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ORIGINAL PAPER

Chitooligosaccharides antagonize the cytotoxic effect of glucosamine

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Abstract Chitooligosaccharides (COS) are partially hydrolyzed compounds derived from chitosan that exhibit a number of biological activities, including antitumor, antibacterial and antifungal properties. In this work, we examined the cytotoxicity of pure COS and oligomers A, B and C (solutions composed of different amounts of COS) produced by enzymatic hydrolysis using a crude enzyme extract produced by the fungus *Metarhrizium anisopliae*. The antiproliferative effect of these molecules was analyzed using tumor cell lines (HepG2 and HeLa cells) and in a normal cell line (3T3). The antioxidant activity was analyzed in several in vitro experiments. Glucosamine showed higher toxicity (approximately 92%) to all cell lines studied. However, the oligomers obtained after hydrolysis demonstrated no toxic effects on the normal cells (3T3). Furthermore, we showed that a small amount of other COS can decrease the cytotoxic effect of glucosamine against 3T3 cells, indicating that glucosamine could be used as an antitumor drug in the presence of other COS. In addition, different effects were found in antiproliferative assays, which depended on the COS composition in the oligomers (A, B and C), showing that a combination of them may be essential for developing antineoplastic drugs. Superoxide anion scavenging was the main antioxidant activity demonstrated by the COS and oligomers. This activity was also dependent on the oligomer composition of the chitosan hydrolysates. Further work will identify the ideal proportions of COS and glucosamine for maximizing the effects of these biological activities.

Keywords Glucosamine · Chitosan · *Metarhrizium anisopliae* · HeLa · HepG2 · 3T3

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Introduction

Chitin, a naturally abundant polysaccharide, is the main component of the exoskeleton of crustaceans and insects and consists of a 2-acetamido-2-deoxy-β-D-glucose through a β (1 → 4) linkage. Chitosan is the *N*-deacetylated derivative of chitin, although this *N*-deacetylation is almost never complete (Majeti and Kumar 2000). Chitin and chitosan are of commercial interest due to their high nitrogen content (6.89%) compared to synthetically substituted cellulose (1.25%). This makes chitin a useful chelating agent (Muzzarelli 1973). As most present-day polymers are synthetic materials, their biocompatibility and biodegradability are much more limited than those of natural polymers, such as cellulose, chitin, chitosan and their derivatives. However, these naturally abundant materials also exhibit a limitation in their reactivity and ability to be processed (Zikakis 1984; Illum 1998). In this respect, chitin and chitosan are recommended as suitable functional materials because these natural polymers have excellent properties, such as biocompatibility, biodegradability, non-toxicity adsorption (Majeti and Kumar 2000).

The production of chitin and chitosan is currently based on crab and shrimp shells discarded by the canning industries in Oregon, Washington, Virginia and Japan and by various fishing fleets in the Antarctic. Several countries possess large unexploited crustacean resources (e.g., Norway, Mexico and Chile) (Muzzarelli 1984). To produce 1 kg of 70% deacetylated chitosan from shrimp shells, 6.3 kg of HCl and 1.8 kg of NaOH are required in addition to nitrogen, process water (0.5 t) and cooling water (0.9 t). Important items for estimating the production costs include transportation, which varies depending on labor and location. In India, the Central Institute of Fisheries Technology, Kerala, initiated research on chitin and chitosan. From their investigation, they found that dry prawn waste contained 23% chitin, and dry squilla contained 15% chitin (Madhavan and Nair 1974). They have also reported that the chitinous solid waste fraction of the average Indian landing of shellfish ranges from 60,000 to 80,000 tons (Majeti and Kumar 2000).

Chitosan exhibits antitumor (Qin et al. 2002), antimicrobial (Yang, Chou and Li 2005) and antimutagenic (Kogan et al. 2004) activity. Although chitosan displays very strong biological activities, its insolubility in water and high molecular weight are drawbacks in a number of applications (Shen et al. 2009). Recent studies of chitosan have increased the interest of its conversion to chitooligosaccharides (COS) because these compounds are water-soluble, have low molar mass and have several functional properties, such as antitumor (Prashanth and Tharanathan 2005; Huang et al. 2006) and antioxidant activity (Chen et al. 2005). Furthermore, they act as free radical scavengers (Je, Park and Kim 2004) and exhibit anti-hepatotoxic (Chen et al. 2005) and anti-angiogenic activity (Prashanth and Tharanathan 2005).

The degradation of *O*-glycosidic bonds in chitosan using different methods favors the production of COS with different degrees of polymerization as well as the number and sequence of glucosamine (GlcN) and GlcNAc units. These methods include acid hydrolysis (Il'ina and Varlamov 2004), enzymatic hydrolysis (Kuroiwa et al. 2002) and oxidative degradation (Shirui et al. 2004). Enzymatic hydrolysis has been proposed as the preferred method for the production of bioactive COS for the past few decades. A host of chitosanlytic enzymes (chitosanases) have been obtained from different microorganisms, such as fungi (Muzarelli et al. 1994; Kim et al. 1998) and bacteria (Lee et al. 1996; Varum et al. 1996).

The search for compounds that can inhibit tumor cell proliferation has resulted in the emergence of cytotoxicity tests that measure viability and in vitro cell proliferation. In vitro methods have a number of advantages over their in vivo counterparts, such as limiting the number of experimental variables, obtaining significant data more easily and often requiring a shorter test period (Rogero et al. 2003). A number of researchers have studied the antitumor activity of oligomers obtained from chitosan and their mechanisms for inducing cell death (Pae et al. 2001; Huang et al. 2006). Accordingly, the aim of this study was to assess cytotoxicity and cell proliferation in 3T3 (fibroblast), HepG2 (hepatocellular carcinoma) and HeLa (adenocarcinoma) cell lines treated with either pure oligosaccharides acquired from Seikagaku Corp. (Japan) or a mixture of oligosaccharides obtained from the enzymatic hydrolysis of chitosan using a crude enzyme extract produced by the fungus *Metarhizium anisopliae*. Their respective antioxidant activities were then evaluated in vitro.

Materials and methods

Reagents

Chitosan (85% deacetylated, molecular weight (MW): 90–190 Da) and standard glucosamine were purchased from Sigma-Aldrich (MO, USA). Chitosan oligomer standards (dimers, trimers, tetramers, pentamers and hexamers) were acquired from Seikagaku Corp. (Japan). The other reagents used were of the highest quality available.

Preparation of chitosan

Water-soluble chitosan was obtained using the modified Yabuki et al. (1987) method.

Chitooligosaccharide production

Chitosan oligomer production was achieved by enzymatic hydrolysis using the crude extract of chitosanlytic enzymes from the fungus *M. anisopliae* (Dou et al. 2007). The reactions were carried out in a mixture containing a total volume of 1.0 ml (0.5 ml chitosan solution, 10 mg/ml and 0.5 ml crude enzyme extract). The reactions were incubated at 55°C for 10, 30 and 40 min. The reaction was interrupted by immersing the tube in boiling water for 10 min (Assis et al. 2010). The hydrolysates, denominated here as hydrolysate A, B, and C were analyzed in HPLC.

HPLC analysis of the hydrolysates

Chitosan oligosaccharides were detected by High Performance Liquid Chromatography (HPLC) using a Shim-Pack CLC-NH_2 column (Shimadzu Co., Japan). Oligomer analysis was performed in HPLC with an acetonitrile (60%) mobile phase, a flow of 0.8 ml/min and a RI detector. $GlcN_{n=1-6}$ peaks were identified and estimated using a standard calibration curve (1–10 mg/ml) according to Liang's

equation (Liang et al. 2007). The total number of oligomers obtained by enzymatic hydrolysis was considered to be 100% for the calculation of oligomer proportions.

Cell culture

Embryonic 3T3 fibroblasts (ATCC CCL-164) were provided by Prof. Carmen Ferreira (Department of Biochemistry, UNICAMP, Brazil). Hepatocarcinoma HepG2 (ATCC–HB8065) and cervical adenocarcinoma HeLa cells (ATCC CCL-2) were also used in this work. The cells were grown in DMEM supplemented with 10% newborn calf serum (CUTILAB, Campinas-SP, Brazil) and penicillin/streptomycin (1 μg/ml) (Sigma-Aldrich, St. Louis, USA). The cells were incubated at 37°C for 72 h in a humidified incubator containing 5% CO_2.

Cell proliferation assay

The in vitro proliferation assays were conducted using 0.1 ml (5×10^3 cells) of each cell suspension transferred to 96-well polystyrene plates. Cells were allowed to adhere for 12 h. Before the addition of chitosan oligomers, the medium containing non-adhered cells was removed, and the oligomer-containing medium (from 0.1 to 1 mg/ml) was then added and tested along with a control containing no oligomers. Cell growth after 72 h of incubation was assessed using the MTT method.

MTT assay

After treatment, the cytotoxic effect of chitosan oligomers on HepG2, HeLa and 3T3 was determined using the MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide)] method described by Mosmann (1983) with modifications. Briefly, the cells were washed with PBS at 37°C and supplemented with 100 μl of serum-free medium containing 0.5 mg/ml of MTT in each well. After 4 h of incubation, the culture medium was removed, and 100 μl of isopropyl alcohol was added to each well for formazan solubilization. The plates were agitated for 10 min and absorbance was measured with a plate-reading spectrophotometer at 570 nm (Denizot and Lang 1986). Absorbance of the treated cells was compared with that of the control, and cells exposed only to normal medium were considered 100% viable.

Determination of total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by chitooligosaccharides and subsequent formation of a green phosphate/Mo (V) complex at acidic pH (Smiirnoff and Cumbes 1989). The tubes containing chitooligosaccharides and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95°C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as ascorbic acid equivalent.

Hydroxyl radical scavenging activity assay

The scavenging activity of chitooligosaccharides against hydroxyl radicals was investigated using Fenton's reaction ($Fe^{2++}{\cdot}H_2O_2 \rightarrow Fe^{3++}{\cdot}OH^- + OH^-$). These results were expressed as an inhibition rate. Hydroxyl radicals were generated using a modified method (Dasguptaa and De 2007) in 3 ml sodium phosphate buffer (150 mM, pH 7.4), which contained 10 mM $FeSO_4{\cdot}7H_2O$, 10 mM EDTA, 2 mM sodium salicylate, 30% H_2O_2 (200 ml) and varying chitooligosaccharide concentrations. In the control, sodium phosphate buffer replaced H_2O_2. The solutions were incubated at 37 C for 1 h, and the presence of hydroxyl radicals was detected by monitoring absorbance at 510 nm.

Superoxide radical scavenging activity assay

The assay was based on the capacity of chitooligosaccharides to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the riboflavin–light–NBT system (Bilan et al. 2007). Each 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 mM riboflavin, 100 mM EDTA, 75 mM NBT and 1 ml of sample solution. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after a 10 min illumination from a fluorescent lamp. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes with the reaction mixture were kept in the dark and served as blanks.

Reducing power

The reducing power of the samples was quantified as later described (Wang et al. 2008). Briefly, 4 ml of reaction mixture, containing different sample concentrations in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1% w/v) at 50°C for 20 min. The reaction was terminated with the addition of TCA solution (10% w/v). The solution was then mixed with distilled water and ferric chloride (0.1% w/v) solution, and the absorbance was measured at 700 nm. The result was expressed as a percentage of activity seen with 0.2 mg/ml of vitamin C.

Ferric chelating

The ferrous ion chelating ability of the samples was investigated according to the method described by previous studies (Costa et al. 2010). Briefly, the reaction mixture containing $FeCl_2$ (0.05 ml, 2 mM) and ferrozine (0.2 ml, 5 mM) was shaken and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm and compared against a blank.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was done via one-way Anova using SIGMAStat version 2.01 software. Student–Newmans–Keuls post-tests were performed for multiple group comparison. In all cases statistical significance was determined at $P < 0.05$.

Results and discussion

Chitosan hydrolysate composition

Chitosan hydrolysates contain a mixture of oligomers. The percentage of each chitooligosaccharide is presented in Table 1. Chitosan monomer concentration increases with hydrolysis time, whereas the concentration of tetramers and pentamers decrease. Trimer percentage remains practically constant over the different hydrolysis times.

Oligosaccharide production during enzymatic hydrolysis of chitosan is influenced by the hydrolytic capacity of the enzyme used (non-specific enzymes or chitosanases). According to Cabrera and Custem (2005), the hydrolysis potential (enzymatic activity) and stability of each enzyme differ according to the conditions of which it is submitted and its microbial origin. In this work, the conditions used for oligomer production were determined in prior assays that indicated an ideal chitosan concentration of 10 mg/ml, enzymatic hydrolysis temperature of 55°C and pH 5.5 (data not shown). Liang et al. (2007) determined chitin oligomer production, using a crude enzyme extract of *Bacillus amyloliquefaciens* V656, which showed moderate endo and exochitinase activity. The selective production of glucosamine (GlcN) from chitinolytic material was accompanied by the cooperative action of both enzymes. Trimers $(GlcN)_3$, tetramers $(GlcN)_4$ and pentamers $(GlcN)_5$ were also produced.

Table 1 Effects of hydrolysis time on the yield of COS: $(GlcN)_{1,3,4,5}$

Compound	(GlcN) (%)	$(GlcN)_3$ (%)	$(GlcN)_4$ (%)	$(GlcN)_5$ (%)
A	75	11	7	6
B	81	11	8	[a]
C	88	12	[a]	[a]

Chitosan was hydrolysed by the raw extract of an enzyme a solution from the fungus *M. anisopliae* at 55°C for 10, 30 and 40 min

Total concentration of olygomers was considered 100%

[a] Not detected

Assessment of cell proliferation

We investigated the antiproliferative activity of chitosan oligomers in normal (3T3) and tumor cells (HepG2 and HeLa) using the MTT assay. In this study, normal cells are used as standard to compare the antiproliferative activity of chemical mixtures. In this study, 3T3 cells (embryonic fibroblasts) were used to assess proliferation and cell toxicity.

As shown in Table 1, only COS glucosamines, trimers, tetramers and pentamers are present in the compounds A, B and C. Thus, the effect of these COS on cell proliferation was also analyzed. Figure 1 shows the antiproliferative activity (%) of cells treated for 72 h using these COS at concentrations ranging from 0.1 to 1 mg/ml.

Figure 1 shows the results obtained when the cells were treated with chitosan hydrolysates (compounds A, B and C). The hydrolysates A and B did not cause significant inhibition of 3T3 and HepG2 cell proliferation. Only compound C inhibited proliferation (~20%). Furthermore, HeLa proliferation was inhibited by chemical mixtures. Higher activity was observed when compound A was used at 0.5 mg/ml; in this case the rate of inhibition was about 60%.

The trimers, tetramers and pentamers showed a weak antiproliferative activity. However, the latter showed a high antiproliferative activity against 3T3 cells (~90%). The higher activity was observed with glucosamine. This compound (0.8 mg/ml) inhibited proliferation of all cell lines o the same degree (about 90%).

Chitosan oligomers have attracted considerable attention due to their antitumor properties (Qin et al. 2002; Shen et al. 2009; Huang et al. 2006), which was first reported in 1970 (Cabrera and Custem 2005). This activity was suggested because of the cationic properties exerted on amino acid clusters. It would subsequently be accepted that molecular weight also plays an important role in their antitumor activity (Qin et al. 2002). According to our results obtained for the pure oligomers, the variations exhibited by COS in different cell lines indicate that their activity varies depending on the specific properties of each cell. In addition, an increase in the degree of polymerization did not lead to further inhibition of cell proliferation. In fact, glucosamine was more potent than other COS.

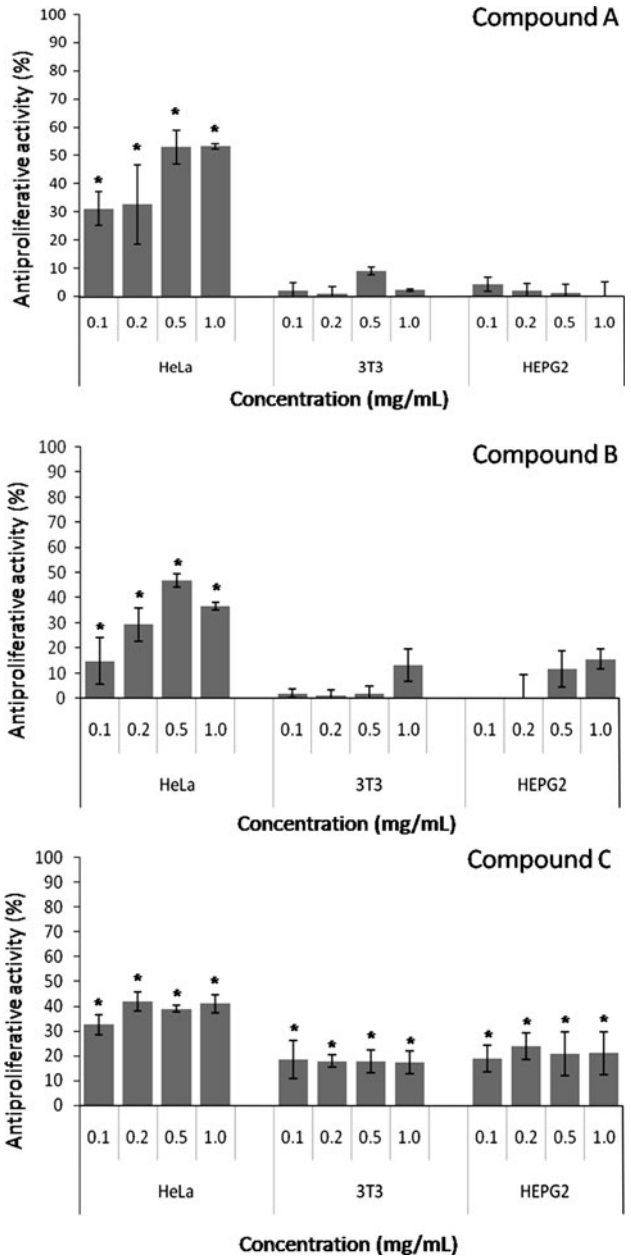

Fig. 1 Antiproliferative effects of oligomer mixtures on HepG2, HeLa and 3T3 cells. The cells were treated with different concentrations of the oligomer mixture: compound A [(GlcN), $(GlcN)_3$, $(GlcN)_4$, $(GlcN)_5$] compound B [(GlcN), $(GlcN)_3$, $(GlcN)_4$] and compound C [(GlcN), $(GlcN)_3$] for 72 h. In the absence of these compounds, the reduction of MTT was considered as being 0.0%. * $P < 0.05$

Glucosamine, a naturally occurring amino sugar, is an important carbohydrate component of many glycoproteins, glycolipids and glycosaminoglycans (Liu and Zeng 2009). Few studies have shown any toxic effects of glucosamine against cancer in vivo or in vitro. Quastel and Cantero (1953) administered glucosamine to tumor-bearing rats and observed retraction of the nucleus and cytoplasm and marked eosinophilia in the neoplastic tissue within 2 h. Rubin et al. (1954) reported that the incubation of Sarcoma 37 cells with D-glucosamine caused extensive cell degeneration. Fjelde et al. (1954) observed that glucosamine caused marked inhibition of epidermoid carcinoma cell growth in tissue culture. Using histological examinations they observed striking nuclear alterations and cytoplasmic granulation in neoplastic cells treated with glucosamine. Molnar and Bekesi (1972) verified cytoplasmic and nuclear alterations in Ehrlich cells exposed to glucosamine. The cytotoxic mechanisms of glucosamine are complex and seem to be involved in the concatenation of several different effects (Liu and Zeng 2009). The most prominent and rapid alterations are produced in the cell membrane. Two hypotheses have been suggested for its antitumor activity: the first theory is that glucosamine causes tumor cell lysis through depletion of cell nucleotides; the second postulates that structural disorganization occurs in the cell membrane system (Molnar and Bekesi 1972; Friedmann and Skehan 1980).

Several works have shown that glucosamine exhibits low toxicity against normal tissues (Rubin, Springer and Hougue 1954). However, in this study glucosamine showed high antiproliferative activity on normal 3T3 cells when used in high concentration (0.5 and 1.0 mg/ml). These results corroborate Mori et al. (1997), who determined that high concentrations of glucosamine inhibited the cell proliferation of L929 mouse fibroblasts in culture. The greatest challenge in cancer chemotherapy is to develop selective drugs that are toxic for neoplastic cells without affecting normal host tissue (Friedmann and Skehan 1980). Glucosamine could have potential as an antineoplastic drug, but it is essential to block its toxicity in normal cells.

The compounds produced by enzymatic hydrolysis are composed of a mixture of oligomers, and according to the results of this study, all are composed mainly of glucosamine (from 75 to 88%). However, even in high concentration, the antiproliferative activity of these compounds against HeLa cells was lower than pure glucosamine (Fig. 2). In addition, when the antiproliferative effect was assayed with 3T3 and HepG2 cells, only compound C showed an antiproliferative effect, whereas compounds A and B showed a significant effect. These data indicate that the COS present in compounds A and B seem to impede the mechanism of glucosamine toxicity. Further work will be needed to identify the mechanism by which these COS exert their effect.

Antioxidant activity

Antioxidant activity was evaluated by assaying total antioxidant capacity (TCA), hydroxyl and superoxide radical scavenging ability, ferric chelating power and reducing

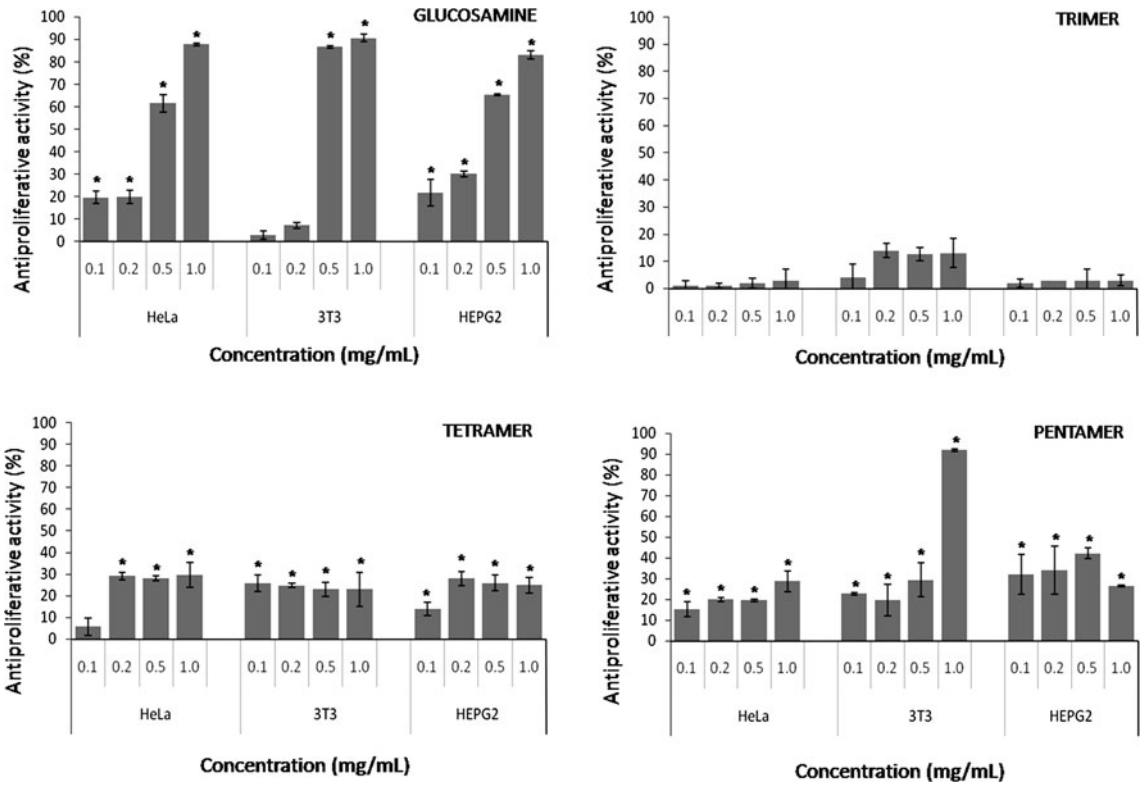

Fig. 2 Antiproliferative effects of oligomers on HepG2, HeLa and 3T3 cells. The cells were treated with different concentrations of (GLcN), $(GLcN)_3$, $(GLcN)_4$, $(GLcN)_5$ oligomers for 72 h. In the absence of these compounds, the reduction of MTT was considered as being 0.0%. * $P < 0.05$

power. Total antioxidant activity (expressed in equivalents of ascorbic acid) demonstrated that the pentamer was the compound that exhibited the highest total antioxidant activity (62.74 mg/g equivalents of ascorbic acid) (Fig. 3). None of the chitosan hydrolysates (A, B, and C) showed significant total antioxidant activity ($P > 0.05$).

Hydroxyl radical and superoxide anion scavenging and ferric chelating in the presence of COS are shown in Table 2. Any single compound analyzed had neither hydroxyl radical scavenging nor ferric chelating activity. However, all compounds showed superoxide radical scavenging activity. The pentamer was the pure oligomer that exhibited the highest activity (53%) with approximately 1.6-fold less than gallic acid, at 0.5 mg/ml. All compounds obtained from chitosan hydrolysis showed superoxide anion scavenging activity; the greatest activity was exhibited by hydrolysate A (58%) with approximately 1.5-fold less than gallic acid, at 0.5 mg/ml. The IC_{50} values found in this assay were: glucosamine (2.2 mg/ml), trimer (1.4 mg/ml), tetramer (3.4 mg/ml), pentamer (0.6 mg/ml), A (0.05 mg/ml), B (0.9 mg/ml), and C (1.6 mg/ml).

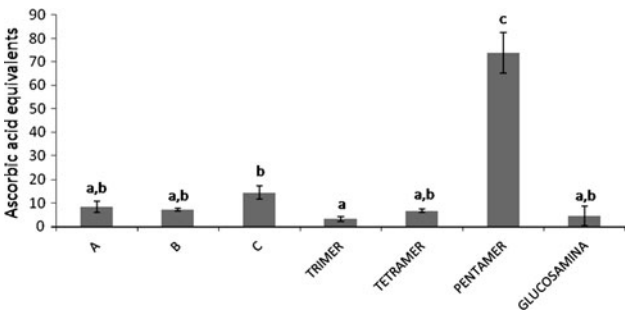

Fig. 3 Total antioxidant capacity of COS using compound A [(GlcN), $(GlcN)_3$, $(GlcN)_4$, $(GlcN)_5$], compound B [(GlcN), $(GlcN)_3$, $(GlcN)_4$] and compound C [(GlcN), $(GlcN)_3$]. The results are expressed as ascorbic acid equivalents. Data are expressed as means ± standard deviation. *Different letters* indicate a significant difference between COS via one-way Anova followed by Student–Newman–Keuls test ($P < 0.05$)

Reducing power was expressed as a percentage, using ascorbic acid as control (0.2 mg/ml), and the data are shown in Fig. 4. The sugars are arranged from highest to lowest activity, which ranged from 30 to 92%. Thus, two groups were clearly distinguishable; the first group included compounds A and B, glucosamine, tetramer and trimer; the second group included compound C (92%) and the pentamer (72%).

The beneficial properties of COS have recently been recognized and exploited in the development of nutritional food (Qin et al. 2006). The beneficial health effects of COS can be largely attributed to its antioxidant activity (Xu et al.

Table 2 Hidroxil and superoxide radical scavenging and Ferric chelating activity of COS

COS	Concentration (mg/ml)	Inhibition (%)		
		O_2	OH	Fe^{++}
Glucosamine	0.10	20.5 ± 0.0	[a]	[a]
	0.20	24.7 ± 8.5	[a]	[a]
	0.50	33.6 ± 9.2	[a]	[a]
	1.00	32.0 ± 7.0	[a]	[a]
Trimer	0.10	3.2 ± 2.5	[a]	[a]
	0.20	9.1 ± 4.1	[a]	[a]
	0.50	16.2 ± 8.0	[a]	[a]
	1.00	36.6 ± 1.9	[a]	[a]
Tetramer	0.10	3.5 ± 2.1	[a]	[a]
	0.20	3.1 ± 3.5	[a]	[a]
	0.50	16.4 ± 2.2	[a]	[a]
	1.00	14.2 ± 1.2	[a]	[a]
Pentamer	0.10	45.7 ± 3.9	[a]	[a]
	0.20	44.2 ± 0.2	[a]	[a]
	0.50	48.9 ± 6.9	[a]	[a]
	1.00	53.1 ± 1.2	[a]	[a]
Compound A	0.10	48.5 ± 0.0	[a]	[a]
	0.20	51.8 ± 4.2	[a]	[a]
	0.50	57.1 ± 8.6	[a]	[a]
	1.00	58.0 ± 8.8	[a]	[a]
Compound B	0.10	5.3 ± 0.0	[a]	[a]
	0.20	42.6 ± 8.3	[a]	[a]
	0.50	41.6 ± 6.4	[a]	[a]
	1.00	48.2 ± 6.8	[a]	[a]
Compound C	0.10	15.5 ± 3.1	[a]	[a]
	0.20	28.1 ± 8.6	[a]	[a]
	0.50	38.9 ± 0.2	[a]	[a]
	1.00	40.0 ± 6.4	[a]	[a]
Gallic acid	0.10	30.0 ± 3.0	12.6 ± 1.0	[a]
	0.20	40.0 ± 4.1	44.6 ± 2.7	[a]
	0.50	73.1 ± 2.5	63.7 ± 4.0	[a]
	1.00	87.1 ± 3.1	94.5 ± 6.0	[a]
EDTA	0.10	[a]		31.3 ± 0.7
	0.20	[a]		37.8 ± 2.7
	0.50	[a]		45.4 ± 1.1
	1.00	[a]		66.8 ± 0.8

[a] Not detected

2010). Any single compound analyzed has neither hydroxyl radical scavenging nor ferric chelating activity. However, all showed superoxide anion scavenging activity. Previously, it was reported that the scavenging mechanism of chitosan is due to the fact that the free radical can react with the residual free amino group NH_2 to form ammonium groups NH_3^+ by absorbing a hydrogen ion from solution (Xie et al. 2001). This can explain superoxide anion scavenging activity of the COS as well as the compounds A, B and C. The more potent superoxide anion scavenging activity was found with compound A ($P < 0.05$). In addition, the activity of the compounds C and B did not show a significant difference ($P < 0.569$). Compound A is the only one that presents pentamers in its composition, which can explain its high activity in comparison to compounds B and C. This is consistent with the observation that the pentamer was the pure oligomer that exhibited the highest activity (53%) (Table 2).

All the molecules analyzed here displayed reducing power, with compound C showing stronger reducing power. It has been previously reported that there was a direct correlation between antioxidant activity and

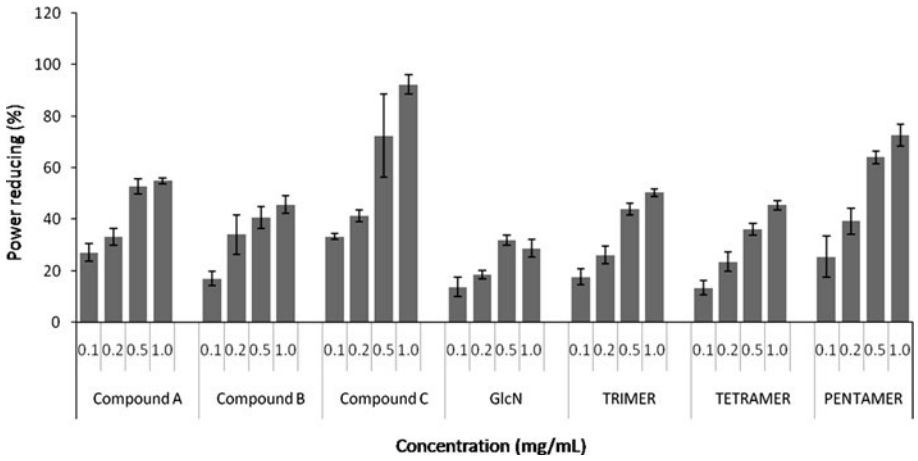

Fig. 4 Reducing power of COS using compound A [(GlcN), $(GlcN)_3$, $(GlcN)_4$, $(GlcN)_5$], compound B [(GlcN), $(GlcN)_3$, $(GlcN)_4$], and compound C [(GlcN), $(GlcN)_3$]. Data are expressed as means ± standard deviation. Reducing power is expressed as a percentage of activity as compared to 0.2 mg/ml of ascorbic acid

chitooligosaccharides reducing power. The reducing activities were usually related to the development of reductones. Reductones were reported to be terminators of free radical chain reactions by donating a hydrogen atom. Irrespective of the stage in the oxidative chain, in most cases where the antioxidant action is assessed, most non-enzymatic antioxidative activity is mediated by redox reactions (Costa et al. 2010). Thus, several researchers have reported that the antioxidant activity was concomitant with the reducing power. Zhang et al. (2010) showed that chitooligosaccharides with high hydrogen donating ability displayed excellent reducing power. Therefore, the presence of NH_2 groups in COS is probably responsible for its reducing activity. In addition, our data suggest that the reducing power of COS contributes to the antioxidant activity observed in this work.

Conclusions

Glucosamine has a higher antiproliferative activity against tumor cells. However, it is also active against normal cells. For the use of this monosaccharide as an antitumor drug, it is important to block or decrease its antiproliferative effect against normal cells. The oligomers produced by the raw extract of the enzyme from the fungus *M. anisopliae* show that the COS mixtures exert a pronounced effect on HeLa cells but not on HepG2 cells. Furthermore, the oligomers obtained (A, B, C) demonstrated no toxic effects on the normal cells (3T3). Additionally, we showed that a small amount of other COS can decrease the cytotoxic effect of glucosamine on 3T3 cells, which indicates that glucosamine could be used as an antitumor drug in the presence of other COS. In addition, different effects were found in antiproliferative assays, depending on the COS composition in the compounds (A, B and C), showing that the combination between them may be essential for developing antineoplastic drugs. In addition to the antiproliferative effect of COS on tumor and non-tumor cells, we studied the in vitro antioxidant properties that these compounds exhibit. Superoxide anion scavenging ability was the main antioxidant activity exhibited by COS. This activity was also dependent on the oligomer composition in the chitosan hydrolysates. Further work will be needed to identify the ideal proportions of COS and glucosamine for several biological activities of interest.

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