Research Note

Antimicrobial Activity of Native Chitosan, Degraded Chitosan, and O-Carboxymethylated Chitosan

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ABSTRACT

The antimicrobial activity of native chitosan was compared to that of lipase-degraded chitosan. The effects of O-carboxymethylated (O-CM) substitution on native (molecular weight, 120; degree of deacetylation, 84.71%) and lipase-degraded chitosans were also investigated. The antimicrobial activity of native chitosan was more extensive than that of lipase-degraded chitosan; however, lipase-degraded chitosan was still highly effective and more water-soluble. O-CM chitosan derived from degraded chitosan was more effective than O-CM chitosan derived from native chitosan. O-CM substitution enhanced lipase-degraded chitosan’s antimicrobial activity without reducing its solubility.

Chitosan (poly-β-1,4-linked glucosamine) is a cationic polysaccharide made from alkaline N-deacetylation of chitin. As one of the most abundant natural polymers, it has attracted much attention in various fields as a result of its biological activity, biocompatibility, and biodegradability as well as its low toxicity (23). It has been used for wastewater purification (9), for the chelation of transition metals (13), to produce edible coatings for fruit and vegetables (14), and to produce packaging films (16). However, food and medical applications for this polysaccharide have been limited by its low solubility in aqueous media (6).

Chitosan hydrolysate can be prepared by acid hydrolysis (5) or by enzyme hydrolysis (8, 18). The enzymatic method can be used under mild conditions, does not create environmental problems (12), and has a minimal effect on the chemical nature of the reaction product, since there are minimal side reactions. There are several reports of the use of different enzymes, such as lipase, lysozyme, chitinase and chitosanase, in this procedure (1, 19).

Chitosan’s antifungal and antimicrobial activities are believed to originate from its polycationic nature (17). Binding with anionic sites in proteins results in selective antimicrobial activities against fungi or bacteria (7). Researchers have shown that the antimicrobial activities not evident in chitin were mainly dependent on the types of functional groups in chitosan and the molecular weight (MW) of the base chitosan (7, 11, 22, 23).

Chitosan contains three types of reactive functional groups, an amino group, a primary hydroxyl group, and a secondary hydroxyl group, at the C-2, C-3, and C-6 positions of the glucosamine residue, respectively (2). Chemical modifications of these groups have provided numerous useful materials in different fields of application (10, 21). O-Carboxymethylated (O-CM) chitosan is thought to play an important role in the antimicrobial activity of chitosan because of the substitution of the hydroxyl group in the C-6 position of chitosan with the acetyl group, which enhances the protonation of the amine group in the C-2 position in the presence of the new carboxyl ion (11). Moreover, since O-CM chitosan can be dissolved in water over a wide pH range (16), it has a much broader application as an antimicrobial agent than chitosan does.

There has been little research on the antimicrobial activity of chitosan substitution with regard to different MWs. The objective of the present research was to measure the antimicrobial activity of different chitosan molecules with various MWs and at various concentrations. In addition, the effects of O-CM substitution on native and lipase-degraded chitosans were investigated. The antimicrobial effectiveness of these materials was tested on various gram-positive and gram-negative bacteria.

MATERIALS AND METHODS

Materials. All chemicals used in this study were obtained from commercial sources and were of the highest purity available. Native chitosan (MW, 120; degree of deacetylation, 84.71%) was purchased from Kimitsu Co. (Tokyo, Japan). Escherichia coli O157:H7 strain ATCC 43894, Staphylococcus aureus KCTC 1916, Bacillus subtilis KCTC 1021, and Salmonella Typhimurium KCTC 1925 were obtained from the Graduate School of Biotechnology, Korea University (Seoul, Korea). Biochemical reagents of beef extract, peptone, and agar powder were obtained from Difco Laboratories (Sparks, Md.).

Preparation of degraded chitosan. Degraded chitosan was prepared enzymatically from native chitosan with the use of lipase from Rhizopus japonicus as described by Shin et al. (19). Five fractions, with MWs ranging from 30 to 50, were obtained, and...
one major fraction with an MW of 30 was selected for its antimicrobial activity.

**Preparation of O-CM chitosan.** Ten grams of native or degraded chitosan was mixed with 13.5 g of NaOH in 100 ml of a solution of H₂O/isopropanol (1:4) for 1 h in a water bath (Thermoregulator, Comabiotech Co., Korea) set at 50°C. Fifteen grams of monochloroacetic acid in 20 ml of isopropanol was added to a solution consisting of chitosan suspended in water. The resulting suspension was incubated for a further 4 h at 50°C. The solid was filtered and rinsed and then vacuum dried at room temperature. The O-CM chitosans derived from native and degraded chitosan were characterized by Fourier transform infrared spectroscopy (Jasco, Tokyo, Japan).

**Determination of MWs of chitosan.** High-performance size exclusion chromatography, together with a multianalogue laser light scattering–refractive index (MALLS-RI) system, was used to determine the MWs of degraded chitosan, O-CM chitosan derived from native chitosan, and O-CM chitosan derived from degraded chitosan. The system consisted of a pump (P2000, Spectra System, San Jose, Calif.), an injection valve (Model 7021, Rhodyne, Cotati, Calif.), a guard column (TSK PWH, Tosoh Corp., Tokyo, Japan), one SEC column (TSK Gel 3000PW, 7.8 × 600 MM, Tosoh Corp.), a MALLS photometer (Dawn DSP-F, Wyatt Technology, Santa Barbara, Calif.), and an RI detector (Shodex SE71, Tokyo, Japan). The columns and the detector were maintained at 25 and 35°C, respectively. The mobile phase (water with 0.02% NaN₃) was filtered first through a 0.2-μm filter and then through a 0.1-μm cellulose acetate filter. The flow rate of the mobile phase was 0.5 ml/min, and the sample injection volume was 500 μl.

**Antimicrobial assessment.** The antibacterial activities of the various chitosans against both gram-positive and gram-negative strains were evaluated by the optical density (OD) method. The cultures were grown on nutrient agar (Difco) and expanded in nutrient broth (Difco). During incubation, the turbidity of the medium was measured every 2 h at 610 nm with the Bioscreen C (Labsystems Co., Finland). The OD values were corrected by subtracting the initial OD from the final OD.

**Statistical analysis.** Analysis of variance was carried out with SAS software (Statistical Analysis Systems Institute, Cary, N.C.), and least significant difference comparisons were carried out to determine significant differences ($P < 0.01$).

**RESULTS AND DISCUSSION**

The MW of degraded chitosan was 30, that of O-CM chitosan derived from native chitosan was 435.5, and that of O-CM chitosan derived from degraded chitosan was 78.6 according to the MALLS MW analysis. Native chitosan showed growth inhibition for all strains (Fig. 1) and demonstrated more extensive antimicrobial activity than degraded chitosan did ($P < 0.01$). However, degraded chitosan was still highly effective and was more water-soluble than native chitosan was. Degraded chitosan’s increased water solubility may give it a wider application for commercial use.

O-CM chitosan derived from degraded chitosan was more effective as an antimicrobial agent than O-CM chitosan derived from native chitosan was ($P < 0.01$), and O-CM-chitosan derived from degraded chitosan was more effective as an antimicrobial agent than degraded chitosan was ($P < 0.01$). Thus, O-CM substitution of degraded chitosan enhanced its antimicrobial activity without lessening its solubility. In contrast, O-CM chitosan derived from native chitosan was less effective as an antimicrobial agent than native chitosan was ($P < 0.01$). O-CM chitosan derived from degraded chitosan was an effective antimicrobial agent at lower concentrations as well for all bacteria except *E. coli* (Fig. 2). Only at the higher concentrations (0.1 and 0.5%) was this chitosan effective against *E. coli*. 
Hirano and Nagano (4) demonstrated that chitosan’s inhibition of the growth of bacteria and fungi depended on the MW of the chitosan. An increase in positive charges on chitosan will foster more interaction with negative charges on the membrane proteins, thus promoting the antimicrobial activity of chitosan. Chitosan’s antimicrobial activities (which are not evident in chitin) are related to its typical molecular structure (7). Sudarshan et al. (20) argued that the growth-inhibitory activity of chitosan markedly increased with the lengthening of the polymer, while Groboillot et al. (3) held that the cross-linked chitosan did not inhibit bacterial growth, suggesting that only soluble chitosan is inhibitory to such growth.

According to Liu et al. (11), antimicrobial activity increases with MW in the range of 5 to 91.6 and decreases with MW in the range of 91.6 to 1,080. This finding may be explained by the MW distribution’s relation to solubility and the number of protonated amine groups in solution. As the MW of the chitosan increases, so does the charge intensity, with the NH$_3^+$ content increasing until it reaches the cutoff point, at which the MW is 91.6. After this cutoff point is reached, there is a corresponding decrease in the charge intensity as the MW of the chitosan continues to increase.

O-CM chitosan derived from degraded chitosan was more effective as an antimicrobial agent than degraded chitosan was, possibly because its MW had increased but was still <91.6. In contrast, O-CM chitosan derived from native chitosan was less effective as an antimicrobial agent than native chitosan was, even though both have MWs of >9.16. Since an excessive concentration of amino groups on O-CM chitosan derived from native chitosan promoted a chitosan derivative bearing carboxymethyl-$\beta$-cyclodextrin. Carbohydr. Polym. 9:29–34. Groboillot, A. F., C. P. Champagne, and G. D. Darling. 1993. Membrane formation by interfacial cross-linking of chitosan for microencapsulation of Lactococcus lactis. Biotechnol. Bioeng. 42:1157–1163.


