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Investigation of the Antifungal Activity and Mechanism of Action of LMWS-Chitosan

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Chitosan, a cationic polysaccharide, has been widely used as a dietary supplement and in a variety of pharmacological and biomedical applications. The antifungal activity and mechanism of action of low molecular weight water-soluble chitosan (LMWS-chitosan) were studied in fungal cells and vesicles containing various compositions of fungal lipids. LMWS-chitosan showed strong antifungal activity against various pathogenic yeasts and hyphae-forming fungi but no hemolytic activity or cytotoxicity against mammalian cells. The degree of calcein leakage was assessed on the basis of lipid composition (PC/CH; 10:1, w/w). Our result showing that LMWS-chitosan interacts with liposomes demonstrated that chitosan induces leakage from zwitterionic lipid vesicles. Confocal microscopy revealed that LMWS-chitosan was located in the plasma membrane. Finally, scanning electron microscopy revealed that LMWS-chitosan causes significant morphological changes on fungal surfaces. Its potent antibiotic activity suggests that LMWS-chitosan is an excellent candidate as a lead compound for the development of novel anti-infective agents.

Keywords: LMWS-chitosan, antifungal activity, confocal microscopy, electron microscopy

Chitosan is a cationic polysaccharide produced by alkaline *N*-deacetylation of chitin, with the sugar backbone of chitosan composed of β-1,4-linked glucosamine units. It exhibits a wide variety of biological activities, including antitumor activities [21], immunostimulating effects [5], cholesterol-lowering effects [20], antimicrobial effects [14], wound healing effects [17], antifungal activities, and free radical scavenging activities [15]. Chitosan possesses several unique properties such as nontoxicity, biocompatibility, and biodegradability, which make it an attractive candidate for pharmaceutical and biomedical applications [4, 6]. Owing to its many biological functions, chitosan has attracted much attention from researchers; however, the application of chitosan is limited by its insolubility. Many investigators have modified chitosan with water-soluble groups or salts in an effort to overcome the problem. However, there are certain disadvantages to these forms of chitosan, including decrease of the positive charge and cytotoxicity. Therefore, in our previous study, we investigated the *in vitro* and *in vivo* molecular weight-dependent absorption phenomena of chitosan using fractionated water-soluble chitosan (chitosan lactate) [3].

Antimicrobial agents have become indispensable in the modern health care system, assisting and complementing the natural immune system. Moreover, the appearance of antibiotic-resistant strains has made the search for more potent and efficient antibiotic agents necessary. The therapeutic potential of many natural products that are important components of the innate immune system and their synthetic derivatives are currently being investigated. Natural products that control cell proliferation and protect against pathogen invasion are essential components of the vertebrate and invertebrate defense systems [2, 12].

In this study, we report the results of an investigation of the antifungal activity of low molecular weight water-soluble chitosan (LMWS-chitosan) against fungal cells. We also discuss the importance of LMWS-chitosan, demonstrate its antifungal activity using lipid compositions and a membrane probe, and highlight its ability to repair cell membrane damage.

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Materials and Methods

Materials, Microbial, Strains, and Antifungal Assay

CH (Cholesterol) (Type V, from *E. coli*) and FITC (fluorescein isothiocyanate) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PC (egg yolk L-α-phosphatidylcholine) was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Calcein was acquired from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were of analytical grade. Buffers were prepared in double-glass-distilled water. Low molecular weight water-soluble chitosan was supplied from KittoLife Co.

Aspergillus fumigatus (ATCC 6145), *Aspergillus parasiticus* (ATCC 6598), and *Fusarium oxysporum* (ATCC 16909) were obtained from American Type Culture Collection; *Fusarium solani* (KCTC 6326) and *Penicillium verrucosum* var. *verrucosum* (KCTC 6265) were obtained from the Korean Collection for Type Cultures; and *Botrytis cinerea* (KACC 40573) was obtained from the Korean Agricultural Culture Collection.

The fungal strains were grown at 28°C in potato dextrose broth (PDB) medium. The fungal cells (2×10^5 cells/ml) were seeded in 100 µl of potato dextrose broth per well in 96-well microtiter plates, mixed with 10 µl of the serially diluted chitosan solution, and incubated for 24 h at 28°C. Ten µl of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution [5 mg/ml MTT in phosphate-buffered saline (PBS), pH 7.4] was added to each well, and the plates were incubated further at 37°C for 4 h. Thirty µl of 20% (w/v) sodium dodecyl sulfate (SDS) containing 0.02 M HCl was then added, and the plates were incubated at 37°C for 16 h to dissolve the formazan crystals that had formed [9, 16].

The turbidity of each well was measured at 570 nm using an Emax microtiter plate reader (Molecular Devices Emax, CA, U.S.A.). All assays were performed in triplicate.

To visualize the fungicidal effect, morphological changes were examined by phase contrast light microscopy using an ECLIPSE TE300 microscope (Nikon, Japan).

hRBC (Human Red Blood Cell) Hemolysis and Cytotoxicity

The hemolytic activities of each chitosan were assessed using hRBCs from healthy donors collected on heparin. The fresh hRBCs were rinsed three times in PBS *via* 10 min of centrifugation at 800 ×*g*, and resuspended in PBS. The chitosans dissolved in PBS were then added to 100 µl of the stock hRBCs suspended in PBS (final RBC concentration, 8% v/v). The samples were incubated with agitation for 60 min at 37°C and then centrifuged at 800 ×g for 10 min. The absorbance of the supernatants was assessed at 414 nm. The controls for zero hemolysis (blank) and 100% hemolysis comprised hRBCs suspended in PBS and 1% Triton X-100, respectively. Each measurement was conducted in triplicate.

The human keratinocyte HaCaT cell line was obtained from Dr. N. E. Fusenig (Heidelberg, Germany). Cells cultured in 75 cm^2 plastic flasks were grown in Dulbecco's modified Eagle's 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. The cells were then cultured at 37°C in a humidified chamber in an atmosphere containing 5% CO_2.

The percentage of growth inhibition was evaluated using an MTT (Sigma) assay for the measurement of viable cells. A total of 4×10^3 cells/well were seeded onto a 96-well plate for 24 h, treated with various concentrations of the tested chitosans, and then incubated for an additional 24 h at 37°C. Subsequently, 10 µl of MTT was added to each well at a concentration of 5 mg/ml, and the cells were incubated for an additional 4 h. The supernatants were aspirated, and 100 µl of DMSO was added to the wells in order to dissolve any remaining precipitate. The absorbance was then measured at a wavelength of 570 nm using an EL_X800 reader (Bio-Tek Instruments, Inc., Winooski, VT, U.S.A.). Three replicates were generated for each test chitosan.

Calcein Leakage Measurement

Calcein-encapsulated small unilamellar vesicles (SUV) composed of phosphatidylcholine and cholesterol (10:1, w/w) were prepared by reverse-phase ether evaporation methods [10, 22] using 70 mM calcein in 10 mM sodium phosphate buffer (SPB), pH 7.4, as a low salt buffer. The vesicles that formed initially were extruded through a Nucleopore filter of 0.1 mm. To remove free CF dye, the vesicles were passed through a Bio-Gel A 0.5-m (Bio-Rad, Richmond, U.S.A.) column (1.5×30 cm) using eluting buffer. The separated SUV fractions, following appropriate dilution to a final concentration of 100 µM phosphate, were mixed with chitosan and filled with buffer to a final volume of 2 ml in a cuvette at 25°C. The time-course leakages of calcein from the SUVs were monitored by measuring the fluorescence intensity at 520 nm and excited at 480 nm on a spectrofluorometer (Perkin-Elmer LS55). The apparent percent leakage value at the fluorescence intensity, F, was calculated by the following equation:

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

F_t denotes the fluorescence intensity corresponding to 100% leakage after the addition of 10 µl of 10% Triton-X100 and represents the fluorescence of the intact vesicle.

Confocal Laser Scanning Microscopy (CLSM)

Intracellular localization of the fluorescein isothiocyanate (FITC)-LMWS-chitosan (10,000) in *C. albicans* and *F. oxysporum* were analyzed by confocal laser scanning microscopy. *C. albicans* and *F. oxysporum* cells were inoculated into 3 ml of yeast medium and incubated at 28°C for 12 h. The cells were then diluted 1:50 in Saubouraud dextrose broth medium (SB medium; 1% bacto peptone, 4% glucose) and incubated at 28°C for 3 h to enrich the population

Table 1. Antifungal activities of LMWS-chitosan.

MIC (mg/ml)	MW 1,000	MW 3,000	MW 5,000	MW 10,000
C. albicans	1.56	1.56	<0.04	<0.04
T. beigelli	<0.04	<0.04	<0.04	<0.04
S. cerevisiae	<0.04	<0.04	<0.04	<0.04
A. fumigatus	>2.5	<0.04	<0.04	<0.04
A. parasiticus	>2.5	<0.04	<0.04	<0.04
B. cinerea	0.08	<0.04	<0.04	<0.04
F. solani	<0.04	<0.04	<0.04	<0.04
F. oxysporum	<0.04	<0.04	<0.04	<0.04
P. verrucosum	0.16	0.08	<0.04	<0.04

The fungal strains were grown at 28°C in potato dextrose broth (PDB) medium. The fungal cells (2×10^5 cells/ml) were seeded in 100 µl of potato dextrose broth per well in 96-well microtiter plates, mixed with 10 µl of the serially diluted chitosan solution, and incubated for 24 h at 28°C. The turbidity of each well was measured at 570 nm using an Emax microtiter plate reader. All assays were performed in triplicate.

of exponentially growing cells. The number of cells was adjusted to 10^6 cells/ml by dilution in SB medium. Stationary phase cells were used instead of exponential phase cells in some cases. FITC-LMWS-chitosan (10,000) was added to 100 μl of the cell suspension at a concentration 6.25 μM, and the cells were incubated at 28°C for 15 min. The cells were pelleted by centrifugation at 10,000 rpm for

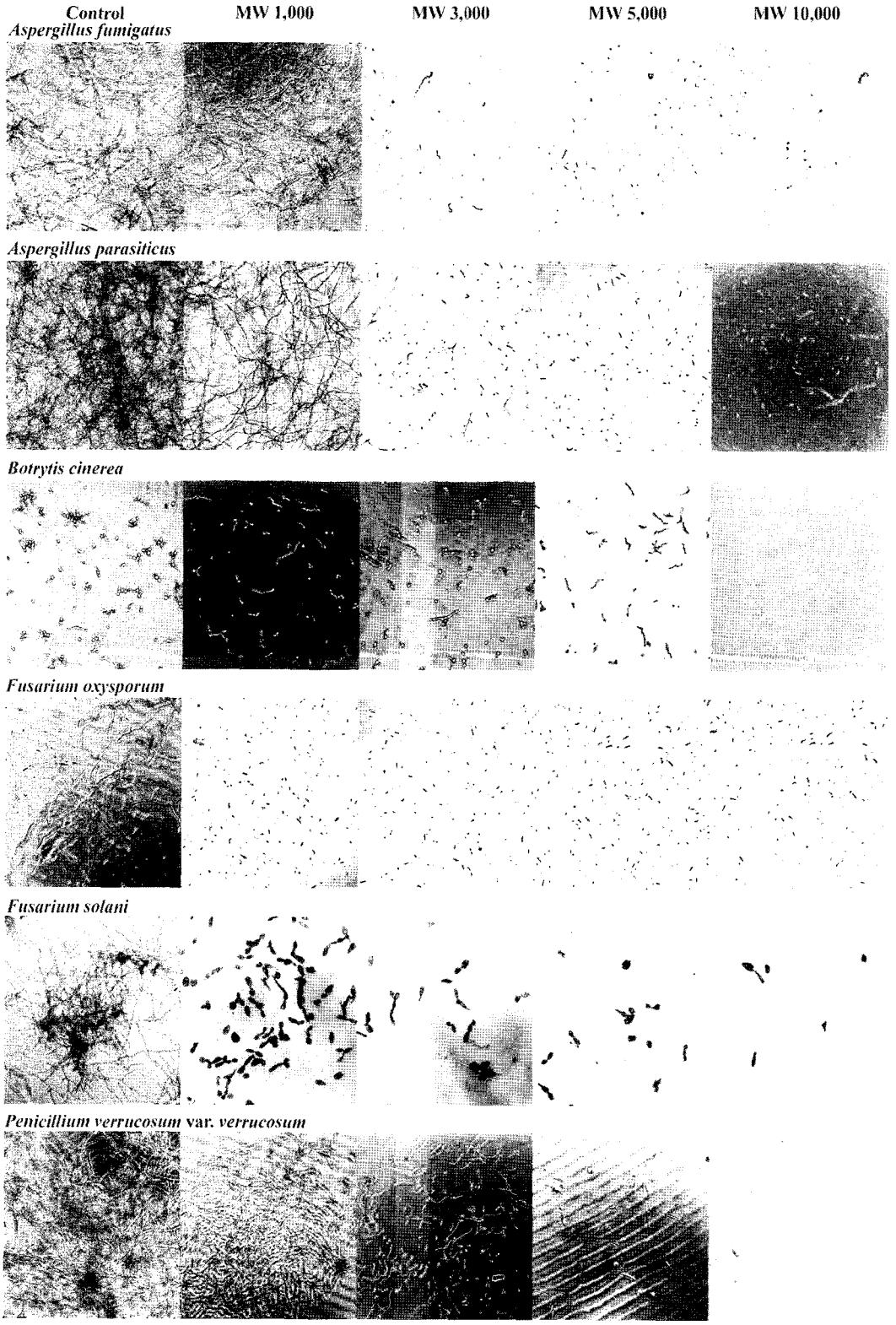

Fig. 1. Antifungal activity of LMWS-chitosan against various fungal strains.
Yeast cells were suspended at approximately 2×10^5/ml in PDB. LMWS-chitosan (0.039 mg/ml) was added, and the reaction mixture was incubated for 24 h at 30°C.

5 min and washed three times with ice-cold PBS buffer. Intracellular localization of FITC-P5 was examined by the Leica TCS 4D connected to a confocal laser scanning microscope (LSM 510META, Zwiss, Germany).

Electron Microscopic Examination of Fungal Membranes

Midgrowth phase *C. albicans* and *F. oxysporum* were resuspended at 10^8 CFU/ml in sodium-phosphate buffer (pH 7.4) supplemented with 100 mM NaCl and then incubated at 28°C with LMWS-chitosan (10,000). The cells were fixed with equal volumes of 4% glutaraldehyde and 1% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2). After fixation for 3 h at 4°C, the samples were centrifuged at 150 ×*g* and washed twice with 0.05 M cacodylate buffer (pH 7.2). The samples were dehydrated with sequential treatments with 50%, 70%, 90%, 95%, and 100% ethanol. After lyophilization and gold coating, the samples were examined by scanning electron microscopy (Hitachi, Tokyo, Japan).

Results and Discussion

Effect of Antifungal Activities of the LMWS-Chitosan

In our previous study, soluble-chitosan with a free amine group was prepared without the use of acetic acid by the novel salts-removal method [11]. In this reaction, triethylamine (TEA) is used to remove a lactic acid bound to an amine group at the C-2 position and to protect the OCH_2OH group at the C-6 position of chitosan. TEA initially reacts with chitosan, which exhibits strong acidity, and the amine group in TEA interacts with the H of the amine group of chitosan and is substituted with lactic acid ($CH_3CHOHCOO^-$). Therefore, a free amine exists at the C-2 position of chitosan, and the OCH_2OH group at the C-6 position remains protected by TEA. Finally, to remove TEA at the C-6 position and an unreacted compound, the reaction mixture was treated with 0.001 N HCl aqueous solution, resulting in LMWS-chitosan with a free amine at the C-2 position and an OCH_2OH group at the C-6 position. This LMWS-chitosan was analyzed by FTIR, ^{1}H-NMR, ^{13}C-NMR, and MALDI-TOF mass spectrum analyses [11].

The antifungal activities of the LMWS-chitosan were assayed against representative pathogenic yeast, which commonly infests immunocompromised individuals, as well as human and plant pathogenic hyphae-forming fungi. The MICs of the LMWS-chitosan toward all tested fungal cells are shown in Table 1. The antifungal activity of LMWS-chitosan (1, 3, 5, and 10 kDa) was examined against various human and plant pathogenic fungi using the MTT assay (Table 1 and Fig. 1) [1, 7, 18, 19, 24]. The antifungal activity of LMWS-chitosan (10 kDa) was stronger than that of the other LMWS-chitosan (1, 3, and 5 kDa) against all tested fungal cells. As shown in Fig. 1, light microscopy confirmed that LMWS-chitosan (10 kDa) strongly inhibited the growth of *A. fumigatus*, *A. parasiticus*, *B. cinerea*, *F. solani*, *F. oxysporum*, and *P. verrucosum* (Fig. 1).

Table 2. Cell survival/viability of the LMWS-chitosan against the human cell line HaCat cell.

Chitosan (2.5 mg/ml)	MW 1,000	MW 3,000	MW 5,000	MW 10,000
Cell survival viability (%)	100	100	100	100

Hemolytic activities of the LMWS-chitosan were tested using a highly diluted (8%) hRBC suspension [8]. The LMWS-chitosan showed no hemolytic activity at their respective MICs (data not shown). The potential lytic effects were assessed *via* MTT assays in the culture media of LMWS-chitosan-treated cells. The cell viability assay of the LMWS-chitosan designed in this study against HaCaT cell lines was determined *via* MTT assay. None of the LMWS-chitosans tested showed any cytotoxic activity (Table 2). This suggests that LMWS-chitosans represent good candidates for the development of novel antibiotic agents.

Although the overall antibiotic mechanisms of chitosan have not been clearly elucidated, the disruption of the cell structure has been discussed [13, 23]. Therefore, the antibiotic effects of LMWS-chitosan were further investigated by testing their lipid membrane-disrupting activities using a calcein-entrapped artificial liposomal vesicle (PC/CH; 10:1, w/w). The result indicated that the membrane-disrupting

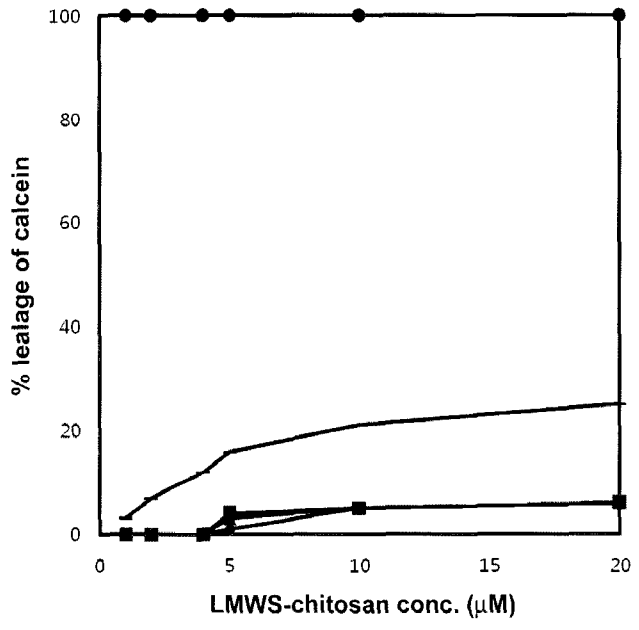

Fig. 2. LMWS-chitosan-induced release of calcein from liposomes.

Calcein-containing liposomes composed of the indicated lipids were prepared and quantified as described in the Materials and Methods section. Liposome suspensions containing 100 μM lipids (PC/CH=10/1) were incubated with molecular weight 1,000 (■), 3,000 (◆), 5,000 (▲), 10,000 (-), and positive control melittin (●), respectively. 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.

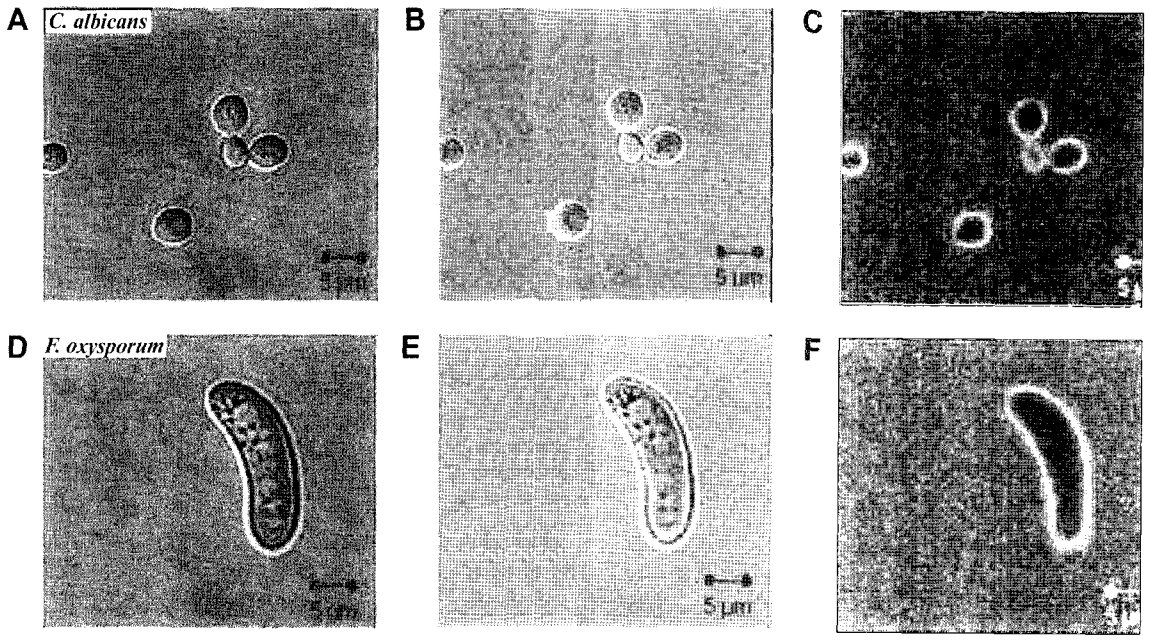

Fig. 3. Confocal fluorescence microscopy of *C. albicans* (**A**, **B**, and **C**) and *F. oxysporum* (**D**, **E**, and **F**) cells treated with FITC-LMWS-chitosan (10,000).

Cells treated with fluorescein isothiocyanate (FITC)-labeled LMWS-chitosan (10,000) were incubated for 15 min at 28°C. Visualization and localization of the labeled LMWS-chitosan (10,000) was performed using a confocal laser scanning microscopy. **A** and **D**, fluorescence image of the FITC-labeled-LMWS-chitosan (10,000) in fungal cells; **B** and **E**, bright field image of the FITC-labeled-LMWS-chitosan (10,000) in fungal cells; and **C** and **F**, the merged image of the FITC-labeled-LMWS-chitosan (10,000) in fungal cells.

activity decreased with decreasing molecular weight (Fig. 2). Thus, the membrane-disrupting ability of the LMWS-chitosans is correlated to their molecular weight, in that the LMWS-chitosans with larger molecular weights showed stronger membrane-disrupting abilities.

These results suggest that the abilities of model-membrane perturbation can be estimated by the molecular weight of the LMWS-chitosan.

LMWS-chitosan was labeled with FITC and visualized under confocal microscopy to examine the target sites of LMWS-chitosan in *C. albicans* and *F. oxysporum*. The reaction of the isothiocyanate group in FITC with the primary amine group at the C-2 position resulted in the FITC-labeling of LMWS-chitosan. The FITC content in the chitosan was measured using a fluorescence plate reader. Typically, 0.8–0.9% of FITC was attached to an amine group at the C-2 position. FITC had no effect on the antimicrobial activity of LMWS-chitosan. The FITC-labeled LMWS-chitosan penetrated the cell membrane and accumulated in the plasma membrane of the cell immediately after addition to the cells (Fig. 3). This result suggested that the major target site of LMWS-chitosan is the plasma membrane of the microorganism.

The morphological changes induced by the LMWS-chitosan were examined using SEM. Untreated *C. albicans* and *F. oxysporum* had a normal smooth surface (Figs. 4A and 4C), whereas cells treated with LMWS-chitosan for 4 h showed cell surface disruption (Figs. 4B and 4D, respectively). The SEM observations provided morphological evidence of the potent permeabilizing activity of the LMWS-chitosan.

In summary, LMWS-chitosans have an affinity for plasma membrane lipids, and their specificities for microbial membranes in many cases have been shown to be related to the positive charges of the chitosan, which favors

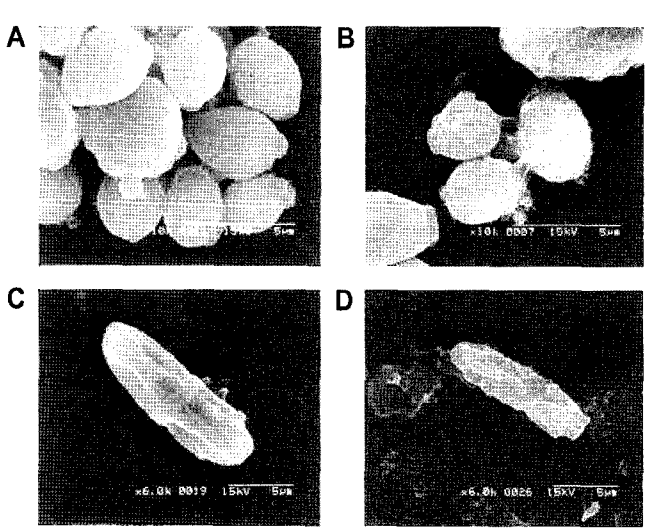

Fig. 4. Morphological changes in the intact cells.

C. albicans (**A** and **B**) and *F. oxysporum* (**C** and **D**) were treated with LMWS-chitosan (10,000) at their respective MICs. **A** and **C**: untreated cells; **B** and **D**: treated cells.

interaction with the exposed anionic component of the microbial plasma membrane. LMWS-chitosan, which exhibits potent antifungal activity without hemolytic activity, may have potential as specific pharmacological agents and as a model for the study of the development of a novel therapeutic agent.

Acknowledgments

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