Antifungal activity of chitosan oligomer prepared using chitosanase of *Aeromonas media* KLU 11.16

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Abstract. Chitosan oligomer is a hydrolysis product of chitosan which can be prepared by enzymatic method, including chitosanase that may be produced by bacteria. Previously, chitosanase from *Aeromonas media* KLU 11.16 has been purified and characterized. The aim of this study was to analyze the bioactivity of chitosan oligomer prepared by using *Aeromonas media* KLU 11.16 chitosanase, as antifungal. Chitosan oligomer was prepared from shrimp waste chitosan, and hydrolyzed by 8U chitosanase per gram chitosan. The reaction time was for 2, 3 and 4 hours at 30°C. The oligomer produced then determined their yields and viscosities; and was identified by using a thin layer chromatography (TLC). Antifungal activity test against *Aspergilus flavus*, *A. niger*, *Eurotium amstelodami* and *Emericella nidulans* was performed on selected oligomers based on consideration of yield and TLC result, with oligomer concentrations of 0; 50; 100 and 200 ppm. The result showed that hydrolysis chitosan by *Aeromonas media* KLU 11.16 chitosanase for 4 hours reduced the viscosity from 187.5 to 13 cPs, and produced oligomers 96.14% of yield. The oligomers identified by TLC were monomer (1 unit) to hexamer (6 unit), and showed their antifungal activity against the four fungi tested. The best inhibition was showed by 200 ppm of oligomer against *A. flavus*.

Keywords: *Aeromonas media* KLU 11.16, antifungal**,** chitosanase, chitosan oligomer

1. Introduction

Chitosan oligomers are low molecular weight chitosans which have various bioactivities and potential applications in the food, pharmaceutical, agricultural and environmental industries. One of the bioactivities of chitosan oligomers is as antifungal, which can be used as a food preservative.

In Indonesia, food deterioration caused by fungal infestation is a common problem. The available antifungal preservatives officially used for food are generally produced from chemicals or synthetic preservatives. The use of natural preservatives for food is generally done traditionally based on hereditary knowledge.

Chitosan oligomers are commonly produced for research purposes. They are prepared by enzymatic method which involves chitinolytic enzymes. Over the past few years, our laboratory has explored microbial chitinolytic enzymes, particularly chitosanases, to investigate the potential bioactivity of the chitosan oligomers produced from the hydrolysis process of chitosan using these enzymes. One of the potential chitinolytic enzyme was produced by isolate KLU 11.16. The isolate was identified as *Aeromonas media*, and the chitosanase produced has been purified and characterized. However, the

application of the enzyme to produce chitosan oligomers and the bioactivity of the product has not been reported. This research was intended to study the potency of chitosan oligomers prepared by chitosanase from *A. media* KLU 11.16 as antifungal against several molds which are detrimental to food or harm for human.

Over the last few decades, chitosan oligomers have received an increasing interest due to their various bioactivity for human health and potential applications in the food, pharmaceutical, agricultural and environmental industries [1]. It is soluble in water and low molecular weight derivatives, exhibit an enormously wide range of biological activities such as to be an antifungal [2-5], antitumor [6-10], antimicrobial [11, 12] and anti-inflammatory agent [13, 14].

Antifungals can be used as a food preservative. Fungi are the main cause of deterioration of food and feedstuffs which lead to a lowering in yield, and a lowering in the quality and economic value of agricultural products [15]. Fungi may produce mycotoxins, that are harm and capable of causing disease and death in humans and animals, such as aflatoxin, ochratoxin, patulin, etc. [16]. Several toxigenic fungi found in food as well as fish products [15-19]. They infest the food, produce their metabolites and contaminate the foods. The most popular mycotoxigenic fungi is *Aspergillus* sp., mainly *A. flavus.*

Aspergillus flavus is well known as a aflatoxin producer, a harmful and poisonous toxin having carcinogenic effects [16]. Other Aspergilluses which potentially can produce mycotoxins are *Aspergillus niger* and *Emericella nidulans*, which was known previously as *Aspergillus nidulans*. Even though *A. niger* has generally regarded as safe (GRAS) status and is frequently used in biotechnology or industrial fermentation, it has the potential to produce two groups of potentially carcinogenic mycotoxins: fumonisins and ochratoxins [20]. Meanwhile, *E. nidulans* produces sterigmatocystin, a carcinogenic mycotoxin which is a precursor of the aflatoxins [21]. This fungus is commonly found in cereals and cereal products.

In Indonesia, food deterioration caused by fungal infestation is a common problem. The available antifungal preservatives officially used for food are generally produced from chemicals or synthetic preservatives. The use of natural preservatives for food is generally done traditionally based on hereditary knowledge. However, exploration of natural ingredients for food preservatives is widely investigated to obtain the safe and effective one, including the use of plant extracts for fish preservation [22], chitosan for fish and tofu preservation [23, 24] and determination the antibacterial activity of various plant extracts [25] as well as marine and fisheries products, including chitosan or its derivatives [26].

Chitosan oligomers can be prepared by enzymatic method which involves chitinolytic enzymes. Exploration of microbial chitinolytic enzymes conducted by our laboratory showed that chitosan oligomers produced by using the enzymes had various activities such as antibacterial and antitumor [27] as well as antifungal [28]. We had another potential chitinolytic enzyme produced by *Aeromonas media* KLU 11.16 [29, 30] which had been purified and characterized [31]. Thus, it should be further studied on its applications and evaluation of their products bioactivities.

2. Materials and Methods

2.1. Materials

Materials used in this study were *Aeromonas media* KLU 11.16 isolated from shrimp waste, chitosan from shrimp waste obtained from Bogor Agricultural University, fungi isolated from fish floss by Indriati which was collected by Microbiology Laboratory at Research Center for Marine and Fisheries Product Processing and Biotechnology, Jakarta, Indonesia. The fungi isolates were *Aspergilus flavus*, *A. niger, Eurotium amstelodami* and *Emericella nidulans.*

2.2. Methods

The research steps consist of production of chitosanase in 5 L fermenters, preparation of soluble chitosan, production of chitosan oligomers, identification of chitosan oligomers and antifungal assay.

2.3. Production of chitosanase

Chitosanase was produced following Chasanah *et al* [30] by cultivating *Aeromonas media* KLU 11.16 in a 5L-fermenter with a working volume of 1L of liquid Minimal Synthetic Medium (MSM) at 30^{o}C, 100 rpm for 48 h. The MSM used contained 0.5% colloidal chitin prepared from the chitin (Sigma) by method of Trudel and Asselin [32]; 0.1% K_2HPO_4; 0.01% $MgSO_4.7H_2O$; 0.1% NaCl; 0.7% $(NH_4)_2SO_4$; 0.05% *yeast extract* and 0.1% tryptone. The culture broth was then centrifuged at 9.820 x g, 4^{o}C for 15 min., and the supernatant was collected as extracellular chitosanase. The enzyme was then concentrated by 10X using an ultrafiltration with 10 kDa molecular weight cut off (MWCO). Both retentate and permeate as well as crude enzymes were assayed of their activity.

2.4. Chitosanase and protein assay

The chitosanase activity was assayed [31]. One unit of activity was defined as the formation of 1 µmoL D-glucosamine per minute under the assay conditions. The enzyme activity was presented as U/mL enzyme volume or U/mg protein for specific activity. Protein content was determined based on the Lowry method with bovine serum albumin as the standard [33].

2.5. Production of chitosan oligomers

Chitosan oligomers were produced enzymatically by reacting 2% soluble chitosan prepared previously using shrimp waste chitosan with a degree of deacetylation (DD) 51.44%. The concentration of chitosanase used was 8U per g chitosan in the total volume of 50 mL. Hydrolysis was carried out at 30^{o}C as optimal temperature for working enzyme [3] for 2; 3 and 4 h. The hydrolysate produced at the 2; 3 and 4-h was measured their viscosities using a viscometer (Brookfield Synchro-lectric). The initial soluble chitosan viscosity as well as 4h-hydrolysate without enzyme addition was presented as controls. The reaction was stopped by addition NaOH 0.25 M until pH 7. The solution was then centrifuged at 153 *g*, room temperature for 20 min., followed by freeze drying the supernatant containing chitosan oligomer. Identification of the chitosan oligomer was carried out using thin layer chromatography (TLC) with 2 standards used, i.e. chitosan oligomer solution containing 1-6 unit from Seikagaku Corp. and D-glucosamine HCl from Sigma.

2.6. Identification of chitosan oligomer

Chitosan oligomer was identified using TLC [34]. The mobile phase of TLC containing of 1-propanol, aquadest and 25% ammonia (20:7:3) was prepared, then 10 µL samples as well as standards with the concentration of 1% were spotted on a *Silicagel* 60 F_{254} (Merck) plate. The spot of TLC was visualized by spraying solution of 0.1% ninhydrine in 1-butanol prior to drying at 105^{o}C for 10 min.

2.7. Antifungal assay

Antifungal activity of chitosan oligomers was carried out against *Aspergilus flavus*, *A. niger, Eurotium amstelodami* and *Emericella nidulans* using agar diffusion method [35]. The isolates were sub-cultured on malt extract agar (MEA) slants for 5-7 days. Composition of MEA medium was including malt extract, yeast extract, neutralized bacteriological peptone, agar bacteriological, and artificial salt water containing NaCl, KCl, Na_2SO4, $MgCl_2.6H_2O$, and $CaCl_2$. Fresh cultures were inoculated on semi solid agar containing 0.2% bacteriological agar and 0.05% tween 80 [36]. The fungal spores agar was spotted on MEA medium which was added aseptically with 3 concentration of chitosan oligomers (50 ppm, 100 ppm, dan 200 ppm) in a different petri dish. The dishes were incubated at 28^{o}C for 4-7 days and diameters of growth zones were measured (3 replicates for each treatment).

2.8. Statistical analysis

The statistical significance of the difference between mean values in each treatment was determined by a single factor ANOVA (analysis of variance) followed by the student's t-test for determining which treatments resulting in a significant effect. P-values less than 0.05 were considered as significant values.

3. Results and Discussion

3.1. Production of enzyme and chitosan oligomer

Activity of chitosanase produced by *A.medi*a KLU 11.16 is presented in table 1. Concentration of crude enzyme by using ultrafiltration (10 kDa MWCO) increased the enzyme specific activity about 1.7 times. This was due to separation of enzymes from small molecules of protein by ultrafiltration. Similar results were reported [31] that ultrafiltration (10 kDa MWCO) of chitosanase from *Stenotrophomonas maltophilia* KPU 2123 MWCO) increased the enzyme specific activity by 1.76 times. However, the different result was occurred in other study using chitosanase from *Stenotrophomonas maltophilia* KPU 2123 [37]. The increase of chitosanase specific activity after ultrafiltration reached 5.6 times. Concentration process by ultrafiltration is affected by condition of process, such as pH, enzyme concentration, temperature; the characteristics of the equipment; and the type and pre-treatment of membranes [38].

Table 1. Activity of chitosanase produced by *A.media* KLU 11.16.

Sample	Protein (mg/mL)	Activity (U/mL)	Specific activity (U/mg)
Crude enzyme	0.167	0.1294	0.775
Rentetate of utrafiltration	0.782	1.0223	1.307
Permeate	0.189	0.1256	0.663

Hydrolysis process of chitosan produced hydrolysate that was low in viscosity (table 2). The highest decrease in viscosity occurred in the first 2-h hydrolysis, reached about 86%. At 4-h enzymatically hydrolysis, the viscosity of hydrolysate produced was 13.00 cPs (~6.9 % of initial viscosity), while viscosity of control (treatment without enzyme addition) was 133.35 cPs (~71.1% of initial viscosity). This result showed that the enzyme had an important role in decreasing the viscosity. The enzyme may contain chitinase, chitosanase or other chitinolytic enzymes, since it was used as the crude enzyme. Meanwhile, the hydrolysate contained at least 1-6 chitooligosaccharides as shown in TLC result (figure 1).

Specification of chitosan oligomer produced by enzymatic hydrolysis is affected by factors, including: chitosan degree of deacetylation, enzyme activity and ratio enzyme to substrate, hydrolysis process condition, etc. Hydrolyzing chitosan by crude chitosanase from *Stenotrophomonas maltophilia* KPU 2123 (activity of 1.004 U/ml or 1.4 U/mg protein) reduced the viscosity of chitosan hydrolysate up to 95% in 4 h of hydrolysis time [3]. Meanwhile, higher viscosity reduction of chitosan (up to 99.5%) on 16-h hydrolysis using the similar enzyme (activity of 0.459 U/mL) in a larger scale (10-15 times capacity) [39]. Hydrolysis of chitosan (DD 74.6%) using pepsin decreased 86% of viscosity after 1 h [40].

Yield of chitosan oligomer tended to increase with the increasing of reaction time, showing that the enzyme was still working to degrade chitosan until the end of observation time (table 3). However, the rate of increase in yield is slower which may be caused by a decrease in enzyme activity. Hydrolysis of swollen chitin by *Aeromonas* sp. GJ-18 crude enzyme produced about 78% mixed of GlcNAc and $(GlcNAc)_2$, identified by HPLC [41]. Whereas, 64~77% GlcNAc was produced in hydrolysis process of chitin by *A. hydrophila* H-2330 crude enzyme [42]. The lower yield later mentioned was related to the HPLC used to separate and identify oligomer produced which was more accurately compared to determination of yield conducted in this research.

Table 2. Viscosity of chitosan hydrolysate during hydrolysis.

Hydrolysis time (h)	Viscosity (cPs)
2	26.00±0.70
3	17.75±0.35
4	13.00±0.70
Control (4 hrs without enzyme)	133.35±0.21
Soluble chitosan 2%	187.5

Table 3. Yields of chitosan oligomer during hydrolysis.

Hydrolysis time (h)	Yields (%)
2	82.17±0.21
3	90.26±0.16
4	96.14±0.06

3.2. Identification of chitosan oligomer

Identification of hydrolysate by Thin Layer Chromatography indicated that the hydrolysis products was predicted contained di- to tetrachitooligosaccharide (figure 1). The figure showed clear spots on the standard, but not for the sample. The sample's spots look slightly blurry and unclear. This is probably due to the purity of the standard compared to the samples. Some factors may affect the product purity, including the purity of the ingredients used, and the presence of other products resulting from the reaction. In this case, we used crude enzyme for producing chitosan oligomers. This enzyme might contain salts derived from medium. Another ingredient was soluble chitosan which was prepared by using a buffer or salt solution. Thus, chitosan oligomers produced contained salts that interfered on TLC visualization. Better quality of chitosan oligomers might be acquired through the use of purified enzyme as well as purifying chitosan oligomer produced.

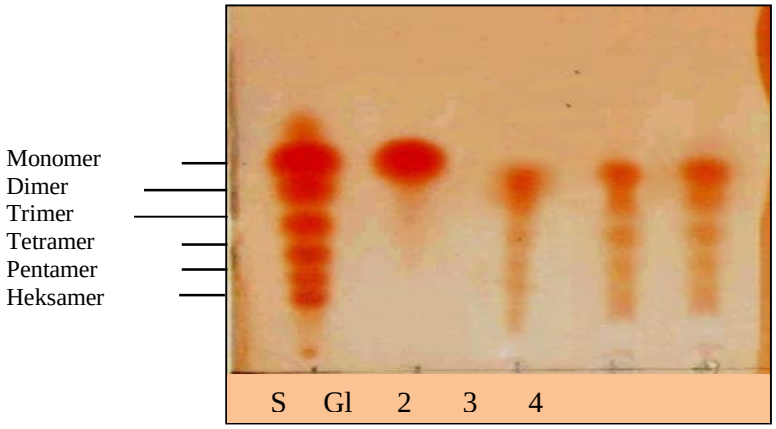

Figure 1. TLC profiles of chitosan oligomer produced in the hydrolysis of soluble chitosan by crude chitosanase from *A. media* KLU 11.16, S = Chitosan oligomers standard (Seikagaku Corp), Gl = lucosamine, 2; 3; 4 = visualization results of chitosan oligomers containing dimer – tetramer.

Aeromonas sp. was reported as chitinolytic bacteria producing chitinase [43, 44], chitosanase [30, 45]. However, publication related to the application of these enzymes for hydrolyzing chitin/chitosan is still limited. *Aeromonas hydrophila* chitinase was more specific toward the N-acetyl-β-D-glucosaminidic bonds in partially N-acetylated chitosan [43].

3.3. Anti-fungal activity of chitosan oligomer

Antifungal activity of chitosan oligomers against *A. flavus, A. niger, E. amstelodami* and *E. nidulans* is shown in figure 2. It was found that concentration of chitosan oligomers gave a significant fungal inhibition rate ($p<0.05$), except for *E. nidulans* at the concentration of 100 and 200 ppm. Higher concentrations of oligomers expressed a higher rate of inhibition; with the highest inhibition rate occurring on *A. flavus* at 200 ppm, which reached around 92%. It indicated that chitosan oligomers inhibited those fungi growth in a concentration-dependent manner. However, the effectiveness of inhibition chitosan oligomers against each fungus was not compared, because we did not quantify the fungi growth or determine minimum inhibitory concentration (MIC) of the chitooligosaccharides.

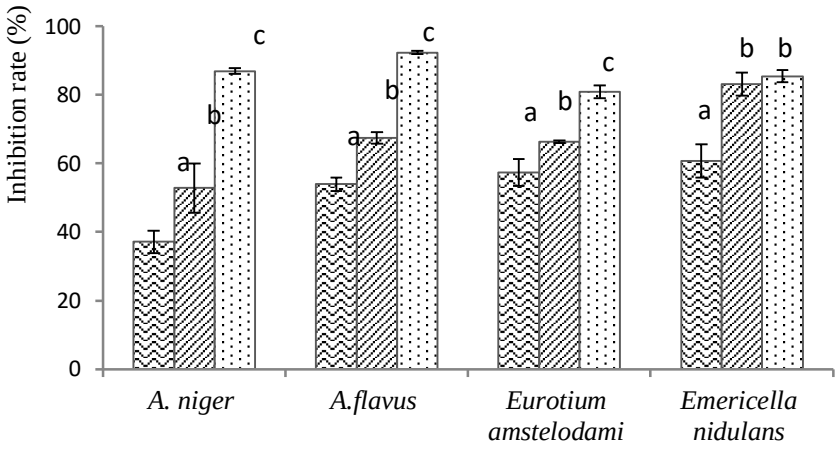

Figure 2. Inhibition rate of chitosan oligomers against *A. flavus, A. niger, E. amstelodami* and *E. nidulans* at various concentrations, ▨ 50 ppm ▨ 100 ppm ▨ 200 ppm.

Low molecular weight chitosan (LMWC) has potential for the antifungal agent. The anti-fungal effect of LMWC was due to the interaction of chitosan with lipids in the plasma membrane. Chitosan increased intracellular oxidative stress, then passed through the plasma membrane and killed cells in an energy-dependent manner. Thus, the composition of the fungal plasma-membrane affected the sensitivity against chitosan [46]. They also investigated that fungi with a higher content of polyunsaturated fatty acids might be more sensitive to chitosan. Effects of molecular weight and concentration of chitosan on antifungal activity against *A. niger*, that the antifungal activity of chitosan is mainly due to the inhibition of DNA transcription to RNA [47]. Other factors that may influence the effectiveness of chitosan oligomers as antifungals were the chitosan type and degree of polymerization, target microorganism, nutrient composition of substrates or broth, and environmental conditions [48].

Antifungal effects of chitosan oligomer on some deteriorative fungi of fishery products [28]. They found that chitosan oligomer inhibited the growth of *A. niger, A. flavus*, fungus isolates B12 and C13. Meanwhile, growth inhibition of the dermatophyte *Trichophyton rubrum* caused by chitosan oligomers was investigated [4], and they found that chitosan oligomers inhibited the growth of *Botrytis cinerea*, also known as gray mold, a necrotrophic fungus in numerous fruit and vegetable crops [49].

4. Conclusion

Preparation of chitosan oligomer by using chitosanase from *Aeromonas media* KLU 11.16 produced hydrolysate containing at least a mixture of chitosan monomer to hexamer. The chitosan oligomer inhibited the growth of *A. flavus, A. niger, Eurotium amstelodami* and *Emericella nidulans.* The inhibition of fungi growth was a concentration-dependent manner with the rate of inhibition reaching more than 80% at 200 ppm.

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