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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 2122–2126

Cytotoxic activities of water-soluble chitosan derivatives with different degree of deacetylation

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Received 26 September 2005; revised 30 December 2005; accepted 17 January 2006
Available online 3 February 2006

Abstract—Chitosans with different degree of deacetylation (DD) (90% and 50% deacetylated chitosan) were prepared by N-deacetylation followed by grafted onto chitosan to form water-soluble aminoethyl-chitosan (AE-chitosan), and dimetylaminoethyl-chitosan (DMAE-chitosan), diethylaminoethyl-chitosan (DEAE-chitosan). In the present study, cytotoxic activities of the chitosan derivatives were evaluated using three tumor cell lines and two normal cell lines, and structure–activity relationship was suggested. The cytotoxic activity was dependent on their DD and substituted group.

Chitosan, which is a copolymer consisting of β-(1 → 4)-2-acetamido-D-glucose and β-(1 → 4)-2-amino-D-glucose units, is derived from chitin by deacetylation in the presence of alkali. Due to their biocompatibility and less toxic nature, it has been developed as new physiologically bioactive materials since they possess various biological activities such as antibacterial activity,[1–4] hypocholesterolemic activity,[5] antitumor activity,[6] immuno-stimulating effect,[7] antioxidant activity,[8] and antihypertensive activity.[9] Although chitosan has very strong functional properties in many areas, the water-insoluble property of chitosan is disadvantageous for its wide application. In the research field of chitosan, therefore, chitosan derivatives with water-soluble and functional property have been developing for pharmaceutical and new drug candidate. Cytotoxic drugs continue to play a major role in cancer therapy. However, cytotoxic drugs offer produce side effects, especially through the destruction of lymphoid and bone marrow cells.[10] Therefore, strategic improvements in cancer therapy are needed to improve efficacy while decreasing side effects. Several literatures exist regarding the effects of antitumor activities of chitosan and its oligomers.[11,12] However, cytotoxic activity of chitosan derivatives for anticancer agents is little information available until now. Most biological activities of chitosan are attributed to their free amino group. Therefore, in the present study, we modified C_6 position of chitosan with different degree of deacetylation and investigated cytotoxic activities of water-soluble chitosan derivatives.

Chitosans with different degree of deacetylation were prepared as described in a previous report, and the degree of deacetylation designated as 90% and 50%.[13] Water-soluble AE-chitosan, DMAE-chitosan, and DEAE-chitosan with 0.63–0.92 degree of substitution were designated as AE-chitosan (90%), DMAE-chitosan (90%), and DEAE-chitosan (90%) prepared from 90% deacetylated chitosan, and AE-chitosan (50%), DMAE-chitosan (50%), and DEAE-chitosan (50%) prepared from 50% deacetylated chitosan (Scheme 1).[14]

HeLa (cervix cancer), HT1080 (human fibrosarcoma), A549 (lung cancer), MRC-5 (human lung fibroblast), and ECV304 (human endothelial) cell lines were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, NY, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS), and maintained at 37 °C under a humidified atmosphere with 5% CO_2.

Cells were cultured for 1 day in 96-well plates at the density of 20,000 cells/well in DMEM supplemented with 10% FBS. The next day, various concentrations of the

Keywords: Water-soluble; Chitosan derivatives; Cytotoxic activity.
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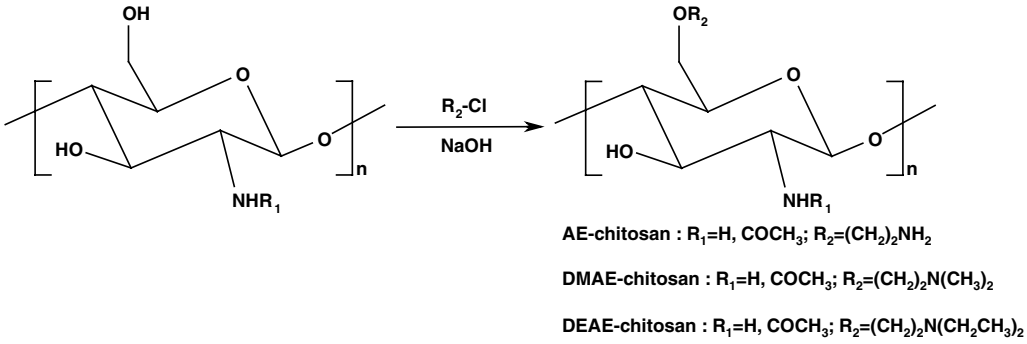
doi:10.1016/j.bmcl.2006.01.060



Scheme 1. Synthesis and chemical structure of chitosan derivatives.

chitosan derivatives were treated, and controls were carried out with cells treated with an equivalent volume of serum-free medium without derivatives. Cell viability was identified using the MTT assay on the second day of culture.

The results presented here suggest that chemically modified chitosan derivatives showed to significantly suppress the growth toward HeLa, A549, and HT1080 tumor cells. Figure 1A exhibits that 90% deacetylated chitosan derivatives exhibited dose-dependent inhibitory effects on the proliferation of the HeLa cell line. Among the derivatives, the DEAE-chitosan (90%) was highly tumor suppressive and the AE-chitosan (90%) showed moderate activity. At the concentration of 62.5 μg/mL, the DEAE-chitosan (90%) killed around 83% of HeLa cells, and the IC_{50} value of DEAE-chitosan (90%) was 16 μg/mL. DEAE-chitosan (50%) also exerted dose-dependent inhibitory effect on the proliferation (Fig. 1B). However, cytotoxic activity of DEAE-chitosan (50%) was lower than that of DEAE-chitosan (90%). The IC_{50} value of DEAE-chitosan (50%) was 26 μg/mL. In the A549 cell line, chitosan derivatives also suppressed cell proliferation, DEAE-chitosan derivatives showing the highest antiproliferation activity (Fig. 2). The IC_{50} values of DEAE-chitosan (90%) and DEAE-chitosan (50%) were 51 μg/mL and 93 μg/mL. As shown in Figure 3A, DEAE-chitosan (90%) exhibited inhibition of proliferation on HT1080 cell, and an IC_{50} value was 63 μg/mL. However, DEAE-chitosan (50%) showed a low cytotoxic activity (Fig. 3B). In cytotoxic activity, chitosan does not have the potential to suppress the growth of tumor cell line (data not shown). When comparing the IC_{50} values of reported chitosan derivatives and other polysaccharides (20–2500 μg/mL), we can suggest that DEAE-chitosan (90%) has similar cytotoxic effects as reported chitosan derivatives, and much higher than values reported for other polysaccharides ($IC_{50} \sim$ 2500 μg/mL).[15,16]

According to the results, the cytotoxic activity of chitosan derivatives was in the order of DEAE-chitosan > AE-chitosan > DMAE-chitosan > chitosan, and activity was dose-dependent. That is, the cytotoxic activity increased with increasing hydrophobicity, indicating that there is a hydrophobic interaction between DEAE group and the tumor cell surface. In spite of low hydrophobicity, however, AE-chitosan exhibited

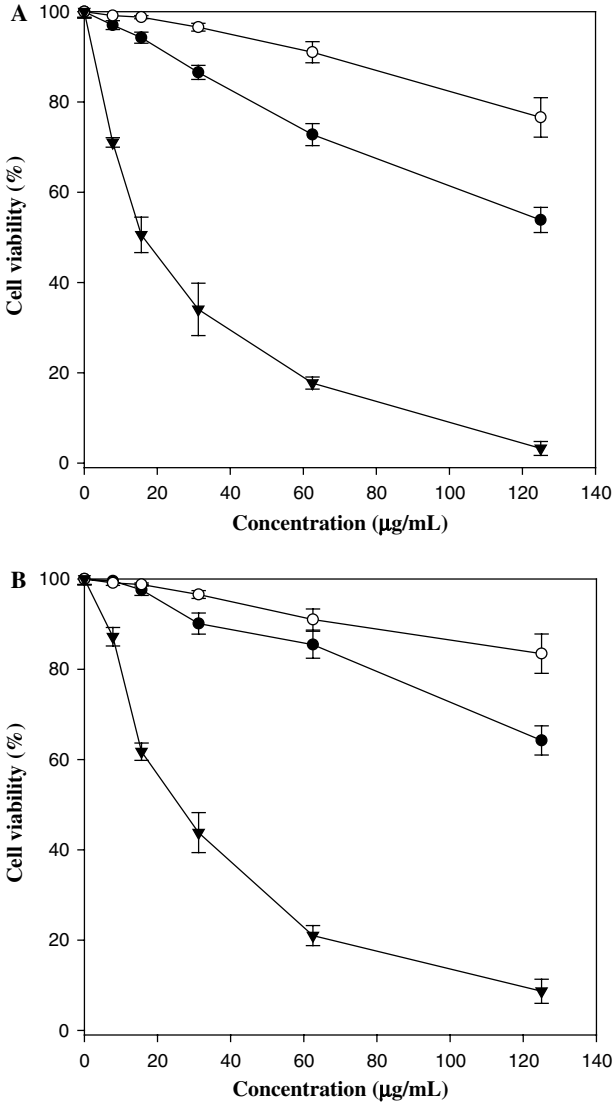


Figure 1. Cytotoxic activities of chitosan derivatives against HeLa cell line. (A) 90% deacetylated chitosan derivatives; (B) 50% deacetylated chitosan derivatives. AE-chitosan, (-●-); DMAE-chitosan, (-○-); and DEAE-chitosan, (-▼-). Values represent means ± SE (n = 3).

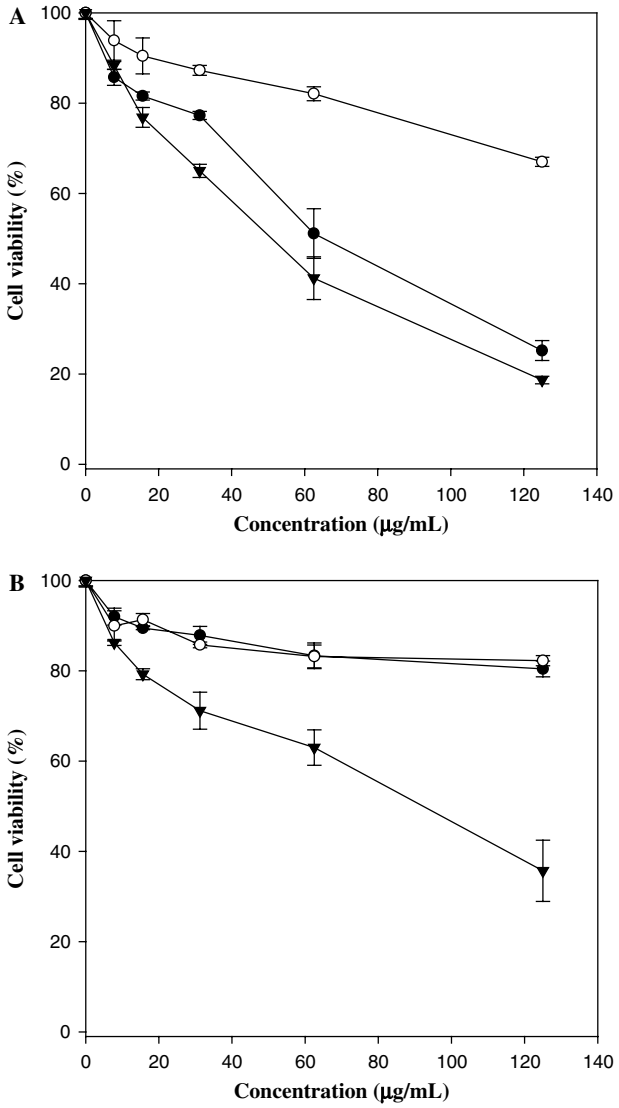


Figure 2. Cytotoxic activities of chitosan derivatives against A549 cell line. (A) 90% deacetylated chitosan derivatives, (B) 50% deacetylated chitosan derivatives. AE-chitosan, (-●-); DMAE-chitosan, (-○-); and DEAE-chitosan, (-▼-). Values represent means ± SE (n = 3).

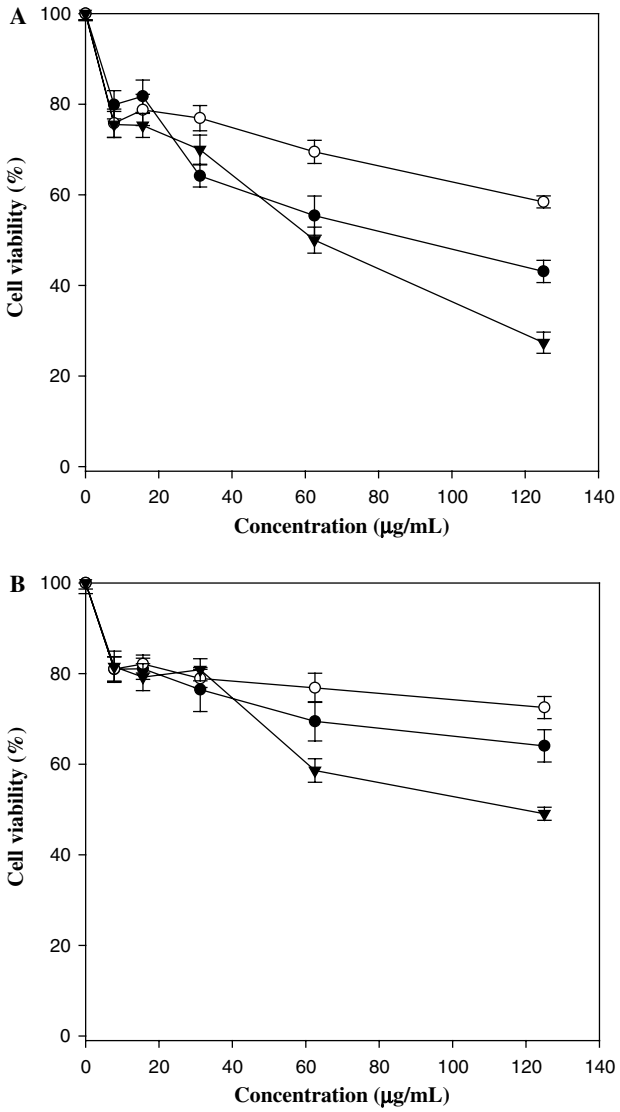


Figure 3. Cytotoxic activities of chitosan derivatives against HT1080 cell line. (A) 90% deacetylated chitosan derivatives; (B) 50% deacetylated chitosan derivatives. AE-chitosan, (-●-); DMAE-chitosan, (-○-); and DEAE-chitosan, (-▼-). Values represent means ± SE (n = 3).

higher cytotoxic activity than DMAE-chitosan. The amino group in chitosan has a pK_a value of ~6.5, thus, chitosan is positively charged. That cytotoxic result is caused by increased cationic charge of chitosan derivative due to free amino group, indicating that there is an electrostatic interaction between AE-chitosan and the negatively charged functional residues on the tumor cell surface such as mucosal membranes. Moreover, all chitosan derivatives derived from 90% deacetylated chtiosan revealed excellent cytotoxic activity than that of chitosan derivatives derived from 50% deacetylated chtiosan. These results also supported that the cytotoxic activity of chitosan derivatives was dependent on their cationic character. Major factor affecting the cytotoxic activity of the chitosan derivatives in this study is not only hydrophobicity according to the results of DEAE-chitosan and AE-chitosan but cationic charges compared with DEAE-chitosan (90%) and DEAE-chitosan (50%). However, several literatures reported on the importance of cationic character related to cytotoxic effects on tumor cell line. Chitosan nanoparticles and copper-loaded chitosan nanoparticles revealed cytotoxic activities against various tumor cell lines. They have different zeta potential that is surface charge, copper-loaded chitosan nanoparticles with high zeta potential exhibited excellent cytotoxic activity than chitosan nanoparticles.[17] In addition, nanoparticle size was also affecting its cytotoxic activity. As a kind of cationic polymer, the surface charge of chitosan derivatives is the major factor affecting its cytotoxic activity due to the electrostatic interaction between the negatively charged groups of the tumor cells and the positively charged amino groups of the chitosan.[10] In summary, increasing of hydrophobic moiety and cationic character is important factor to develop anticancer drugs

using chitosan. Additionally, molecular weight of chitosan should be considered.

We also evaluated cytotoxic effects of chitosan derivatives using two human normal cell lines as MRC-5 and ECV304. The results showed that chitosan derivatives exhibited less cytotoxic effects than those of tumor cell lines (Figs. 4 and 5). It means that the cytotoxicities of chitosan derivatives used in this study were specific to tumor cell lines. Table 1 shows IC_{50} values of chitosan derivatives against different cell lines. DEAE-chitosan (90%) has the highest potential to suppress the growth of tumor cells, and the potential is dependent on their tumor cell lines. Therefore, this result leads to the application of DEAE-chitosan (90%) in a further study involving a broad spectrum of malignant tumors, and detailed mechanism of DEAE-chitosan (90%) is now under investigation.

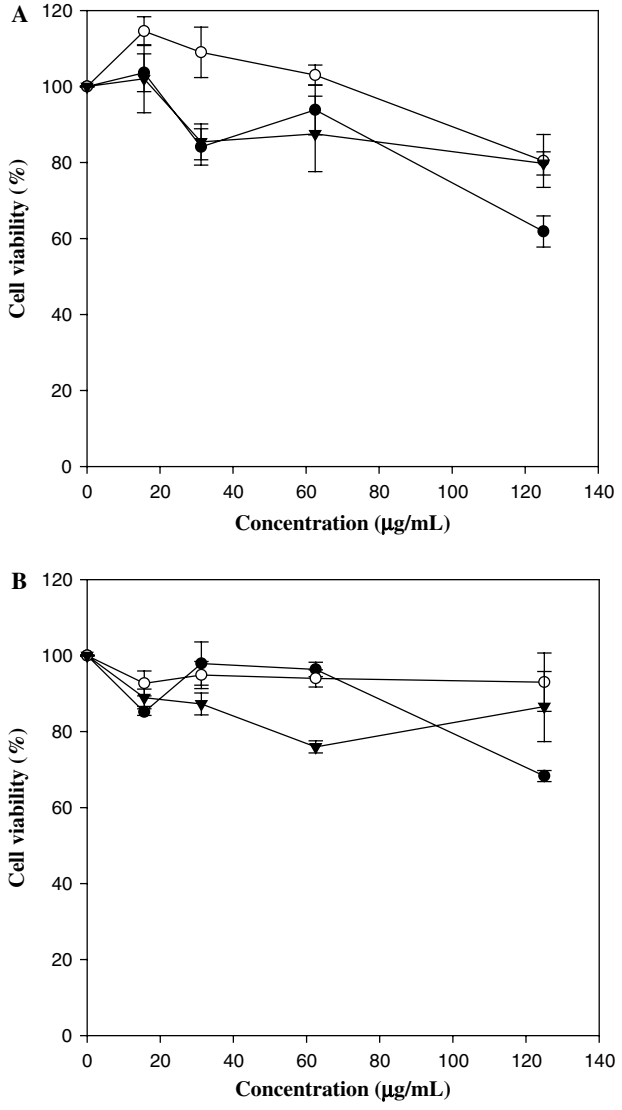


Figure 4. Cytotoxic activities of chitosan derivatives against MRC-5 cell line. (A) 90% deacetylated chitosan derivatives; (B) 50% deacetylated chitosan derivatives. AE-chitosan, (-●-); DMAE-chitosan, (-○-); and DEAE-chitosan, (-▼-). Values represent means ± SE (n = 3).

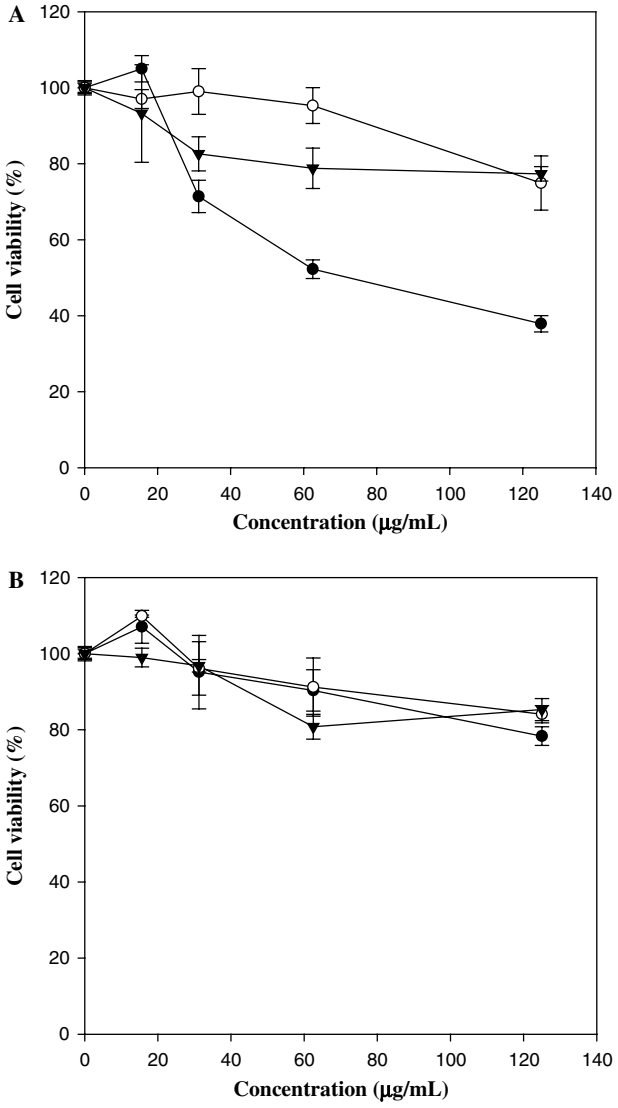


Figure 5. Cytotoxic activities of chitosan derivatives against ECV304 cell line. (A) 90% deacetylated chitosan derivatives; (B) 50% deacetylated chitosan derivatives. AE-chitosan, (-●-); DMAE-chitosan, (-○-); and DEAE-chitosan, (-▼-). Values represent means ± SE (n = 3).

Table 1. Cytotoxic activities of DEAE-chitosan derivatives against different cell lines

Panel of cell lines	Cell line	Compound/cytotoxicity (IC_{50}, μg/mL)	
		DEAE-chitosan (90%)	DEAE-chitosan (50%)
Cervix cancer	HeLa	16 (±4)	26 (±4)
Lung cancer	A549	51 (±4)	93 (±5)
Human fibrosarcoma	HT1080	63 (±3)	126 (±5)

Acknowledgment

This research was supported by a grant (P-2004-01) from Marine Bioprocess Research Center of the Marine Bio 21 Center funded by the Ministry of Maritime Affairs & Fisheries, Republic of Korea.

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