Original Article

Antimicrobial effect of Chitooligosaccharides Produced by Chitosanase from *Pseudomonas* CUY8

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The aim of this study was to investigate the antimicrobial effect of chitooligosaccharides prepared by chitosanase from *Pseudomonas* CUY8. Antimicrobial activities of different degrees of deacetylation (DD) and polymerization (DP) of chitooligosaccharides against various species of bacteria and fungi were measured. The antimicrobial effects of chitooligosaccharides compared with chitosan and chitosanase were evaluated. Inhibitory diameter of chitooligosaccharides at the concentration of 0.1% with DP 4 was 19 ± 0.20mm, and inhibitory activity with DD 90% was 79± 2.1%, which were higher than other DP and DD, respectively. The results showed that antimicrobial activities of chitooligosaccharides increased with increase of DD, but decreased with increase of DP. Chitooligosaccharides, chitosan and chitosanase all showed significantly stronger antimicrobial activities against bacteria than fungi (p<0.001). Antimicrobial activities of chitooligosaccharides were significantly higher than that of chitosan (p<0.05), but insignificantly lower than that of chitosanase (p>0.05).

Key Words: chitooligosaccharides, antimicrobial effect, chitosanase, chitosan

Introduction

Chitosan is one of the most abundant renewable polysaccharides prepared from chitin by deacetylation.[1] Chitosanase is found in many organisms, including actinomycetes, fungi, plants, and bacteria. In plants, chitosanases are believed to be used in defense against pathogenic fungi. In microorganisms, chitosanases are important for maintenance of the ecological balance.[2-4]

Recently, chitosan and chitosanase are attracting a wide attention in their potential application in medicine, industry and agriculture.[5-6] Also, chitooligosaccharides have received much more interest, because they are not only water-soluble but also possess distinctive biological activity, such as antitumor, antifungal and antibacterial activities, immuno-enhancing effects, and promote host defense against infection of certain pathogens in mice.[7-11] Chitooligosaccharides obtained by enzymatic hydrolysis of β-1，4-glycosidic bonds of chitosan have been applied to medicament, foodstuff and cosmetic industry.[12]

In previous study, chitooligosaccharides with different degrees of polymerization were prepared through hydrolysis of colloidal chitosan by chitosanase from *Pseudomonas* CUY8.[13] In this study, We investigated the antimicrobial effects of chitooligosaccharides compared with chitosan and chitosanase against four species of bacteria and six species of fungi, which are mostly putrefactive microorganisms in food and aquatic preservation.

Materials and methods

Materials

Chitosan was purchased from Zhejiang Yuquan, Ltd. (China). Chitosanase (115.90 units/mg protein) and chitooligosaccharides were pepared from *Pseudomonas* CUY8 in our laboratory.[13] Test strains were obtained from Shanghai Municipal Center for Disease Control & Prevention (China), which included four bacteria (*Escherichia coli* ATCC 1150, *Staphylococcus aureus* ATCC 6538P, *Streptococcus lactis* KCTC 1950, *Bacillus subtilis* KCTC 1028), and six fungi (*Saccharomyces cerevisiae* ATCC 4126, *Rhodotorula bacarum* ATCC 7025, *Mucor circinelloides* ATCC 1216, *Rhizopus apiculatus* ATCC 11996, *Penicillium charlesii* ATCC 20841, *Aspergillus niger* ATCC 1015).

Preparation of chitooligosaccharides

Chitooligosaccharides were prepared through hydrolysis of colloidal chitosan by chitosanase from *Pseudomonas* CUY8. First, 30.0mL of enzyme solution (4.0units/mL) dissolved in 30.0mL of 0.05M acetate buffer (pH 5.0) was added to 30.0mL of 1.0% (w/v) colloidal chitosan. Then, the mixture was incubated for 30 minutes at 55℃. The reaction was stop by heating at 100℃ for 5 minutes. A 10.0mL of 0.25M sodium hydroxide (NaOH) solution was added to the mixture, and then the mixture was centrifugated for 20min at 1,000×g. The precipitated chitosan was removed and the supernatant containing chitooligosaccharides was collected.[14-15]

The amounts of degrees of deacetylation (DD) and polymerization (DP) of chitooligosaccharides were determined by the methods of Ruixun Lin and Jianmin Wu.[16-17]

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Assays for antimicrobial activity

Antimicrobial activities of chitooligosaccharides, chitosan and chitosanase were examined as follows. The medium (nutrient agar medium for bacteria and potato agar medium for fungi) was distributed in aliquots of 20 mL, and autoclaved at 120℃ for 20 min, then cooled to 45℃ before the addition of each test strains (10^5 CFU/mL). Target cells were seeded into the molten medium and then poured into petri dishes. A stainless steel punch was used to make wells (5 mm in diameter) on the solidified agar. Sterile solutions of chitosan, chitooligosaccharides, and chitosanase at the same concentration of 0.1% were added to the wells, respectively. Acetate buffer (pH 6.0) was used as a control. The agar plates were incubated at 37℃ for 24h for bacteria and 30℃ for 72h for fungi, respectively. Inhibitory activity was indicated by a clear zone surrounding the well, and three independent assays were performed in duplicate. The diameters of clear inhibition zones delimited were measured in millimeters.[18]

The assays of bactericidal or fungicidal activity were carried out by colony count on incubated agar plates. To a flask was added, 1.0mL of test strain (10^5 CFU), 1.0mL of the sterile sample solution (1.0 mL of acetate buffer for control) and 3.0mL of 0.05M acetate buffer (pH 6.0). This reaction flask was incubated with shaking, at 37℃ for 1h for test bacteria and 30℃ for 3h for test fungi, respectively. 1.0mL of the reaction mixture was added to the agar medium on the petri dish, and then incubated, at 37℃ for 24h for test bacteria and 30℃ for 72h for test fungi, respectively. After incubation, the colonies were counted to indicate bactericidal or fungicidal activity which was calculated by the following equation: Inhibitory (bactericidal or fungicidal) activity (%)={(C-T)/C}×100, where C is the numbers of colonies counted as the control and T is the numbers of colonies obtained from each tested sample.

Minimum inhibitory concentration (MIC) was tested by two-fold serial broth dilution as follows. Bacteria culture (10^5 CFU/mL) containing 1.0 mL of test sample solution grown in 5.0 mL tryptic soy broth medium was incubated at 37℃ for 18h. Fungi culture containing the same sample grown in 5.0 mL nutrient broth was incubated at 30℃ for 72h. Experiments were conducted for three independent assays in triplicate. MIC was defined as the lowest concentration of the tested sample at which the cell growth was not visible with microscopy.

Statistical analysis

The data analyses were performed using a SPSS software program. The values were reported as mean ± SD in all the results. P values were two tailed and P <0.05 was considered as significant.

Results

Table 1 shows the minimum inhibitory concentrations of chitooligosaccharides against bacteria and fungi. The MICs of chitooligosaccharides against bacteria and fungi were less than 0.12% and 0.15%, respectively. The MIC values of chitooligosaccharides in bacteria groups were 0.12%, except for the case of *E. coli* (0.08%). In fungi groups, the MIC values ranged from 0.13% to 0.15%.

Table 2 shows the mean ± standard deviation for inhibitory diameters of different degrees of polymerization (DP) of chitooligosaccharides against bacteria and fungi. The results showed that inhibitory diameters decreased with means values of 25mm, 25mm, 24mm, 23mm, 17mm and 16mm in bacteria groups and 13mm, 13mm, 13mm, 12mm, 8mm and 6mm in fungi groups with different DP of 4, 5, 6, 7, 8 and 9.

Table 1. Minimum inhibitory concentrations of chitooligosaccharides

Groups	Test strains	MIC (%)
Bacteria	*E. coli*	0.08±0.00
	S. aureus	0.12±0.01
	S. lactis	0.12±0.00
	B. subtilis	0.12±0.01
Fungi	*Rhodotorula bacarum*	0.13±0.01
	Sac. cerevisiae	0.13±0.00
	Mucor circinelloides	0.15±0.00
	Rhizopus apiculatus	0.15±0.00
	P. charlesii	0.14±0.01
	A. niger	0.15±0.01

Table 2. Antimicrobial effects of different degrees of polymerization of chitooligosaccharides against bacteria and fungi[a]

Groups	Test strains	Inhibitory diameters (mm)					
		DP					
		4	5	6	7	8	9
Bacteria	*E. coli*	26±0.1	26±0.1	26±1.1	25±0.1	18±1.2	16±1.1
	S. aureus	26±0.1	26±1.2	25±0.0	25±0.0	19±0.1	16±0.0
	S. lactis	24±0.1	24±1.1	23±0.2	21±0.0	16±1.3	15±1.0
	B. subtilis	23±0.0	23±0.2	22±0.0	22±0.1	16±1.1	15±1.0
Fungi	*Rhodotorula bacarum*	14±2.0	14±1.2	14±0.0	13±0.1	12±0.0	7±1.0
	Sac. cerevisiae	14±1.0	14±1.1	14±1.0	13±0.1	11±0.1	9±1.1
	Mucor circinelloides	13±0.1	13±1.1	13±1.0	11±1.1	6±0.2	5±1.0
	Rhizopus apiculatus	13±0.2	13±1.0	12±1.1	12±0.1	7±0.0	6±1.0
	P. charlesii	12±1.1	12±0.1	12±1.0	11±0.0	7±1.0	6±1.0
	A. niger	12±1.0	11±1.1	11±0.0	10±0.2	6±1.2	5±1.1

[a] Different degrees of polymerization of chitooligosaccharides were obtained by enzymatic hydrolysis with chitosanase from *Pseudomonas* CUY8.

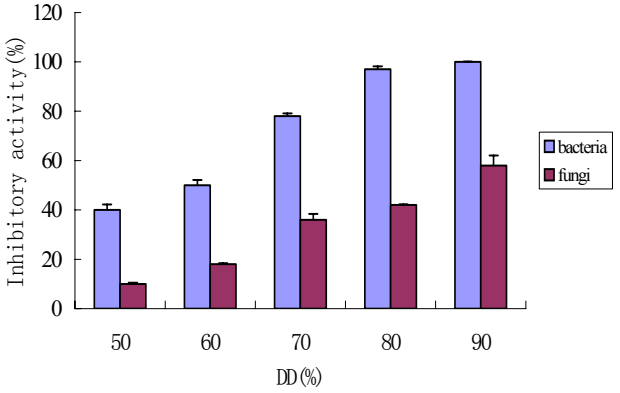

Figure 1. Antimicrobial effects of different degrees of deacetylation of chitooligosaccharides against bacteria and fungi

Figure 1 shows the inhibitory activity of different degrees of deacetylation (DD) of chitooligosaccharides against bacteria and fungi. Inhibitory activity increased with means values of 40%, 50%, 78%, 97% and 100% in bacteria groups and 10%, 18%, 36%, 42%, and 58% in fungi groups with different DD of 50%, 60%, 70%, 80%, and 90%.

Different inhibitory diameters of chitosan, chitooligosaccharides and chitosanase at the same concentration of 0.1% are shown in Figure 2. Inhibitory diameters of chitosan, chitooligosaccharides(DD value = 90%, DP value = 4) and chitosanase were 21±1.00mm, 26±1.80mm, and 26.5±1.70mm in bacteria groups and 9.2±0.80, 11.8±1.60mm and 12.8±1.20mm in fungi groups, respectively.

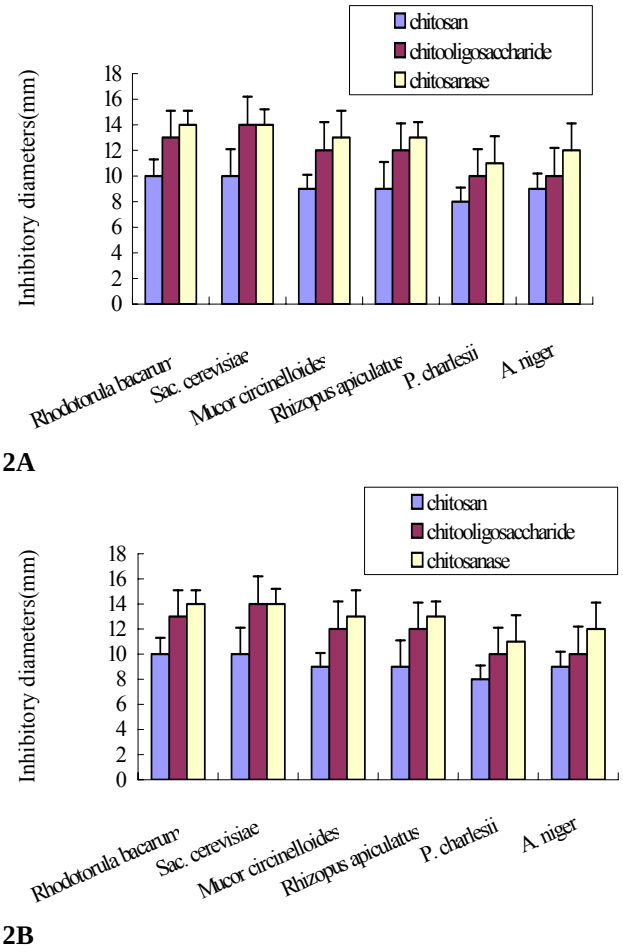

Figure 2. Antimicrobial activities of chitooligosaccharides by chitosanase from *Pseudomonas* sp. compared with chitosan and chitosanase. Results are given as the means of three trials in triplicate.

Discussion

Our results showed that antimicrobial effects of chitooligosaccharides against bacteria were higher than fungi. MIC of chitooligosaccharides against bacteria was significantly lower than fungi, with levels of 0.11±0.02% and 0.14±0.01% ($p<0.05$).

The antimicrobial effects of chitooligosaccharides had been shown to be greatly dependent on their degrees of polymerization. Among the different DPs, chitooligosaccharides of DP 4 had the strongest antimicrobial effect, which inhibitory diameter was higher than other DPs significantly ($p<0.05$), except DP 5 ($p>0.05$). From the results, degree of deacetylation of chitooligosaccharides is an important factor governing the inhibition of bacteria and fungi. For effective inhibition, DD values should be higher than or around 80%, with inhibitory activity against bacteria exceeding 97%. Inhibitory activity of chitooligosaccharides of DD 90% was significantly higher than other DD values ($p<0.05$). In addition, chitooligosaccharides with different degrees of deacetylation and polymerization showed excellent antimicrobial activities for all the bacteria examined, but they generally showed slight suppression for the growth of the examined fungi. Inhibitory activities of chitooligosaccharides with different DDs and DPs against bacteria were significantly higher than fungi ($p<0.05$).

The data indicated that antimicrobial effect of chitooligosaccharides increased with increase of DD, but decreased with increase of DP. Chitooligosaccharides with low degrees of polymerization, as small molecules, are very easy to penetrate through the cell membrane of microorganisms, interact with DNA in cytoplasm and karyon, lead to DNA replication mistake and result in suppression of the growth of microorganisms[19]. Antimicrobial activity of chitooligosaccharides increased with increase of DD, because chitooligosaccharides with high DD possess a lot of free amines. The free amines interact with the negatively charged residues at cell membrane. The reaction is to adsorb, congregate and precipitate the cells, subsequently result in death of cells.[19]

Chitooligosaccharides, chitosan and chitosanase all showed significantly stronger antimicrobial activities against bacteria than fungi ($p<0.001$). Inhibitory diameters of chitooligosaccharides were significantly higher than that of chitosan ($p<0.05$), but insignificantly lower than that of chitosanase ($p>0.05$). Chitosan is a kind of high molecular polymer, with positive charge which interact with the negatively charged residues of macromolecules at the surface of microorganisms, and inhibit the growth of microorganisms. However, chitosan, as a higher molecule, possesses lower water solubility which result in decrease of inhibitory activity. In addition, with respect to antimicrobial activity, chitosanase is superior to chitosan because chitosanase can hydrolyze chitosan in cell wall of microorganisms and inhibit the growth of

microorganisms. The water-soluble chitooligosaccharides may be advantageous as antimicrobial agents compared to water-insoluble chitosan. Hirano et al reported that inhibitory activities of chitooligosaccharides (DP ranging from 2 to 8) against some plant pathogens (*Fusarium oxyporam, Phomopsis fukushi*, *Allernaria alternata*) were higher than that of chitosan.[20] Similar results were also obtained in the present work. Chitooligosaccharides effectively inhibited growth of putrefactive microorganisms in food and aquatic preservation and their effects were higher than that of chitosan.

Our results showed that antimicrobial effects of chitooligosaccharides were similar to chitosanase's. Antimicrobial avtivities of chitooligosaccharides produced by chitosanase from *Pseudomonas* CUY8 depend on their concentrations, degrees of deacetylation and polymerization. This study suggested that chitooligosaccharides and chitosanase from *Pseudomonas* CUY8 have the potential application to food and aquatic preservation.

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