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Stability and antibacterial activity of chitosan solutions affected by storage temperature and time

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Abstract

Stability of chitosan (M_w of 2025 and 1110 kDa) solutions and their antibacterial activity against gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and gram-negative (*Salmonella enteritidis* and *Escherichia coli*) bacteria were investigated at 4 and 25 °C after 15-week storage. Viscosity of chitosan solutions (1% (w/v) in 1% (v/v) acetic and/or lactic acid) decreased with increased storage time and temperature. After 15-week storage, the decrease in viscosity ranged from 44 to 48% and 81 to 90% of the initial viscosity value, respectively, at 4 and 25 °C. Antibacterial activity of chitosan solutions (1% (w/v) in 1% (v/v) acetic acid) before and after 15-week storage differed depending on the molecular weight of chitosans, the storage temperature, and the bacteria. In general, chitosan solutions before storage showed higher antibacterial activity than those after 15-week storage. Chitosan solutions stored at 25 °C possessed similar or weaker antibacterial activity compared with those at 4 °C.

Keywords: Chitosan solution; Viscosity; Storage stability; Antibacterial activity

1. Introduction

During the past several decades, chitosan has been receiving increased attention for its commercial applications in biomedical, food, and chemical industries (Sandford & Hutchings, 1987). Chitosan is now widely produced commercially from crab and shrimp shell wastes with a molecular weight (M_w) usually in the range of 10^5–10^6 Da and viscosity reaching or exceeding 1500 cP (No & Meyers, 1995; Sugano, Yoshida, Hashimoto, Enomoto, & Hirano, 1992; Van Ornum, 1992).

Recently, chitosan and its oligomers have attracted notable interest due to their biological activities, that is, antimicrobial (Kendra & Hadwiger, 1984; No, Park, Lee, & Meyers, 2002; Sudarshan, Hoover, & Knorr, 1992), antitumor (Suzuki et al., 1986; Tokoro et al., 1988), and hypocholesterolemic functions (Sugano et al., 1992). Recent studies on antibacterial activity of chitosan and its oligomers have revealed that chitosan is more effective in inhibiting growth of bacteria than chitosan oligomers (Jeon, Park, & Kim, 2001; No et al., 2002; Uchida, Izume, & Ohtakara, 1989). Furthermore, the antibacterial effect of chitosan and its oligomers is reported to be dependent on its molecular weight or viscosity (Cho, Chang, Lee, Jeong, & Lee, 1998; Jeon et al., 2001; No et al., 2002; Uchida et al., 1989). For example, Uchida et al. (1989) found that chitosan hydrolysate, which was slightly hydrolyzed with chitosanase, was more effective as an antibacterial agent than was native chitosan and its oligomers. Cho et al. (1998) reported that the antibacterial activity of chitosan against *Escherichia coli* and *Bacillus* sp. increased with decreased viscosity from 1000 to 10 cP.

Chitosan has been approved for use as a food additive in Japan and Korea since 1983 and 1995, respectively (KFDA, 1995; Weiner, 1992), and thus considerable attention has been given on the use of chitosan as an antibacterial agent to improve shelf-life of foods. In general, chitosan is applied to food in a solution state after dissolving in acetic or lactic acid. Preparation of chitosan solution, especially from high M_w chitosans, requires several hours due to its high viscosity. For commercial applications, it would be practical to prepare chitosan solutions in bulk and to store them for further use. During storage, specific characteristics of chitosan, that is, viscosity or molecular weight, may, however, be altered. Thus, change in viscosity of chitosan solution must be monitored since it may influence other functional properties of the chitosan solution.

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The objectives of the present research were to evaluate stability (via viscosity) of chitosan solutions under 4 and 25 °C storage conditions for 15 weeks and to compare antibacterial activities of chitosan solutions before and after 15-week storage against gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and gram-negative (*Salmonella enteritidis* and *E. coli*) bacteria. For this, two chitosans (M_w of 2025 and 1110 kDa) and two organic acids (acetic and lactic) as a chitosan solvent were used. Only chitosan solutions prepared with acetic acid were used for the antibacterial activity study.

2. Materials and methods

2.1. Materials

Two commercial chitosans with a molecular weight (M_w) of 2025 and 1110 kDa were obtained from Kitto Life (Seoul, Korea). These chitosans were acid soluble, white-colored powder prepared from crab leg shell.

2.2. Preparation of chitosan solutions and storage test

Four chitosan solutions (2 $M_w \times 2$ organic acids) were prepared. Each solution was prepared by dissolving chitosan in 1% organic acid (acetic or lactic acid) at a 1% concentration, and 220 mL of the solution was then placed in a 250 mL screw-capped brown glass bottle and stored in an incubator (DuRi Co., model DF-95B, Gyeonggi-do, Korea) at 4 and/or 25 °C for 15 weeks. Samples (15 mL) were taken at 1 or 2-week intervals for determination of viscosity. Triplicate experiments were conducted.

2.3. Measurement of viscosity and pH

Viscosity of chitosan solutions was determined with a Brookfield viscometer, model LVDV-II+ (Brookfield Engineering Labs., Stoughton, MA). Measurements were made using a small sample adapter at 5 rpm (for 2025 M_w) or 20 rpm (for 1110 M_w) in the solution (8 mL) at 25 ± 0.3 °C, and reported in centipoise (cP) units. pH was measured with a pH meter (Mettler Delta 320, Halstead, UK).

2.4. Microorganisms and assays for antibacterial activity of chitosans

Four bacteria were tested for antibacterial activity of chitosan solutions (1% (w/v) in 1% (v/v) acetic acid) before and after 15-week storage. These included two gram-positive bacteria (*L. monocytogenes* KCCM 12255 and *S. aureus* KCCM 12255) and two gram-negative bacteria (*S. enteritidis* KCTC 12400 and *E. coli* ATCC 11775).

Antibacterial activity of chitosan was assayed as follows: each chitosan solution was added to Mueller Hinton broth (MHB, Merck, Darmstadt, Germany) to give a final chitosan concentration of 0.05%. The pH of the broth was adjusted to 5.9 with 1 N HCl and/or 1 N NaOH. The 0.05 mL of each bacterium was inoculated into 10 mL of MBH containing 0.05% chitosan and incubated at 37 °C for 24 h with constant shaking at 100 rpm. Viable cell counts (log CFU/mL) were made on tryptic soy agar (Difco, Detroit, MI) by a pour plate method after incubation at 37 °C for 48 h.

2.5. Statistical analysis

All experiments were carried out in triplicate, and average values were reported. Means of the main effects were separated by Duncan's multiple-range test using the SPSS (Statistical Package for Social Sciences, SPSS, Inc., Chicago, IL) software package.

3. Results and discussion

3.1. Storage stability of chitosan solution

Fig. 1 shows changes in viscosity of chitosan (2025 kDa) solution over storage time at 4 and 25 °C. The viscosity decreased with increased storage time, as also observed by previous workers (Moorjani, Khasim, Rajalakshmi, Puttarajappa, & Amla, 1978; Sophanodora & Hutadilok, 1995; Yusoff, Alimuniar, & Agusnar, 1995). However, the decreasing rate of viscosity was more noticeable at higher temperature in our study, regardless of the acids used. At 25 °C, the viscosity decreased rapidly by 46% (acetic acid as a solvent) and 43% (lactic acid) of the initial value (563 and 581 cP, respectively) after 1 week of storage. The viscosity then decreased gradually by 81% (acetic acid) and 82% (lactic acid) of the initial value after 15 weeks of storage. On the other hand, the viscosity at 4 °C decreased gradually by 46% (acetic acid) and 47% (lactic acid) of the initial value after 15 weeks of storage. The trend of the decreasing rate of viscosity was comparable between acetic and lactic acids. The viscosity decrease observed over time

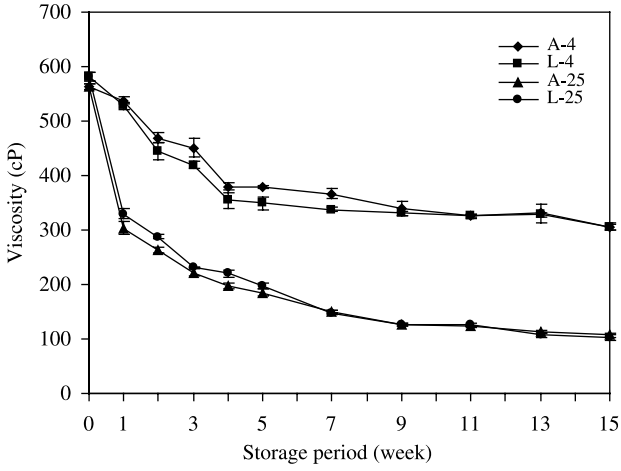

Fig. 1. Changes in viscosity of chitosan (2025 kDa) solution during 15-week storage at 4 and 25 °C. A-4 and L-4, chitosan was dissolved in acetic and lactic acid, respectively, and stored at 4 °C; A-25 and L-25, chitosan was dissolved in acetic and lactic acid, respectively, and stored at 25 °C.

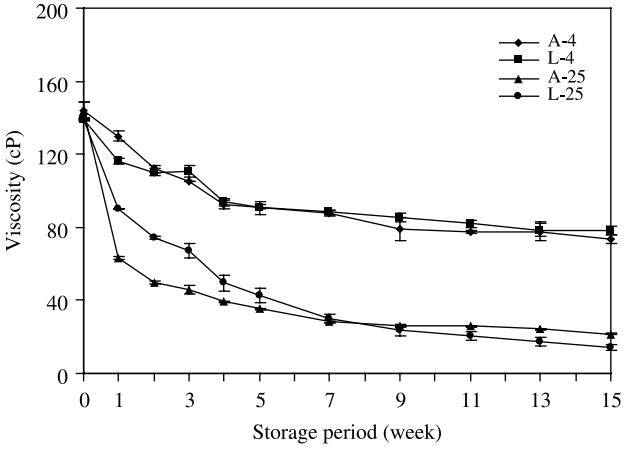

Fig. 2. Changes in viscosity of chitosan (1110 kDa) solution during 15-week storage at 4 and 25 °C. A-4 and L-4, chitosan was dissolved in acetic and lactic acid, respectively, and stored at 4 °C; A-25 and L-25, chitosan was dissolved in acetic and lactic acid, respectively, and stored at 25 °C.

(Fig. 1) is probably due to partial degradation of chitosan by organic acid solutions (Jun, Kim, No, & Meyers, 1994; Yusoff et al., 1995).

Changes in viscosity of chitosan (1110 kDa) solution over storage time at 4 and 25 °C (Fig. 2) showed a similar trend as observed in Fig. 1 for the chitosan (2025 kDa) solution. However, the viscosity at 25 °C decreased more rapidly with acetic acid (by 55% of the initial value) than lactic acid (by 35%) after the first week of storage. At 25 °C, the viscosity decreased by 85% (acetic acid) and 90% (lactic acid) of the initial value (141 and 139 cP, respectively) while at 4 °C, the viscosity decreased by 48% (acetic acid) and 44% (lactic acid) of the initial value after 15 weeks of storage.

Sophanodora and Hutadilok (1995) reported that the viscosity of chitosan solution (1% (w/v) in 1% (v/v) acetic acid) rapidly decreased from 2500 to 1500 cP within 24 h of the experiment at room temperature. At the 10th day, the viscosity decreased to one-fifth of the original. Similarly, Moorjani et al. (1978) also observed a rapid decrease in viscosity of chitosan solution (1% (w/v) in 1% (v/v) acetic acid) from about 4000 to 1800 cP in one day under storage conditions of room temperature. However, such drastic decrease in viscosity was not observed in the present study and neither by No, Kim, Kim, Kim, and Meyers (1999). In this present study, the viscosity of chitosan solution (1% (w/v) in 1% (v/v) acetic acid) decreased by 46% (chitosan with 2025 kDa) and 55% (1110 kDa) of the initial value after 1 week of storage at 25 °C. No et al. (1999) also observed decrease in viscosity of chitosan (1670 kDa) solution (1% (w/v) in 1% (v/v) acetic acid) by about 30% after 1 week of storage at 23 °C. Differences in viscosity decrease among these studies may be due to differences in chitosan product used and storage conditions.

Acetic acid is a commonly used organic acid for solubilizing chitosan (No & Meyers, 1995) and promotes acidic hydrolysis more effectively than does lactic acid (Muzzarelli, Tomasetti, & Ilari, 1994; No, Nah, & Meyers, 2003). However, such a trend was not observed in the present study, except for the first 3 weeks of storage for chitosan (1110 kDa) solution at 25 °C (Fig. 2). The pH values of chitosan solution prepared with lactic acid were slightly lower than that of chitosan solution prepared with acetic acid. The pH values of chitosan solutions stored for 15 weeks was slightly higher than that of chitosan solutions before storage (Table 1).

Table 1
pH of chitosan solutions (1% (w/v) in 1% (v/v) organic acid) before and after 15 weeks of storage at 4 and 25 °C

Chitosan solvent	M_w (kDa) of chitosan	pH before storage	pH after 15 weeks of storage	
			4 °C	25 °C
Acetic acid	2025	3.81	4.09	3.91
	1110	3.93	4.08	3.98
Lactic acid	2025	3.28	3.52	3.44
	1110	3.33	3.53	3.42

Data from Figs. 1 and 2 indicate that under the present experimental conditions, the viscosity of chitosan solution decreases by 44–48% of the initial value at 4 °C and 81–90% at 25 °C after 15 weeks of storage. Therefore, it is anticipated that functional properties of chitosan solution before and after 15 weeks of storage may differ since the physicochemical characteristics of chitosan influence its final functional properties (No & Meyers, 1995).

3.2. Antibacterial activity of chitosan solution

Chitosan (2025 and 1110 kDa) solutions prepared with acetic acid were used for the antibacterial activity study. Table 2 shows antibacterial activity of chitosan solutions (1% (w/v) in 1% (v/v) acetic acid) before and after 15 weeks of storage at 4 and 25 °C. Overall, chitosans markedly inhibited growth of most bacteria tested; however, the inhibitory effects differed depending on the molecular weight of chitosans, the storage conditions of chitosan

Table 2
Antibacterial activity (log CFU/mL)[a] of chitosan solutions (1% (w/v) in 1% (v/v) acetic acid) before and after 15 weeks of storage at 4 and 25 °C

Bacteria	M_w (kDa) of chitosan	Initial	Control	Before storage	After 15 weeks of storage	
					4 °C	25 °C
L. monocytogenes	2025	6.29	8.45	ND[b]	ND	ND
	1110			ND	2.73	1.30
S. aureus	2025	7.58	8.65	2.22a	3.00b	3.60c
	1110			0.60a	2.47b	2.48b
S. enteritidis	2025	6.29	8.67	4.00b	4.08b	3.67a
	1110			0.48a	2.30b	4.09c
E. coli	2025	6.98	8.80	5.44a	6.29b	6.40b
	1110			5.93a	6.13b	6.30c

Means with different lowercase letters (a–c) within each row indicate significant difference ($P < 0.05$).

[a] Viable cells after incubation without (control) and with 0.05% chitosan for 24 h at 37 °C.

[b] ND, not detected.

solution, and the tested bacteria. Chitosan generally showed stronger bactericidal effects for gram-positive bacteria than for gram-negative bacteria in the presence of 0.05% chitosan, as also observed by Jeon et al. (2001) and No et al. (2002).

In comparison of chitosan solutions before and after 15 week-storage, the former generally showed higher antibacterial activity than the latter, with some exceptions. Growth of *L. monocytogenes* was completely suppressed by chitosan (2025 kDa), irrespective of storage time and temperature. Chitosan (2025 kDa) solution stored at 25 °C showed higher antibacterial activity against *S. enteritidis* than chitosan solution before storage, although the difference in growth reduction was less than 1 log.

Results from Figs. 1 and 2 indicated that decrease in viscosity was more noticeable at higher storage temperature. This may also suggest that antibacterial activity of chitosan solution may differ with storage temperature of 4 and 25 °C. Table 2 revealed that the inhibitory effects differed with the molecular weight of chitosan, the storage temperature of chitosan solution, and the type of bacterium. Most chitosan solutions exhibited comparable or weaker antibacterial activity at higher storage temperature. However, antibacterial activity of chitosan (1110 kDa) against *L. monocytogenes* and of chitosan (2025 kDa) against *S. enteritidis* seemed to increase at higher storage temperature.

Uchida et al. (1989) observed that chitosan hydrolysate, slightly hydrolyzed with chitosanase, was more active as an antibacterial agent than native chitosan and chitosan oligomers. Cho et al. (1998) reported that the antibacterial activity of chitosan against *E. coli* increased with decreased chitosan viscosity using an enzymatic hydrolysis. In the present study, the viscosity of chitosan solution decreased with increasing storage time and temperature. However, noticeable increases in the antibacterial inhibitory effects of chitosan with decreased viscosity were not observed at 0.05% chitosan concentration. Allan et al. (1984) reported that *S. aureus* was negligibly inhibited at a chitosan concentration of 0.1% and that *E. coli* was only slightly affected at a level as high as 1.0%. However, in the present study, *S. aureus* was effectively inhibited by 5–6 log cycles and *E. coli* was inhibited by 2–3 log cycles even by treatment with stored chitosan solutions at a 0.05% concentration.

4. Conclusion

This study demonstrated that viscosity of chitosan solution decreases with increased storage time and temperature. The viscosity of chitosan solution decreased by 44–48% of the initial value at 4 °C and 81–90% at 25 °C after 15 weeks of storage. These results document the instability of chitosan solution under storage conditions of 25 °C.

Change in viscosity of chitosan solution during storage may influence its functional properties. Results on antibacterial activity of chitosan solutions before and after storage for 15 weeks at 4 and 25 °C indicated that the inhibitory effects differed with the molecular weight of chitosan, the storage time and temperature of chitosan solution, and the type of bacterium. In general, chitosan solution before storage showed higher antibacterial activity than chitosan solution after 15-week storage. Chitosan solutions stored at 25 °C possessed similar or weaker antibacterial activity compared with chitosan solution stored at 4 °C. Thus, it is recommended that chitosan solutions be freshly prepared if intended for use as an antibacterial agent for improved shelf-life of foods. However, it is worthwhile to note that chitosan (2025 kDa) possessed similar bactericidal effect against *L. monocytogenes*, irrespective of storage time and temperature under the present experimental conditions. Significant implication is seen for use of chitosan with high antibacterial activity as a preservative to prevent health hazards associated with consumption of foods contaminated with *L. monocytogenes*.

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