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Effect of Chitosan Acetate on Bacteria Occurring on Neungee Mushrooms, *Sarcodon aspratus*

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Minimal growth inhibitory concentrations (MICs) of chitosan acetate (M.W. 60 kDa) on heterotrophic bacteria (strains MK1, S, and R) isolated from the soft-rotten tissues of Neungee mushroom (*Sarcodon aspratus*) were measured. The slimy substance produced by the MK1 strain was responsible for the diseased mushroom's appearance. The S and R strains were members of the *Burkholderia cepacia* complex. These strains showed different levels of susceptibility toward chitosan acetate. The MIC of chitosan acetate against the MK1 and S strains was 0.06%. The MIC against the R strain was greater than 0.10%. Survival fractions of the MK1 and S strains at the MIC were 3×10^{-4} and 1.4×10^{-3} after 24 h, and 2×10^{-4} and 7×10^{-4} after 48 h, respectively. Survival fractions of the R strain after 24 and 48 hr at 0.1% chitosan acetate were 1×10^{-2} and 6.9×10^{-3}, respectively. Compared to the MK1 and S strains, the low susceptibility of the R stain towards chitosan acetate could be due to the ability of the R strain to utilize chitosan as a carbon source. Thirty-eight percent of Neungee pieces treated in a 0.06% chitosan acetate solution for 2~3 second did not show any bacterial growth at 4 days, whereas bacterial growth around untreated mushroom pieces occurred within 2 days. These data suggest that chitosan acetate is highly effective in controlling growth of indigenous microorganisms on Neungee. The scanning electron micrographs of the MK1 strain treated with chitosan revealed a higher degree of disintegrated and distorted cellular structures.

KEYWORDS : Antibacterial activity, Chitosan acetate, Heterotropic bacteria, Minimal inhibitory concentration (MIC), Neungee mushroom, *Sarcodon aspratus*

Neungee mushroom (*Sarcodon aspratus*, [Berk] S. Ito, fragrant hedgehog mushroom), an uncultivable mushroom, is generally recognized in Korea and Japan as a top-ranked delicacy, owing to its exclusive aroma and taste (Jeong *et al*., 2001; Song *et al*., 2003). This mushroom was reported as being protein rich, with a protein content of about 31.3% (Park, 1983). For a few hundred years Neungee has been used as a folk medicine for treating dyspepsia in Korea. There are reports on the superior meat digestive capacity of Neungee mushrooms (Park, 1986; Shin *et al*., 2007; Yang *et al*., 1989) as compared with other edible mushrooms such as *Lentinus edodes, Tricholoma mastudake*, etc.

Despite the popularity of the Neungee mushroom as a delicacy, its supply is usually far less than its demand because of the limited production and labor-intensive process of field collection (Kim *et al*., 2000). Moreover, an apparent problem associated with Neungee mushrooms is their market quality deterioration caused by bacterial growth during shipping (Fig. 1). Lee and Koo (2007) investigated the browning and deteriorating problem of Neungee mushrooms and identified four heterotrophic bacterial isolates from the soft-rotten tissues. Among the four heterotropic bacterial isolates, a slow grower (the P strain) belongs to the genus *Methylobacterium*, the R and S strains are members of the *Burkholderia cepacia* complex, and the MK1 strain, which produces a copious mucoid substance, was a novel bacterium. The MK1 strain drew our interest since the bacterial colony was surround with its own exopolysaccharide and had a similar appearance as that seen on the soft-rotten tissue of the mushrooms.

Chitosan is a β-1,4 linked polymer of glucosamine (GlcN), which is a deacetylated derivative of chitin (N-acetylglucosamine), and has been recognized for its antimicrobial, antitumor, hypolipidemic, hypocholesterolemic, and immune-stimulating activities (Jeon and Kim, 1997; Jeon *et al*., 2005; Kim and Lee, 1998; No *et al*., 2002; Yun *et al*., 1999). Because of its safety in human and animal consumption, application of chitosan has been widely used in pharmaceutical, cosmetic, food, and feed industries (Jeon *et al*., 2000; Kim and Jeon, 1997; Kim and Lee, 1998; No, 1998; Rinaudo, 2006). The antimicrobial activity of chitosan and chitosan derivatives has been well investigated (Chung *et al*., 2003; Helander *et al*., 2001; Park *et al*., 2003; Peng *et al*., 2005), and chitosan is widely considered as an effective substance for controlling the growth of various microorganisms including prokaryotic bacteria (Jeon *et al*., 2005; Lee *et al*., 2001; Yun *et al*., 2008) and eukaryotic fungi (Allan and Hadwiger, 1979;

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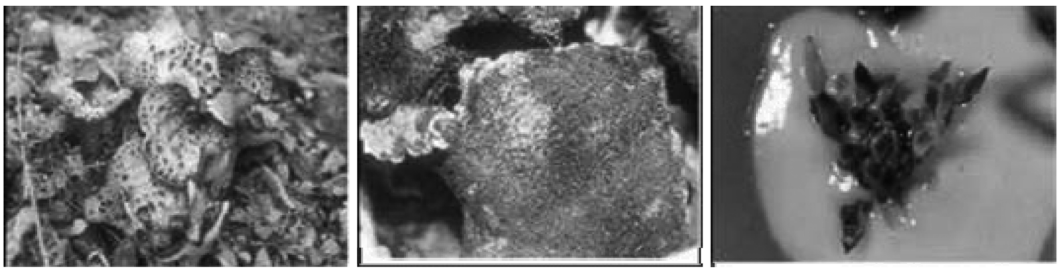

Fig. 1. Photos of Neungee mushroom, *Sarcodon asparatus*. *S. aspratus* fruit bodies on an oak forest floor (a), young healthy spines of gills of *S. aspratus* (b), and diseased gills surrounded by glistening white mucoid substance (c).

Hahn and Nam, 2004; Yun *et al.*, 1999). The antibacterial activity of chitosan is variable based on its molecular size and the nature of its chemical residues (Lee *et al.*, 2001; No *et al.*, 2002; Yun *et al.*, 1999).

In this study, the antibacterial activity of water-soluble chitosan acetate against the three fast growing bacterial isolates (MK1, R, and S strains) was investigated in order to obtain an appropriate measurement for the control of bacterial growth on Neungee after harvest.

Materials and Methods

Chitosan acetate and bacterial strains. A solution of 5.0% chitosan acetate in acetic acid (Chitopol A Green®, Chembio Co., Jincheon, Chungbuk, Korea) with a molecular weight of ca. 60 kDa was used in this study. Three fast growing heterotrophic strains (MK1, R, and S) isolated from the soft-rotten tissue of Neungee mushroom were used for investigating the antibacterial effect of chitosan. Bacteria cultures (OD_{600} = 0.6~0.8) for seeding were prepared in Luria-Bertani broth (LB; 1% tryptone, 0.5% yeast extract, and 1% sodium chloride) overnight at 30°C and 150 rpm. All chemicals and medium constituents of the highest purity were obtained from Difco Lab. (Detroit, MI, USA).

Determination of the minimum inhibitory concentration (MIC) of chitosan. MICs of chitosan acetate against bacterial strains were measured as follows: chitosan solutions were prepared in 5% (v/v) acetic acid and each solution was added to LB medium at a final concentration of 0.00~0.08% (w/v). To prevent clumping of chitosan during bacterial culturing, LB Medium was adjusted to pH 6, and then an aliquot of the overnight-bacterial culture (OD_{600} = 0.6~0.8) was seeded to give OD_{600} = 0.02~ 0.04 in LB broth containing different concentrations of chitosan and incubated at 30°C and 150 rpm. Bacterial growth was recorded every 6 hr for 3 days by measuring OD_{600} (Libra S22, Biochrom, UK) (Yun *et al.*, 2008). Triplicate readings for each sample were obtained, and each experiment was repeated at least three times.

Determination of survival fraction of bacteria. The antibacterial activity of chitosan was further investigated by obtaining the survival fraction of each bacterium in LB medium contained MIC and 1/2 MIC chitosan solutions. To 50 *ml* of LB broth supplemented with chitosan, 1.5 *ml* of an overnight culture (OD_{600} = 0.6~0.8) was added to yield 10^6~10^7 cells per ml and incubated for 48 hr at 30°C and 150 rpm. Bacteria in the presence of chitosan acetate after 24 and 48 hr of incubation were enumerated by counting colony forming units (CFUs) on LB agar. The survival fraction of each strain at each concentration of chitosan was expressed as: CFU with chitosan at given time/CFU without chitosan in culture broth.

Effect of chitosan acetate on bacteria occurring on the surface of mushrooms. Pieces of fresh Neungee mushroom were dipped in 0.02, 0.04, and 0.06% chitosan acetate for 2~3 second and allowed to drip dry. The pieces were placed on potato dextrose agar (0.4% potato starch, 2% dextrose, and 1.5% agar; pH 5.6 ± 0.2) and incubated at 30°C for several days. The number of mushroom pieces showing bacterial growth were then tallied.

Utilization of chitosan acetate by bacterial strains as a carbon source. An overnight culture (OD_{600} = 0.6~0.8) was inoculated into minimal medium (0.7% potassium phosphate dibasic, 0.2% potassium phosphate monobasic, 0.1% ammonium sulfate, 0.05% sodium citrate, and 0.01% magnesium sulfate, pH 6.0) containing 0.06% chitosan to give an OD_{600} = 0.02~0.04, and then incubated at 30°C and 150 rpm. Bacterial growth resulting from consuming chitosan acetate as a sole carbon source was examined by constructing growth curves with optical densities of culture broth at OD_{600} (Libra S22, Biochrom, UK) recorded every 6 hr for 3 days. Triplicate readings of each sample were obtained.

Scanning electron microscopy (SEM) of MK1 treated with chitosan acetate. A 24-hr culture of MK1 was diluted in sterilized saline solution. The diluted bacterial culture was spread on LB agar, and an aliquot of chitosan

solution (0.06%) was added to a stainless cup (7 mm in diameter) placed on the LB agar plate. LB agar plates were then incubated at 30°C for 24 hr. An agar block (0.5 mm × 0.5 mm) was removed from the border between the bacterial growth and growth-inhibited zones as the specimen for SEM and prepared as previously described (Hunter, 1984). The bacterial cells on the agar block were fixed with 2.5% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.0) for 12 hr at room temperature and then washed with the same buffer three times. Further fixation of the specimen was performed by treatment with 1% osmium tetraoxide solution for 3 hr and then washing with 100 mM potassium phosphate buffer (pH 7.0). The intracellular water in bacterial cells was then sequentially replaced with ethanol (10% increments from 30 to 100%). Finally, to eliminate ethanol from the agar specimen, the specimen was placed in an isoamyl acetate solution for 15~20 min twice and then the specimen was dried for 24 hr. The specimens were subjected to SEM (LEO1530, Carl Zeiss, Germany) after gold coating (SC7640, Polaron, UK).

Results and Discussion

The R and S strains, the member of *Burkholderia cepacia* complex, seemed to be associated less frequently with the soft tissue of Neungee mushrooms because these bacteria are very common in nature. However, it has been claimed that the MK1 strain, which produces a copious mucoid substance, seemed to be more responsible for the diseased appearance of Neugnee mushrooms (Lee and Koo, 2007). The growth curve of the MK1 strain in the LB medium containing different concentrations of chitosan acetate, as shown in Fig. 2, suggested that its growth was significantly affected by chitosan. The growth rate of MK1 in the presence of 0.04% chitosan dropped to 0.064 h^{-1} compared to 0.223 h^{-1} in the absence of chitosan. The delayed growth of MK1 in the presence of chitosan probably resulted in the prolonged time period prior to entering stationary phase. The optical density of the MK1 culture containing 0.06% chitosan showed only an insignificant increase up to 36 hours and thereafter remained constant. This slow increase in optical density likely resulted from either the growth of MK1 prior to responding to antimicrobial action of chitosan or the exopolysaccharide (EPS) released from the MK1 cells due to the effectivenss of chitosan. We determined the MIC of chitosan acetate against MK1 as 0.06% (v/v). The growth rates of the S strain in the presence of 0.02 and 0.04% chitosan (0.152 h^{-1} and 0.145 h^{-1}, respectively) were rather similar to each other, despite the growth in the stationary phase quite differed from each other (Fig. 3). The S strain in the presence of 0.04% chitosan entered the stationary phase at 18 hours; thereafter, a slight but steady increase in optical density suggested the S strain gained tolerance toward chitosan following exposure. Since there was no bacterial growth in the presence of 0.06% chitosan for the length of the culture time tested, this concentration was considered as the MIC toward the S strain. Although the R and S strains were identified as members of the *Burkhoderlia cepacia* complex (Lee and Koo, 2007), the growth patterns of these strains in the presence of chitosan acetate were dissimilar, as shown in Fig. 3 and 4. The R strain showed a fair amount of growth at both 0.06 and 0.08% chitosan, although the amount of growth and rate were somewhat reduced at increased chitosan concentrations (Fig. 4). Steady growth of R occurred even at 0.1% chitosan, suggesting resistance of the R stain to chitosan and the possibility that chitosan was acting as a carbon source for R growth.

In brief, the MICs of chitosan toward the MK1 and S strains were about 0.06% and toward R were higher than 0.1%. To assure the antibacterial activity of chitosan against MK1, S, and R strains, survival fractions of these bacteria in the presence of 1/2 MIC and MIC of chitosan were obtained as depicted in Table 1. The survival fractions of MK1, S, and R strains varied depending on chitosan concentrations, as one would expect. The survival fractions of

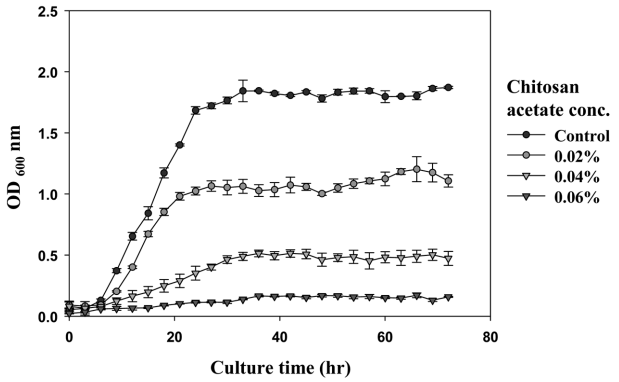

Fig. 2. Growth of the MK1 strain at different concentrations of chitosan acetate in LB broth. This figure was a representative result among three separate experiments.

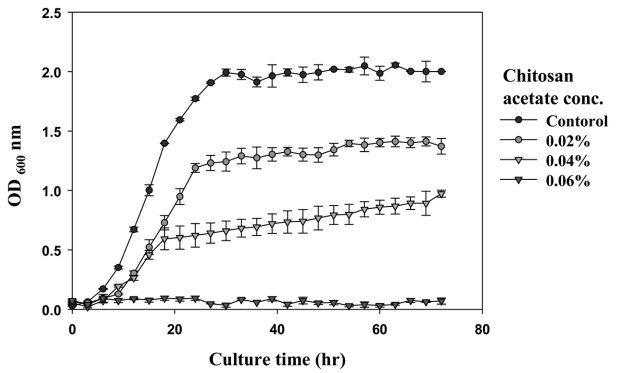

Fig. 3. Growth of the S strain at different concentrations of chitosan acetate in LB broth. This figure was a representative result among three separate experiments.

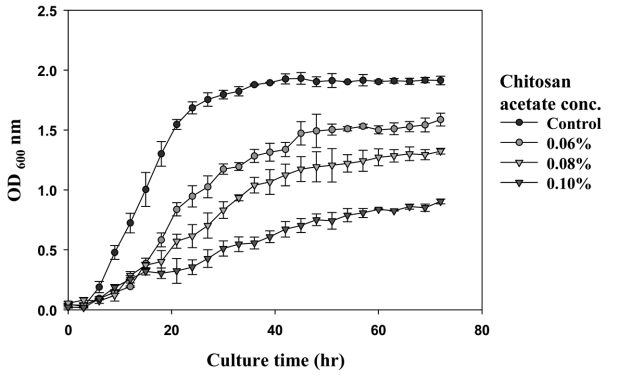

Fig. 4. Growth of the R strain at different concentrations of chitosan acetate in LB broth. This figure was a representative result among three separate experiments.

MK1 at 1/2 MIC and MIC of chitosan were 2.75×10^{-2} and 3×10^{-4}, respectively, at 24 h and remained the same at 48 h. This suggested that the antibacterial effect of chitosan toward MK1 would occur within 24 hr. However, the survival pattern of the S and R strains differed from that of MK1. For instance, the survival of the S strain at MIC of chitosan (0.06%) was reduced to 7×10^{-4} at 48 hr, which was a 2-fold decrease from 1.4×10^{-3} at 24 hr. These data suggested that chitosan exhibited its antibacterial effect toward S and R strains over a prolonged time period. One would assume that the resistance of R strain to chitosan, as presented in Fig. 4, would result in a 2.3-fold increase in R cells, even at 0.1% chitosan. These data suggested that chitosan has significant antibacterial activity against the bacterial strains isolated from the soft tissue of Neungee mushrooms, with the most and least activity against the MK1 and R strains, respectively.

There was a delay in the appearance of bacterial growth around the pieces of the freshly harvested Neungee mushroom that were dipped very briefly in chitosan solution, as

Fig. 5. No bacterial growth around pileus pieces dipped in 0.06% chitosan acetate solution and placed on PDA up to 4 d (a) and 2 d control (b).

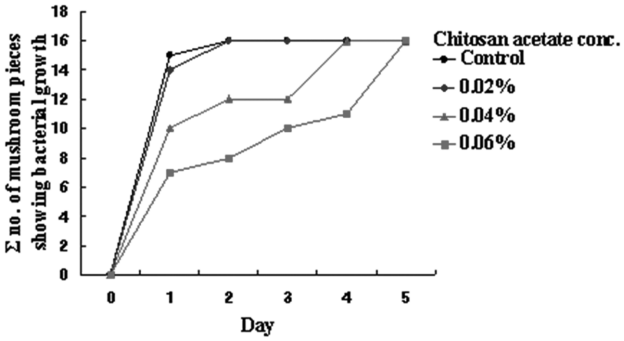

Fig. 6. Number of instances of bacterial growth around pileus pieces of Neungee. The pileus pieces of Neungee mushroom dipped in different concentrations of chitosan acetate were placed on PDA.

shown in Fig. 5 and 6. While the control mushroom pieces (no chitosan treatment) shown 100% bacterial growth at 2 days, delayed bacterial growth was recorded on chitosan treated mushroom pieces; the higher the chitosan concentration the longer growth delay. For instance, there was no bacterial growth observed for 6 out of 16 mushroom pieces (38%) treated with 0.06% chitosan even at 4 days. This suggested that chitosan would be effective in controlling quality depreciation of Neungee mushrooms caused by the

Table 1. Survival fractions of the MK1, S, and R strain at their MIC and 1/2 MIC of chitosan acetate

Conc. of chitosan acetate	Strain	Bacterial growth					
		CFU/*ml* (24 hr)	Cell increase (fold)*	Survival fraction**	CFU/*ml* (48 hr)	Cell increase (fold)*	Survival fraction**
No chitosan	MKI	1.6×10^{9}***	500		2.2×10^{9}	688	
0.03%		4.4×10^{7}	14	2.75×10^{-2}	5.8×10^{7}	18	2.63×10^{-2}
0.06%		4.2×10^{5}	0.1	3.0×10^{-4}	3.3×10^{5}	0.1	2.0×10^{-4}
No chitosan	S	8.5×10^{8}	340		1.5×10^{9}	600	
0.03%		9.0×10^{7}	36	1.05×10^{-1}	2.6×10^{8}	104	1.73×10^{-1}
0.06%		1.2×10^{6}	0.5	1.4×10^{-3}	1.1×10^{6}	0.4	7.0×10^{-4}
No chitosan	R	2.1×10^{9}	233		2.9×10^{9}	322	
0.05%		1.9×10^{8}	21	9.04×10^{-2}	1.8×10^{8}	20	6.21×10^{-2}
0.10%		2.1×10^{7}	2.3	1.0×10^{-2}	2.0×10^{7}	2.2	6.9×10^{-3}

*CFU at given incubation time/CFU of inoculum (MK1: 3.2 × 106, S: 2.5 × 106, R: 9.0 × 106).
**(CFU with chitosan/CFU without chitosan).
***Average of triplicate.

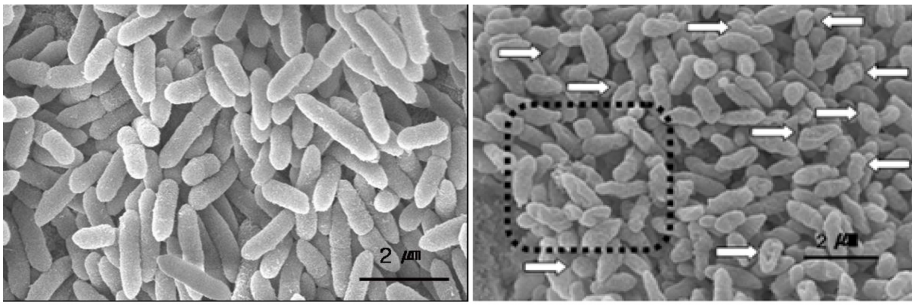

Fig. 7. Scanning electron micrographs of the MK1 strain, (a) normal cells, (b) cells affected by chitosan acetate. White arrows indicate disintegrated cellular structure. Dotted region indicates distorted cells covered with some substance.

indigenous bacteria.

In the minimal basal medium supplemented with chitosan as a sole carbon source, only the R strain (the least susceptible to chitosan) seemed to utilize chitosan as a carbon source, since the optical density of the culture after a 12 hr lag phase began to slowly increase. However, this growth was rather limited, reaching an OD_{600} 0.2~0.3 at stationary phase (data not shown). This observation suggested that the tolerance of the R strain at higher concentrations of chitosan might be due to its ability to utilize it as a carbon source.

The MK1 strain grown in the presence at sub-MIC of chitosan acetate revealed a high number of damaged cells via SEM analysis; showing shrinkage along with disintegrated or irregular structure and some cells covered with an unidentified substance as seen in Fig. 7. Extensive alterations of MK1 cells by chitosan were confirmative of the substantial inhibitory effect of chitosan. Similar reports on structural damage to various bacteria treated with chitosan were previously reported in the literature (Yun *et al*., 1999, Jeon *et al*., 2005; Helender *et al*., 2001; Yun *et al*., 2008).

Chitosan, a natural nontoxic biopolymer, has been widely used in particular food and feed industries, owing to its safety for human and animal consumption (Kim and Jeon, 1997; No, 1998; Jeon *et al*., 2000; Kim *et al*., 2006; Rinaudo, 2006). Since a thin coating of fruits with chitosan is beneficial for keeping freshness by preventing water reduction and/or inhibiting microbial growth, chitosan is applied to prolong the storage of fruits and vegetables such as tomato, cucumber, bell pepper, strawberry, citrus, jujube fruit, etc. (El Ghaouth *et al*., 1992a, b; Chien *et al*., 2006; Zhang and Quantick, 1997; Zhong and Wenshui, 2006). An improved storage quality of eggs coated with chitosan was previously reported (Kim and Hur, 2005). Also, it has been suggested that chitosan is an ideal preservative coating for *Agaricus bisporus* since chitosan coating markedly lowered the maturity index of fresh produce, possibly attributable to its antifungal activity and film forming properties (Kim *et al.*, 2006).

Most mushrooms are highly perishable and tend to lose quality within a few days after harvest. Since Neungee mushrooms under usual shipping and marketing conditions face quality deterioration by indigenous bacterial growth, a proper and safe measure to prevent indigenous bacteria growth post-harvest is needed to maintain their market value. The substantial inhibitory effect of chitosan on bacteria occurring on Neungee mushrooms suggests that the application of chitosan acetate might be useful for maintaining mushroom quality. It would be worthwhile to develop a proper coating method for applying chitosan to Neungee mushrooms so as to prolong their market value.

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