Research Note

Antimicrobial Activity of a Low-Molecular-Weight Chitosan Obtained from Cellulase Digestion of Chitosan

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ABSTRACT

A water-soluble chitosan hydrolysate with high activity against *Escherichia coli* was obtained during cellulase digestion of chitosan for 18 h. This 18-h hydrolysate is composed of low-molecular-weight chitosan (LMWC), with a molecular weight of 12.0 kDa, and chitooligosaccharides, which are composed of sugars with a degree of polymerization of 1 to 8. LMWC has a strong activity at 100 ppm against many pathogens and yeast species, including *Bacillus cereus*, *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhii, and *Saccharomyces cerevisiae*, while the chitooligosaccharides have much weaker antimicrobial activity than does LMWC. Accordingly, the antimicrobial activity against *E. coli* in the 18-h hydrolysate proved to come mainly from the presence of LMWC.

Chitosan, a partially deacetylated chitin (poly-β-(1→4) N-acetyl-D-glucosamine) can inhibit the growth of a wide range of microorganisms (1, 2, 4–8, 12, 14, 15, 18, 19). Chitosan can only be dissolved in acids and some organic solvents, and various water-soluble chitosan hydrolysates with antimicrobial activity have recently been prepared (3, 8–11, 20–22).

There is disagreement about the antimicrobial activity of chitooligosaccharides in chitosan hydrolysates. Kendra and Hadwiger (11) first demonstrated that chitooligosaccharides with a degree of polymerization (DP) equal to or greater than 6 had strong fungicidal activity. Some reports demonstrated that chitooligosaccharides with DPs of 2 to 8 (8, 3 to 6 (9), or 4 to 7 (21) had antimicrobial activity. However, Ueno et al. (22) concluded that chitooligosaccharides with molecular weights less than 2.2 kDa did not have antimicrobial activity. Jeon et al. (10) reported that chitooligosaccharides with molecular weights of 1 to 10 kDa are crucial for antibacterial activity. In a previous study, we digested chitosan with cellulase, and the water-soluble hydrolysate product contained chitooligosaccharides with DPs of 1 to 8 and had strong activity against many foodborne pathogens (20). In this paper, the water-soluble hydrolysate with strong antimicrobial activity was fractionated into low-molecular-weight chitosan (LMWC) and a chitooligosaccharide mixture. The antimicrobial activity of these two fractions against *Escherichia coli* and other pathogens and yeasts was evaluated.

**MATERIALS AND METHODS**

**Chitosan preparation.** Shrimp chitosan was prepared from shrimp (*Solenocera melontho*) shells on the basis of a previous report (20). The chitosan was 90% deacetylated (D90), as measured by the colloid titration method (17).

**Enzymatic hydrolysis of chitosan.** Chitosan D90 was enzymatically hydrolyzed according to the method of Tsai et al. (20), with some modifications. The chitosan solution (4.5% [wt/vol]) was hydrolyzed with cellulase (10 U/g, technical grade, from *Trichoderma viride*, Challenge Bioproducts Co., Yuenlin, Taiwan) in 0.5 N acetic acid–bicarbonate buffer (pH 5.2) at 55°C. At intervals, 50 ml of the hydrolysate was sampled, boiled for 15 min, adjusted to pH 7.0, and then centrifuged (12,000 × g for 30 min). One milliliter of the supernatant was filter sterilized (0.2 µm; Gelman Sciences, Ann Arbor, Mich.) and used for antibacterial testing against *E. coli* using optical density measurement. The rest of the supernatant was added to an equal volume of methanol to separate the LMWC and the chitooligosaccharides with DPs less than 16, according to the protocol of Muraki et al. (13).

**Chitosan molecular weight and chitooligosaccharide analyses.** The average chitosan molecular weight was analyzed by high-pressure liquid chromatography (HPLC) using a TSK gel G5000PWXL column (7.8 by 300 mm; Tosoh Co., Tokyo, Japan) (16). The sugar compositions of the chitooligosaccharides were analyzed using HPLC with a Nucleosil 5 NH2 column (4.6 by 250 nm; Vercopak Inc., Taipei, Taiwan) (20).

**Analysis of *E. coli* inhibition.** Ten microliters of filter-sterilized, neutralized hydrolysate was added to a tube containing 10 ml of nutrient broth (NB; Difco Laboratories, Detroit, Mich.); 100 µl of *E. coli* CCRC 10674 culture was then inoculated into the tube to yield an initial cell density of 10⁵ CFU/ml, and the tube was incubated at 37°C. At various time intervals, the optical density of the tube was measured at a wavelength of 660 nm. The experiment was run in duplicate.

**Antimicrobial testing.** Except for *Listeria monocytogenes* LM-LM, which was originally isolated from a contaminated chicken purchased from a retail store and kindly provided by Dr. S. C. Chen at the National Taiwan Ocean University in Taiwan,
TABLE 1. Antimicrobial effects of chitosan with 90% deacetylation (D90), low-molecular-weight chitosan (LMWC), hydrolysate, and chitooligosaccharide mixture at 100 ppm against various microorganisms

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Control</th>
<th>Chitosan D90</th>
<th>LMWC</th>
<th>Hydrolysate</th>
<th>Chitooligosaccharide mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram (+) bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus CCRC 10603</td>
<td>6.71 ± 0.12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6.61 ± 0.12</td>
</tr>
<tr>
<td>Listeria monocytogenes LM-LM</td>
<td>7.69 ± 0.02</td>
<td>ND</td>
<td>3.56 ± 0.24</td>
<td>3.84 ± 0.06</td>
<td>7.35 ± 0.05</td>
</tr>
<tr>
<td>Staphylococcus aureus CCRC 12652</td>
<td>7.39 ± 0.08</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.17 ± 0.03</td>
</tr>
<tr>
<td>Gram (-) bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aeromonas hydrophila CCRC 13881</td>
<td>8.16 ± 0.06</td>
<td>7.95 ± 0.13</td>
<td>5.15 ± 0.36</td>
<td>7.38 ± 0.07</td>
<td>7.73 ± 0.26</td>
</tr>
<tr>
<td>Escherichia coli CCRC 10674</td>
<td>8.29 ± 0.11</td>
<td>ND</td>
<td>3.82 ± 0.25</td>
<td>4.92 ± 0.14</td>
<td>7.44 ± 0.08</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa CCRC 10944</td>
<td>7.73 ± 0.10</td>
<td>ND</td>
<td>ND</td>
<td>2.36 ± 0.05</td>
<td>4.39 ± 0.27</td>
</tr>
<tr>
<td>Salmonella enterica serovar Typhi CCRC</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>10746</td>
<td>7.96 ± 0.14</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.90 ± 0.21</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus CCRC 10806</td>
<td>7.31 ± 0.12</td>
<td>4.97 ± 0.15</td>
<td>7.25 ± 0.12</td>
<td>7.49 ± 0.03</td>
<td>7.41 ± 0.04</td>
</tr>
<tr>
<td>Yeast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida albicans CCRC 20513</td>
<td>7.31 ± 0.09</td>
<td>4.65 ± 0.12</td>
<td>7.25 ± 0.04</td>
<td>7.35 ± 0.02</td>
<td>7.35 ± 0.06</td>
</tr>
<tr>
<td>C. magnoliae CCRC 21703</td>
<td>7.17 ± 0.06</td>
<td>6.44 ± 0.07</td>
<td>7.04 ± 0.20</td>
<td>7.09 ± 0.11</td>
<td>7.22 ± 0.07</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae CCRC 21671</td>
<td>7.36 ± 0.03</td>
<td>ND</td>
<td>1.65 ± 0.16</td>
<td>7.19 ± 0.07</td>
<td>7.03 ± 0.03</td>
</tr>
<tr>
<td>S. cerevisiae CCRC 21687</td>
<td>6.46 ± 0.13</td>
<td>ND</td>
<td>ND</td>
<td>1.72 ± 0.20</td>
<td>5.95 ± 0.08</td>
</tr>
</tbody>
</table>

* Hydrolysate, chitosan hydrolysate obtained by cellulase digestion of D90 for 18 h; LMWC and chitooligosaccharide mixture, components separated from 18-h hydrolysate by methanol extraction.

b Data are mean ± standard deviation (n = 3).

c ND, not detectable.

According to the protocol of Tsai et al. (20), the freeze-dried samples of chitosan, chitosan hydrolysate, LMWC, or the chitooligosaccharide mixture at 100 ppm were added into NB (or NB plus 3% NaCl for Vibrio parahaemolyticus or yeast-malt broth [Difco] for yeast) and incubated at 37°C for 24 h or at 25°C for 48 h for yeast.

All cultures listed in Table 1 were purchased from the Culture Collection and Research Center (CCRC, Hsinchu, Taiwan). All of the microorganisms were subcultured twice in NB (or NB plus 3% NaCl for Vibrio parahaemolyticus or yeast-malt broth [Difco] for yeast) and incubated at 37°C for 24 h or at 25°C for 48 h.

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According to the protocol of Tsai et al. (20), the freeze-dried samples of chitosan, chitosan hydrolysate, LMWC, or the chitooligosaccharide mixture at 100 ppm were added into NB (or NB plus 3% NaCl). After inoculation of the test bacteria and incubation at 37°C for 24 h, the surviving bacteria were detected by plating on nutrient agar (Difco) plates and counting the colonies. The protocol for the antifungal test was similar, except that yeast-malt broth and yeast-malt agar were used, and the cultures were incubated at 25°C for 48 h. The experiment was performed in triplicate.

RESULTS AND DISCUSSION

Technical grade cellulase was used to degrade chitosan D90. The effect of the chitosan hydrolysate following cellulase digestion on the growth of E. coli is shown in Figure 1. The highest antibacterial activity of the hydrolysate was observed with the 18-h hydrolysate. This hydrolysate was composed of LMWC and a DP of 1 to 8 chitooligosaccharide mixture. The molecular weight of LMWC is 12.0 kDa, which is much smaller than the original chitosan D90 reactant (molecular weight, 228.3 kDa). The sum of the concentrations of the polymerized chitooligosaccharides in the chitooligomer mixture was 83.49 mg/ml, among which the concentrations of the DP 1 to 8 chitooligosaccharides were 35.21, 0.61, 20.43, 1.77, 10.79, 8.59, 2.58, and 3.51 mg/ml, respectively.

At 100 ppm, the effects of the chitosan D90, chitosan D90 hydrolysate, LMWC, and chitooligosaccharide mixture on the growth of various microorganisms are shown in Table 1. Chitosan D90 had the strongest antimicrobial activity, followed in decreasing order by the LMWC, hydrolysate, and chitooligosaccharide mixture. After a reaction with chi-
tosan D90 for 24 h, no viable cells were detected for *B. cereus*, *E. coli*, *L. monocytogenes*, *S. aureus*, *P. aeruginosa*, *Salmonella enterica* serovar Typhi, or two strains of *S. cerevisiae*. LMWC showed weaker activity than chitosan D90 in which no viable cells were found for *B. cereus*, *S. aureus*, *P. aeruginosa*, *S. enterica* serovar Typhi, or *S. cerevisiae* CCRC 21687. Except when the CFU concentrations of *S. aureus*, *P. aeruginosa*, and *S. enterica* serovar Typhi were decreased by 4.22, 3.34, and 3.06 log CFU/ml respectively, the chitooligosaccharide mixture had little activity against the rest of the microorganisms tested.

Antimicrobial activity was observed in various chitosan hydrolysates, which were prepared either by acid hydrolysis (8, 11, 22) or by enzymatic degradation (3, 9, 10, 20, 21). DP 1 to 6 chitooligosaccharides were detected in these chitosan hydrolysates. Historically, the chitooligosaccharides were credited with the antimicrobial activity of the hydrolysate (8, 9, 11, 20, 21). In this study, we have proven that the LMWC is responsible for the antimicrobial activity, rather than the chitooligosaccharides, of the hydrolysate. The chitooligosaccharides with a DP of 1 to 8 had little antimicrobial activity.

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REFERENCES


