Biofilm inhibition and attenuation of virulence properties of *Staphylococcus aureus* by low molecular weight chitooligosaccharides

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ABSTRACT

To combat the pathogenesis *of Staphylococcus aureus*, inhibition of biofilm formation and attenuation of virulence properties are considered as a promising solution. The active agents which are highly favored in up-to-date therapies should be biocompatible, biodegradable, non-toxic, and cost-effective. Having been found owning most of the aforementioned properties, chitosan-a natural biopolymer derived from chitin is favorably employed. The limitation in water solubility of chitosan is improved by structural modification to become chitooligosaccharides (COS), thus becoming more attractive in the recent research developments. In the present study, we investigated the antibiofilm and anti-virulence effectiveness of COS of different molecular weights dissolved in neutral water towards *S. aureus*. Results showed that low molecular weight COS of size 1-3 and 3-5 kDa exhibited biofilm inhibition and suppressed the bacterial hemolytic activity as well as H_2O_2 resistance properties. The study was evidenced by the crystal-violet staining and microscopic observation. Congo-red staining results showed that inhibition of amyloid protein (a virulence factor) production at high concentrations of low-molecular-weight COS. The present study provides a new insight for further exploiting chitosan into water-soluble low-molecular-weight COS as a safe and cost-effective drug for the treatment of *Staphylococcus aureus* infections.

STUDY BACKGROUND AND OBJECTIVES

Applications of chitosan as an antimicrobial and antibiofilm compound are considered as promising approaches due to its biodegradability, biocompatibility, non-allergenicity, non-toxicity, economical friendliness and cost-effectiveness [1]. However, the poor water solubility and high viscosity limit chitosan applications [2]. These limitations can be overcome using the chemically- or enzymatically-modified form of chitosan with reduced molecular weight named as chitooligosaccharides (COS) [3]. By becoming more water-soluble and less viscous, the high degree of deacetylation and polymerization and the polycationic nature of COS have led to such a great diversity of COS applications [4]. Hence, COS of different molecular weights was employed in the present study to screen for the antibacterial, antibiofilm and anti-virulence against *S. aureus*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The *Staphylococcus aureus KTCC 1916* was obtained from Korean Collection for Type Cultures (KCTC, Daejeon, South Korea). The culture media for the growth of *S. aureus* include tryptic soy broth and tryptic soy agar. Temperature was used 35°C under aerobic condition.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of each COS sample was determined by the broth microdilution method by using overnight grown *S. aureus* cell culture with the initial turbidity of 0.05 at 600 nm [5].

Biofilm Assays

The procedure for biofilm assay used in this study was adopted from the previous study [5]. Briefly, the overnight grown cell culture was diluted in TSB (1:100) and added to a 96-well microtiter plate. Different subinhibitory concentrations of COS (16-512 µg/ml) were added to the diluted bacterial culture and incubated at 35°C for 24 h in static condition. After incubation period, planktonic cells had been discarded and the attached cells were washed with distilled water, followed by staining with aqueous crystal violet (0.1%) and incubation at room temperature for 20 min. Following the dye removal, the plate was washed, mixed with 95% ethanol and the OD value was measured at 570 nm wavelength. Simultaneously, the growth of bacterial cells within the biofilm in the presence of each COS was estimated by OD value at 600 nm.

Microscopic visualization of biofilm cells

Scanning electron microscope and fluorescence microscope was carried out according to the procedure and described earlier [5].

Congo-red staining assays

The *S. aureus* colony was streaked on the Congo Red agar plates, which was prepared from TSB agar plates and Congo Red dye (40 µg/ml) (Sigma-Aldrich Co., St. Louis, MO, USA). The plates were incubated at 35°C for 24 h and the change in appearance of the red-colored colonies is considered as an indicator for production of amyloid fiber

Hemolytic and hydrogen peroxide activity of *S. aureus*

S. aureus cell culture grown overnight was mixed with different concentrations (32-512 µg/ml) of each COS in the 96-well microtiter plate. The cell culture (50 µl) obtained from the treated and non-treated samples was mixed with 950 µl of diluted sheep red blood cells (RBCs) and incubated at 35°C for 1 h under agitating condition (180 rpm). The OD of the supernatant containing lysed RBCs was determined at 543 nm.

For the H_2O_2 activity the aliquots (100 µl) of each treated cell culture with the COS were mixed with an equal volume of 1.5% H_2O_2 in the fresh titer plate and then the plate was incubated under shaking condition (567 cpm) in titer plate reader at 35°C for 1 h. After incubation, the mixed cell culture was serially diluted and spread plated onto the surface of TSB agar plate. The plates were incubated for 24 h at 35°C. The viability percentage was calculated by dividing the obtained CFU from the H_2O_2-treated sample to the non-treated sample (control).

RESULTS

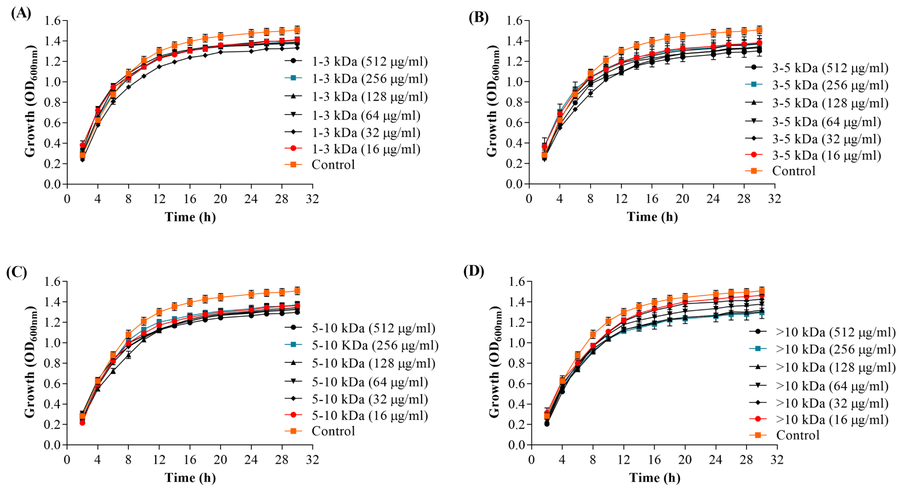

Fig. 1 Growth curve of *S. aureus* in the presence of different molecular weights of COS.
(A) Growth in the presence of 1-3 kDa COS, (B) Growth in the presence of 3-5 kDa COS,
(C) Growth in the presence of 5-10 kDa COS, and (D) Growth in the presence of >10 kDa COS

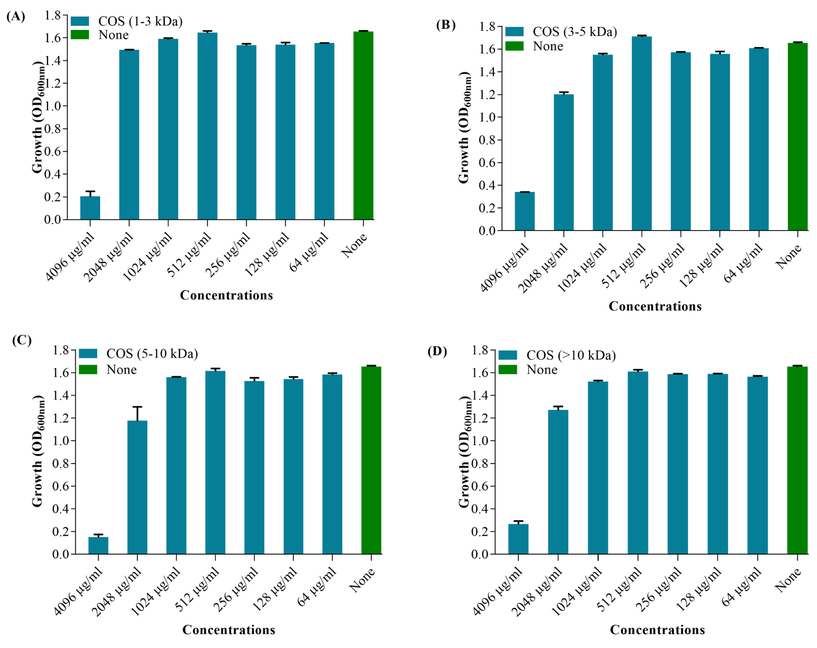

Fig. 2 Determination of inhibitory concentration of different molecular weights of COS against *S. aureus*

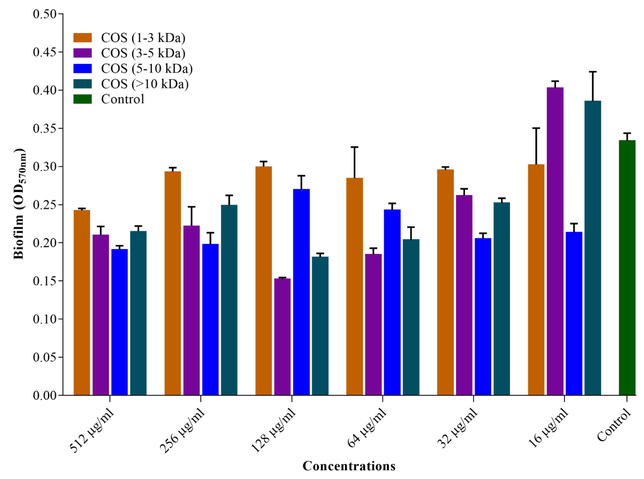

Fig. 3 Biofilm inhibition properties of different molecular weights of COS to *S. aureus*

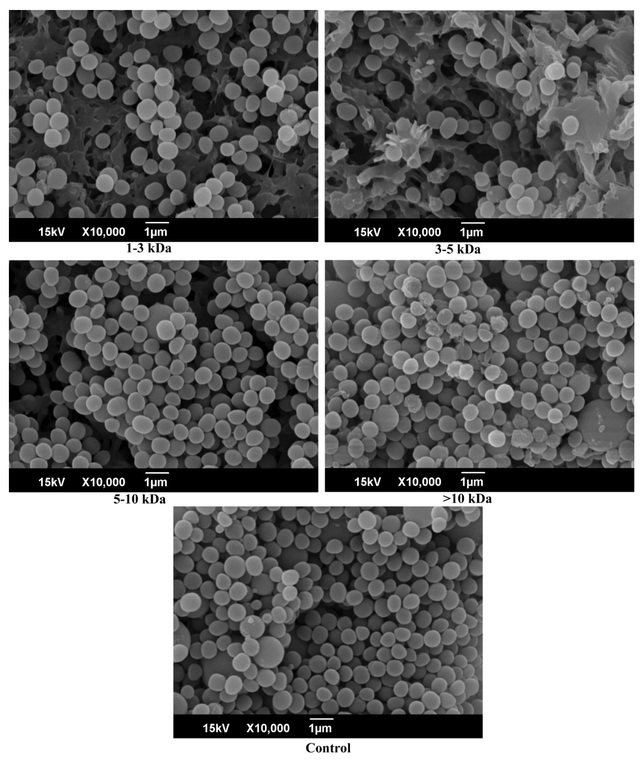

Fig. 4 Determination of the changes in the biofilm structure by scanning electron microscope

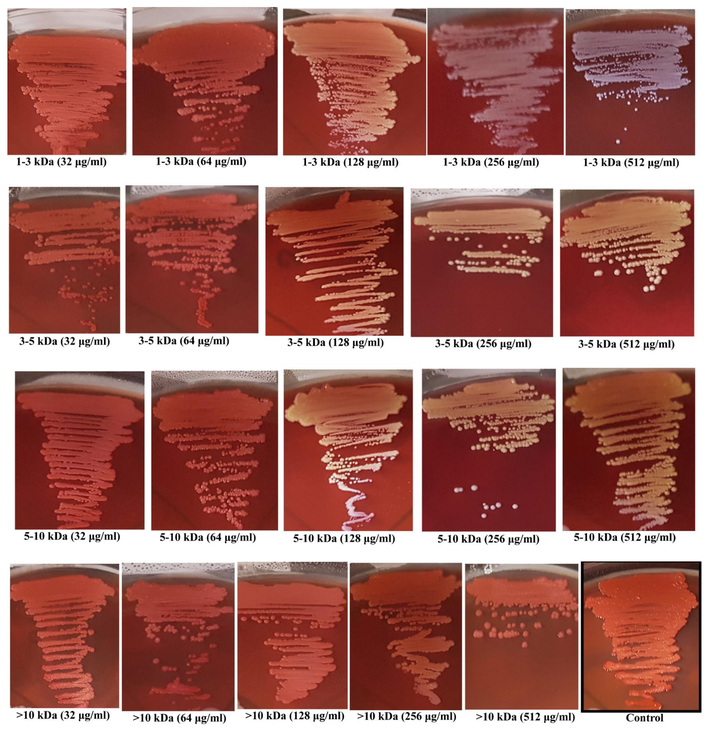

Fig. 5 Congo Red staining of the cell culture on the TSB agar plate

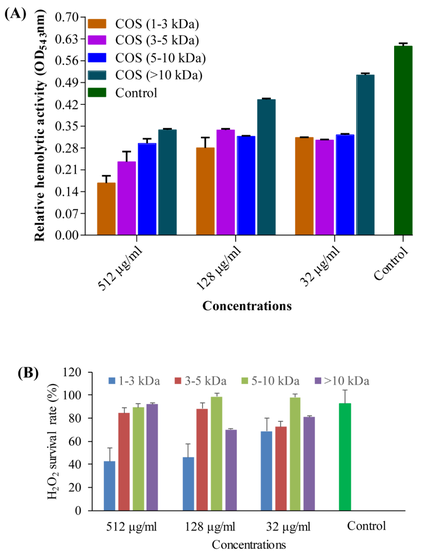

Fig. 6 Effect of different concentrations of each COS on the hemolytic (A) and H_2O_2 resistance (B) properties of *S. aureus*

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