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Antioxidant activity of chitooligosaccharides upon two biological systems: Erythrocytes and bacteriophages

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ABSTRACT

Most of the reports to date on the antioxidant capacity of chitosans and chitooligosaccharides (COS) are based on strictly chemical methods. When studying antioxidants with potential *in vivo* applications, the method used to evaluate the antioxidant activity should be representative of the conditions in which the antioxidant might have a protective effect. In this work we evaluate the antioxidant activity of two COS mixtures and a low MW chitosan (LMWC) upon two biological oxidizable substrates – erythrocytes and phages, subjected to accelerated oxidation conditions.

Our results suggest that COS/LMWC can be used as antioxidants in biological systems. All the tested compounds reduced either the hemolytic and DNA damage, by inhibiting H_2O_2- and AAPH-radicals. However, the results obtained for these biological assays did not reveal a dose dependence, contrary to the chemical assay, suggesting that the protective concentrations should be established, in order to prevent enhancement of the oxidative damage – i.e. a prooxidant effect.

1. Introduction

There is increasing evidence showing the involvement of oxidative stress in many diseases, aging and in food deterioration (Smith, Sayre, Monnier, & Perry, 1995; Wang & Joseph, 1999; Wu, Chiou, Chang, & Wu, 2004). Reactive oxygen species (ROS), including superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide, are often by-products of metabolic processes in cells or of environmental sources, and uncontrolled ROS can easily react with a wide range of essential biomolecules, causing damage and ultimately leading to cell death (Anselmo & Cobb, 2004; Baek et al., 2008; Waris & Ahsan, 2006). Scavengers of free radicals are preventive antioxidants, as they can break the oxidative sequence by delaying or preventing ROS generation, and by competing for existing radicals and removing them from the reaction, thus protecting biomolecules from damage (Kim & Rajapakse, 2005; Xiong, Li, Jin, & Chen, 2007). Consequently, the use of antioxidants as preventive and as therapeutic agents is of great interest. In recent years, there has been an increasing interest in finding natural antioxidants from different sources for dietary, pharmacological and cosmetic uses in order to replace synthetic ones.

Chitooligosaccharides (COS) are derivatives of chitosan, typically differing from native chitosan by having a molecular weight of 10 kDa or less. They have recently attracted attention as potential therapeutic agents, owing to a variety of reported positive biological activities (Eaton, Fernandes, Pereira, Pintado, & Malcata, 2008; Kim & Rajapakse, 2005), and have shown potential as scavenging agents, due to their ability to abstract hydrogen atoms from free radicals (Huang, Mendis, & Kim, 2005; Vårum, Ottøy, & Smidsrød, 1994). This ability has been reported as directly correlated with their structural properties – namely that the amino and hydroxyl groups can react with unstable free radicals to form stable macromolecule radicals (Je, Park, & Kim, 2004; Kim & Rajapakse, 2005). Furthermore, their ready uptake by cells and the intestine, in addition with their claimed low toxicity, make chitooligosaccharides very promising compounds for use as natural antioxidants (Chae, Jang, & Nah, 2005; Fernandes et al., 2008).

Although several studies on chitosans and COS already reported antioxidant activity, so far most reports are based on methods which measure the capacity of a molecule to reduce a stable artificial free radical: scavenging of DPPH or ABTS radicals, carbon cen-

Abbreviations: ROS, reactive oxygen species; Hb, hemoglobin; AAPH, 2,2′-azobis (2-amidinopropane) hydrochloride; PFU, plate forming units; ATCC, American type culture collection; ABTS, [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)]; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

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tered radical scavenging, hydroxyl or superoxide radical assays, and metal ion chelating (Anraku et al., 2009; Feng, Du, Li, Wei, & Yao, 2007; Guzman, Saucedo, Revilla, Navarro, & Guibal, 2003; Park, Je, & Kim, 2004; Pasanphan & Chirachanchai, 2008; Rao, Chander, & Sharma, 2008). In addition, lipid peroxidation and protein oxidation inhibition assays, as well as a DNA protection assay by agarose gel electrophoresis have been also used to study chitosan or COS scavenging ability (Huang et al., 2005; Prashanth, Dharmesh, Rao, & Tharanathan, 2007; Yan et al., 2007). When studying antioxidant activities, the method used should be representative of *in situ* conditions. Therefore, in the work described here, the antioxidant activity of two COS mixtures and of a low MW chitosan (LMWC) has been evaluated using two biological oxidizable substrates – erythrocytes and phages, subjected to accelerated oxidative conditions.

2. Materials and methods

2.1. Materials

Chitooligosaccharide mixtures characterized by two distinct molecular weights – <3 and <5 kDa and possessing a degree of deacetylation in the range 80–85% were purchased from Nicechem (Shanghai, China). Low MW chitosan with 107 kDa and possessing a degree of deacetylation in the range 80–85%, was obtained from Sigma–Aldrich (Sintra, Portugal). All the compounds were derived from crab shells. The stock preparations of phage P22 and *Salmonella typhimurium* were obtained according to ATCC indications, Ref. 19585 B1. All the chemicals used in this work were purchased from Sigma–Aldrich (Sintra, Portugal).

2.2. Total antioxidant capacity

ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation assay ($ABTS^{\bullet+}$). The radical scavenging capacity of both COS mixtures and of LMWC was evaluated against ABTS radical cations generated by a previously reported protocol (Gião et al., 2007). This method is based on the ability of antioxidant molecules to quench the long-lived $ABTS^{\bullet+}$ species, a blue-green chromophore with a characteristic absorption at 734 nm. The addition of antioxidants to the preformed radical cation reduces it to ABTS, causing a loss of color. The assay was performed in triplicate. Results were expressed by plotting % of inhibition versus compound concentration, and were also expressed as equivalent concentration of ascorbic acid (in mg/mL) for purposes of standardization.

2.3. Bacteriophage P22/S. typhimurium system assay

The antioxidant capacity of both COS mixtures and of LMWC was evaluated via the biological system P22/*S. typhimurium,* as described previously by Gião et al. (2009).

Firstly the effect of 250 mM of H_2O_2 on the action of the phage on *S. typhimurium* was determined. Like the later assays, this was done by incubating the phage with the oxidant for 20 min, taking aliquots each 5 min. The activity of these treated phages on *S. typhimurium* was then determined by plate counting, giving results in PFU mL^{-1}. The effects of the potential antioxidants were reported relative to the effect of H_2O_2 alone. The phages were treated as described above, but in the presence of both the H_2O_2 and the COS mixture or LMWC under study at several different concentrations (1.0, 0.1, 0.05, 0.01, 0.005 and 0.001 mg/mL). The antioxidant effect is reported as the difference between the log PFU mL^{-1} measured with the compound and the oxidant, and that measured with only the oxidant.

2.4. Assay system for hemolysis

Blood was obtained from healthy volunteers by venipuncture and collected into tubes containing EDTA as an anticoagulant. Samples were immediately centrifuged at 405*g* for 10 min; plasma and buffy coat were carefully removed and discarded. RBCs were washed three times with phosphate buffered saline (PBS; 125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) at 4 °C and finally resuspended in PBS, to obtain an RBC suspension at 2% (v/v) hematocrit.

The hemolysis assays were performed by using H_2O_2 or 2,2′-azobis (2-amidinopropane) hydrochloride – AAPH as the oxidant agent at a final concentration of 7.5 mM (or 60 mM), with or without ascorbic acid at its physiological concentration (in accordance with the hematocrit used – 3 μM). In all sets of experiments ($n = 4$), a negative control (RBCs in PBS, with no oxidant) was used, as well as compound controls (RBCs in PBS, with each compound at each concentration). We calculated the hemolysis induced by the compounds at each concentration, and used this as a baseline result. These values were below 3% hemolysis for all concentrations and compounds tested (Fernandes et al., 2008). The results are presented as percentages relative to the control hemolysis values. All control and sample tests were run in duplicate. Incubations of RBC suspensions were carried out at 37 °C for 3 h, under gentle shaking. Hemolysis was determined spectrophotometrically, according to the protocol described in Ko, Hsiao, and Kuo (1997). After the incubation period, an aliquot of the RBC suspension was taken out, diluted with 20 volumes of saline and centrifuged (1180*g* for 10 min). The absorption of the supernatant (A) was read at 540 nm. To yield the absorption of a complete hemolysis (B), the RBC suspension was treated with 20 volumes of ice cold distilled water and, after centrifugation the absorption of the supernatant was measured at the same wavelength. The percentage of hemolysis was then calculated as $(A/B) \times 100$.

In order to analyze Hb oxidation**,** visible absorption spectra (500–700 nm) were measured using hemolysates incubated with different concentrations of COS/LMWC for 3 h, either in the presence of AAPH (60 mM) or H_2O_2 (7.5 mM), as described by Colado Simão et al. (2006).

2.5. Statistical analyses

Mean values and standard deviations were calculated from the experimental data obtained, and analysis of variance (ANOVA) was applied at a 5% level of significance, using compound concentration and MW as main factors. Pairwise comparisons were done using the Bonferroni test, at the same level of significance.

3. Results

The results of the total antioxidant capacity assay using ABTS are shown in Fig. 1. These results were also calibrated based on the antioxidant capacity of ascorbic acid, and this is shown in Table 1. All the three studied compounds displayed antioxidant activities as they were able to scavenge the $ABTS^{\bullet+}$. All three compounds displayed a positive correlation between the concentration of compound added and the level of inhibition. These results show that in terms of TAC value, the <3 kDa and <5 kDa COS had almost identical effects, while LMWC showed lower activity at all concentrations (maximum activities were 0.301 and 0.298 gL^{-1} equivalent of ascorbic acid for <3 and <5 kDa, and 0.223 gL^{-1} for LMWC).

The inactivation curve of P22 bacteriophage, which was attained by adding 250 mM of H_2O_2, clearly shows a reduction in the number of available phages to infect *S. typhimurium* during the course of the experiment (Fig. 2). After 20 min, a 3-log cycle

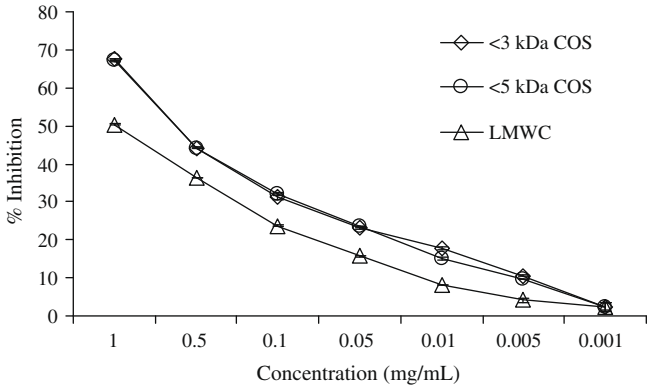

Fig. 1. Concentration–response curve for inhibition of the absorbance of $ABTS^{\cdot+}$ at 734 nm after 6 min, at different concentrations of COS (<3 and <5 kDa) and LMWC.

Table 1
Total antioxidant capacity expressed as equivalent concentration of ascorbic acid (average ± standard deviation) of the three compounds, determined at 6 min.

Concentration (mg/mL)	<3 kDa COS	<5 kDa COS	LMWC
TAC (mg/mL)			
1.0	0.3010 ± 0.0045	0.2986 ± 0.0051	0.2237 ± 0.0056
0.5	0.1968 ± 0.0013	0.1968 ± 0.0003	0.1614 ± 0.0020
0.1	0.1398 ± 0.0019	0.1424 ± 0.0009	0.1055 ± 0.0003
0.05	0.1029 ± 0.0016	0.1045 ± 0.0010	0.0698 ± 0.0013
0.01	0.0794 ± 0.0045	0.0669 ± 0.0006	0.0358 ± 0.0007
0.005	0.0472 ± 0.0013	0.0431 ± 0.0007	0.0183 ± 0.0001
0.001	0.0107 ± 0.0003	0.0109 ± 0.0003	0.0103 ± 0.0007

reduction in the initial number of phages was achieved. This 3-log reduction after 20 min was used as a control in order to analyze the antioxidant activity of both COS compounds and the LMWC. By adding 1.0 mg/mL of any of the compounds under study, an enhancement in the oxidant effect of H_2O_2 upon the phage was observed (Fig. 3). A somewhat decreased effect was observed for both COS mixtures at 0.1 mg/mL, while LMWC simply did not present any effect at that concentration. However, none of the compounds at these concentrations exhibited themselves damage to the phage. A significant protection of phage P22 against oxidation was observed when 0.05 or 0.01 mg/mL of COS were added (statistically different, for both concentrations, from LMWC values, $p < 0.05$). At these concentrations both COS compounds led to an enhancement of phage numbers of around 1 log cycle, which indicates a protection rate of approximately 90%. For concentrations below 0.01 mg/mL the antioxidant activity was absent for both COS, however, LMWC still maintained a slight protective effect (ca. 0.1-log cycle at 0.005 mg/mL).

In the presence of H_2O_2, all the three compounds significantly protected RBCs from oxidative-induced hemolysis at concentrations between 0.05 and 0.005 mg/mL (statistically similar between them, $p > 0.05$). The strength of antioxidant activity was in the order LMWC >3 kDa COS >5 kDa COS (as shown in Fig. 4). At lower concentrations the protection by all the three compounds decreased considerably (to around 10%, but only statistically different from 0.01 mg/mL, $p < 0.05$). For the highest tested concentration (1.0 mg/mL), the hemolysis inhibition was so little as to be almost meaningless ($p < 0.005$, when compared with hemolysis inhibition provided at 0.05 and 0.005 mg/mL); nevertheless, LMWC showed three times higher inhibition at this concentration than both COS compounds ($p > 0.05$).

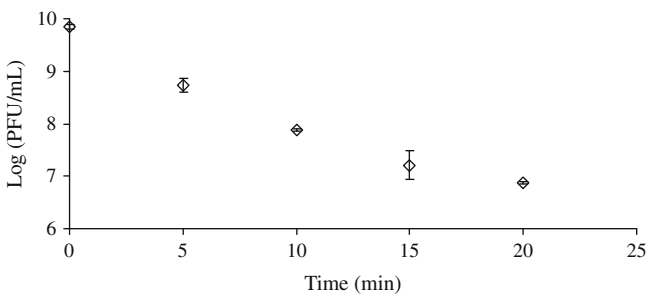

Fig. 2. Bacteriophage inactivation curve, obtained with the oxidant system (250 mM H_2O_2) on the P22 bacteriophage.

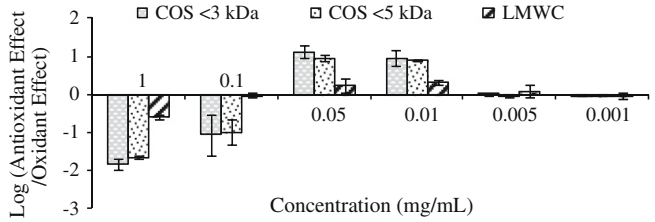

Fig. 3. Results of bacteriophage/*S. typhimurium* assays, expressed as the difference between log PFU mL^{-1} measured in the presence of the oxidant and that measured in the presence of the oxidant plus the antioxidant system.

In order to simulate *in vivo* conditions, the compounds were also tested in the presence of 3 μM ascorbic acid. Only when the concentrations were between 0.05 and 0.005 mg/mL, was some additive protection effect observed. Only the results within this range are shown in Fig. 5, since the results at concentrations above 0.05 and below 0.005 mg/mL were not promising, even with the compounds alone (Fig. 4). The mixture of <3 kDa COS (0.05 mg/mL) and ascorbic acid showed the highest protection (almost 55%), and was the only result significantly different to ascorbic acid alone ($p < 0.05$). Within this concentration range all the other results were similar to the effect of ascorbic acid by itself (39.5%). In addition, ascorbic acid plus any of the COS mixtures at 1 mg/mL potentiated the oxidant effect of H_2O_2 (data not shown).

When AAPH was used as oxidative agent (Fig. 6), all the three compounds significantly protected the RBCs from hemolysis, except at the highest concentration studied. At this concentration (1 mg/mL), LMWC showed a small protective effect (5% – statistically not significant when compared with all other LMWC concentrations tested) and the COS compounds potentiated the oxidation slightly ($p < 0.005$). Once again, the highest inhibition was conferred by concentrations between 0.05 and 0.005 mg/mL. Within the active concentration range, the <3 and <5 kDa COS compounds showed similar activities ($p > 0.05$) and the LMWC a somewhat lower activity. The highest protection rate was achieved by <3 kDa COS at 0.005 mg/mL – 24.9% hemolysis inhibition, while LMWC never attained an inhibition above 14.0%.

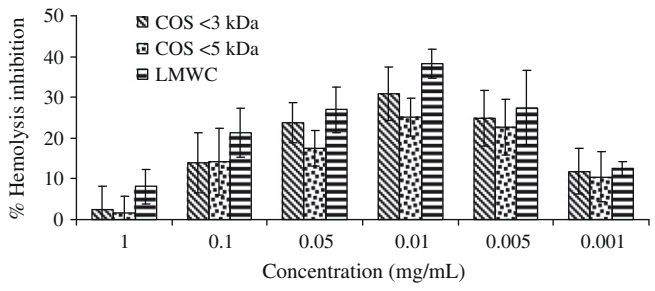

Fig. 4. Percentage of inhibition of lysis of RBCs at 2% hematocrit incubated for 3 h with 7.5 mM of H_2O_2 and COS/LMWC at different concentrations. Mean (error bars represent range) of four determinations for each duplicate.

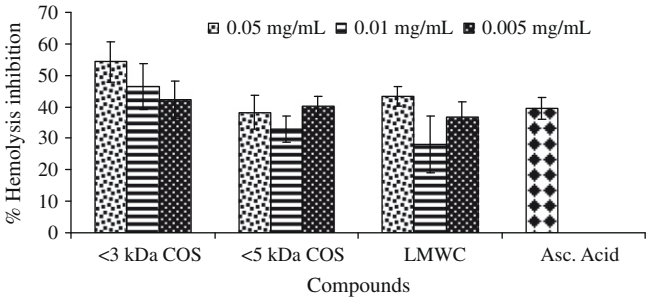

Fig. 5. Percentage of inhibition of lysis of RBCs at 2% hematocrit incubated for 3 h with 7.5 mM of H_2O_2 and COS/LMWC at different concentrations, in the presence of ascorbic acid (3 μM). Mean (error bars represent range) of four determinations for each duplicate.

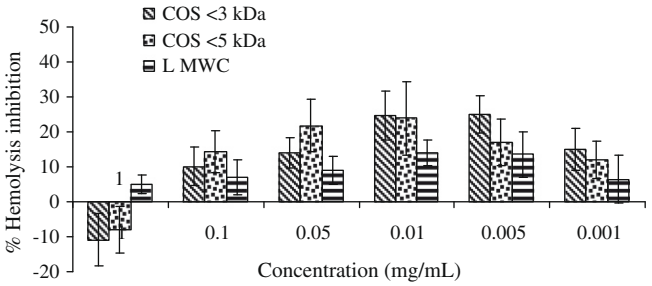

Fig. 6. Percentage of inhibition of lysis of RBCs at 2% hematocrit incubated for 3 h with 60 mM of AAPH and COS/LMWC at different concentrations. Mean (error bars represent range) of four determinations for each duplicate.

The results obtained on inhibition of hemolysis by AAPH in the presence of ascorbic acid are shown in Fig. 7. In the presence of ascorbic acid, the potentiation of AAPH oxidant effect at high concentrations was not observed (data not shown); nevertheless, the protection rate observed was lower than that observed for ascorbic acid alone (36.5% by <3 kDa COS; 34.3% by <5 kDa; 52.9% by ascorbic acid). Similarly to the protection against H_2O_2, the best protection rates were observed at the compound concentrations between 0.05 and 0.005 mg/mL. At 0.005 mg/mL, <3 kDa COS showed a hemolysis inhibition of 68.7%, while <5 kDa COS and LMWC presented relatively lower percentages – 59.0% and 61.5%, respectively. In general, with or without ascorbic acid, <3 kDa COS presented the highest protection against AAPH. However, none of the results showed significant differences to that afforded by ascorbic acid alone ($p > 0.05$).

By performing spectral scans of RBC lysates (obtained after 3 h of incubation at 37 °C in the presence of AAPH or H_2O_2 and with or

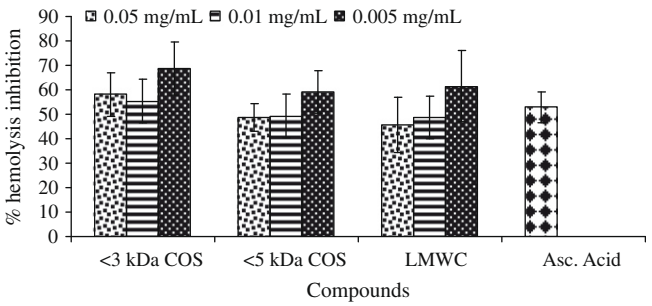

Fig. 7. Percentage of inhibition of lysis of RBCs at 2% hematocrit incubated for 3 h with 60 mM of AAPH and COS/LMWC at different concentrations, in the presence of ascorbic acid (3 μM). Mean (error bars represent range) of four determinations for each duplicate.

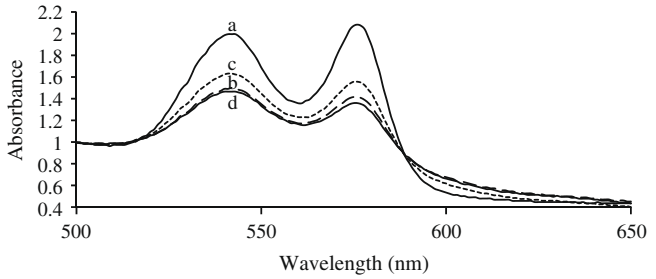

Fig. 8. Spectral scans (500–650 nm) of lysed RBC suspensions, obtained after 3 h of incubation in: (a) PBS; (b) the presence of AAPH (60 mM); (c) the presence of AAPH (60 mM) with 0.005 mg/mL of <3 kDa COS; (d) the presence of AAPH (60 mM) with 0.005 mg/mL of LMWC.

without the compounds under study), changes could be observed in the oxy-hemoglobin peaks (540 and 578 nm) when compared with the control assay (RBCs without the oxidative agents). Representative spectra are compared in Fig. 8. When AAPH was added, a considerable decrease in the size of the oxy-hemoglobin peaks was evident, even with the addition of COS or LMWC. However, a higher level of oxy-hemoglobin remained when any of the COS was added at 0.05 or 0.005 mg/mL. On the contrary, when 1.0 mg/mL was used, the oxy-hemoglobin peaks were lower than those of AAPH (data not shown). Regarding LMWC, oxy-hemoglobin peaks were always lower than those following treatment by AAPH alone, independently of the concentration tested. When the analysis was carried out using H_2O_2 as the oxidant, the reduction in size of both oxy-hemoglobin peaks was greater (data not shown). Although a small protection could be seen with the addition of COS (at 0.05 or 0.005 mg/mL) or LMWC (for all concentrations), none of the compounds effectively protected hemoglobin from oxidation. In fact, a meta-hemoglobin peak (630 nm) appeared in all cases, except in the control. Both COS at 1.0 mg/mL increased to a small extent the hemoglobin oxidation (data not shown).

4. Discussion

The results of the ABTS assay showed clearly that the three compounds possess the capacity to reduce $ABTS^{\cdot+}$ in a dose-dependent manner. In addition, it suggests that this capacity is greater in COS, as in every concentration tested (down to 0.005%), a 10% higher inhibition percentage was measured, compared to inhibition by LMWC. Other authors have described similar results for similar (chemical) assays: Several authors have shown dose-dependent radical scavenging by COS (Huang et al., 2005; Feng et al., 2007) and some authors also found that greater molecular weight COS compounds were more effective, matching our results (Feng et al., 2007). Park et al. (2004) reported that the scavenging activity was dependent on the number of amino groups available (degree of acetylation). Considering that the three tested compounds possess a similar degree of acetylation, the dose-dependent activity observed is justified by the increasing amount of amino groups with increasing concentrations. However, for the same concentration, the difference observed between both COS compounds and the LMWC suggests that an important role may be associated to the chain length.

Bacteriophage populations can be progressively inactivated by a number of environmental stresses. This inactivation can be revealed by assaying the viable phage population prior to and following stress (Gião et al., 2009). Phage inactivation can represent a sensitive approach to measure the effects of oxidative stress on the biological integrity of DNA. Alternatively, it might also reflect damage to the proteinaceous phage capsid, such that the DNA cannot be delivered. Our results showed that both tested COS can

effectively protect phage P22 against oxidative damage by H_2O_2, for a certain range of concentrations (0.05–0.01 mg/mL). Higher concentrations seem to potentiate the H_2O_2 action. It is well-established that for nearly all antioxidant compounds studied, prooxidant effects are found when the compounds are used at very high concentrations (Gião et al., 2009; Otero, Viana, Herrera, & Bonet, 1997; Park et al., 2008). In light of the scarcity of knowledge of the antioxidant activity of chitooligosaccharides, the mechanism of such prooxidant effects is unknown.

The protection effect exhibited by LMWC was lower than the attained by COS; nevertheless, it was shown be more effective at lower concentrations (0.005 mg/mL) and to potentiate H_2O_2 effect, to a lesser extent, at higher concentrations.

In order to further elucidate the antioxidant properties of COS/LMWC in human cells, RBC were selected as a metabolically simplified model system. These blood cells can act as cumulative markers of oxidative stress *in vitro* and *in vivo*, providing information about oxidative damage suffered and accumulated by the cell over its lifespan or during *in vitro* assays. If ROS are overproduced outside or within the erythrocyte, or if the endogenous antioxidant defenses are impaired, an oxidative stress condition will develop, inducing oxidative damage on erythrocyte constituents, including the membrane and Hb, which may lead ultimately to hemolysis.

We tested the efficacy of COS/LMWC as inhibitors of H_2O_2- and AAPH-induced RBC hemolysis. Hydrogen peroxide crosses the RBC membrane and is known to react with Hb, generating highly reactive radical species including hydroxyl radicals (Ko et al., 1997). Our results suggest that COS and LMWC have the capacity to protect RBC from such H_2O_2-induced hemolysis, by scavenging hydroxyl radicals. However, similarly to the results observed for the phage assay, the level of inhibition obtained for this system was lower than those reported by others authors that used strictly chemical methodologies: for example, Chien, Sheu, Huang, and Su (2007) showed that at 1.0 mg/mL, a 12 kDa chitosan exhibited 90.5% scavenging activity toward hydrogen peroxide, while ascorbic acid and a 95 kDa chitosan presented 87.4% and 75.0%, respectively. On the contrary, at this concentration we obtained the lowest inhibition rate measured (lower than 10% for any of the three compounds). A possible justification could be the adhesion and/or aggregation reactions between RBCs and COS, observed for COS concentrations above or equal to 0.1 mg/mL, as directly observed in our previous work (Fernandes et al., 2008). These reactions between COS (at higher concentrations) and RBCs may lead to weakness of the RBC membranes, since it interferes with some RBC membrane proteins that are responsible for the cytoskeleton maintenance (Fernandes et al., 2008).

When RBC suspensions are prepared for the assays, the exogenous plasmatic antioxidant defenses are washed out during the manipulation. Therefore, in order to understand the effective protection of compounds against oxidative-induced injury in RBCs, their activity was also tested in the presence of the most important extracellular antioxidant, the ascorbic acid. It is important to bear in mind that antioxidant substances work together *in vivo*, not alone. In the presence of a physiological concentration of ascorbic acid, no synergetic effect was observed, but an additive one. The protection achieved by the mixture of either COS or LMWC with ascorbic acid may be considered as a good indicator for physiological activity, since at moderate concentrations, they significantly protected the RBCs from H_2O_2-induced oxidation, especially the <3 kDa COS.

In contrast to H_2O_2, AAPH-radicals attack RBC membrane components, causing membrane damage (peroxidation of phospholipids) eventually leading to hemolysis (Colado Simão et al., 2006; Ko et al., 1997). AAPH is a hydrophilic radical initiator producing peroxyl/alkoxyl radicals at physiological temperatures in the presence of molecular oxygen, which can induce lipid peroxidation (Volpi & Tarugi, 1997). Such peroxyl radicals have been identified as the main secondary carriers of biological damage (Anraku et al., 2009). Our results suggest that COS or LMWC have the ability to protect cells from these radicals (when in moderate concentrations – 0.05–0.005 mg/mL), but were less effective than against H_2O_2-induced oxidation. However, the combination of COS/LMWC with ascorbic acid showed a very promising effect on the protection of human cells from these radicals (ca. 60–70%), attaining values more comparable to those reported by chemical methods. Although there is no information on the sites of attack by peroxyl radicals on chitosans, several studies reported a higher scavenging activity upon these radicals by chitosans with higher deacetylation degree (Anraku et al., 2009; Park et al., 2004), suggesting importance of the D-glucosamine units in the process.

Finally, the Hb oxidation assays revealed that none of the compounds tested effectively protected Hb from oxidation by H_2O_2. However, oxidation of Hb by AAPH was considerably reduced by both COS mixtures. The absence of protection against H_2O_2 probably results from the fact that Hb oxidation processes occur intracellularly, where COS or LMWC cannot play their protective effect (Ko et al., 1997). The H_2O_2-generated radicals that COS/LMWC is not able to eliminate outside the RBC may cross the RBC membrane and react with Hb. On the other hand, as AAPH-radicals attack the membrane but do not cross the membrane, the Hb oxidation only occurred after RBC have been lysed. Therefore, the external COS or LMWC molecules were able to scavenge AAPH-radicals preventing Hb oxidation.

5. Conclusions

Our results suggest that chitooligosaccharides can be used as antioxidants in biological systems. All the tested compounds reduced both the hemolytic and DNA damage, by inhibiting H_2O_2- and AAPH-radicals. However, on the contrary to the results obtained with chemical methodologies, our data suggested that COS/LMWC concentrations have to be reasonably low in order to prevent enhancement of the oxidative damage. Furthermore, the comparison of the results using different methodologies clearly highlights the need for testing antioxidant properties of biological substrates; chemical methods alone may suggest misleadingly high antioxidant activities.

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