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Chitin oligosaccharides inhibit oxidative stress in live cells

Dai-Nghiep Ngo [a], Moon-Moo Kim [b], Se-Kwon Kim [a,c,*]

[a] Department of Chemistry, Pukyong National University, Busan 608-737, South Korea
[b] Department of Chemistry, Dong-Eui University, Busan 614-714, South Korea
[c] Marine Bioprocess Research Center, Pukyong National University, Busan 608-737, South Korea

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Abstract

The aim of this research is to identify the cellular antioxidant effects of chitin oligosaccharides (NA-COS; M_w 229.21–593.12 Da) produced by acidic hydrolysis of crab chitin. The inhibitory effect of NA-COS on myeloperoxidase (MPO) activity in human myeloid cells (HL-60) and oxidation of DNA and protein in mouse macrophages (Raw 264.7) were identified. Furthermore, their direct radical scavenging effect by 2′,7′-dichlorofluorescein (DCF) intensity and intracellular glutathione (GSH) level were significantly increased in a time dependent manner, respectively. These results suggest that NA-COS act as a potent antioxidant in live cells.

Keywords: Chitin oligosaccharides (NA-COS); Antioxidant; Free radical scavenging; Glutathione; Reactive oxygen species

1. Introduction

In order to protect oxidation of cellular biomolecules in biological systems, equilibrium between oxidants formation and endogenous antioxidant defense mechanisms exists. If this balance is disturbed, it can produce oxidative stress (Kang et al., 2005). This state of oxidative stress can result in injury to all the important cellular components, thus the reactive oxygen species (ROS) generated excessively in tissues can cause cell death. Furthermore, ROS have direct or indirect relationships with oxidation of cellular biomolecules and play an important role in many diseases such as cancer, arthritis, neurodegenerative, diabetes, hypertension, inflammation and aging (Calabrese et al., 2005; Je, Park, & Kim, 2004). Therefore, the researches to find antioxidant compounds from natural resources have evoked a great interest are necessary. One of these compounds is chitin and its derivatives.

Chitin, a polymer of *N*-acetyl glucosamine, (1-4)-linked 2-acetamido-2-deoxy-β-D-glucan, is widely distributed among invertebrates and crustaceans as structural material in their exoskeletons and fungal cell walls (Jeon & Kim, 2000). Chitin and its derivatives have important biological properties that have potentials for a wide variety of applications such as immunostimulant (Seferian & Martinez, 2000), enzyme inhibitory (Je & Kim, 2005), antimicrobial, anticancer, anticholesteremic (Kim, Ngo, & Rajapakse, 2006), anticoagulant (Vongchan, Sajomsang, Kasinrerk, Subyen, & Kongtawelert, 2003) and wound-healing agents (Freier, Koh, Kazazian, & Shoichet 2005). However, chitin is an insoluble polymer in water and this property is the major limiting factor for its utilization in living systems. Furthermore, until now, there are few reports on antioxidant activities of chitin oligosaccharides (NA-COS) in live cells. Therefore, in this study, we investigated the inhibitory effects of NA-COS on production of ROS in live cells.

2. Materials and methods

2.1. Materials

Chitin (degree of deacetylation <10%) prepared from crab shells was donated by KEUMHO Chemical Products

* Corresponding author. Address: Department of Chemistry, Pukyong National University, Busan 608-737, South Korea. Tel.: +82 51 620 6375; fax: +82 51 628 8147.
E-mail address: sknkim@pknu.ac.kr (S.-K. Kim).

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Co., Ltd (Korea). Fluorescence probes DCFH-DA and monobromobimane were obtained from Molecular Probes Inc. (Eugene, OR, USA). MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide), 3,3′,5,5′-tetramethybenzidine (TMB), 2,4-dinitro-phenyl hydrazine reagent, agarose, and fetal bovine serum (FBS), were purchased from Sigma Chemical Co. (St. Louis, MO).

Human myeloid cells (HL-60) and Raw 264.7 were obtained from American Type Culture Collection (Manassas, VA, USA). RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) medium, penicillin/streptomycin, and the other materials required for culturing of cells were purchased from Gibco BRL, Life Technologies (USA). All other chemicals were of analytical grade or of the highest grade available commercially.

2.2. Preparation of chitin oligosaccharides (NA-COS)

Chitin was ground to powder and insert into reactor, adding 12 N HCl, stirring at 40 °C for 7 h. After reaction was stopped by adding distilled water, hydrolytic chitin solution was neutralized by 25% NaOH solution, centrifuged at 10,000*g* to remove insoluble residues. Supernatant was desalted and purified using a Micro Acilyzer G3 (Asashi Kasei Corp., Japan), and then the solution was subjected to a spray dryer. Finally, we obtained light yellow powder chitin oligosaccharides (NA-COS).

2.3. Analytical methods

Infrared spectra were recorded as clear signals on KBr plates with a Spectrum 2000 FT-IR spectrophotometer (Perkin-Elmer, USA) and proton and carbon NMR (^{1}H and ^{13}C NMR) spectra were recorded in D_2O on a JNM-ECP-400 (400 MHz) spectrometer (JEOL, Japan). Molecular mass of NA-COS was determined by TOF-MS ES spectrometer.

2.4. Culture of cells and viability determination

HL-60 and Raw 264.7 cells were grown, respectively, in RPMI-1640 and DMEM media containing 10% (v/v) fetal bovine serum, 100 μg/mL penicillin–streptomycin, 5% CO_2, and 95% air at 37 °C.

Cytotoxicity levels of NA-COS on HL-60 and Raw 264.7 cells were measured using MTT method as described by Hansen, Nielsen, and Berg (1989). The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of NA-COS. After 48 h of incubation, cells were rewashed and 50 μL of MTT (5 mg/mL) was added and incubated for 4 h. Finally, 200 μL of DMSO was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the optical density (OD) at 540 nm using an GENios microplate reader (TECAN Austria GmbH, Austria).

2.5. Myeloperoxidase activity assay

Determination of Myeloperoxidase (MPO) released to HL-60, cells were treated with different concentrations of NA-COS and blank (no inhibitor) an assay mixture containing 2 mM H_2O_2 and 1.6 mM 3,3′,5,5′-tetramethybenzidine (TMB) was added and amount of MPO released was measured spectrophotometrically at 655 nm (Naito et al., 1998).

2.6. Membrane protein oxidation assay

The oxidation degree of cell membrane proteins was assessed by determining the content of protein by carbonyl group (Levine et al., 1990). Cultured cells were washed three times with PBS and lysed in lysis buffer without reducing agents. Aliquots of cell lysate were transferred into microtubes and treated with different concentrations of NA-COS. After incubation for 30 min at 37 °C, 0.1 mM $FeSO_4$ and 2 mM H_2O_2 were added to the mixture and continued to incubate for 1 h. Solubilized protein was precipitated by centrifugation after addition of 400 μL of 20% trichloroacetic acid. The pellet was resuspended in 0.2% of 2,4-dinitro-phenyl hydrazine in 2 N HCl and allowed to stand at 25 °C for 40 min. The protein was precipitated again with 20% trichloroacetic acid and the pellet was washed three times with ethanol:ethyl acetate (1:1 v/v) solution. It was then dissolved in 200 μL 6 N guanidine hydrochloride and incubated for 15 min at 37 °C. After centrifugation at 1500*g* for 5 min, absorbance of the supernatant was recorded against a complementary blank at 370 nm using a UV/visible microplate reader. A blank was prepared with a parallel procedure using 2 N HCl alone instead of 2,4-dinitrophenyl hydrazine reagent. The carbonyl group of protein was expressed by comparing with control group.

2.7. Determination of radical-mediated DNA damage

Genomic DNA, which was extracted from Raw 264.7 using standard phenol/proteinase K procedure with minor modification (Sambrook & Russell, 2001), was pre-treated with various concentrations of NA-COS and exposed to $^{\bullet}OH$ using Fenton chemistry as described by Milne, Nicotera, Orrenius, and Burkitt (1993). For that, 40 μL of DNA reaction mixture was prepared by adding predetermined concentrations of test sample (or same volume of distilled water as control), 100 μM final concentration of $FeSO_4$, 0.1 mM final concentration of H_2O_2 and 5 μL of genomic DNA in the same order. Then the mixture was incubated at room temperature for 10 min and reaction was terminated by adding 10 mM final concentration of EDTA. Aliquot (20 μL) of reaction mixture containing about 1 μg of DNA was electrophoresed on a 1% agarose gel for 40 min at 100 V. Gels were then stained with 1 mg/mL ethidium bromide and visualized by UV light

using AlphaEase® gel image analysis software (Alpha Innotech, CA, USA).

2.8. Cellular ROS determination by DCFH-DA

Oxidation-sensitive dye DCFH-DA was used to detect formation of intracellular reactive oxygen species (ROS) as described previously (Engelmann, Volk, Leyhausen, & Geurtsen, 2005). For that, Raw 264.7 cells growing in fluorescence microtiter 96-well plates were labeled with 20 μM DCFH-DA in HBSS (Hanks balanced salt solution) for 20 min in the dark. Cells were then treated with different concentrations of NA-COS and incubated for another 1 h. After washing the cells with PBS for three times, 500 μM H_2O_2 was added. The intensity of fluorescence signal emitted by 2′,7′-dichlorofluorescein (DCF) due to oxidation of DCFH by cellular ROS was detected time dependently ($\lambda_{excitation} = 485$ nm, $\lambda_{emission} = 528$ nm) using a GENios® fluorescence microplate reader (Tecan Austria GmbH, Austria). Following maximum rate of fluorescence increase, each well was normalized to cell numbers using MTT cell viability assay. Effects of treatments were plotted and compared with fluorescence intensity of control and blank groups.

2.9. Measurement of intracellular GSH level

Cellular GSH level was determined using monobromobimane (mBBr) as a thiol-staining reagent (Poot, Verkerk, Koster, & Jongkind, 1986). For this experiment, Raw 264.7 cells were seeded into fluorescence microtiter 96-well plates at a density of 1×10^7 cells/mL and following attachment, treated with different concentrations of NA-COS for 30 min. Cells were then labeled with 40 μM mBBr for 30 min at 37 °C in the dark. After staining, mBBr-GSH fluorescence intensity was measured ($\lambda_{excitation} = 360$ nm, $\lambda_{emission} = 465$ nm) using above mentioned fluorescence microplate reader. The average fluorescence values of cell populations were plotted and compared with blank group in which cells were grown without treatment of NA-COS.

2.10. Statistical analysis

All assays were carried out in triplicate, and results are reported as means ± standard deviation.

3. Results and discussion

3.1. Characteristics of chitin oligosaccharides (NA-COS)

Chitin was hydrolyzed using hydrochloride acid to produce chitin oligosaccharides (NA-COS). The FT-IR spectrum of NA-COS (Fig. 1A) exhibits intense absorption bands at 3358 cm^{-1} (–OH stretch), 2917 cm^{-1} (C–H stretch), 1651 cm^{-1} (C=O stretch, amide I), 1548 cm^{-1} (N–H bend, amide II), 1313 cm^{-1} (C–N stretch, amide III), and 1150–1000 cm^{-1} (pyranose). The FT-IR spectrum of NA-COS is generally similar to FT-IR of some products as chitooligosaccharides, chitin or chitosan with the previous reports (Guinesi & Cavalheiro, 2006; Harish Prashanth & Tharanathan, 2005; Shimoda et al.,1996; Valde & Kiekens, 2004). ^{1}H NMR and ^{13} C NMR spectra were used to confirm for structure of NA-COS, Fig. 1B shows the ^{1}H NMR spectrum of NA-COS measured in D_2O solution at ambient temperature. The resonance at 1.97 ppm is assigned to the three *N*-acetyl protons (Akiyama, Kawazu, & Kobayashi, 1995) and the field peaks at 4.5–4.6 ppm and at 5.0 ppm are corresponded to H-1β and H-1α, respectively. The resonance of H-2 is the peak at 3.34 ppm and the field peak at 3.37–3.77 ppm corresponding to H-3,4,5,6 of monomer of NA-COS (Sugiyama et al., 2001). In the ^{13}C NMR spectrum (Fig. 1C), the main units of NA-COS were obviously characterized by the signals as following at 91.2, 95.9 ppm (C-1α, C-1_β), 54.2, 56.8 (C-2_α, C-2_β), 69.0 (C-3), 76.1 (C-4), 70.8, 71.7 (C-5_α, C-5_β), 60.9 (C-6), 174.1 (C-7) and 22.3 (C-8). In addition, molecular weight (M_w) of NA-COS were determined as 229.21–593.12 Da by TOF mass spectrometry (Fig. 1D). These results showed that 1–4 glucosidic links of chitin were broken, leading to NA-COS with low molecular weight and dissolving in water.

3.2. Effect of NA-COS on cell viability of HL-60 and Raw 264.7

In this study, HL-60 and Raw 264.7 cells were treated with different concentration of NA-COS to determine non-cytotoxic concentration for further experiments. The cell viability data confirmed that NA-COS with MW 229.21–593.12 Da are non-cytotoxic effect on both HL-60 (Fig. 2A) and Raw 264.7 cells (Fig. 2B). Even at high concentrations of NA-COS the reduction in cell viability was not significant. Therefore, we could use NA-COS for our next cellular radical scavenging experiments.

3.3. The inhibitory effects of NA-COS on myeloperoxidase

Myeloperoxidase (MPO) is the most abundant protein in neutrophils and catalyses the conversion of H_2O_2 and chloride into HOCl which is the most potent oxidant acting against pathogens during infection (Saito et al., 1991). However, the excessive release of ROS into the extracellular medium by neutrophils not only kills pathogens but also destroys healthy tissues of the host. Because they oxidize thiols in protein and react with unsaturated fatty acids of membrane lipid, this destabilizes integrity of the cell membrane (Park et al., 2003; Rajapakse, Kim, Mendis, & Kim, 2007). Therefore, potent inhibitor needs to be found to inhibit MPO activity and can control oxidation of biomolecules in neutrophils. In this study, we evaluated whether NA-COS can inhibit MPO activity that in turn helps to prevent oxidation of cellular biomolecules. As shown in Fig. 3, NA-COS exhibited that they can inhibit MPO activity in HL-60 cells. They could reduce MPO

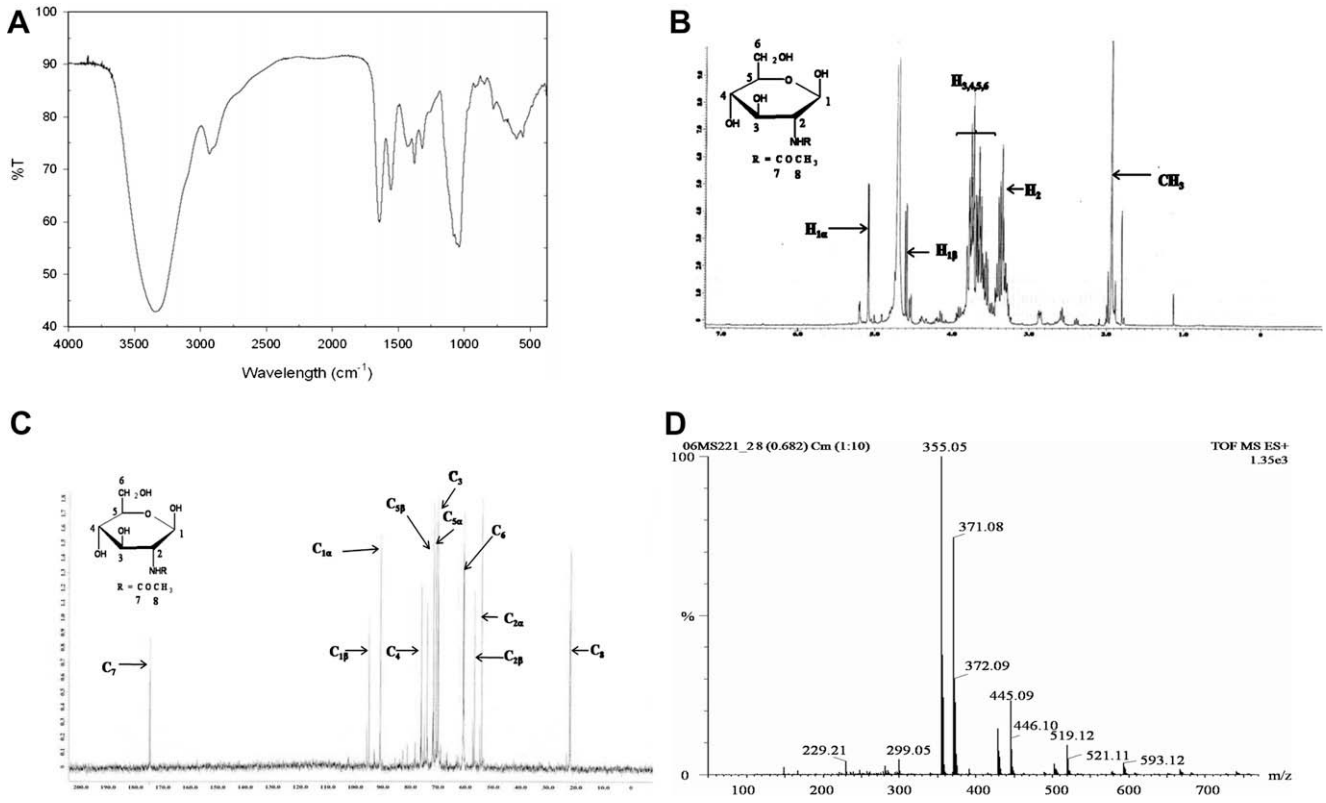

Fig. 1. (A) FT-IR spectra of NA-COS; (B) ^{1}H NMR (D_2O, 400 MHz) spectrum of NA-COS; (C) ^{13}C NMR (D_2O, 400 MHz) spectrum of NA-COS; (D) TOF-MASS spectrum of NA-COS.

activity dose-dependently compared with blank group. Furthermore, NA-COS at 1000 μg/mL, their inhibitory effect on MPO activity is about 52%. Therefore, this result indicates that they act as cellular antioxidant by indirect way in living system.

3.4. Inhibition of membrane protein oxidation

High occurrence of oxidized protein markers has been reported in numerous diseases (Olszowski, Mak, Olszowska, & Marcinkiewicz, 2003). This is because, lysine, proline, arginine and histidine residues in membrane proteins are highly susceptible for oxidative attack by ROS and results in accumulation of their carbonyl moieties (Levine et al., 1990). Furthermore, the compounds inhibiting protein oxidation can play an important role in maintaining cellular integrity and normal biological functions. Therefore, our next interest was to evaluate effects of our material on protein oxidation. In the present study, the inhibitory effect of NA-COS on oxidation cell membrane proteins was carried out according to the method described in the text. The formation of carbonyl moieties was increased when mouse macrophage membranes were exposed to hydroxyl radicals (‘OH). As shown in Fig. 4, protein carbonyl groups in NA-COS treated groups were lower than in oxidation stimulated cell membranes group (control group). Through protein carbonyl groups content, we found that NA-COS can inhibit over 50% oxidation of membrane proteins at 5 μg/mL and about 85% at 1000 μg/mL concentration, compared to control group (Fig. 4). Inclusion of NA-COS into the incubation medium inhibited protein carbonyl group content dose-dependently. We report for the first time that NA-COS can inhibit oxidation of membrane protein in live cells.

3.5. DNA protection activity of NA-COS

Hydroxyl radicals (‘OH) generated by the Fenton reaction are known to cause oxidative breaks in DNA strands to yield its open circular or relaxed forms. In a subsequent experiment, genomic DNA was isolated from Raw 264.7 cells to study protective effects of NA-COS against DNA oxidative damage. In this experiment the combined effect of 100 μM Fe(II) and 0.1 mM H_2O_2 on the integrity of genomic DNA was studied by DNA electrophoresis in the presence or absence of NA-COS. After 10 min of reaction, almost all DNA was degraded in the control group treated only with Fe(II)–H_2O_2. Interestingly, in accordance with previous report of Mendis, Kim, Rajapakse, and Kim (2007) for chitooligosaccharides, NA-COS significantly inhibited oxidative damage of DNA was observed (Fig. 5). The DNA damage at all tested concentrations of NA-COS were inhibited by about 80% based on the intensity of DNA bands. Due to this high inhibitory activity, a clear dose-dependent effect was not observed even at low concentrations. The results of this study clearly explained

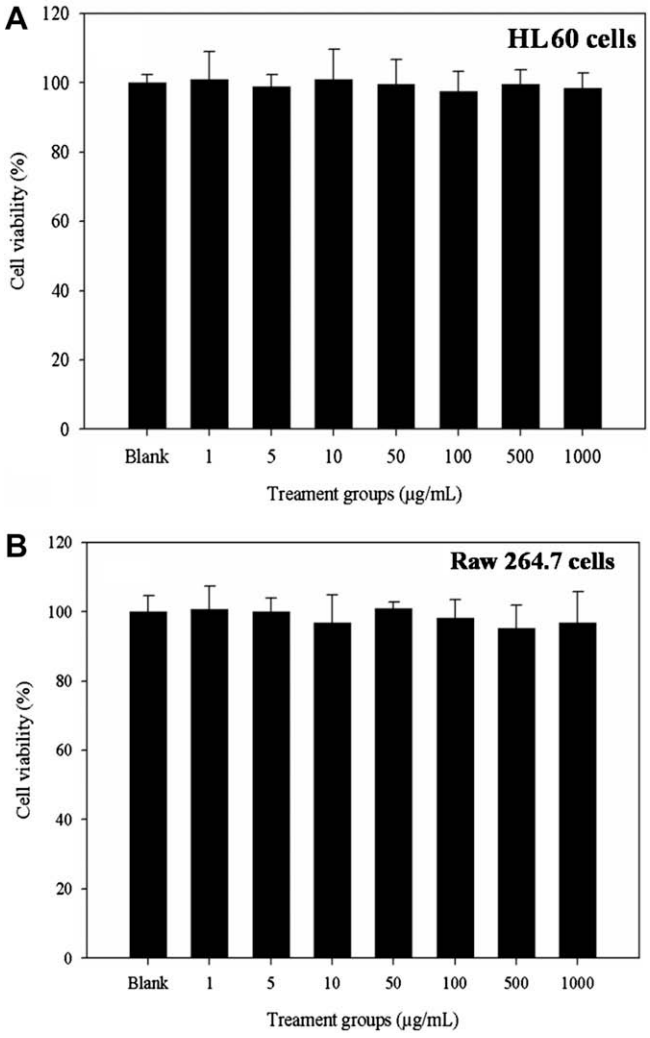

Fig. 2. The cytotoxic effect of NA-COS on viability of HL-60 (A) and Raw 264.7 cells (B). Both cells were treated with NA-COS at the indicated concentrations. After 48 h to treat NA-COS, cell viability was assessed by MTT assay as described in the text. Results are means ± standard error of three independent experiments.

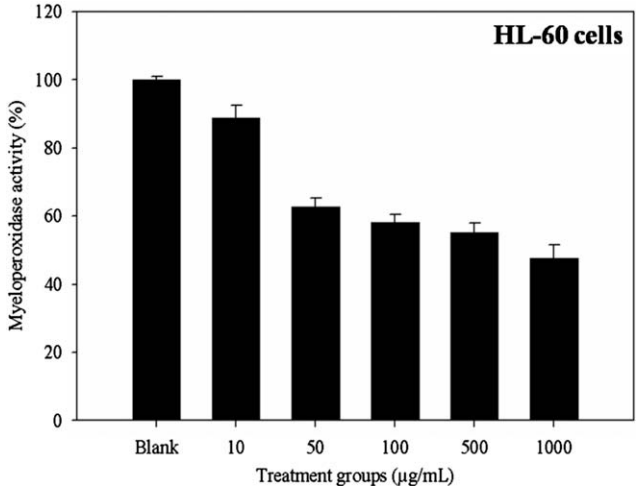

Fig. 3. The inhibitory effect myeloperoxidase activity of NA-COS in HL-60 cells. Cells were treated with different concentrations of NA-COS. An assay mixture containing H_2O_2 and 1.6 mM TMB was added and amount of MPO released was measured spectrophotometrically at 655 nm. MPO activity was compared treated groups with non-treated NA-COS (blank). Results are means ± standard error of three independent experiments.

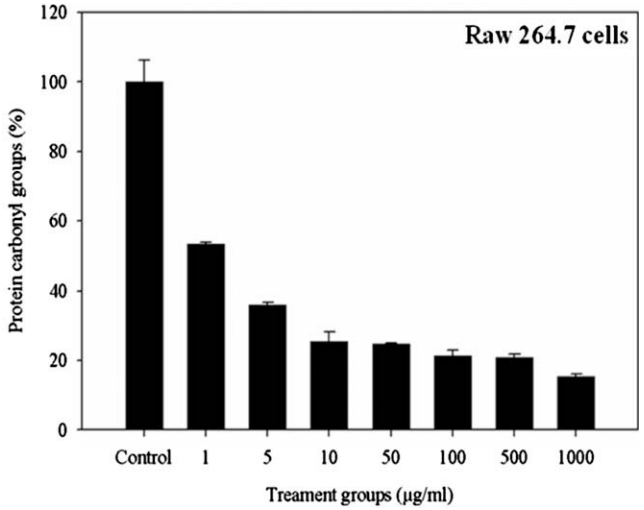

Fig. 4. Assessment of cell membrane protein oxidation. Raw 264.7 cell membranes were treated with various concentrations of NA-COS and protein oxidation was determined by assessing the amount of carbonyl-carbon content as described in the text and compared with ·OH non-treated (blank group) and treated (control group). Results are means ± standard error of three independent experiments.

that NA-COS can prevent oxidative damage to DNA when DNA was exposed to ·OH generated by Fe(II)/H_2O_2. Fe^{2+}-catalyzed conversion of H_2O_2, that is a major route to the synthesis of ·OH in biological systems. The ·OH highly reacted with all components of the DNA molecule, damaging both the purine and pyrimidine base and also deoxyribose backbone cause lesion for DNA (Valko et al., 2007). This lesion increased with increment of the free radical attacked on DNA of cells, which involved in mutagenesis, cancer and aging. However, NA-COS can contribute to prevent these diseases by their inhibitory effect against DNA oxidation in live cells.

3.6. Cellular radical scavenging effect of NA-COS

We were interested in studying the direct scavenging effects of NA-COS on cellular radicals to confirm their ability to scavenge free radicals in a cellular environment. For that, Raw 264.7 cells were labeled with fluorescence probe 2′,7′-dichlorofluorescien diacetate (DCFH-DA) as described in Section 2. DCFH-DA is a specific probe for reactive oxygen species (ROS). The principle of this assay is that DCFH-DA diffuses through cell membrane and is enzymatically hydrolyzed by esterase to become 2′,7′-dichlorofluorescien (DCFH), which reacts with ROS to form DCF, a fluorescent product. When DCFH-DA labeled cells were DCFH-DA to DCFH and traped inside cell and they incubated with H_2O_2 for 30 min, the rapid increment in DCF fluorescence intensity indicated

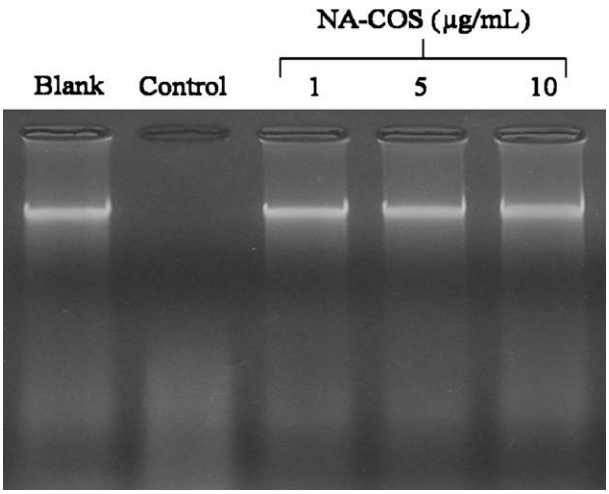

Fig. 5. DNA oxidative damage protection by NA-COS. Genomic DNA from Raw 264.7 cells was pre-treated with NA-COS and exposed to ·OH using Fenton chemistry. After 10 min, reaction mixture containing about 0.5 μg of DNA was electrophoresed on a 1% agarose gel for 40 min at 100 V and visualized by UV light after stained with 1 mg/mL ethilium bromide.

oxidation of DCFH by intracellular radicals (Fig. 6). The monitoring of DCF fluorescence intensities followed a time for 3 h revealed that radical-mediated oxidation increased according to incubation time.

However, the pre-treatment with NA-COS decreased the DCF fluoresce time dependently. All three concentrations of NA-COS (10, 100 and 1000 μg/mL) exhibited the free radical scavenging effect even only after 30 min of incubation comparing with control (H_2O_2 treatment and non-NA-COS) as shown in Fig. 6. According to the previous report of Rajapakse et al. (2007), chitooligosaccharide (COS), hydrolytic product of chitosan, and a synthesized product

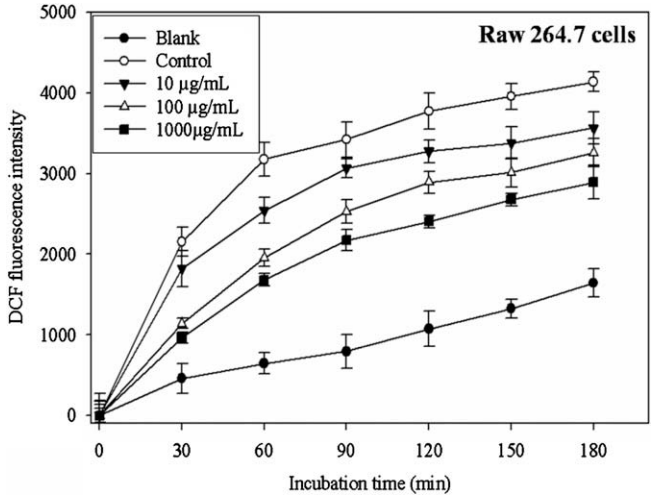

Fig. 6. The intracellular ROS generation level of cells was measured by the 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) which is compound to detect produced H_2O_2. Raw 264.7 cells were treated with 20 nmol/mL DCFH-DA, and then, the cells were treated with different concentration of NA-COS for 1 h. Results are means ± standard error of three independent experiments.

by modification COS, carboxylated-COS exhibited potential antioxidant activity in dose- and time-dependent manner. Like this, our results showed that their antioxidant activity were different with various concentrations of NA-COS, and the free radical scavenging effect of NA-COS at 1000 μg/mL concentration was higher than others. Therefore, we could confirm that NA-COS scavenged the free radical in dose- and time-dependent manner in live cells.

3.7. NA-COS mediated induction of intracellular glutathione level

After we studied direct antioxidant effects of NA-COS on biomolecules, further studies were carried out to investigate their effect on cellular antioxidant system. Here we focused on the effects of NA-COS in regulating cellular GSH level, which is important for maintaining intracellular redox status, using a thiol reactive fluorescent dye mBBr. We typically found that the level of mBBr-GSH fluorescence in NA-COS treated cells was higher than that of in untreated cells (Fig. 7) and increased with incubation time. It is well-known that increased GSH level protects cell death either by elimination of free radicals or by conjugation with toxicants (Sanchez-Reus, Peinado, Molina-Jimenez, & Benedi, 2005). However, measurements at incubation short times did not ameliorate the induction of GSH level in treated cells and it increased clearly after 2 h incubation. Furthermore, at low concentrations of NA-COS did not exhibit an increment in GSH level clearly comparing with hight their concentrations. This result showed that the intracellular GSH level was increased slightly in time and dose-dependent manner by NA-COS.

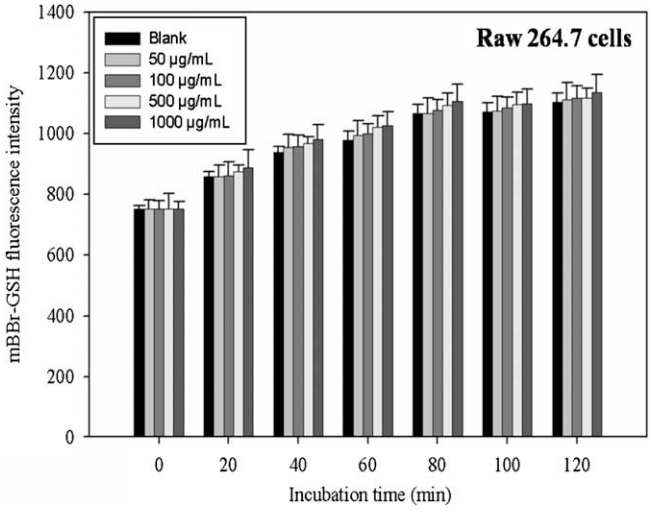

Fig. 7. The effect of NA-COS on regulation of GSH level in Raw 264.7 cells. Cells were treated with different concentrations of NA-COS and incubated for 30 min. Cellular GSH level was determined using mBBr as a thiol-staining reagent according to the method described in the text measuring mBBr-GSH fluorescence intensity at $\lambda_{excitation} = 360$ nm and $\lambda_{emission} = 465$ nm. The average fluorescence values of cell populations were plotted and compared with blank group (non-treated NA-COS). Results are means ± standard error of three independent experiments.

4. Conclusions

The present research showed that NA-COS have free radical scavenging effect in cellular system. They can inhibit myeloperoxidase activity in order to decrease free radicals or oxidation on DNA and membrane protein. Furthermore, they also stimulate increment intracellular GSH level. Based on the results, our conclusion is that NA-COS have potential free radical scavenging effects by both indirect and direct way to inhibit and prevent biological molecular damage of free radicals in live cells. They can be used as a scavenger to control the radical induced damage to cellular systems and promise further applications in future.

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