Note

Antimicrobial Activity of Chitosan with Different Degrees of Acetylation and Molecular Weights

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The antimicrobial activity of chitosan with different molecular weights (MWs) and different degrees of acetylation (DA) was examined to clarify how the activity depends upon the molecular structures. We determined minimum growth inhibitory concentrations (MICs) of various types of chitosan in L broth or its modified medium against bacteria and yeasts. The MICs against Gram-positive bacteria were relatively low for chitosan preparations with higher MWs and that against Gram-negative bacteria were at their minimum at around a MW of 11,000 to 30,000. An increase in the DA of the amino group in the chitosan molecule caused less activity. The antimicrobial activity varied with the genus as well as species of bacteria and yeasts. For application of chitosan as a preservative to food, pharmaceuticals, and other materials, the data obtained here on the characteristics and antimicrobial spectrum may be useful.

Key words: Antimicrobial activity/Chitosan/Degree of acetylation/Degree of polymerization.

Chitosan is a linear D-glucosamine polymer via β-1,4 linkage and obtained by deacetylation from chitin, which is contained in crustacean and arthropod cell walls. This polymer has been utilized in a variety of industries as an adsorbent, chelating agent, wetting carrier, and so on, due to its polycationic property and activity of chelating with metal ions.

Chitosan has been known to possess antimicrobial activity substantially at pH values lower than 6.0 (Allan and Hadwiger, 1979; Cuero, 1999; Roller and Covill, 1999; Sudarshan et al., 1992; Uchida, 1988; Wang, 1992). The activity of chitosan has been reported to depend upon the MW (Hirano and Nagao, 1989; Jeon et al., 2001; Sekiguchi et al., 1994; Tanigawa et al., 1992; Tokura et al., 1997; Uchida, 1988). To date, however, the chitosan preparations used for comparison of these properties seem to have a narrow range of MW and not to be well defined chemically. Although the MW of native chitosan ranges from 100,000 to 1.2 million, mildly degraded chitosan demonstrates a stronger antimicrobial effect than native chitosan, whereas extensively degraded products, chitooligosaccharides, are less active against bacteria (Jeon and Kim, 2000; Rhoades and Roller, 2000; Uchida, 1988) and fungi (Hirano and Nagao, 1989; Kendra and Hadwiger, 1984; Uchida, 1988). The antimicrobial activity of chitosan also depends upon the DA of the amino group in the molecule (Tanigawa et al., 1992; Uchida, 1988). However, the preparations, which have been used so far for these experiments, have been obtained by deacetylation of chitin, which may cause partial depolymerization of the chitosan molecule. The

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antimicrobial spectrum of chitosan is relatively broad, although bacteria more than fungi seem to be generally sensitive to the action of chitosan (Papineau et al., 1991; Rhoades and Roller, 2000: Roller and Covill, 1999). However, the antimicrobial activity varies between and within genera of bacteria, yeasts and fungi, and the reason for these differences is unclear.

In spite of the above studies, it can be said that the characteristics of chitosan have not been clearly and systematically evaluated yet. In this study, we examined the antimicrobial activities of preparations of this polymer with different MW ranges and different extents of acetylation and non-deacetylated chitin as well as chitooligosaccharides.

The following chitosan and chitooligosaccharides preparations were used in this study. SKD (a MW range, 300,000 - 400,000; the degree of deacetylation [DDA], 100) and SD10 (MW, 86,000; DDA, 100) were purchased from Dainichiseika Kogyo Co., Ltd. D-AcHITOSANs FP1 (a MW range, 20,000 - 30,000; DDA, 97), FP2 (MW, 11,000; DDA, 100) and FP3 (MW, 6,000; DDA, 100) were obtained from Dainichiseika Kogyo Co., Ltd. D-Glucosamine HCl was supplied by Wako Pure Chemical Co., and chitobiose 2HCl, chitotriose 3HCl, chitotetraose 4HCl, chitopentaose 5HCl and chitohexaose 6HCl were purchased from Seikagaku Kogyo Co., Ltd. A series of partially N-acetylated chitosan preparations were chemically synthesized according to Hirano’s method (Hirano et al., 1976). Ten g of the SKD preparation, which is chitin that has been completely deacetylated (SKD), was dissolved in 5% (w/w) aqueous acetic acid solution, and the resulting viscous solution was diluted with 750ml of methanol. A series of methanol solutions (50ml each) of acetic anhydride (0.761, 1.522, 2.219, 3.551, 3.994 and 5.389g) were prepared and then each of those was added drop-wise to the SKD solution. After being stirred for 3h at room temperature, the reaction mixture was adjusted to pH 11.2 - 11.9 with 2 mole l-1 NaOH. After the resultant precipitate was washed with water, by lyophilization gave N-acetylated chitosan preparations as a colorless powder. The DDA value (%) was estimated by the IR method (Shigemasa et al., 1996) and molecular weight was determined by means of gel permeation chromatography (column: Asahipak GS-510H, GS-310M, GS-220H; temperature: 50°C; carrier: 0.1M acetate buffer at pH4.8 containing 0.1M NaCl; detector: RI) through calculation on the basis of pullulan calibration. We finally obtained chitosan preparations with a DDA of 74, 55, 43, 29, 23 and 9%. The MWs for first two preparations could not be determined because of difficulty in solubilization, but those for others were in the MW range of ca. 260,000 - 400,000. These chitosan, N-acetylated chitosan and chitooligosaccharide preparations were dissolved at a final concentration of 1% (w/v) in 0.1M acetic acid and the pH was adjusted to 6.0, unless otherwise stated, with 1 mole l-1 HCl.

"Bacillus subtilis" 168, Staphylococcus aureus 209P, Escherichia coli W3110, and Pseudomonas aeruginosa ATCC23268 were cultivated in LB medium (10g Tryptone peptone, 5g yeast extract and 5g NaCl per liter) supplemented with 0.1mM MnCl₂ at 37°C for 18h with shaking at 120rpm. Lactobacillus acidophilusIFO13951, Lactobacillus caseiIFO15883 and Lactobacillus brevisIFO3960 were grown in LB medium supplemented with 20g glucose per liter for 18h without shaking. These first two strains of lactic acid bacteria were cultivated at 37°C and the last one at 30°C. Saccharomyces cerevisiae IFO0234 and Candida utilisOUT6020 were cultivated in the same medium as that for lactic acid bacteria but at 30°C with shaking at 120rpm. The medium used for lactic acid bacteria and yeasts was the same LB as that for the above bacteria except for the supplemented glucose in order to compare antimicrobial activity and consider the interaction of chitosan with its components.

The MIC was determined as follows. The microbial cells cultivated as described above were harvested by centrifugation (3,900 × g for 5min at 4°C) and resuspended in distilled water. Test tubes (18mm × 150mm), which contained 2.0ml each of LB either with or without glucose as described before and chitosan at different concentrations, were inoculated with 20 μl of the above cell suspension to get a final density of 10⁶ to 10⁷ cells per ml. After 18h of incubation of yeasts and L. brevis at 30°C and other bacteria at 37°C, the turbidity due to cell growth was visually checked. At this time, in the absence of chitosan preparation, cells of bacterial and yeast strains tested here were confirmed to grow sufficiently. Therefore, the MICs obtained here for different microorganisms seemed to be reasonable.

We attempted to confirm the validity of application of MIC test in this study, since a precipitate was produced by adding chitosan to the test tube containing growth medium. After incubation at 37°C for 18h of two test tubes both containing the E. coli inoculum (2 × 10⁸ - 3 × 10⁸ cells per a total final volume of 2.02ml) and also the above SKD chitosan preparation at 0.02 (non-inhibitory level) and 0.04% (w/v) (MIC), respectively, the top liquid fraction was separated with a Pasteur pipette from the bottom precipitate, and then sterile distilled water (1ml) was added to the precipitate for suspension. After that, samples were withdrawn from both fractions, diluted
appropriately and then plated on LB agar. To enumerate total cell number, samples were taken out from both tubes. Viable counts were estimated after these LB plates were incubated for about 18h at 37°C. As a result, at 0.04% (w/v) of chitosan, the total viable cell number was decreased after the incubation. The viabilities in the liquid and precipitate fractions were 97 and 3% of the total, respectively, for the 0.02% (w/v) tube and those were 18 and 82%, respectively, for the 0.04% (w/v) tube. At the MIC, even though a substantial level of bacterial cells could be found in the upper liquid, it is possible that many grown cells were possibly located at the precipitate. Therefore, to examine whether the viable cells were properly counted for the 0.04% (w/v) tube, we separated the precipitate from the liquid phase and dispersed it in approximately 1ml of 0.1M acetic acid. When the resultant suspension was observed with a microscope, the cell number was at an undetectable level (much less than the order of 10⁸ cells per ml). From these results, the MIC value seems to be unaffected by the precipitate formed and it is supported that the MIC test can be applied for the investigation of the antimicrobial property of chitosan.

At first, the effect of pH on the MIC of chitosan preparations with a MW range of 300,000 — 400,000 against B. subtilis 168 was examined using LB medium. At initial pHs 5.5, 6.0 and 6.5, the MIC values were 0.04, 0.04 and 0.06% (w/v), respectively. The final concentration of acetate in the medium was 10mM at pH 5.5, which was not inhibitory for bacterial growth.

We examined the MICs of chitosan and chitooligosaccharides, which possess different MWs but no or almost no acetylated groups, against B. subtilis 168, S. aureus 209P, E. coli W3110 and P. aeruginosa ATCC23268 in LB medium at an initial pH of 6.0 (Table 1). For gram-positive bacteria, higher MW chitosan preparations had strong activities, whereas those with MWs of 20,000 — 30,000 and 11,000 were the most active against gram-negative bacteria. Chitooligosaccharides as well as D-glucosamine were ineffective against all bacteria tested.

The effect of acetylation of the chitosan molecule on the antibacterial activity was examined at a pH of 6.0, using chitosan preparations with a MW range of 300,000 — 400,000, to know the importance of the free amino group in the molecule for the antibacterial activity (Table 2). As a result, an increase in the de-
TABLE 3. Antimicrobial activity of chitosan preparations with MWs of 86,000 and 300,000—400,000 against lactic acid bacteria and yeasts at pH 6.0.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MW</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>86,000</td>
<td>300,000</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>0.006*</td>
<td>0.004</td>
</tr>
<tr>
<td>L. brevis</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>L. casei</td>
<td>0.004</td>
<td>0.01</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>C. utilis</td>
<td>0.004</td>
<td>0.004</td>
</tr>
</tbody>
</table>

* % (w/v).

The degree of acetylation caused a reduction in the inhibitory potency of chitosan. Since chitosan is inhibitory at lower pHs, chitosan preparations with MWs of 86,000 and 30,000—400,000 were also examined for their MICs against lactic acid bacteria and yeasts (Table 3). The effectiveness of chitosan was found to depend upon the species among lactic acid bacteria and upon the genus among yeasts. L. brevis IFO3960 and S. cerevisiae IFO0234 were relatively resistant to the chitosan preparations tested, to extents similar to those for B. subtilis, E. coli and P. aeruginosa, whereas L. acidophilus IFO13951, L. casei IFO15883 and C. utilis OUT6020 were rather sensitive. The turbidity monitoring of the control culture untreated with chitosan during a period of 18h demonstrated good growth for these microorganisms (more than 10⁹ cells per ml). Also, the addition of acetate buffer at pH 6.0 used as a solvent had no substantial effects on the growth of these strains at a final maximum concentration of 10mmole l⁻¹.

Since the pKa of chitosan is approximately 6.3, at pH values tested here, the chitosan molecule is cationic. In agreement with other papers (Cuero, 1999; Roller and Covill, 1999; Sudarshan et al., 1992; Tsai and Su, 1999; Wang, 1992), the pH dependency of MIC obtained here supports the idea that the amino group has a critical role in the antimicrobial activity. Low pH enhances the antimicrobial activity of chitosan probably due to an increase in the positive charge of the free amino group in the molecule.

Several authors have so far reported that mildly hydrolyzed chitosan is more active than native chitosan (Hirano and Nagao, 1989; Jeon et al., 2001; Rhoades and Roller, 2000; Sekiguchi et al., 1994; Shimojoh et al., 1996; Tanigawa et al., 1992; Uchida, 1988). According to papers published previously, the antimicrobial activity of chitosan varies with types, genera and species of microorganisms (Jeon et al., 2001; Rhoades and Roller, 2000; Roller and Covill, 1999; Sekiguchi et al., 1994; Tokura et al., 1997; Wang, 1992). Such differences may result from various factors. To date, chitosanase production by microbial cells (Cuero, 1999), chemical composition of the cell envelope structure (Fang et al., 1994; Helander et al., 2001; Sudarshan et al., 1992; Tsai and Su, 1999; Uchida, 1988; Ueno et al., 1997), and nutrient components in the growth medium, upon which microbial cells depend and also which affect the interaction with chitosan molecules (Sudarshan et al., 1992) have been proposed as those factors. When chitosan attacks cells, it first should interact with the cell envelope. The reason why we observed different patterns of antibacterial activity of chitosan against gram-positive bacteria and gram-negative bacteria may be the difference in the envelope structures of the cells. The outer membrane present in the latter type of bacteria as an outermost layer may somewhat affect the interaction with the chitosan polymer (Helander et al., 2001).

The reason why lactic acid bacteria and yeasts respond differently to chitosan in spite of being in the...
same genus or kind remains to be examined. Since the degree of chitosan sensitivity of lactic acid bacteria well corresponded well to the final value of pH of the medium after cultivation for the MIC test, being decreased to pH3.4 for both *L. casei* and *L. acidophilus*, whereas only pH5.2 for *L. brevis* (Shigemoto et al., unpublished data), the amount and kind of acids produced via metabolism may increase the solubility and/or inhibitory action of chitosan. Such pH conditions cannot be applied to yeasts tested here, since the pH values were 4.6 for both *C. utilis* and *S. cerevisiae*. Since the MIC against *S. cerevisiae* was also unaffected by the aeration condition for cultivation, it seems unlikely that the ability of *C. utilis* to re-spire aerobically contributes to a high sensitivity to the antimicrobial action of chitosan. We cannot figure out the reason for this difference at present. There is a similar difference in the chitosan sensitivity between *Candida* sp. and *Rodotorula* sp. (Rhoades and Roller, 2000).

Although the detailed mechanism of the bactericidal action of chitosan remains unclear, chitosan is a promising, naturally occurring preservative available for protection of various materials from microbial attack, although at relatively low pH values. Several practical attempts to apply chitosan has been made so far, especially in the preservation of foods. A typical example of its practical application in Japan is its use to preserve a short term-fermented salted Chinese cabbage (Uchida, 1988). Together with data of other workers, our results obtained here should be useful toward the development of further practical applications of chitosan.

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