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Research Article
Biological Activity of Native and Low Molecular Weight Chitosan obtained by Steam Explosion Process

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Abstract

Background and Objective: Low molecular weight chitosan (LWCS) was interestingly used because of it's solubility and has good functional properties like antioxidant and antibacterial activity. This study aimed to evaluate antioxidant and antibacterial activity of chitosan and low molecular weight chitosan. **Materials and Methods:** Low molecular weight chitosan was obtained by physical and chemical hydrolysis using steam explosion process with steam pressure at 6 bar, temperature at 160°C and concentration of phosphotungstic acid at 0.1% w/v. The antioxidant activity was confirmed by radical DPPH scavenging activity, chelating metal ion value, inhibitory lipid peroxidation and antibacterial activity was confirmed by diffusion methods. **Results:** LWCS had antioxidant activity higher than native chitosan on radical scavenging, chelating ion value and inhibition of lipid peroxidation. LWCS had higher inhibitory effect as antibacterial than native chitosan against tested bacteria, there were *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium*, *Streptococcus aureus* and *Bacillus subtilis*. **Conclusion:** It was concluded that LWCS had more powerful antioxidant and antibacterial activity than native chitosan.

Key words: Antibacterial, antioxidant, low molecular weight chitosan, native chitosan, chelating ion, steam explosion process

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nowadays, development research of chitosan (polymeric β-1,4-N-acetylglucosamine) was being interested. Chitosan is a cationic polysaccharide made from alkaline N-deacetylation of chitin[1]. It was naturally abundant, renewable, low production cost, has excellent properties such as biocompatible, biodegradable and non-toxic[2–6]. Chitosan was interesting to explore in many field such as food and nutrition, biotechnology, material science, drugs and pharmaceuticals[3,7–10]. Chitosan have been studied due to its benefits in several health fields. Chitosan can reduce obesity risk, improve intestinal health and improve the lipid profile[11–13]. Chitosan also have several biological activities such as antioxidant, antibacterial and antitumor[10,14–19].

However, the insolubility of chitosan make limited application[20]. Some researchers are interested to increasing the solubility of chitosan through the degradation process to produce low molecular weight chitosan (LWCS). LWCS has high solubility[20–22], antioxidant[16,23–25], antibacterial[20,24,26] and antimutagenetic activity[16]. LWCS provides more accessible amine and hydroxyl groups that act as ligands to chelating pro-oxidant metal catalyst (e.g., Fe^{2+}). The antioxidant activity of low molecular weight chitosan were possible act as radical scavenging, metal chelating, reducing power and reduce lipid oxidation[16,25,27]. LWCS adsorbed effectively in microbial cell and disrupted cell membranes causing severe leakage of cell constituents and ultimately cell death, this mechanism provide as antimicrobial and anti tumor activity[28-30]. LWCS has powerful antioxidant and antibacterial activity but the study is limited. This study was aimed to examine the biological activity of native chitosan and low molecular weight chitosan obtained. In this research, antioxidant and antibacterial activity was analyzed using DPPH radical scavenging, chelating ion, lipid peroxidation inhibitory effect and diffusion methods.

MATERIALS AND METHODS

Preparation of chitosan: Chitosan was obtain from shrimp shells waste according to Sugiyanti *et al.*[31]. The molecular weight (MW) was $55.7x10^4$ Da for native chitosan and 4800 Da for LWCS.

DPPH radical scavenging assay: The antioxidant activities was performed by 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH)-scavenging assay according to Brand-Williams *et al.*[32]. The percentage of DPPH• Radical scavenging activity (RSA) was calculated by equation as follows:

$$\text{Radical scavenging activity}(\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Metal ions chelating activity: The chelating metal ion Fe^{2+} ability of sample was studied due to Kim[33] method. Fe^{2+} chelating activities were calculated using the following equation:

$$\text{Chelation activity}(\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Inhibitory lipid peroxidation method: Inhibitory lipid peroxidation in this research were determination by Ferric Thiocyanate (FTC) methods and Thiobarbituric Acid (TBA) methods. This methods were done according to Kikuzaki and Nakatani[34]. The inhibition of lipid peroxidation by FTC and TBA methods were calculated as follows:

$$\text{Inhibition}(\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where, A_0 is the maximum absorbance of blanko and A_1 is the maximum absorbance of sample.

Antibacterial activity method: Antibacterial activity was obtained by agar well diffusion method according to Shanmugam *et al.*[35] with some modification in the tested bacteria, in this research using five species of bacteria (clinical isolates) (Gram-positive: *Streptococcus aureus*, *Bacillus subtilis*; Gram-negative: *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) obtained from Food and Nutrition Culture Collection (FNCC), Centre for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta, Indonesia.

Minimum Inhibitory Concentrations (MIC): The MIC value of chitosan and LWCS were determined by Kaya *et al.*[26] and Shanmugam *et al.*[35] with slight modification. Growth media in

this method was using nutrient broth (NB). Each sample diluted to its solute, which is sterile distilled water for LWCS and acetic acid 1% (v/v) for chitosan. The dilution was made in seven concentration of each sample, 10; 5; 2.5; 1.25; 0.62; 0.31 and 0.16 mg mL^{-1}, respectively. The MIC value is set as the lowest concentration that is still able to form a clear area around the well.

Statistical analysis: The data are reported as the Mean±standard deviation (SD) in triplicated, analyzed by SPSS version 17.0 (SPSS Inc.). The statistical significant of differences determined by one way ANOVA and continued by Duncan's multiple range test ($p<0.05$).

RESULTS

Scavenging of DPPH radical: The antioxidant activities of chitosan and LWCS samples were measured by DPPH• radical scavenging as shown in Fig. 1. The scavenging activity of chitosan and LWCS at 1, 5 and 10 mg mL^{-1} were 0.94, 7.23, 16.28% for chitosan and 25.69, 36.44 and 53.57% for LWCS, respectively.

Metal ions chelating activity: Data in Fig. 2 showed the chelating metal value of chitosan and LWCS in several concentration. The results showed that chitosan had chelating activity value 5.44, 9.99 and 17.84% at 1, 5 and 10 mg mL^{-1} concentration, respectively. While, LWCS showed the higher chelating value than native chitosan in the same concentration, there were 39.52, 46.34 and 67.44% at 1, 5 and 10 mg mL^{-1}, respectively. The increasing concentration showed the increase chelating value activity both on LWCS and chitosan.

Lipid peroxidation inhibition activity: The lipid peroxidation inhibitory value of native chitosan by FTC method was 5.34, 15.19 and 20.13% in 1, 5 and 10 mg mL^{-1}, respectively. It was lower than the lipid peroxidation inhibitory value of LWCS, there were 29.31, 40.10 and 53.75% in 1, 5 and 10 mg mL^{-1}, respectively as shown in Fig. 3. This research also showed that LWCS had higher lipid peroxidation inhibitory effect by TBA value compared by native chitosan. The TBA value of native chitosan were 1.29, 5.84, 10.44%, in 1, 5 and 10 mg mL^{-1}. While the TBA value of LWCS were 10.52, 41.03 and 65.91% at 1, 5 and 10 mg mL^{-1}, respectively as shown in Fig. 4. Both of chitosan and LWCS showed the increases lipid peroxidation inhibitory effect in the increases concentration.

Antibacterial effect: LWCS had higher inhibitory power than native chitosan sample as shown in Table 1. At 10 mg mL^{-1} concentration, chitosan showed the inhibitory zone 8.50; 12.06; 16.99; 9.11 and 13.33 mm. While the inhibitory zone of

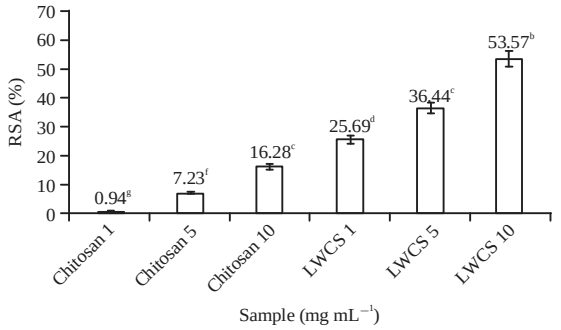

Fig. 1: DPPH radical scavenging activity (RSA%) of BHT, chitosan and LWCS sample

Values are given as the Means of the triplicates measurements, different superscript indicate statistically significant differences ($p<0.05$)

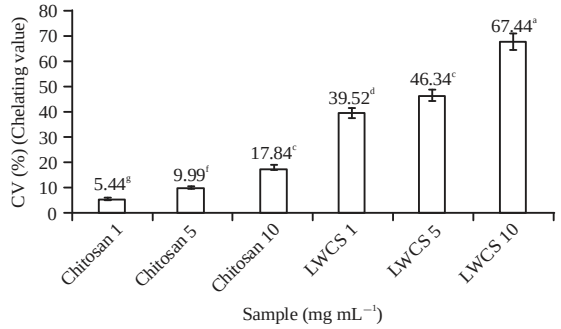

Fig. 2: Metal ion chelating activity of chitosan and LWCS

Values are given as the Means of the triplicates measurements, different superscript indicate statistically significant differences ($p<0.05$)

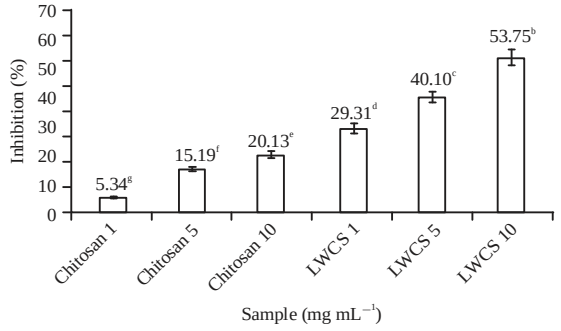

Fig. 3: The percentage inhibition value of chitosan and LWCS samples by FTC methods

Values are given as the Means of the triplicates measurements, different superscript indicate statistically significant differences ($p<0.05$)

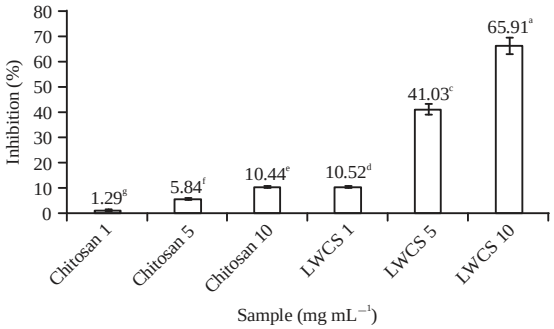

Fig. 4: The percentage inhibition value of chitosan and LWCS samples by TBA value methods
Values are given as the Means of the triplicates measurements, different superscript indicate statistically significant differences ($p<0.05$)

Table 1: Inhibitory effect of chitosan and LWCS against tested bacterial

	Diameter (mm)	
Tested bacteria	CH	LWCS
E. coli	8.50^{d}	18.50^{e}
P. aeruginosa	12.06^{c}	15.50^{d}
S. typhimurium	16.99^{c}	18.00^{d}
S. aureus	9.11^{b}	11.11^{c}
B. Subtilis	13.33^{c}	27.50^{d}

Values are given as the mean of the triplicate measurements. Different letters within the same row indicate statistically significant differences ($p<0.05$), LWCS: Low molecular weight chitosan and CH: Chitosan

Table 2: Minimum inhibitory concentration (MIC) percentage of chitosan and LWCS against tested the bacterial

		MIC (mg mL^{-1})	
Groups	Tested bacteria	LWCS	CH
Gram positive	*B. subtilis*	0.16	0.16
	S. aeruginosa	0.16	0.16
Gram negative	*E. coli*	0.16	0.16
	P. aeruginosa	0.16	2.5
	S. typhimurium	0.16	0.16

Values are given as the mean of the triplicate measurements., LWCS: Low molecular weight chitosan and CH: Chitosan

LWCS were 18.50, 15.50, 18.00; 11.11 and 27.5 mm against *E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *B. subtilis*, respectively. The Minimum Inhibitory Concentrations (MIC) of chitosan were 0.16 mg mL^{-1} in the tested bacteria except *P. aeruginosa* (2.5 mg mL^{-1}), while LWCS had the MIC value at 0.16 mg mL^{-1} in the five tested bacteria as shown in Table 2.

DISCUSSION

In this research, the scavenging activity of DPPH radical in LWCS was higher than native chitosan. The scavenging activity of LWCS derived by ammonium group in the chitosan unit, which can react with •OH radical through its H^+ ions. Moreover, the •OH radical can react with the residual free amino groups NH_2 to form stable macromolecule radicals. The NH_2 groups can form ammonium groups (NH_{3+}) by absorbing hydro ion from the solution then reacting with OH^+ via addition reaction[1].

The research by Anraku *et al.*[23] also shown that low molecular weight chitosan had higher antioxidant activity than the high molecular weight chitosan. Chang *et al.*[16] also had degraded chitosan from the molecular weight of 300 kDa to produce six degraded chitosan with molecular weights of 156.0, 72.1, 29.2, 7.1, 3.3 and 2.2 kDa. The result was chitosan with the lowest molecular weight significantly increased the antioxidant scavenging activity against 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical. Chien *et al.*[36] also produced low molecular weight chitosan (12 kDa) that exhibited stronger scavenging activity toward DPPH radicals and higher ferrous ion chelating activity compared to medium and high molecular weight chitosan (95 and 318 kDa, respectively). Yen *et al.*[14] reported that chitosan had DPPH radical scavenging activity about 28.4-52.3% at 10 mg mL^{-1} concentration.

In this research LWCS presented higher chelating activity than native chitosan. That because LWCS provide more amine and hydroxyl groups. The amine group from chitosan structure can provide multiple binding sites to form complexes[37] with Fe^{2+}. Kong *et al.*[38] had reported that chitosan have high ability as chelating agent on chelating metal ion Cu^{2+} and Ni^{2+}. Chien *et al.*[36] also reported that LWCS exhibited an excellent ferrous ion-chelating capacity of approximately 78.8% at a concentration of 0.4 mg mL^{-1}, larger than HWCS and MWCS .Jung and Zhao[39] showed that LWCS with molecular weight 4-5 kDa has higher chelating metal value (22.04%) than the high molecular weight that are 22-300 kDa.

In this research LWCS have more powerful inhibition of lipid peroxidation compared by native chitosan through FTC and TBA methods. LWCS exhibit greater capacity in scavenging free radicals and chelating ion. Its antioxidant mechanism could be explained by the primary amino groups of chitosan forming a stable fluorosphere with volatiles aldehydes such as malondialdehyde, which derived from lipid oxidations. Low molecular weight chitosan have higher amino groups than the high molecular weight chitosan. Kim and Thomas[7] studied the oxidation inhibitory activity of chitosan depend on its molecular weight and concentrations. Chitosan with low molecular weight (30 kDa) shows the high

antioxidant activity indicated with its lower TBARS value compared by the higher molecular weight (90 and 120 kDa, respectively), although in low concentration. Lee and Lee[37] also reported that the amine group of chitosan structure can provide multiple binding sites to form complexes with Fe^{2+}. Matsugo *et al.*[40] reported that water-soluble chitosan effectively inhibit TBARS (thiobarbituric acid reactive substances) formation in t-butylhydroperoxide and benzoyl peroxide induced lipid peroxidation.

Antibacterial effect of LWCS in this research greater than chitosan. This is because LWCS has more cationic sides than chitosan oligomers. Thatte[41] also reported that high molecular weight chitosan (greater than 500 kDa) has lees effective antibacterial activity than the lower molecular weight chitosan. This was related to the large viscosity of chitosan in high molecular weight, so chitosan is difficult to diffuse. No *et al.*[42] studied there are a tendency that the antibacterial activity can increase by decreasing the molecular weight of chitosan only applies in gram negative bacteria and it does not apply to Gram-positive. The study also mentions that antibacterial activity against *E. coli* (Gram-negative) will increase when the chitosan molecular weight decreases. This is caused by chitosan with a small molecular weight easier to enter the cell and interfere with cell metabolism.

The minimum inhibitory concentration (MIC) of chitosan and LWCS ranging from 0.16-2.5 mg mL^{-1} depend on the isolate of tested bacteria. In this research LWCS show the lower MIC especially in *P. aeruginosa*. Bacteria Gram-positive does not have an outer membrane so it easily facilitate antibacterial compounds to find its targets[43]. No *et al.*[42] found that chitosan have greater antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. This is caused by the simple cell wall structure of gram positive bacteria, it was single layered with low lipid content (1-4%) makes it easier for bioactive components to enter the cell. The cell wall structure of gram negative bacteria was more complex, there are three layered consists of an outer layer of lipoprotein, a middle layer of lipopolysaccharide which acts as a barrier the entries of bioactive components such as antibacterial and an inner layer of peptidoglycan with high lipid content (11-12%).

CONCLUSION

LWCS was produced by hydrolysis through steam explosion process catalyzed by phosphotungstic acid. The process was breakdown the chitosan molecular binding and improve its biological value, such as antioxidant and antibacterial. The results showed enhanced antioxidant and antibacterial activities of LWCS. LWCS shows higher radical scavenging, chelating ion activity and reducing lipid oxidation than chitosan originally. LWCS also showed higher antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Streptococcus aureus* and *Bacillus subtilis*. The antioxidant and antibacterial activity increases with the increase concentration.

SIGNIFICANCE STATEMENT

This study discover the low molecular weight chitosan have more antioxidant and antibacterial activity that can be beneficial for development of food and pharmaceutical industry applications. This study will help the researcher to uncover the critical areas of low molecular weight chitosan that many researchers were not able to explore. Thus a new theory on antioxidant and antibacterial activity of low molecular weight chitosan may be arrived at.

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