

Molecular Weight Determination of Chitosan with Antibacterial Activity Using Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry Analysis

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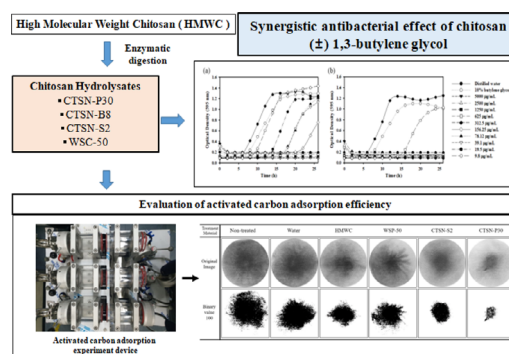
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Abstract: The antibacterial effect of chitosan with different molecular weights was investigated to define their potent biological activity. Both chitosan hydrolysates designated to CTSN-P30 and CTSN-B8 derived from the hydrolysis of high molecular weight chitosan showed significant synergistic antibacterial effects toward *Staphylococcus aureus* in the presence of 1,3-butylene glycol. The concentration-dependent antimicrobial effect is very important in determining the minimum inhibitory concentration (MIC) of both CTSN-P30 and CTSN-B8. Each MIC₅₀ of CTSN-P30 and CTSN-B8 was determined to be about 25 and 50 µg/mL, respectively. Among chitosan hydrolysates, CTSN-P30 can effectively protect the membrane from the penetrating of fine dust which may contain unspecified microorganisms. Also, CTSN-P30, which showed very significant antimicrobial activity, was confirmed to be a hydrolysate containing (GlcNAc)₃, and other hydrolysates consisting of (GlcN)₅ to (GlcN)₁₅ using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis. These results demonstrated the feasibility of CTSN-P30 and 1,3-butylene glycol to use as a potent antibacterial and find a dustproof agent for the preparation of various industrial products.

Keywords: antibacterial effect, chitosan, fine dust, 1,3-butylene glycol, molecular weight.



1. Introduction

For several decades, chitosan with different molecular weights, deacetylation degree, and chemically modified have been considered the most potent biological material in various research fields. Although many reports demonstrated the significant properties of chitosan and its relative materials for anti-oxidant, antibacterial,¹⁻³ anti-fungal,⁴ wound healing,⁵ and pharmaceutical activities,⁶⁻⁹ there are no reports on the evaluation of a single molecule bigger than octa-glucosamine. Perhaps it may not be able to prepare efficiently this size of single-molecule using so far known methods. So far, many researchers have been trying to develop several possible approaches including chemical, enzymatic, and combination processes to solve these problems out for a long time.⁹⁻¹¹ Therefore, further investigation for fine-tuning the reaction conditions for the preparation of target molecules is critically

necessary. Since we develop a potent and powerful method for preparing chitosan hydrolysates ranging approximately from 2 to 10-kDa from the hydrolysis of high molecular weight chitosan (HMWC), the further chance of separation of a single molecule among the hydrolysates of chitosan that can be used as a potent biological agent may be closely located to us.

In recent, we have been suffering from air contamination with fine dust, which is strongly concerned as out-coming air-pollutants flying inside to our country from neighbor countries and/or produced themselves by some domestic industry power-plants.^{12,13} Since many reports described the direct or indirect effects of fine dust on human beings and all lives, controlling and removing fine dust were big issues in Korea, including in some Asian countries. Therefore, amongst natural compounds without or less toxic including water-soluble polysaccharides isolated from photosynthetic marine microalgae *Tetraselmis* species,¹⁴ chitosan with a particular molecular weight ranging from 2-30 kDa has recently been considered as particular agents.^{10,15} Preparing measures to effectively remove fine dust and various microorganisms that have not yet been clearly identified is an important issue facing Korea and other Asian countries in particular. Not only can it cause various health problems importantly in respiratory diseases and lung cancer, but it also can bring severe problems in the culture, economics, and environment. Besides, fine dust that contains particulate matter (PM), heavy metal ions,

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and unidentified microorganisms could be spread through the worldwide nations freely and widely. As demonstrated in the previous reports, various effects of PM depending on the size and microorganisms were accounted as unpleasant results from power-plants and human-being.^{16,17} Furthermore, it can cause some inconvenient disputes between neighboring nations because of either it happened in nature or some of the industrial pollutants. Whatever happened people living in these areas have to protect themselves from unexpected exploring and infection by fine dust and unpleasant microbial species. Upon investigating the properties of chitosan and its hydrolysates in the preliminary study, we found some significant microbial growth inhibitory and fine dustproof effects in the presence of 1,3-butylene glycol, which has a potent antibacterial activity.^{4,18} Therefore, this study aims to elucidate the feasibility of developing a potent antimicrobial and find a dustproof agent using chitosan with different molecular weights. To the best of our knowledge, this first reports both synergistic antimicrobial and fine dustproof effects of chitosan with 1,3-butylene glycol.

2. Experimental

2.1. Materials

Both high molecular weight chitosan (HMWC, >2,000-kDa) and water-soluble chitosan (WSC, 300-kDa)-chloride salt with 95% deacetylation degree were kindly provided from the Sokchomul-san Co. Ltd (Sokcho, Korea). Chemicals such as copper sulfate pentahydrate, sodium hydroxide, alpha-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), and 4-Hydroxybenzhydrazid (PHABAH) were purchased from Sigma Chemical co. (St. Louis, MO, USA) for the determination of reducing sugar concentration derived from the hydrolysis HMWC, and its molecular weight as a matrix for matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis. Chitosanase obtained and purified from *Streptomyces griseus* was purchased from Sigma Chemical co. (St. Louis, MO, USA), used for the preparation of chitosan hydrolysates. All other chemicals including 1,3-butylene glycol (Sigma Chemical co., St. Louis, MO, USA) used in this study were first grade for the quantification and chemical reactions.

2.2. Degradation of HMWC

For the preparation of hydrolysates, dissolved HMWC (2 g) being homogeneous in 2% acetic acid (100 mL) at room temperature for 2 h to make 2% (w/v) of chitosan solution. Chitosanase (5.0 Unit) suspended in 50 mM Tris-HCl buffer (pH 7.5) was added into the chitosan solution and reacted at wide temperatures ranging from 4 °C to 60 °C for up to 24 h in a temperature dependence manner. To define the end-point of the enzyme reaction, reaction samples (100 mL) in each time point were collected in 1.5 mL tubes and heated at 100 °C for 5 min on a heat block to quench the enzyme reaction. After heating, tubes containing the hydrolysates obtained from the degradation of HMWC by enzyme action were transferred onto the ice for cooling down. WSP-50 having a molecular weight of about

5.0-kDa was also obtained under the same treatment conditions and method as HMWC. To delete undigested HMWC, WSC, and denatured enzymes, further separation of samples was performed by centrifugation at 13,000 rpm for 5 min.

2.3. Characterization of chitosan hydrolysates

Efficiency for the hydrolysis based on temperature dependence was determined by increasing the reducing sugar contents using PHABAH (4-Hydroxy-benzhydrazid) method. In brief, 0.025 g PHABAH was dissolved slowly and gently in 10 mL NaOH (0.5 mM) as described in the previous study.^{19,20} The degree of the hydrolysis of HMWC by the enzyme reaction performed under various conditions was quantitatively determined by the colorimetric method at a wavelength of 405 nm using a UV-spectrophotometer (Infinite M200 Pro Nanoquant, TECAN, Austria). The relative concentration of reducing end sugars derived from the enzymatic hydrolysis of HMWC was estimated by comparing to the values of standardization observed with glucosamine ranging from 0 to 1 mM.^{19,20}

2.4. MALDI-TOF mass spectrometry analysis of chitosan hydrolysates

For the analysis of the molecular weights of chitosan hydrolysates, both 2,5-Dihydroxybenzoic acid (DHB) and alpha-cyano-4-hydroxycinnamic acid (CHCA) were used as the matrixes. The mass range of chitosan hydrolysates was set from 499 to 10,000 m/z with MS reflector positive ion mode, as the operating mode based on the instructor's recommendation. The exact molecular weight and range were determined by the number of peaks given and the size of m/z. Calibration of the date was evaluated in TOF/TOF6 peptides cal mix – mass tolerance +/- 10 ppm following the instructor's manner, described in the previous study.²¹

2.5. Growth inhibitory effect of chitosan hydrolysates on microorganisms

Chitosan hydrolysates derived from the enzyme action under various conditions were added into LB-medium (pH 7.0) with wide concentrations ranging from 0 to 1% (w/v) as the final concentration. The initial bacterial cell number of *S. aureus* was adjusted to be 2×10^3 /mL in the LB medium. Pre-cultured 150 mL of bacterial cells were further divided into 8 wells of 96-well plate in the presence or absence of chitosan hydrolysates. All samples were then placed onto a microplate reader (Infinite M200 Pro Nanoquant, TECAN, Austria) and cultured continuously for monitoring the effect of chitosan hydrolysates on bacterial growth at 37 °C for 24 h. The bacterial growth inhibitory effect of chitosan hydrolysates was determined compared to the control test performed in the absence of chitosan hydrolysates.

The MIC₅₀ or MIC₉₀ of chitosan hydrolysates was determined with various concentrations ranging from 0 to 1 mg/mL toward *S. aureus*. Bacterial growth in the presence or absence of chitosan hydrolysates was monitored using a UV-spectro-

photometer (Infinite M200 Pro Nano-quant, TECAN, and Austria) for 24 h at 37 °C. The value of MIC₅₀ or MIC₉₀ was expressed as the mean ± standard deviation (S.D.) of three independent experiments to determine the potency of antibacterial activity.

2.6. Disc diffusion assay for antibacterial activity

Antibacterial activity of the chitosan hydrolysates was determined by following the previous studies with slight modification.^{3,22,23} Briefly, bacterial cells (5×10^7 /mL) pre-cultured in LB medium for 8 h at 37 °C were mixed well in 50 mL of fresh LB medium containing 1.5% (w/v) of agar. The mixture consisting of bacterial cells and LB medium was gently poured onto a disposal Petri-dish in a clean bench. While solidifying the medium, 10 mL of chitosan hydrolysates in 2% acetic acid were loaded to a paper disc (0.8 cm/diameter). After loading chitosan hydrolysates or 2% acetic acid used as the control to paper-disc, placed the disc very carefully onto the solidified agar medium in the plate. After confirming the disc attached to the medium, closed the lids and placed these plates at 37 °C for 1-2 days. Time after the bacterial cell growth inhibitory effect of chitosan hydrolysates was monitored and recorded by a photograph.

2.7. Find dust-proofing effect of chitosan hydrolysates

An assembled tester consisting of selective tubes with nylon clothes was set up to collect fine dust made by air-blow with very fine activated carbon. Nylon clothes coated with chitosan hydrolysates by spraying were air-dried for 10 min at room temperature. Activated carbon in the chamber was blown under 6 Barr for 5 seconds to the nylon clothes with or without treating chitosan hydrolysates through the tubes by high-pressure injection of nitrogen gas. The quantity of the air passed activated carbons used as air contaminants through the nylon clothes was measured using a portable machine called Air Quality Monitor (BR-SMART 126, Bramc, USA), as described in the previous study.²⁴ The removal effect of chitosan hydrolysates on activated carbon tested as fine dust was also monitored by a bright-reverse phase microscope (ECLIPSE TS100, Nikon, Japan), and then imaged by comparing to values of the control test.

2.8. Statistical analysis

For the statistical analysis, average values showed as the means and standard deviations ($\pm$ S.D.) from at least three different experiments. The significance of the values obtained through all experiments was determined compared with student *t*-tests ($p < 0.05$).

3. Results and discussion

3.1. Enzymatic hydrolysis of HMWC for preparation of chitosan hydrolysates

For several decades, various kinds of methods were developed and applied to prepare a potent bioactive size of chitosan hydrolysates. It is quite easy to just digest the high molecular weight

chitosan (HMWC) using either chemicals or enzymes to obtain chitoooligosaccharides (COS) ranging from mono-glucosamine (GlcN)₁ to Penta-glucosamine (GlcN)₅. Upon investigating the significance of those molecules in the determination of biological activity, the bigger size of molecules than (GlcN)₆ are consistently being required for further many studies. As described in the previous study, COS showed remarkable significance in immune-stimulating activity.²⁵ Moreover, there are many reports on applying COS to characterize their biological activities such as antioxidant, antibacterial and anticancer.⁶ However, less is known about the potent agent using higher linked glucosamine longer than (GlcN)₆. There is no outstanding method to subject as a powerful tool for the preparation of these molecules that can be derived from the hydrolysis of HMWC. Therefore, it is worth working to develop a suitable method for the hydrolysis of HMWC. Hence, we currently tried to establish the potentially applicable method to solve these problems for preparing different molecular weights of chitosan derived from the enzymatic hydrolysis of HMWC.

Eventually, many studies reported on the determination of the optimal conditions for the enzyme to elucidate the mode of action. Herewith a question arose what is the optimal condition for an enzyme? Because it has to reconsider depending on the target molecule that has the most potent biological activity that we want to take care of. As a result, we found that a commercialized chitosanase purified from *S. griseus* used in this study showed temperature dependence by monitoring the increase of reducing end sugars derived from the hydrolysis of HMWC. As is expected, COS was derived from the hydrolysis of HMWC in rapid enzyme reaction under the optimal conditions that were suggested by the provider. However, we were not interested in the preparation of the low molecular weight of COS. Therefore, we have been challenged to produce a bigger size of chitosan hydrolysates than (GlcN)₆ to expand the possibility of applications. As the result, we obtained three samples using as major products designated to CTSN-P30 treated at 4 °C, CTSN-B8 treated at room temperature, CTSN-S2 derived at 30 °C in a continuous process, as well as WSC-P50 derived from the parental material called water-soluble chitosan chloride-salt (WSC) at room temperature, respectively. The proposed molecular weight of chitosan hydrolysates was estimated as the average molecular weight based on the reducing sugar contents, the results of HPLC, and MALDI-TOF mass analyses, as described in the previous study.^{26,27} Under these conditions, the product yields (%) of these molecules were determined to be 45.6 ± 0.45 (CTSN-P30), 36.3 ± 0.38 (CTSN-B8), 16.3 ± 0.32 (CTSN-S2), and 48.3 ± 0.65 (WSC-P50), respectively. Based on these experiments, we propose that such a harsh condition might be useful for the preparation of chitosan hydrolysates that are derived in partial digestion. As described in the previous study, partially digested chitosan called medium molecular weight chitosan (MMWC) revealed significant antibacterial activities.^{2,3,28} Therefore, our data suggest that we could make some different sizes of chitosan hydrolysates including COS under non-sensible conditions for the enzyme reaction. In current, we continuously strive to prepare valuable chitosan hydrolysates with a much narrow range of molecular weight than MMWC for the development of potent biomedical or pharmaceutical agents compared to that of COS' biological activities.^{8,10,15}

3.2. MALDI-TOF mass spectrometry analysis of chitosan hydrolysates CTSN-P30

To elucidate the size and distribution of the hydrolysates CTSN-P30, the countable molecular weight of each molecule was determined by MALDI-TOF mass spectrometry analysis. Although it is not possible to determine the quantitative unit, it is a very powerful analysis technique to determine the molecular weight size range. As shown in Figure 1, CTSN-P30 was identified as chitosan hydrolysates mainly consisting of glucosamine (GlcN)₃ to (GlcN)₁₅. In general, the intensity of sensitivity at which small molecules are detected when constant energy is provided is higher than that of large molecules, the main size is determined to be (GlcN)₆ to (GlcN)₁₂. Herewith, a closer look in the data analysis reveals complex hydrolysates such as (GlcNAc)₃, and (GlcN)₆ that also exists as a single molecule are combined with (GlcNAc)_n, n=1~3. In terms of their respective molecular weights, (GlcNAc)₃ is corresponding to 647, and values of (GlcN)₆ + (GlcNAc)_n, n=1~3 are corresponding to 1,210, 1,412, and 1,614 m/z, respectively. This is considered to be a hydrolysates generated in the process of hydrolysis by enzymes since the degree of deacetylation of the HMWC used as a raw material was about 95%. Although it is not intended to describe the mechanism of action of the enzyme, CTSN-P30 mainly contains 6 to 15 units of glucosamine, and also contains hetero-oligomer hydrolysates. The molecular weight range of the molecules constituting CTSN-P30 can be predicted to be approximately 1-3 kDa, although it is difficult to compare the antibacterial activity between pure glucosamine molecules and mixed molecules with the current technology. Nevertheless, it can be estimated that chitosan hydrolysates CTSN-P30 having such molecular weight exhibit excellent antibacterial activity. One question may arise here. The problem is how to determine a more accurate molecular weight size and range without a standard material. The reason is that it is not possible to obtain a uniform or purified product much bigger than (GlcN)₆, although

the average molecular weight of chitosan hydrolysates can be determined by comparing the values by HPLC, viscosity, and quantification of reducing sugar, respectively. This is because there is no example of producing, separating, and purifying a single molecule of 1 kDa or more by any known methods so far. Therefore, it is very important to secure a technology capable of efficient production and separation or purification of chitosan hydrolysates having a molecular size for precise analysis. As described in several papers,^{29,30} most of the chitosan-degrading enzymes, their mechanism of action, and final product are only about hydrolysates smaller than (GlcN)₆. For another reason, the difference in the sensitivity of the peak and the molecular weight range was confirmed by using a matrix, alpha-cyano-4-hydroxycinnamic acid (CHCA) that mainly used in the analysis of peptides which are protein degradation products. Although it was not used as an appropriate matrix, it did not show a significant difference in sensitivity compared to the matrix 2,5-Dihydroxybenzoic acid (DHB) (data not shown). Infrequent, but in some cases, a continuous peak of DHB itself may be non-specifically confirmed when DHB is used as a matrix. Therefore, it is necessary to check the detection of only DHB for conditioning before analyzing the sample. No clear reason has been revealed, as a result of checking whether the size of the continuous peak derived from DHB is the same as the m/z value of the sample, 1216, 1228, and 1232, which have similar sample analysis values to 1,200 m/z, were identified as major peaks. However, in the case of CHCA, there is no confirmed example of its continuous peak, so peaks of about 300 m/z or less are mainly identified. Therefore, in this study, as a result of checking with two different matrices, CTSN-P30 is confirmed to be 1,210 m/z, which means a value composed of (GlcN)₆ + GlcNAc₁. Additionally, as one unit becomes larger, the difference between the sizes of GlcN and GlcNAc is 161 and 203 (± 1 or 2) m/z, respectively. On the other hand, the size change of the continuous peak of DHB itself is about 100 m/z, which is thought to act as a background

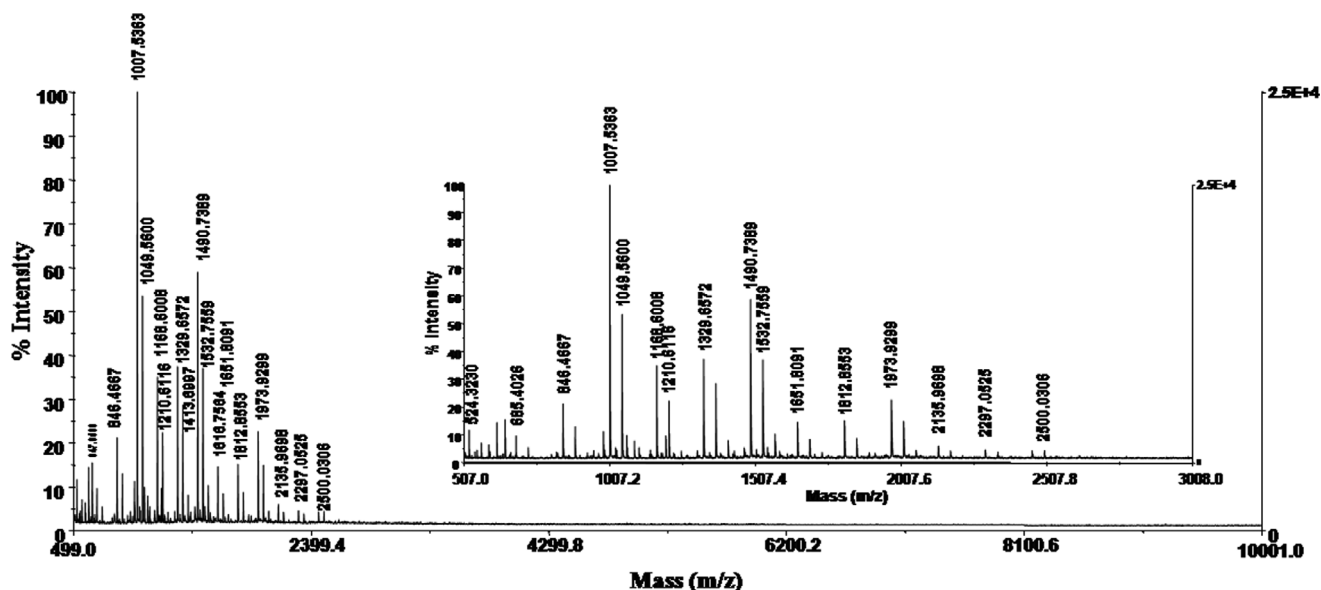


Figure 1. MALDI-TOF mass spectrometry analysis of chitosan hydrolysate CTSN-P30. Amongst chitosan hydrolysates, the molecular weight of CTSN-P30 derived from the hydrolysis of HMWC (1%, w/v) in acetic acid (2%) based on reactions with different temperatures was determined by MALDI-TOF mass spectrometry analysis ranging from 499 to 10,000 m/z. To observe the main peak range in more detail, the range of the figure was expanded from 600 to 3,000 m/z (inset). 2,5-Dihydroxybenzoic acid(DHB) was used as a matrix substance.

that can slightly affect the resolution in the sample analysis result. Therefore, selection of a matrix for sample analysis and multifaceted analysis is very important.

Therefore, through efforts to produce a narrow range of molecules with higher efficiency, we have great expectations for securing technology and utilizing such molecules as basic and applied research material. Besides, although one of the useful methods in determining the molecular size of the hydrolysates is MALDI-TOF mass analysis, quantitative evaluation is practically impossible, so unfortunately, the analysis or interpretation is limited. Therefore, calculating the average molecular weight of chitosan and its hydrolysates using other physicochemical methods according to the absence of a standard substance may lead to an error that misses a crucial clue. However, the fact that we are striving to reduce the margin of error as much as possible in a variety of ways can be said to be very valuable.

3.3. Antibacterial activity of chitosan hydrolysates

3.3.1. Growth inhibitory effect of chitosan hydrolysates on microorganisms

To elucidate the biological activity of chitosan hydrolysates CTSN-P30, CTSN-B8, CTSN-S2, and WSC-P50, bacterial growth

inhibitory activity toward *S. aureus* was determined in the presence or absence of 1,3-butylene glycol. As shown in Figure 2, the chitosan hydrolysates CTSN-P30 demonstrated the potent and strongly significant bacterial growth inhibition toward *S. aureus* with a wide range from 156 to 5,000 $\mu\text{g/mL}$ (Figure 2(a)). However, our data demonstrated that CTSN-P30 showed a much stronger growth inhibitory activity in the presence of 10% 1,3-butylene glycol, presenting the decrease of the minimum inhibitory concentration (MIC_{90}) from 156 to approximately 10 $\mu\text{g/mL}$ (Figure 2(b)). It becomes an about 15-fold increase in the activity. Furthermore, it is critically important to note that the time-lag with 10 $\mu\text{g/mL}$ of CTSN-P30 reaching the exponential phase was remarkably changed from 10 h to 16 h in the presence of 10% 1,3-butylene glycol. However, CTSN-B8 has shown comparably higher activity than that of CTSN-P30 in the absence of 10% 1,3-butylene glycol under the same range of concentration at 156 $\mu\text{g/mL}$ (Figure 3(a),(b)). Besides, CTSN-S2 and WSC-P50 showed similar activity for CTSN-B8 estimated in the minimum inhibitory concentration (MIC_{90}) against *S. aureus* in the presence or absence of 10% 1,3-butylene glycol (Figure 4, 5). Therefore, our data demonstrated that bacterial growth inhibitory activity was affected by CTSN-P30 in the presence of 1,3-butylene glycol. Taken together, the antimicrobial activity of the

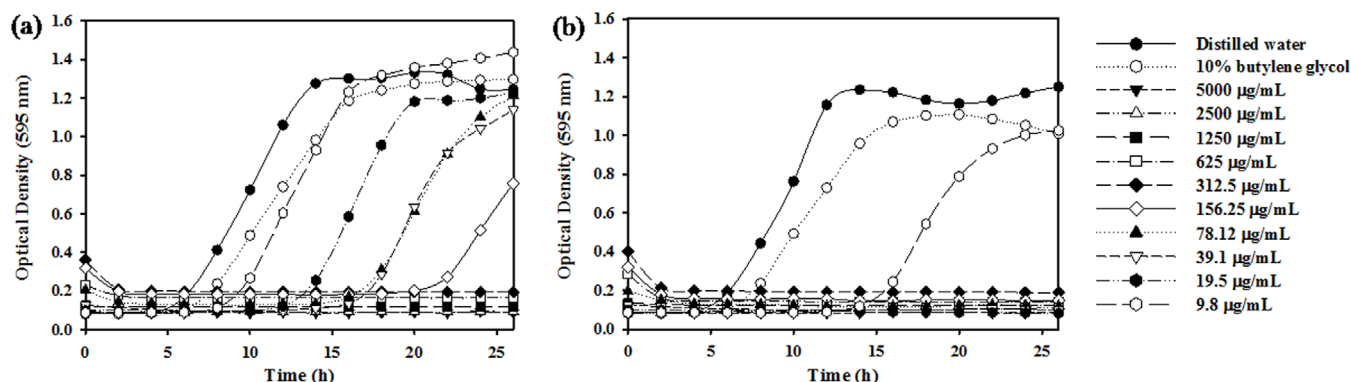


Figure 2. Influence of chitosan hydrolysates on bacterial growth. Chitosan hydrolysates designated to CTSN-P30 derived from the hydrolysis of high molecular weight chitosan (HMWC) by the temperature depending enzyme reaction was subjected to monitor the inhibitory effect on bacterial growth of *S. aureus* in the absence (a) or presence of 1,3-butylene glycol (b). CTSN-P30 with wide concentrations ranging from minimum 0 as the control to the maximum 1% (w/v) as the final concentration was applied to the culture medium, respectively. The initial number of *S. aureus* was adjusted to be approximately 2×10^3 / mL in the LB medium. The inhibitory effect of CTSN-P30 on the growth of *S. aureus* was monitored at 37 °C for up to 24 h.

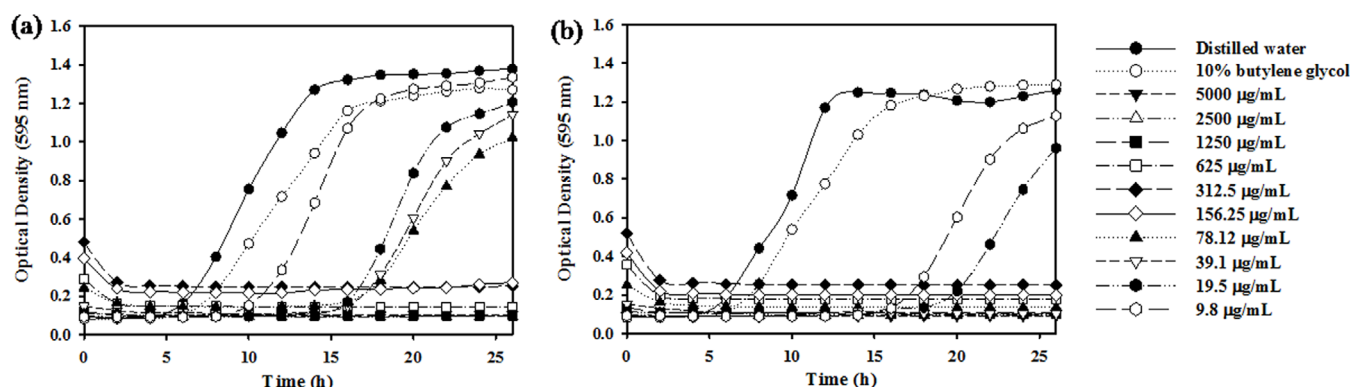


Figure 3. Effect of CTSN-B8 on bacterial growth. One of the chitosan hydrolysates designated to CTSN-B8 was subjected to elucidate the inhibitory effect on bacterial growth of *S. aureus* in the absence (a) or presence of 1,3-butylene glycol (b), as tested the assay with CTSN-P30 under the same conditions. The initial number of *S. aureus* was adjusted to be approximately 2×10^3 / mL in the LB medium. The inhibitory effect of CTSN-B8 on the growth of *S. aureus* was monitored at 37 °C for up to 24 h.

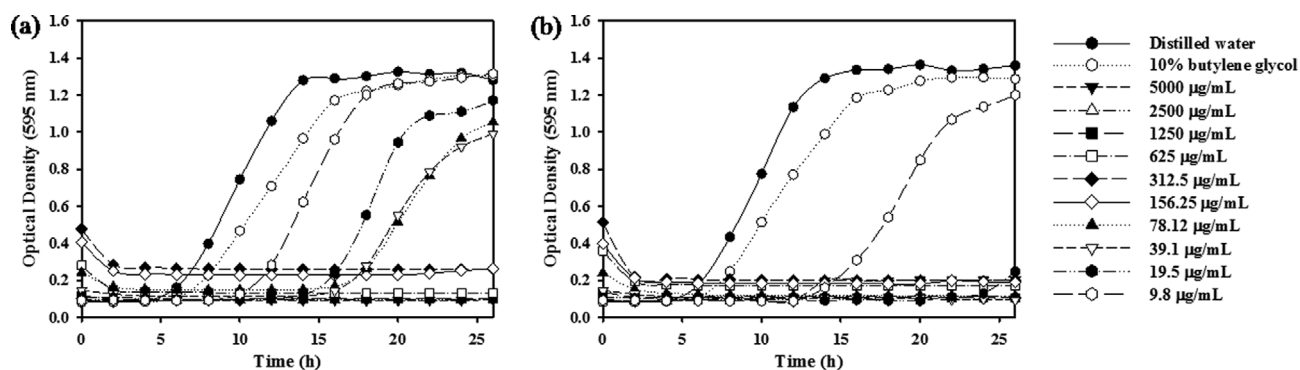


Figure 4. Effect of CTSN-S2 on bacterial growth. Chitosan hydrolysates designated to CTSN-S2 were subjected to elucidate the inhibitory effect on bacterial growth against the *S. aureus* in the absence (a) or presence of 1,3-butylene glycol (b), as tested the assay with CTSN-P30 or CTSN-B8. The initial number of *S. aureus* was adjusted to be approximately 2×10^3 /mL in the LB medium. The inhibitory effect of CTSN-S2 on the growth of *S. aureus* was monitored at 37 °C for up to 24 h.

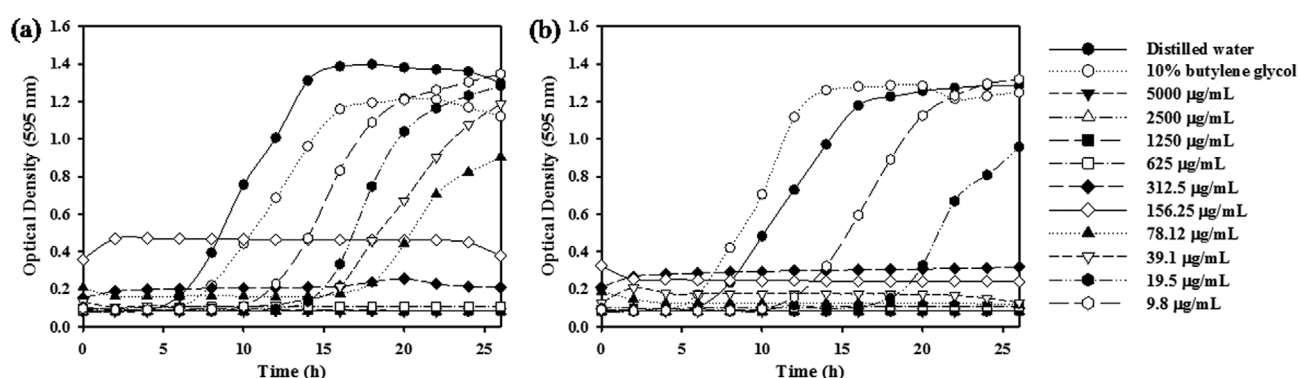


Figure 5. Determination of bacterial growth inhibitory activity of WSP-50. The WSP-50 was prepared from the hydrolysis of water-soluble chitosan prepared in chloride salt and subjected to monitor the inhibitory effect on bacterial growth of *S. aureus* in the absence (a) or presence of 1,3-butylene glycol (b). The inhibitory effect of WSP-50 on the bacterial growth of *S. aureus* was observed under the same conditions described above. Refer to the legend of Figure 1.

chitosan hydrolysates showed a difference in direct effect depending on the molecular weight, as well as the antimicrobial synergistic effect in the presence of 1,3-butylene glycol. Furthermore, WSC-P50 has also shown a similar activity with that of CTSN-B8 and CTSN-S2. It made us hypothesize to bring other concepts that the bacterial growth inhibition by WSC-P50 be related to the solubility of hydrolysates before or after treating enzyme. Currently, it is not possible to conclude the real effect of the size of the molecule of chitosan hydrolysates that may be directly involved in bacterial growth inhibitory activity. As described in the previous study,^{8,10,15} however, the molecular weight of hydrolysates might be the most important factor for determining the

biological activity of chitosan including antibacterial activity,³¹ antioxidant, and other potent activities. In addition, a significant decrease of MIC₉₀ values against *S. aureus* was observed in the presence of 1,3-butylene glycol (10%) as the final concentration (Figure 2, 3 and Table 1). These results demonstrated that chitosan hydrolysates with the specific size of molecular weight had shown remarkable synergistic antimicrobial activity toward *S. aureus* in the presence of 1,3-butylene glycol, indicating some of the potent applications. As we are always concerned about further investigation of the real action of chitosan hydrolysates, some high technical methods for the purification of a singular chitosan hydrolysate bigger than 1 kDa are still being required.

Table 1. Minimum inhibitory concentration (MIC₉₀) of the chitosan hydrolysates in the presence or absence of 1,3-butylene glycol

Chitosan hydrolysates	Average MW (kDa)	1,3-Butylene glycol	<i>S. aureus</i>
			MIC concentration (µg/mL)
CTSN-P30	3.0	-	312.50
		+	19.53
CTSN-B8	0.8	-	156.25
		+	39.06
CTSN-S2	0.2	-	156.25
		+	39.06
WSP-50	5.0	-	156.25
		+	39.06

Taken together, our data suggest that chitosan hydrolysates with different molecular weights ranging from 1 to 5 kDa may be useful for the preparation of commercial products in various application fields.

3.3.2. Disc diffusion assay for antibacterial activity

Based on the determination of MIC_{90} of chitosan hydrolysates in the presence of 1,3-butylene glycol, further disc diffusion assays were performed to elucidate the effect of chitosan hydrolysate CTSN-P30 on bacterial growth. As shown in Figure 6, much wider and clearer zones were observed around the disc when 10 mL of chitosan hydrolysates CTSN-P30 were loaded onto the disc in the presence of 1,3-butylene glycol with wide-ranging concentration. These results are correlated with the results demonstrated in Figure 2-3. However, limiting of concentration of 1,3-butylene glycol on the disc diffusion assay was observed depending on the size of chitosan hydrolysates that might be tightly absorbing 1,3-butylene glycol due to their hydrophobic interaction with each other. On the other hand, there was no significant antibacterial effect observed when both controls of none and 1,2-hexanediol were used (data not shown). Nonetheless, several chitosan hydrolysates applied in this study have shown the potent possibility of utilization with the synergistic influence of 1,3-butylene glycol (Table 2). Again, herewith we emphasize the importance of the molecular weight of chitosan to investigate further biological activity in detail. Taken together, we suggest that chitosan hydrolysates and 1,3-butylene glycol can be selected as potent bioactive materials that might be use-

ful for several industrial applications for developing cleaners or sanitary products.

3.4. Removal effect of fine dust

After observing of antibacterial activity of chitosan hydrolysates, we subjected chitosan hydrolysates to investigate the removal efficiency of activated carbon used as fine dust. Chitosan hydrolysates with different molecular weights dissolved in 1% acetic acid were ubiquitously sprayed on a nylon membrane and were subjected to an air-passing tube in the presence of activated carbon powder. The amount of activated carbon passed through the nylon membrane either coated with a different molecular weight of chitosan hydrolysates or not was optically observed by phase-contrast images compared with that of the control. As shown in Figure 7, we found that one of the chitosan hydrolysates called CTSN-P30 has shown the highest potent affinity toward activated carbon compared to that of HMWC, WSP-50, and CTSN-S2, respectively. Besides, CTSN-P30 showed the more removal efficiency of activated carbon in the presence of 10% of 1,3-butylene glycol as the final concentration. Other samples also showed a more effective removal efficiency of activated carbon in the presence of 1,3-butylene glycol used at the same concentration. Although there were few differences observed, no significant concentration dependence of 1,3-butylene glycol was monitored. Therefore, CTSN-P30 may be the most dominant molecule toward both antibacterial and fine dust-proofing effects. As many times as we described, again our observation showed

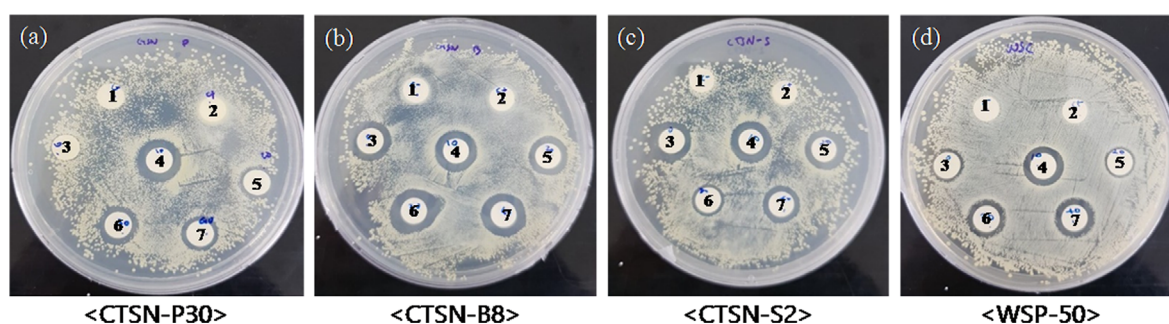


Figure 6. Disc diffusion assay for chitosan hydrolysates. The inhibitory effect of chitosan hydrolysates on bacterial growth of *S. aureus* was monitored with a different molecular weight of chitosan (a: CTSN-P30, b: CTSN-P8, c: CTSN-S2, d: WSP-50), respectively. Acetic acid (1.0%, v/v) was used for the solvent of chitosan and its hydrolysates and 1,3-butylene glycol (10%, v/v) was used as a commercialized agent for antimicrobial chemicals. The size of clear zone around the disc was measured as the mean of antibacterial activity of chitosan hydrolysates. 1. control (1.0% acetic acid); 2. 1,3-butylene glycol (10%); 3. CTSNs (100 μ g/mL); 4. CTSNs (100 μ g/mL) + 10% 1,3-butylene glycol; 5. CTSNs (100 μ g/mL) + 20% 1,3-butylene glycol; 6. CTSNs (100 μ g/mL) + 30% 1,3-butylene glycol; 7. CTSNs (100 μ g/mL) + 40% 1,3-butylene glycol.

Table 2. The disc diffusion assay for evaluation of the chitosan hydrolysates against *S. aureus* in the presence of 1,3-butylene glycol

Chitosan hydrolysates	Diameter of the clear zone (mm)						
	Controls		Chitosan hydrolysates (100 μ g/mL)				
	Distilled water	1,3-Butylene glycol	1,3-Butylene glycol (%)				
			0	10	20	30	40
CTSN-P30	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	10.67 $\pm$ 1.53**	14.00 $\pm$ 1.00*	10.67 $\pm$ 0.58*	11.67 $\pm$ 0.58	10.67 $\pm$ 0.58*
CTSN-B8	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	13.00 $\pm$ 1.00*	14.67 $\pm$ 1.15*	13.00 $\pm$ 0.00	12.67 $\pm$ 1.53**	14.33 $\pm$ 0.58
CTSN-S2	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	13.00 $\pm$ 1.00*	14.33 $\pm$ 0.58	13.00 $\pm$ 1.73**	11.33 $\pm$ 1.53**	12.00 $\pm$ 1.00**
WSP-50	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	10.00 $\pm$ 0.04	12.33 $\pm$ 0.58	11.00 $\pm$ 1.00**	12.00 $\pm$ 0.05	12.00 $\pm$ 1.00**

All values represent the average diameter $\pm$ standard deviation (S.D.) of triplicate tests. Means with different superscripts (*) are significantly different ($P < 0.05$) with the different type of chitosan hydrolysates and the different concentrations of 1,3-butylene glycol.

Treatment Material	Non-treated	Water	HMWC	WSP-50	CTSN-S2	CTSN-P30
Original Image						
Binary value 100						

Figure 7. The fine dust removal efficiency of chitosan hydrolysates. Activated carbon used as an artificial air contaminant in an assembled tester associated with nylon clothes coated with chitosan hydrolysates was blown under 6 Barr for 5 seconds through the tubes by high-pressure injection of nitrogen gas. The number of activated carbons captured on the nylon clothes was monitored by a bright-reverse phase microscope (ECLIPSE TS100, Nikon, Japan), and then imaged by comparing to values of the control test.

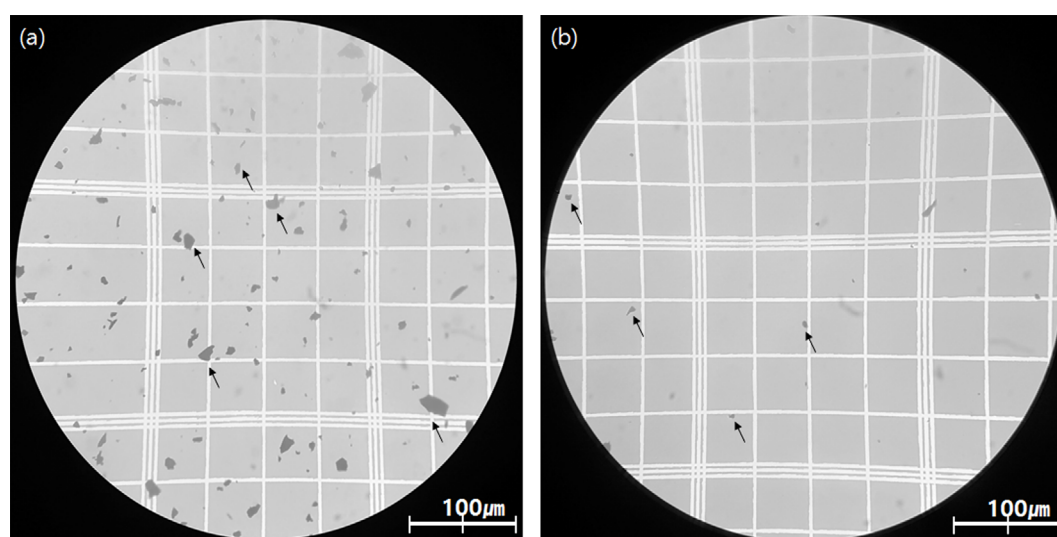


Figure 8. A microscopic analysis of activated carbon captured on a nylon membrane. Activated carbon passed through the nylon membranes either coated with CTSN-P30 (a) or not (b) was observed by using a microscope. Arrows indicated the sizes of activated carbon on the membranes.

the importance of the molecular weight of chitosan that may be involved in a variety of actions for both dust-proofing and protection from microbial infection as demonstrated in earlier studies.^{12,24,25}

The removal efficiency of activated carbon after passing through the nylon membrane either coated with a different molecular weight of chitosan hydrolysates or not was monitored by using a microscope and its values were calculated compared to that of the control test. As shown in Figure 8, several sizes of activated carbon captured on the nylon membrane coated with CTSN-P30 were observed in unspecified features (Figure 8(a)), whereas a very small number of activated carbons were detected on the nylon membrane in the absence of CTSN-P30 (Figure 8(b)). Based on these results, the removal rate of activated carbon of various sizes was confirmed that the CTSN-P30 treated samples showed significantly higher removal rates than the untreated samples (Figure 9). One question arises: how it can

interfere with breathing when wearing a mask when the CTSN-P30 needs to be sprayed on the membrane. If these results are to be used for industrial applications, further research is needed. Herewith, the important reason for using activated carbon is to solve both reproducibility and deviation problems due to severe changes in weather conditions. Subsequently, it is necessary to process some separate experiments under various environmental conditions to evaluate the efficiency of fine dust removal quantitatively. Therefore, to obtain more accurate results, further research is required to prepare several samples to evaluate the efficiency of fine dust removal, as well as environmental pollutants contained therein under various environmental conditions. Collectively, taken together we propose that amongst chitosan hydrolysates CTSN-P30 is critically useful as a potential absorber or preventer of microbial species and fine dust, or from other contaminants in various industrial applications.

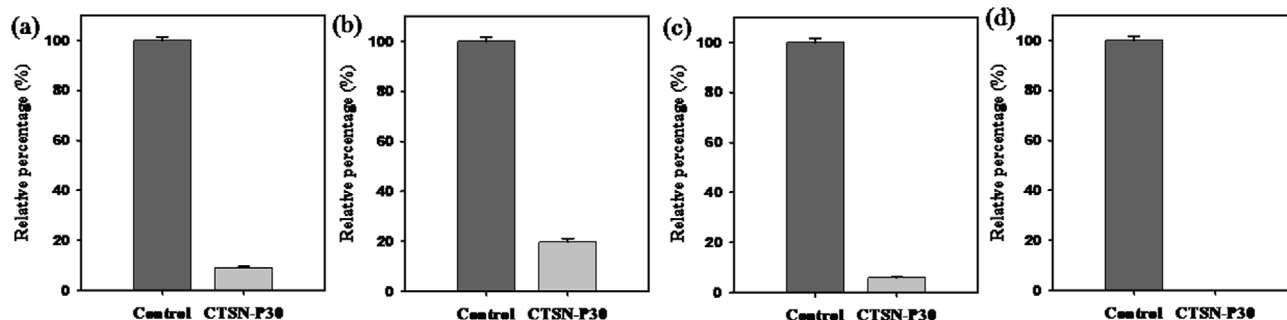


Figure 9. The removal efficiency of activated carbon. Numbers of activated carbon after passing through the nylon membranes in the presence or absence of CTSN-P30 were counted from four different samples. The relative removal efficiency was presented as the average mean of tests and its standard deviation ($\pm$ S.D.). The relative removal percentage (%) of different sizes of particle materials (PM) captured through the nylon membranes coated with CTSN-P30 was calculated by computational analysis. a, total activated carbon mixed with all sizes; b, PM 2.5; c, PM 10; d, >PM 10, respectively.

4. Conclusion

Elucidation of the crucial factors such as molecular weight and deacetylation degree of chitosan and its relative molecules involved in regulating biological activity is critically important. Amongst chitosan hydrolysates, CTSN-P30 prepared in enzyme degradation showed remarkable antibacterial and fine dust proofing influences compared to that of other sizes and different characteristics of chitosan. Not only CTSN-P30 showed antibacterial activity, but also demonstrated the highly significant synergistic influences of fine dust proofing activity in the presence of 1,3-butylene glycol to be 10% as the final concentration, which has been used as one of the cosmeceutical components. The result demonstrated the significance of the size of the molecular weight of chitosan in this study. Although it is not able to define the position of the *N*-acetyl group on the molecule of chitosan hydrolysates due to lack of the purified molecule, further study on the purification and characterization of a single molecule from the mixture of CTSN-P30 is necessary, undergoes in a large scale of the purification in the current. Nevertheless, our data suggest that CTSN-P30 may contain the most suitable size of the molecule as a potent bioactive compound for removing microbes from the various routes of contamination and fine dust in the air.

Authorship contribution: JKP and YJH conceptualized the idea, designed and coordinated the study. SYP performed the preparation of chitosan hydrolysates with different molecular weights, and YHL performed antibacterial and dust-proofing experiments. JKP supervised and edited the manuscript, YHL generated fund, YJH provided technical comments for the study. All authors read and approved the final version of the manuscript before submission.

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