Comparative studies on ability of \(N\)-acetylated chitooligosaccharides to scavenge reactive oxygen species and protect DNA from oxidative damage

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Antioxidants have key role in scavenging free radicals and have been considered as dietary part of health conscious population for reducing the risk of many diseases. In the present study, commercially available \(N\)-acetylated chitooligosaccharides \([\text{GlcNAc})_n; n=2, 3, 5, 6]\) were studied for their antioxidant ability against superoxide, hydroxyl, DPPH radicals and \(\text{H}_2\text{O}_2\) by various radical scavenging assays. The \(N\)-acetylated chitooligosaccharides exhibited radical scavenging activities in the range of 49.1-63% for hydroxyl radical and 41-53.3% for superoxide radical. Their antioxidant ability was at par or better than standard antioxidants like butylated hydroxyanisole and mannitol. These \(N\)-acetylated chitooligosaccharides were also evaluated for their ability to prevent oxidative DNA damage in peripheral blood mononuclear cells exposed to \(\text{H}_2\text{O}_2\). \((\text{GlcNAc})_2, (\text{GlcNAc})_3, (\text{GlcNAc})_5\) and \((\text{GlcNAc})_6\) decreased the oxidative DNA damage due to \(\text{H}_2\text{O}_2\) by 59.5, 55.5, 22.9 and 4.9%, respectively. This antioxidative potential of \(N\)-acetylated chitooligosaccharides can be extended to beneficial health applications.

**Keywords:** Antioxidant, \(N\)-acetylated chitooligosaccharides, comet assay, oxidative DNA damage, oxidative stress, reactive oxygen species

**Introduction**

The identification of low cost natural bioactive resources with antioxidant potential is gaining an increasing interest in the improvement of food quality and human health\(^1\). Further, value added compounds from seafood waste are becoming an innovative alternative to synthetic dietary antioxidants in the global market. Less focused \(N\)-acetylated chitooligosaccharides (COS) have been prioritized in the present research since an extensive attention to the chitosan oligosaccharides has so far been given in many publications. COS are partially hydrolyzed products of chitin, one of the most abundant natural renewable bioresource materials from aquatic environment\(^2\). Besides being water soluble, they are biodegradable, biocompatible and non-toxic\(^3\). They also possess antioxidant\(^4,5\), anti-microbial\(^6\), anti-inflammatory\(^7\), anti-angiogenic and immune-stimulating properties\(^8\). Thus, to enhance the nutritional quality of food and human health, there is a great need to understand the antioxidant potential of COS to scavenge free radicals, especially reactive oxygen species (ROS) by various radical scavenging assays and the protective effects of COS against oxidative DNA damage, which is the root cause of a number of diseases.

Recent studies have focused on ROS, which play a vital role in day to day life style activities. Oxidative stress (OS) is a redox status as a result of imbalance between pro-oxidant and antioxidant\(^9\). ‘OS’ is initiated by free radicals, which are highly reactive, causing injury to the healthy human cells and finally damaging the necessary biomolecules, such as, DNA, proteins, carbohydrates and lipids\(^10,11\). The variety of diseases, \(\text{viz.}\), cancer\(^12\), diabetes mellitus\(^13\), cardiovascular disease\(^14\), neurodegenerative disorders\(^15\) and aging process\(^16\) are caused due to ‘OS’. The popularity of antioxidants, which prevent ‘OS’ and provide value added nutrition to the food by antioxidant therapy, is increasing day by day\(^17\). The antioxidant defense mechanism by scavenging free radicals is offered by many dietary antioxidants (\(\alpha\)-tocopherol, \(\beta\)-carotenes, ascorbic acid, uric acid, etc.) and endogenous enzymes (catalase, superoxide dismutase, glutathione peroxidase/reductase etc.). However, synthetic antioxidants, such as, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have adverse side effects on human health\(^18\).
Thus, attempts have been made to compare the prospective antioxidant potential of N-acetylated chito-oligosaccharides, which would be further beneficial as functional food. Current study was aimed to find out the in vitro free radical scavenging ability of commercially available COS [(GlcNAc)ₙ with degree of polymerization (n=2, 3, 5, 6)] against superoxide, hydroxyl, 2,2-diphenyl-2-picrylhydrazyl (DPPH) radicals and hydrogen peroxide (H₂O₂), which are normally found in the body. Protective effects of COS against oxidative DNA damage induced by H₂O₂ were also investigated by single cell gel electrophoresis (SCGE) or comet assay, which is a well known molecular technique for evaluating DNA damage at individual cell level.

Materials and Methods

Materials
COS [(GlcNAc)ₙ] with degree of polymerization (n=2, 3, 5, 6), phenazine methosulfate (PMS), NADH, nitroblue tetrazolium (NBT), deoxyribose and thiobarbituric acid, and low melting point agarose were purchased from Sigma Aldrich (St. Louis, MO, USA). (GlcNAc)₄ was discontinued by Sigma Company, hence was not included in the comparative studies and was not replaced with same product from other company to maintain uniformity of the preparations. Butylated hydroxyanisole (BHA) and mannitol was procured from HiMedia Laboratories Pvt. Ltd., (Mumbai, India). H₂O₂ (50% v/v) and all other chemicals used for comet assay were of highest analytical grade obtained from Thermo Fischer Scientific (Mumbai, India).

Evaluation of In Vitro Free Radical Scavenging Activity of COS

Superoxide Radical Scavenging Assay
Antioxidant ability of COS was assayed by superoxide radical scavenging activity by the method of Fernandes et al.¹⁹ and Valentao et al.²⁰. This was primarily evaluated by a non-enzymatic method, which was based on PMS/NADH (nicotinamide adenine dinucleotide) system for the generation of superoxide anion and reduction of NBT. The reaction mixture contained NADH (160 µM), NBT (25 µM) in sodium phosphate buffer, pH 7.4 (100 mM) and 200 µL of test sample (COS). The reaction started by addition of PMS (20 µM) followed by incubation at room temperature for 5 min and the absorbance was read at 560 nm against blank without COS and PMS. In control, COS was replaced with buffer. The percent inhibition was determined by comparing the results of control and test samples by formula 1.

Superoxide radical scavenging activity (%) =

\[
\frac{A_c - A_T}{A_T} \times 100
\]  

... (1)

Where Aᵣ is absorbance of control and Aₜ is absorbance of test sample at 560 nm.

Hydroxyl Radical Scavenging Assay
Hydroxyl radical scavenging assay was performed as described by Elizabeth and Rao.²¹ The assay is based on the generation of hydroxyl radicals by the Fe²⁺-EDTA-ascorbate-H₂O₂ system (Fenton reaction), which further attack 2-deoxyribose leading to the formation of thiobarbituric acid reactive substances (TBARS). The degradation product of 2-deoxyribose, malonaldehyde is quantified by its condensation with thiobarbituric acid (TBA). The reaction mixture contained 2-deoxyribose (2.8 mM), FeCl₃ (100 µM), KH₂PO₄-KOH buffer, pH 7.4 (20 mM), EDTA (100 µM), H₂O₂ (1.0 mM), ascorbic acid (100 µM) and 200 µL test sample (COS) in a final volume of 1 mL. The mixture was incubated at 37°C for 1 h. After incubation, 1 mL of 2.8% TCA and 1 mL of 0.6% aqueous solution of thiobarbituric acid (TBA) were added to 0.5 mL of reaction mixture mentioned above. This was heated at 95°C for 15 min, cooled and the absorbance of TBARS formed was measured at 532 nm against reagent blank without deoxyribose. Control sample was devoid of COS. The percent hydroxyl radical scavenging ability was calculated as a result of deoxyribose degradation, which was measured as TBARS. Percentage scavenging of hydroxyl radical was determined by the formula 2.

Hydroxyl radical scavenging activity (%) =

\[
\frac{A_c - A_T}{A_T} \times 100
\]  

... (2)

Where Aᵣ is absorbance of control and Aₜ is absorbance of test sample at 532 nm.

H₂O₂ Scavenging Assay
This assay was performed according to the method of Wei et al.²². The reaction mixture contained 200 µM phenol red, 10 µM H₂O₂ and 200 µL of test compounds (COS), which was incubated at room temperature for 5 min. Then 10 µL of horseradish peroxidase (10 U/mL) was added and further incubated for 5 min for oxidation of phenol red. This reaction was terminated by addition of 10 N NaOH.
The purple colour formed due to oxidation of phenol red was measured at 610 nm by keeping blank without horseradish peroxidase. Control was devoid of COS. The percent scavenging activity of H$_2$O$_2$ was calculated as described for superoxide radical scavenging assay by formula 2.

**DPPH Radical Scavenging Assay**

This assay is based on the reaction with purple coloured stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical, which is determined by the assay of Nagai et al.$^{23}$ The reaction mixture contained 0.1 mL of 1 mM DPPH, 0.8 mL of 99% ethanol and 0.1 mL of the test compound (COS). The solutions were mixed rapidly and the scavenging capacity was measured by monitoring the absorbance at 517 nm. The bleaching of DPPH absorption is representative of the radical scavenging capacity of the test compound. The DPPH radical scavenging activity was estimated by formula 3.

DPPH radical scavenging activity (%) = \[ \frac{A_c - A_f}{A_c} \times 100 \]  

Where \( A_c \) is absorbance of control and \( A_f \) is absorbance of test sample at 517 nm.

**Assessment of Protection of DNA Damage**

The alkaline comet assay was carried out according to the method described by Singh et al.$^{24}$ as previously carried out by Holz et al.$^{25}$ with minor modifications. Briefly, the comet slides were prepared by sandwich method where frosted area of microscopic slides were half pre-coated with 400 µL of 1.5% normal-melting point agarose to enhance the attachment of subsequent layers. A thin layer was obtained with the help of cover glass (22×50 mm$^2$; No.1) and air dried for 15 min. Peripheral blood mononuclear cells (PBMC) were treated with 100 µM of H$_2$O$_2$ (oxidant) for 10 min at 4°C to induce DNA damage. After treatment, the cells were centrifuged at 3000 g for 5 min and the pellet washed with PBS. $2\times 10^6$ cells/mL were then embedded in 1:10 (v/v) proportion in 1% low melting point agarose and 85 µL of this suspension was placed on the top of first agarose layer, covered with the coverglass and kept at 4°C for 5 min to solidify. The cover glass was removed after solidification and 80 µL of 1% low melting point agarose was added as third layer, covered with coverglass and kept at 4°C for another 10 min. After solidification, the cells were lysed for 1 h in prechilled lysis buffer (2.5 M NaCl, 100 mM Na$_2$-EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO & 1% Sarcosine, pH 10.0) at 4°C without coverglass. DNA unwinding was carried out by placing the slides for 20 min in alkaline electrophoresis buffer (300 mM NaOH & 1 mM Na$_2$-EDTA, pH > 13). The electrophoresis was run at 4°C for 25 min (25 V, 300 mA). The slides were gently washed 3 times in neutralization solution (0.4 M Tris, pH 7.5) for 5 min each and then stained with 50 µL of propidium iodide solution (10 µg/mL). All the above mentioned steps were performed in the dark to minimize additional DNA damage. The slides were observed immediately after 15 min of staining under a fluorescent microscope at 100× magnification (Olympus CX41, Olympus optical Co., GmbH, Hamburg, Germany) attached to a ProgRes C5 camera connected to a computer.

To determine the sublethal concentration of H$_2$O$_2$, a dose response experiment was carried out where PBMC were exposed to various concentrations (25, 50, 100 & 200 µM) of H$_2$O$_2$ as described above. The control contained cells with phosphate buffered saline (PBS) throughout the experiments to check the endogenous DNA damage. The probable genotoxic effect of COS at 100 µM was also determined. To evaluate the protective effects of COS against oxidative damage induced by H$_2$O$_2$, cells were initially treated with COS for 10 min at 4°C, centrifuged at 3000 g for 5 min, washed with PBS and then treated with 100 µM of H$_2$O$_2$ for 10 min at 4°C. The cells were washed again with PBS and then processed for comet assay. Cell viability for each treatment was determined by trypan blue dye exclusion assay.

All the experiments were carried out in triplicates. Single cells were analyzed by ImageJ 1.48v analysis software with OpenComet 1.3 as plug-in.$^{26}$ The calculations were done on the basis of random selection of 50 individual cells in triplicates. The induced DNA damage was expressed by one of the most common comet parameters, % DNA in tail$^{27}$ by formula 4.

\[ \frac{\% \text{DNA in tail}}{\text{Intensity of cell DNA}} = \frac{\text{Intensity of tailDNA}}{\text{Intensity of cellDNA}} \times 100 \]  

**Statistical Analysis**

All in vitro radical scavenging tests were performed in triplicates. The results from the assays were expressed as mean±standard deviation (SD) of three measurements (n=3). The data of the comet
Results and Discussion

In Vitro Free Radical Scavenging Activity of COS

Table 1 illustrates the free radical scavenging activity of the COS [(GlcNAc)\_n with n=2, 3, 5, 6] using various radical scavenging assays against hydroxyl and superoxide radicals and \(H_2O_2\), and their antioxidant ability was compared with the standard antioxidants, BHA and mannitol. The hydroxyl radical being highly potent oxidant reacts very fast with almost all biological macromolecules. The initiation of chain reaction occurs when it reacts with cell membrane leading to formation of lipid peroxides, which then further decompose to malondialdehyde (MDA) and cause DNA damage. We observed that (GlcNAc)\_2 and (GlcNAc)\_3 had very low hydrogen peroxide scavenging activity, whereas (GlcNAc)\_5 and (GlcNAc)\_6 did not exhibit this activity as compared to BHA (84.8%).

Thus all the COS analyzed were effective in the scavenging of free radicals in order of hydroxyl radical > superoxide radical > hydrogen peroxide. However, DPPH radical scavenging activity was not observed for any COS. It has been observed that most functions of the COS are clearly dependent on their mol wt\(^{29}\). This suggests that protection against hydroxyl and superoxide radicals by COS is of great significance for their possible use as dietary antioxidants and functional foods contributing to the health benefits.

Effect of COS on Antioxidative DNA Damage

The protective effect of COS on PBMC against oxidative stress induced by \(H_2O_2\) was investigated by comet assay. A linear increase was seen in DNA damage of PBMC with increase in concentration of \(H_2O_2\) as shown in Fig. 1. The cell viability, determined by trypan blue dye exclusion assay, was above 90% for all the concentrations; except for 200 \(\mu M \ H_2O_2\).

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<td>ND 17.40±0.40 57.10±0.20 49.10±0.15 63.00±0.35 61.20±0.30</td>
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<td>29.30±0.20 ND 53.30±0.35 41.00±0.61 51.40±0.30 44.20±0.25</td>
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*% values were calculated from mean±SD of 3 replicates, Nil: No scavenging activity, ND: Not done

Fig. 1—DNA damage of PBMC exposed to \(H_2O_2\). [Cells were treated with different concentrations of \(H_2O_2\) for 10 min at 4°C in dark (n=3×50 PBMC, \(p<0.05\))]

Table 1—In vitro free radical scavenging activity of N-acetylated chitooligosaccharides

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The assay was subjected to one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test by using GraphPad Prism 6.0 (GraphPad Prism software, USA). The test criterion for statistical significance was \(p<0.05\). The graphs were plotted using Origin 6.0 statistical software.
for which it was 72%. The cell viability was limited to ≥75% according to Kiskinis et al\textsuperscript{30}. Based on the results, 100 µM was used as sublethal dose of H\textsubscript{2}O\textsubscript{2} (82.32% DNA in tail) throughout the experiments. Fig. 2 shows the DNA damage due to COS where (GlcNAc)\textsubscript{2}, (GlcNAc)\textsubscript{3}, (GlcNAc)\textsubscript{5} and (GlcNAc)\textsubscript{6} did not show any significant oxidative damage to the cells as compared to the control (13.25, 11, 17.6 & 8.95% DNA in tail respectively). Furthermore, the COS [(GlcNAc)\textsubscript{n}, n=2, 3, 5, 6)] were tested at 100 µM for their DNA protective ability as shown in Figs 3 and 4. Our results indicate that (GlcNAc)\textsubscript{2} and (GlcNAc)\textsubscript{3} could significantly prevent DNA damage. DNA damage decreased to 24.51 and 28.58% for (GlcNAc)\textsubscript{2} and (GlcNAc)\textsubscript{3}, respectively as compared to 84.1% damage induced by H\textsubscript{2}O\textsubscript{2}. A relatively lesser protection was given by (GlcNAc)\textsubscript{5} and (GlcNAc)\textsubscript{6} where DNA damage was 61.24 and 79.25%, respectively. ANOVA followed by Tukey’s multiple comparison test showed significant protection was offered by (GlcNAc)\textsubscript{2}, (GlcNAc)\textsubscript{3} and (GlcNAc)\textsubscript{5} compared to (GlcNAc)\textsubscript{6} (correlation coefficient
0.99 & p<0.05). The inhibitory effect of low mol wt (229.21-593.12 Da) N-acetylated chitooligosaccharides on oxidative DNA damage of human lymphoma U937 was shown by Ngo 

et al31, which correlates with our study. Our attempts to check the protective effects of COS clearly indicate that (GlcNAc)$_2$ and (GlcNAc)$_3$ offer better protection to DNA against oxidative damage as compared to (GlcNAc)$_5$ and (GlcNAc)$_6$. The protective effects of glucosamine and N-acetyl glucosamine have been studied by Jamialahmadi 

et al32 where higher dose of GlcNAc (40 mM) was used. In this study, we could observe the protective effects of COS at relatively lower concentrations (100 μM). This dose would suffice in preventive manner against the oxidative DNA damage by reducing their risk of being pro-oxidants.

**Conclusion**

$N$-acetylated chitooligosaccharides [(GlcNAc)$_n$, $n=2, 3, 5, 6$] can be used as potent antioxidants and in future can serve as neatraeuticals. The protective function of $N$-acetylated chitooligosaccharides against oxidative DNA damage and their *in vitro* free radical scavenging ability holds promise for their demand in functional foods. $N$-acetylated chitin oligosaccharides may be useful to replace the synthetic dietary antioxidants in near future.

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