

ANTIBACTERIAL POTENCY OF CHITOSAN OLIGOMER PRODUCED BY *Bacillus licheniformis* MB-2 CHITOSANASE

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ABSTRACT

Bacillus licheniformis MB-2 chitosanase isolated from hot spring water in Manado, Indonesia, was used to produce chitosan oligomers. Both crude and pure *Bacillus licheniformis* MB-2 chitosanase were used to prepare chitosan oligomers, and the antibacterial activity of the resulting oligomers were tested towards 6 pathogenic bacteria, including *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus*. By contact method, the oligomers, at the MIC value (*Salmonella typhimurium* of 321 ppm, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* (MIC of 402 ppm) and the contact time of 24 h, were able to reduce all pathogenic bacteria tested by 2 to 5 log cycles. Using *Pseudomonas aeruginosa* protease, the oligomers were capable of reducing the protease activity by 64%, indicating that antiprotease might be involved in the antibacterial mechanism by these oligomers.

KEYWORDS: *Bacillus licheniformis* MB-2 chitosanase, oligomer, antibacterial agent, protease inhibitor

INTRODUCTION

Chitosan is a natural polymer containing *N*-acetyl-*D*-glucosamine and *D*-glucosamine residues. Having biodegradable and biocompatible properties, this deacetylated form of chitin polymer has been used widely at various industries such as food, agricultural, pharmaceutical, and waste water treatments. To improve its application, i.e enhancing the water solubility properties, the polymer is usually modified by substituting the functional groups with chemical substances or partially hydrolyzed chemically or enzymatically. Application of enzyme for partially degrading chitosan was more preferable because of the mild and safe process as well as specific reaction, resulting in high quality oligomer products. Specific size oligomer was reported to maintain various prominent biological activities such as antimicrobial properties.

Study on antimicrobial properties of chitosan has been reported by a number of scientists (Sagoo *et al.*, 2002; Helander, 2001; Rhoades & Roller, 2000; Muzarelli *et al.*, 1990; El-Ghaouth *et al.*, 1992). At low pH (<6.3), chitosan polymers showed antifungal activity (Roller & Covill, 1999; Rhoades & Roller, 2000). Partially degraded chitosan or chitosan oligomer was reported to have antibacterial properties against some pathogenic bacteria compared to the native one (Kendra & Hadwiger, 1984). The antibacterial mechanism has been proposed as loosing of barrier function of bacterial cell wall caused by chitosan

binding to the outer membrane of bacteria, and Chung *et al.* (2004) reported that there was a positive relationship between antibacterial activity of chitosan and surface characteristics of bacterial cell wall. Another possible mechanism through inactivation of important enzymes such as protease has never been reported yet.

Bacillus licheniformis MB-2 has been isolated from hot spring water of Manado and be able to produce thermostable chitosanase. In pure form, the enzyme hydrolyzes specifically chitosan, producing pentamer and hexamer of chitooligosaccharide (Chasanah, 2004). Preliminary study on antibacterial properties of oligomers from the crude enzyme using 4 concentration (0.005 to 0.170 U per mg chitosan) showed that oligomer produced by applying 0.1 U per mg chitosan and reaction time of 1 to 3 hours was able to reduce the 6 pathogenic bacteria tested (Meidina *et al.*, 2004). Further study on the sensitivity and mechanism of antibacterial through their potency as anti protease was assessed in this study using *P. aeruginosa* protease. Six pathogenic bacteria frequently associated with food borne infection and intoxication was used.

MATERIALS AND METHODS

Chemicals

Chitin and chitosan (85% deacetylated) were purchased from Sigma. Colloidal chitosan (about 76%

deacetylated) was chemically prepared from chitosan by method of Trudel and Asselin (199). All other reagents were of analytical grade. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli* were obtained from Pertamina Hospital, Jakarta, while *Listeria monocytogenes* and *Bacillus cereus* was from Veterinary Research Institute, Bogor. Commercial antibiotics, i.e kanamycin, ampicillin, and cephotaxim was used as control, obtained in 5 mg vial.

Microorganism and Enzyme Production

Bacillus licheniformis MB-2 was cultivated in medium consisted of 0.24% chitosan, 0.25% casiton, 1% $MgSO_4$, 1.4% K_2HPO_4 , 0.02% $CaCl_2 \cdot 2H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$ (pH7.0), and incubated at 55°C, 120 rpm shaker waterbath. A seed culture (15%) (pH7.0) of the bacteria (18 h) was used. The supernatant containing the enzyme was collected after centrifuging the fermentation broth at 8,000 g for 20 min. The enzyme was further concentrated by addition of 80% saturated ammonium sulphate.

Purification

Cell free supernatant containing 30% saturated ammonium sulphate (75 ml) was applied onto hydrophobic Butyl Sepharose 4 fast flow matrix, which had been previously equilibrated with 30% saturated ammonium sulphate to 0.05 m phosphate bufer (pH6). After sample application, the column was washed with the same buffer, and the eluate was obtained by washing with linear gradient of 15% saturated ammonium sulphate phosphate buffer (0.05 m, pH 6) and 0.05 m phosphate buffer (without ammonium sulphate) with elution rate of 2 ml per h. Each fraction (3 ml) was collected, and the protein and chitosanase activity were measured.

Chitosanase Assay

Chitosanase assay was conducted according to Yoon *et al.* (33), with modifications. The reaction mixture consisting of 100 μ l of 1% colloidal chitosan, 100 μ l 0.05 m phosphate buffer (pH 6) and 100 μ l of the enzyme solution was incubated at optimum temperature for 30 min. The reaction was stopped by incubating the mixture at -10°C for 15 min. The amount of reducing sugar in the mixture was determined by a modified method of Schales (27). An amount of 200 μ l of the solution was further mixed with 1 ml schales reagent and 800 μ l aquadest, and are further heated in boiling water for 15 minutes, centrifuged for 10 min at 3,000 g and the absorbance was read at $\lambda=420$ nm. A

blank was prepared using aquadest. One unit of chitosanase activity was defined as the amount of the enzyme which produces 1 imol of reducing sugar (glucosamine) per minute.

Protein Determination

Protein content was determined based on Bradford method using bovine serum albumin as the protein standard at 0.2 to 1.2 mg protein per ml. The reaction mixture contained 100 μ l of sample, 1 ml of aquadest and 1 ml Bradford reagent. After vortexing the mixture, the absorbance was read at 595 nm. A blank was prepared by substituting sample solution with 100 μ l of aquadest.

Chitosan Oligomer Preparation

Oligomer chitosan was made by reacting the enzyme (0.1 unit per mg chitosan), both crude and pure, to 1 of 85% deacetylated chitosan for 1 hour (Meidina *et al.*, 2004). Oligomer was harvested by boiling the mixture to inactivate the enzyme followed by sentrifugation (10,000 rpm, 10 minutes). The oligomer (supernatant) was sterilized at 121°C, 15 minutes before applied for antibacterial test.

Viability Study of Pathogenic Bacteria

Bacterial viability was analyzed by contact method (Carson & Riley, 1995). Bacterial culture amounting to 10^4 CFU per ml were incubated along with chitosan oligomer, and incubated in medium broth at 37°C. The number of bacteria at 0, 1, 3, 11, and 24 hours were plated and counted as colony forming unit per ml The chitosan oligomer concentration used was 1 and 1.5 MIC value (Meidina *et al.*, 2004)

Identification of Chitosan Oligomer

Chitosan oligomer formed was identified using dual lambda 440 mode HPLC, with 60% acetonitrile solvent in water as the moving phase. Detection was based on retention time. The flow rate speed used was 1 ml per min. The oligosaccharides standard (monomer to hexamer of glucosamine) was used at 1% (w/w).

Chitosan oligomer as anti protease

Protease enzyme was produced by inoculating 1 ose of *Pseudomonas aeruginosa* into LB and incubated for 24 h in 37°C shaker water bath. Extracellular protease was harvested by centrifuging the broth at 10,000 g for 15 min. Protease was assayed using 2% casein according to Bergmeyer *et al.* (1983).

Inhibitor protease was analysed according to Anson
In Imada et al. (1989).

RESULTS AND DISCUSSION

Enzyme and Oligomer Production

Bacillus licheniformis MB-2 chitosanase was harvested at the seventh days of fermentation using 15% starter, at 55°C 120 rpm shaker bath. Crude enzyme was obtained by concentrating the cell free supernatant with 80% saturated ammonium sulphate. Purification was done by hydrophobic interaction column chromatography as previously mentioned, and the relatively pure enzyme (Figure 1, Fraction 2) was collected.

Oligomer chitosan was made by reacting the enzyme, both crude, and pure, to 1 of 85% deacetylated chitosan. Optimal concentration of the crude enzyme effectively retarded the 6 pathogenic bacteria tested was 0.1 IU per mg chitosan with reaction time of 1 to 3 h (Meidina *et al.*, 2004). The diameter of a clear zone as antibacterial indication was about 10 to 19 mm for the 6 pathogenic bacteria.

This concentration was used in this experiment with reaction time of 1 h. For pure enzyme, the same concentration was used. In this experiment, oligomer possessing antibacterial properties was identified based on HPLC analysis. It was found that oligomer mixture were in the form of 2 to 6 unit oligomer.

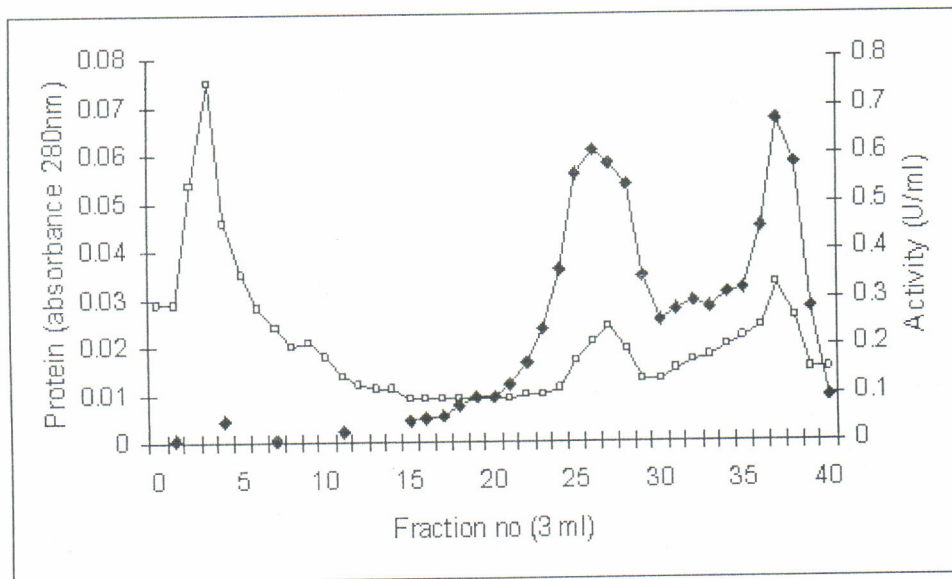


Figure 1. Elution profile of chitosanase and protein during hydrophobic interaction chromatography using Butyl Sepharose 4 FF matrix.
 Remarks: • = chitosanase activity; | = absorbance 280 nm

Antibacterial Study

Previous study by Meidina resulted that MIC value of oligomer produced by the crude chitosanase, was lower (321 to 562 ppm) compared to the polymer chitosan (7,000 to 10,000 ppm), but it was much higher compared to the commercial antibiotics (5 to 100 ppm). The MIC value of *Salmonella typhimurium* was 321 ppm, while the MIC of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* were 402 ppm, and the MIC of *Listeria monocytogenes* and *Bacillus cereus* were 482 and 562 ppm, respectively.

In this study, antibacterial potency of the oligomer was assessed by viability study. The concentration of oligomer used was at the MIC and 1.5 MIC, and the number of bacteria survived was counted during certain

time as indicated at Figure 2 below. Results showed that, in general, the number of all bacteria tested was reduced during 11 hours contact time. It was assumed that during that time all oligomer were already bound to the cells, and increasing bacterial number after that time may indicated the survived bacteria were capable to regain replication. The 1.5 MIC used was not significantly shown different effect to the number of bacterial reduction. Among bacteria tested, *Pseudomonas aeruginosa* was the most sensitive. The number of the *Pseudomonas aeruginosa* was significantly reduced during the first 3 hours contact, while *Escherichia coli* and *Salmonella typhimurium* were significantly reduced by 11 and 24 hours contact. On the other hand, gram positive pathogenic bacteria tested were reduced not as much as those gram negative bacteria. The results was in agree with

previous research results (Chung *et al.*, 2004; Helander *et al.*, 2001). It was reported that there was a positive relationship between antibacterial activity of chitosan and surface characteristics of bacteria cell wall. Gram negative bacteria which has more negative

charged and more hydrophilic were more sensitive to oligomer chitosan compared to the gram positive bacteria. Chitosan oligomer produced by pure chitosanase did not give different inhibition results as the crude one.

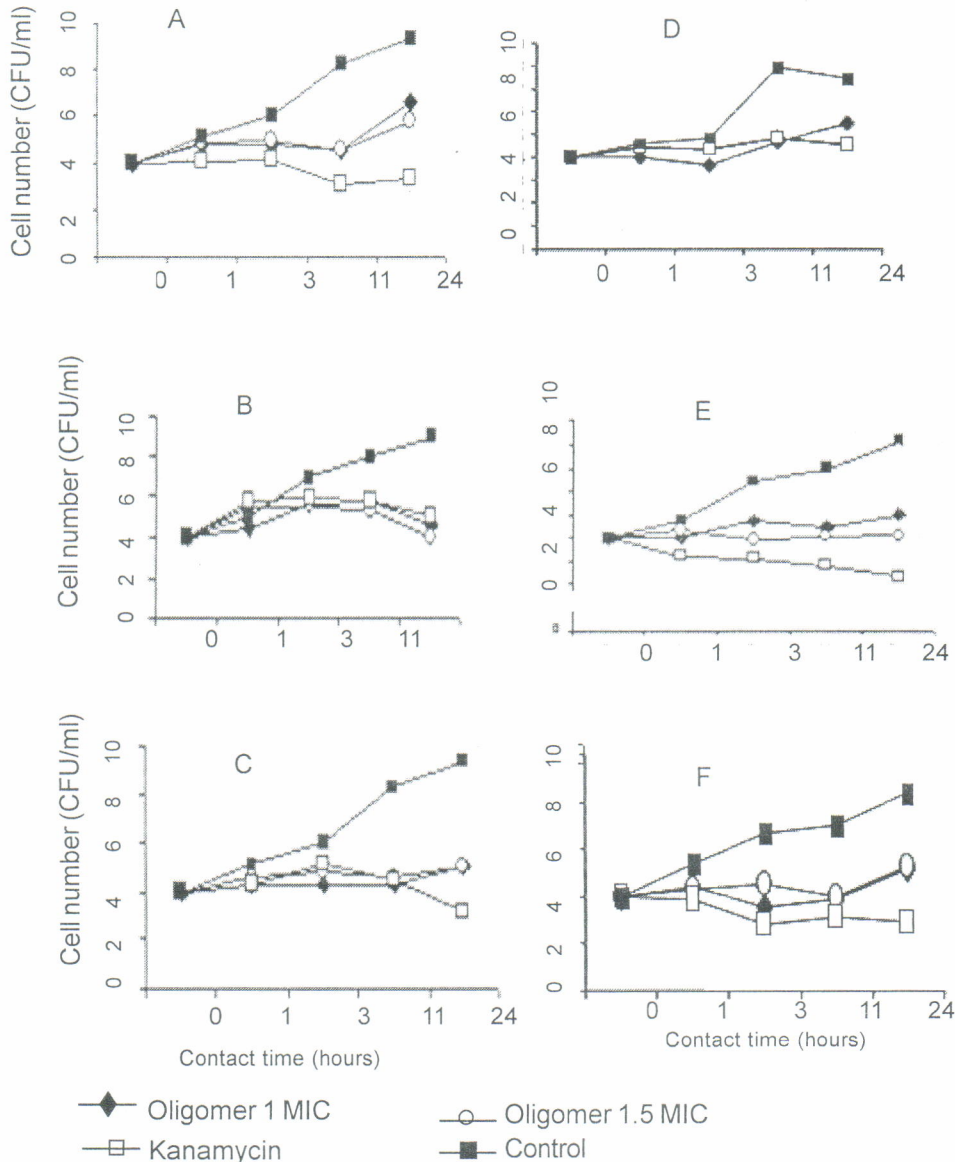


Figure 2. Effect of chitosan oligomer concentration and contact time on to cell viability (A = *Pseudomonas aeruginosa*; B = *Salmonella typhimurium*; C = *Escherichia coli*; D = *Staphylococcus aureus*; E = *Listeria monocytogenes*; F = *Bacillus cereus*).

Anti Protease Activity of the Oligomer

Protease enzymes presented in bacterial cell surface has important role in defensive mechanism. It was hypothesized that protease secreted by pathogenic bacteria was known as toxin, and some other were hydrolytic enzymes capable of degrading

extracellular matrix compounds of host polymer, for their carbon and energy sources.

In this experiment, chitosan oligomer produced by *Bacillus licheniformis* MB-2 was assessed for antiprotease. Results showed that chitosan oligomer at their MIC were able to retard 64% of *Pseudomonas*

aeruginosa protease having activity of 0.09 U per ml, while 25% retardation was obtained when using chitosan oligomer at 0.5 MIC. From the result, it can be deduced that the ability of chitosan oligomer, produced by *Bacillus licheniformis* MB-2 chitosanase, to inhibit pathogenic bacteria might through antiprotease mechanism.

CONCLUSIONS

1. Chitosan oligomers produced by *Bacillus licheniformis* MB-2 chitosanase has capability of reducing the 6 pathogenic bacterial tested by 2 to 5 log CFU per ml during 24 h contact.
2. Gram negative bacteria were more sensitive to the chitosan oligomer than the gram positive one.
3. The oligomers, at MIC value, was able to inhibit *Pseudomonas aeruginosa* protease by 64%, suggesting that the ability to inactivate bacterial protease might be one of the antimicrobial mechanisms performed by chitosan oligomer.

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