molecular Weight Water-Soluble Chitosan (LMWSC). Five grams of chitosan oligosaccharides prepared by enzymolysis were dissolved in 10 mL of phosphate-buffered saline (pH 7.0) to remove the lactic acid salt attached to the C-2 position of the glucose amine units. Approximately 5.2 mL of TEA were then slowly added to the chitosan solution, which was then stirred for 2 h at room temperature. Next, acetone was added and the mixture was centrifuged (supra 30 K, 15000 X g, 20 min, 4 ℃). This process was repeated four times to obtain a pure product. The products were then combined and air-dried. The product was treated with aqueous 0.001 N-HCl for an additional 2 h. Next, acetone was added to this solution and the mixture was centrifuged using the method described above. This process was repeated 5 times and the product was then combined and air dried. To remove residual HCl and other impurities, the product was dissolved in double distilled water and then passed through an activated carbon and ion-exchange resin. The resulting aqueous solution was then lyophilized, which yielded LMWSC with a free amine group. LMWSC was divided into four groups, MW1(1.2 KDa), MW3(2.7 KDa), MW5(6.7 KDa), and MW10(10.5 KDa) based on molecular weight.

The MWs of LMWSC were shown in Table 1.

Microbial Strains. *Staphylococcus aureus* (ATCC 25923) was obtained from the American Type Culture Collection. *Streptococcus epidermidis* (KCTC 3096) and *Pseudomonas aeruginosa* (KCTC 1637) were obtained from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (KRIBB), Daejon, Korea.

Table 1. Molecular Weights of LMWSC Measured by GPC

Sample	Membranes	M_n(Da)[a]	PDI[a]	DDA(%)[b]
MW1	>1 K	1.2 K	1.42	94.55
MW3	1~3 K	2.7 K	1.24	92.96
MW5	3~10 K	6.7 K	1.17	91.32
MW10	10~30 K	10.5 K	1.13	90.17

[a]GPC measurement (0.5 M amonium acetate, pH 5.5, 25 ℃). [b]UV first-order differentiation.

Antibacterial Activity. Microdilution assays were conducted to establish the minimal inhibition concentrations (MIC) and minimal bactericidal concentration (MBC) values of the LMWSC. Briefly, cells were grown to the mid-phase in medium comprised of 10 g/L bactotryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7.0. The LMWSC were then filtered through a 0.22 μm filter and diluted stepwise in medium containing 1% bactopeptone. Each organism to be tested was then suspended at a concentration of 2×10^6 colony formation units (CFU)/mL in growth medium. 100 μL of this solution was then mixed with 100 μL of each two-fold serial solution of LMWSC in a microtiter plate well. Three replicates were conducted for each test sample. The plates were then incubated for 18 h at 37 ℃, after which the MIC was determined based on the lowest concentration of peptide that resulted in no visible growth on the plate.[3] MBC was evaluated using the same test, but was defined as the lowest concentration of peptide that killed 99.9% of the test inoculums.[3]

Cell Line and Culture. The human keratinocyte HaCaT cell line was obtained from Dr. NE. Fusenig (Heidelberg, Germany). Cells that had been cultured in 75 cm^2 plastic flasks were subcultured in Dulbecco's modified Eagle medium (DMEM) supplemented with antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin), 10% fetal calf serum, 1 mM pyruvate, and 4 mM L-glutamine at 37 ℃ in a humidified chamber under 5% CO_2.

Cytotoxicity. Cytotoxicity was evaluated using a MTT (Sigma) assay to measure the percentage of viable cells. A total of 4×10^3 cells/well was seeded onto a 96-well plate for 24 h. The cells were then treated with various concentrations of the peptides and incubated for an additional 24 h at 37 ℃. Next, 10 μL of MTT at a concentration of 5 mg/mL was added to each of the wells and incubated for an additional 4 h. The supernatants were then aspirated and 100 μL of DMSO were added to the wells to dissolve any remaining precipitate. Finally, the absorbance was measured at a wavelength of 570 nm using an EL_X800 absorbance microplate reader.

Calcein Release from Liposomes. The interaction and permeabilization of peptides against liposomes were assayed by measuring calcein leakage. The calcein-entrapped liposomes were prepared as follows. Calcein-entrapped LUVs (large unilamellar vesicles) were prepared by vortexing the dried lipid in a dye buffer solution (70 mM calcein, 10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4). The suspension was then freeze-thawed in liquid nitrogen for nine cycles and extruded through polycarbonate filters (two stacked 0.2 μm pore size filters) 30 times using an Avanti Mini-Extruder (Avanti Polar Lipids Inc., Alabaster, AL). The calcein-entrapped vesicles were then separated from free calcein by gel filtration chromatography on a Sephadex G-50 column. The entrapped LUVs in suspensions containing 100 μM lipids were then incubated with various concentrations of the LMWSC (0.6~20 μM). Next, the fluorescence of the released calcein was assessed using a spectrofluorometer (Perkin-Elmer LS55) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Complete (100%) release was achieved through the addition of Triton X-100 at a final concentration of 1 mM. Spontaneous leakage was determined to be negligible over this time scale. All experiments were conducted at 25 ℃ and the apparent percentage of calcein release was calculated using the following equation:[13]

$$\text{Release}(\%) = 100 \times (F - F_0)/(F_t - F_0)$$

Where, F and F_t represent the fluorescence intensity prior to and after the addition of the detergent, respectively, and F_0 represents the fluorescence of the intact vesicles.

Confocal Laser Scanning Microscopy (CLSM). Intracellular localization of the fluorescein isothiocyanate (FITC)-LMWSC (MW10) in *S. aureus* was analyzed by confocal laser scanning microscopy. *S. aureus* cells were inoculated into 3 mL of bacterial medium and then incubated at 37 ℃ for 12 h. After this incubation period, the cells were washed with 10 mM NAPB and immobilized on a glass slide. FITC-labeled LMWSC were observed using an Olympus IX 70 confocal laser-scanning microscope (Japan). Fluorescent images were obtained using a 488 nm band-pass filter to excite FITC.[14,15]

Results and discussion

LMWSC Preparation and Activity Assay. Chitosan is a natural polycationic polymer that has been widely used as a bioactive material[16] due to its biocompatibility, nontoxicity, and strong positive charge. However, its potential applications are limited due to its poor solubility. Specifically, chitosan can only be dissolved in water containing acetic acid and this solvent may affect other bioactive agents such as peptides or protein drugs, genetic material, or anticancer drugs. To resolve this problem, we prepared a sample of pure LMWSC that contains a free amine group to increase its solubility in water using a novel method developed in our laboratory.[17] In a previous study, we examined the antifungal activity of the water soluble chitosan as assayed against various pathogenic yeasts and found that they were very effective antimicrobial compounds.[18] Although the overall antibiotic mechanisms of chitosan have not yet been elucidated, the results of this previous study indicated that they are associated with the disruption of the cell membrane. Thus, the results described in our previous work suggest that the LMWSC may be good candidates for the development of novel antibacterial agents.[18]

In the present study, chitosan was synthesized at four different molecular weights: (i) MW1, (ii) MW3, (iii) MW5, and MW10. We then examined the effect of molecular weight and charge content on the antimicrobial activity of LMWSC against various pathogenic antibacterial cells. LMWSC killed both gram-positive and gram-negative strains *in vitro* (Table 2).

Bacterial cells were substantially attenuated via the removal of the positive-charged content of MW10.[18] The MW10 concentration that stopped bacterial growth *in vitro* (MIC) was about the same as that required to kill the organisms (MBC). This property suggests that once MW10 has altered the growth of the microbe *in vitro*, an irreversible process leading to death begins to occur. The increase in the positive-charge of the chitosan for MW10 induced a dramatic increase in the selective antibacterial activity, which suggests that this chitosan can be used to better understand the mechanism of action of soluble antibiotic chitosan and in the design of novel antibiotics with enhanced antibiotic activity. Removal of the positive-charge and deletion of the positively-charged

Table 2. Antibacterial and Cytotoxicity of LMWSC

MIC (mg/mL)		MW1	MW3	MW5	MW10	Survival viability (%)[a]
Grampositive strains	*S. aureus*	>2.5	>2.5	>2.5	1.25~2.5	100
	S. epidermidis	>2.5	>2.5	>2.5	0.63~1.25	100
Gramnegative strains	*P. aeruginosa*	>2.5	>2.5	>2.5	<0.078	100

[a]Cytotoxicity at 2.5 mg/mL chitosan.

group was shown to decrease the antibacterial activity of LMWSC.

We then examined the *in vitro* activity of LMWSC (MW1, MW3, MW5, and MW10) against gram-negative and gram-positive bacterial strains by determining the minimum bactericidal concentration (MBC) (Table 3, Figure 1) using the microdilution method.[19,20] In these experiments, MW10 was shown to cause a dramatic increase in antibacterial activity. Indeed, the MIC values of MW10 ranged from <0.078 to 2.5 mg/mL, whereas the MBCs were more bactericidal than the MICs.

Also, potential lytic effects and cytotoxicity of LMWSC against the HaCaT cell line were assessed using the MTT assay.

In these experiments, treatment with the LMWSC did not exert any cytotoxic activity (Table 2), which suggests that MW10 is a promising candidate for the development of novel antibiotic agents.

Mode of Action Assays. Next, the ability of the LMWSC to permeabilize membranes was assessed[18] by measuring the release of the fluorescent marker calcein from liposomes with lipid compositions (Figure 2). The activities of MW10 and melittin were most pronounced against liposomes that contained negatively charged PE/PG (7:3, w/w) (Figure 2). When evaluated at the same concentration, treatment with the other LMWSC (MW1, MW3 and MW5) resulted in the release of less than 10% of the total entrapped calcein. Consistent with these findings, the MICs of the LMWSC (MW1, MW3, MW5, and MW10) toward microbial cells was similar to their CF release (Figure 2). Specifically, the highly active MW10 and melittin resulted in a high level of leakage from PE/PG, while LMWSC (MW1, MW3, and MW5) were practically inactive and resulted in low leakage at these cell membrane compositions.

To examine the target sites of LMWSC in *S. aureus*, MW10 was labeled with FITC and visualized by confocal microscopy. MW10 was labeled by reacting the isothiocyanate group in FITC with the primary amine group at the C-2 position. The FITC content in the chitosan was then measured using a fluorescence plate reader. Typically, 0.8~0.9% of the FITC was attached to the amine group at the C-2 position. In addition, FITC had no effect on the antibacterial activity of MW10. When cells that were treated with the FITC-labeled MW10 were imaged by confocal microscopy, the FITC-labeled MW10 was shown to penetrate the cell membrane and

Table 3. Minimal Bactericidal Concentration of LMWSC

	MBC (mg/mL)	
	Gram-positive	Gram-negative
	S. aureus	*P. aeruginosa*
MW1	>2.5	0.63
MW3	>2.5	>2.5
MW5	>2.5	1.25
MW10	1.25	<0.04

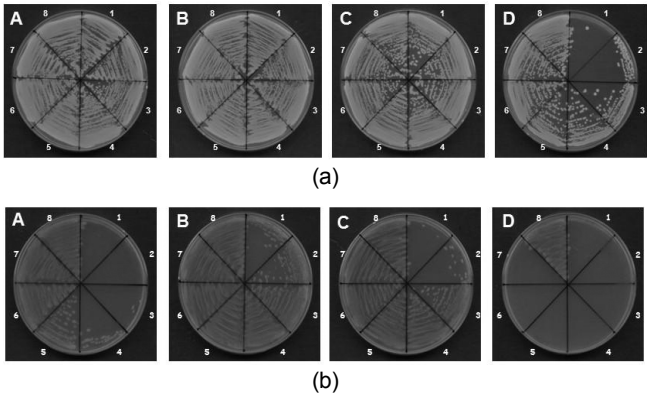

Figure 1. Minimal bactericidal concentration (MBC) of low molecular weight water-soluble chitosans (LMWSC) against gram-positive (a) and gram-negative bacteria cells (b). (a); A: LMWSC MW1 against *S. aureus*, B: LMWSC MW3 against *S. aureus*, C: LMWSC MW5 against *S. aureus*, D: LMWSC MW10 against *S. aureus*. (b); A: LMWSC MW1 against *P. aeruginosa*, B: LMWSC MW3 against *P. aeruginosa*, C: LMWSC MW5 against *P. aeruginosa*, D: LMWSC MW10 against *P. aeruginosa*. 1: 2.5 mg/mL, 2: 1.25 mg/mL, 3: 0.63 mg/mL, 4: 0.32 mg/mL, 5: 0.16 mg/mL, 6: 0.08 mg/mL, 7: 0.04 mg/mL, 8: negative control.

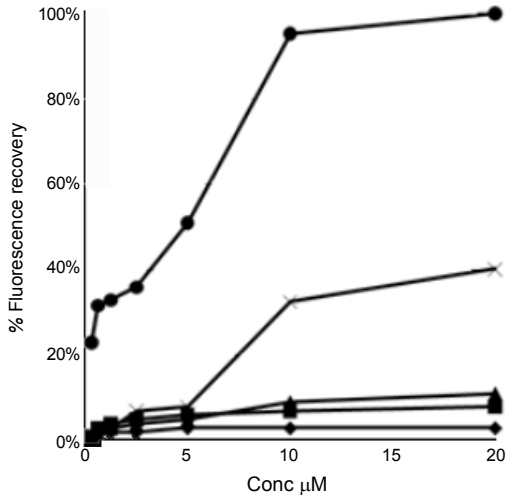

Figure 2. LMWS-chitosan-induced release of calcein from liposomes. Calcein-containing liposomes composed of the indicated lipids were prepared and quantified as described in the Experimental Procedures section. Liposome suspensions containing 100 μM lipids (PE/PG=7/3) were incubated with LMWSC of different molecular weights (MW1) (■), MW3 (◆), MW5 (▲), and MW10 (−), as well as the positive control, melittin (●). The fluorescence of the released calcein was then assessed using a spectrofluorometer (excitation, 480 nm; emission, 520 nm). 100% release was achieved using 1% Triton X-100.

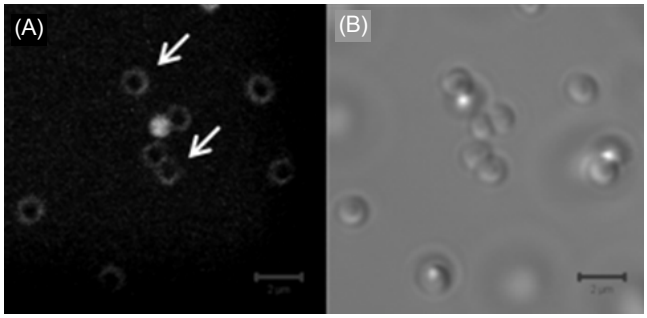

Figure 3. Confocal fluorescence microscopy of *S. aureus* cells treated with FITC-MW10. Cells were treated with fluorescein isothiocyanate (FITC)-labeled MW10 and then incubated for 15 min at 37 ℃. Visualization and localization of the labeled MW10 was performed using confocal laser scanning microscopy. A; fluorescence image of the FITC-labeled-MW10 in bacterial cells, and B; bright field image of the FITC-labeled- MW10 in bacterial cells.

accumulate in the plasma membrane of the cell immediately after addition to the cells (Figure 3). This result suggested that the major target site of MW10 is the plasma membrane of the microorganisms.

Conclusions

Although the results of this *in vitro* study is not entirely sufficient to fully explain the mechanism of action of water-soluble chitosan, the results do suggest that an appropriate balance of electrostatic interactions must regulate the interactions between chitosan and lipid membranes in a complex manner, and thus modulate both their target cell selectivity and biological properties. Further studies are in progress to determine the membrane activity of chitosan in greater detail. Our findings on low molecular weight water-soluble chitosan suggest that certain pharmaceutical therapies could allow bacteria to persist longer in host tissue environments where innate immune responses are normally summoned.

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