

## Cytotoxicity of Chitosan Oligomers Produced by Crude Enzyme Extract from the Fungus *Metarhizium Anisopliae* in HepG2 and Hela Cells

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### Abstract

Chitoooligosaccharides exhibit biological activities, including antitumor, antimicrobial and antioxidant. In this study we used a mixture of chitoooligosaccharides produced by enzymatic hydrolysis in two tumor cell lines and assessed the cell proliferation and cytotoxicity of these compounds. The proliferation of HeLa cells was inhibited around by 60%.

**Keywords:** Chitoooligosaccharides; HepG2 cells; HeLa cells; Cytotoxicity

### Introduction

In recent years, the research for more efficient alternatives in the treatment of infectious and neoplastic diseases has mobilized professionals from a host of different areas. Promising results have emerged from the use of substances produced by microorganisms. Chitosanolytic enzymes from different microorganisms, including fungi [1] and bacteria [2], have been reported and used with excellent results in the production of chitoooligosaccharides (COS)[3]. Chitoooligosaccharides are partially hydrolyzed products of chitosan, within which pentamers and hexamers can be obtained as intermediate reactions [4].

Two methods can be used to obtain chitoooligosaccharides: chemical and enzymatic. Chemical hydrolysis is carried out using high temperatures under acidic conditions and produces a large amount of glucosamine (chitosan monomer), compromising control over the reaction progress. This method produces low pentamer and hexamer yields. Enzymatic hydrolysis has a number of advantages for the production of chitoooligosaccharides and some chitosanases may catalyze hydrolysis under mild conditions and not produce monosaccharides [4].

COS is applied more widely than chitosan in health-care, food, medicine, pesticides and feedstuffs. The anti-tumor activity of COS has been known since 1986 [5], and several mechanisms have been proposed. These include the regulation of immunity [6], the direct killing of tumor cells, or causing tumor cell apoptosis and inhibiting tumor angiogenesis [7].

The aim of this study was to quantify and analyze the chitoooligosaccharides produced during 20 minutes of enzymatic hydrolysis of chitosan produced by fungus *Metarhizium anisopliae* and evaluates cell viability of these compounds in hepatocarcinome (HepG2) and uterine carcinome (HeLa) cells. The 3T3 cell lines, which are fibroblast cells, were used as standard non-toxic concentrations.

### Materials and Methods

#### Fermentation conditions

The fungus *Metarhizium anisopliae* was kindly gifted by Embrapa Recursos Genéticos e Biotecnologia (Brasília/DF-Brazil). Ten milliliters of spore suspension ( $10^7$  spores/mL) from a 5-day culture in PDA medium were transferred using 2 mL of sterile water. Next, this spore suspension was once again transferred to a 250 mL Erlenmeyer containing 90 mL of culture medium consisting of: 0.2% chitosan, 0.1%

$K_2HPO_4$ , 0.05%  $MgSO_4$ , 0.5 % KCl, 0.3% yeast extract, 0.5% peptone, 0.2%  $NaNO_3$ , 0.001%  $FeSO_4$  (pH 5.5); growth took place in a rotating incubator (110 rpm) for 2 days at 25°C. From this suspension, 10 mL was transferred to 90 mL of the same medium. After 48 hours of culture the broth was centrifuged at  $13400 \times g$  for 15 minutes and the supernatant was used to determine the enzymatic activity assay.

#### Enzyme activity

Enzymatic activity was assessed by determining the reducing sugars generated by chitosan hydrolysis. In this case, 500  $\mu$ L of the fermented broth was mixed with 500  $\mu$ L of chitosan solution solubilized in hydrochloric acid (0.1 N). The reaction was carried out for 30 min at 55°C. To terminate the reaction, 2.5 mL of dinitrosalicylic acid was added and then cooled in an ice bath, and quantification of the reducing sugars was performed using a spectrophotometer (Thermo Spectronic) at 600 nm [8] and a standard curve with D-glucosamine. One unit (U) of chitosanase was defined as the amount of enzyme that is capable of releasing 1  $\mu$ mol of reduced sugar equivalent to chitosan D-glucosamine/min.

#### Chitoooligosaccharide production

The hydrolysis reaction of chitosan to obtain the chitoooligosaccharides consisted of mixing a solution of 1% (m/v) soluble chitosan in chloridric acid (pH adjusted with NaOH to 5.5) to the broth fermented at a ratio of 1:1; the mixture was maintained at 55°C for 20 minutes. The reaction was finalized by the thermal inactivation of the enzyme at 100°C for 10 minutes, followed by centrifugation at  $13400 \times g$  for 15 minutes and filtration in a 0.22  $\mu$ m filtering membrane. Detection of glucosamine and of chitosan oligosaccharides from dimer to hexamer was determined by high-performance liquid chromatography using a CLC-NH<sub>2</sub> Shim-Pack column (Shimadzu Co, Japan). Oligomer analy-

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sis was performed in HPLC with 60% acetonitrile as mobile phase and flow rate of 0.8 mL/min and using an RI detector. The peaks of  $(\text{GlcN})_{n=1-6}$  were identified and estimated using a standard calibration curve (1-10 mg/mL) according to Liang's equation [9].

## Cell culture

Embryo 3T3 fibroblast cells (ATCC CCL-164), HepG2 hepatocarcinoma cells (ATCC HB-8065) and cervical adenocarcinoma HeLa cells (ATCC CCL-2) were used for viability and cytotoxicity. The cells grew in DMEM medium (Dulbecco's Modified Eagle Medium) supplemented with 10% newborn calf serum (CUTILAB, Campinas-SP, Brazil) and penicillin/streptomycin (1µg/mL) (Sigma-Aldrich, St. Louis, USA). The cells were incubated in oven at 37°C under moisture atmosphere containing 5% CO<sub>2</sub>. The viability assays were conducted *in vitro* as follows: 100µL (5x10<sup>3</sup> cells) of cell suspension was plated in 96-well polystyrene plates. Cell adhesion was conducted for 12h. Before the addition of chitosan oligomers, the medium with the non-adhered cells was removed and the medium containing the oligomers (from 0.1 to 1 mg/mL) was used. A control without oligomers was also used. After treatment, the cytotoxic effect of the chitosan oligomers in HepG2, HeLa 3T3 cells was determined using the MIT method described by Mosmann [10] with some modifications. The cells were washed with PBS at 37°C and then 100 µL of serum-free medium containing 0.5mg/mL of MTT was added to each well. After 4 hours of incubation, the culture medium was removed and 100 µL of isopropyl alcohol was added to each well for solubilization of the formazan formed. The plates were agitated for 10 minutes and the mean absorbance of the plate was measured in the spectrophotometer at 570 nm [11]. The absorbance of the treated cells was compared with that of the control. The control cells were considered 100% viable, whereas the percentage of cell growth inhibition was calculated for those treated with the hydrolysate. All the analyses were conducted in triplicate.

## Statistic analysis

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

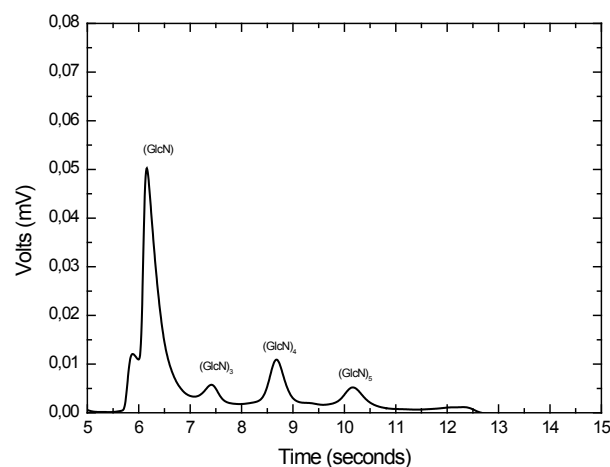
## Results and Discussion

The products of chitosan hydrolysis were analyzed by HPLC. Figure 1 shows the chromatogram obtained for a hydrolysis time of 20 minutes using the crude enzyme extract. This figure also demonstrates the formation of monomers (GlcN), trimers  $(\text{GlcN})_3$ , tetramers  $(\text{GlcN})_4$  and pentamers  $(\text{GlcN})_5$  at concentrations of 6.532; 0.792; 0.395 and 0.352 (mg/mL), respectively. The monomer (glucosamine) shows the highest concentration. Such a polymer profile suggests the presence of enzymes with exo and endochitosanase activity [12,13].

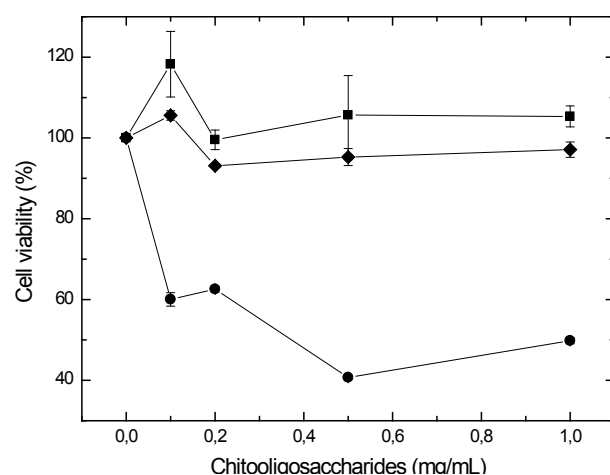
Figure 2 shows the cytotoxicity of the tested compounds in tumor and normal cells. In the MTT assays we used different hydrolysate concentrations containing a mixture of chitoooligosaccharides (0.1 – 1 mg/mL) in the different cell lines.

The cells were treated with different concentrations of the supernatant, for 72 hours. In the absence of these compounds the reduction of MTT was considered as being 100%. The experiment was carried out in 96-well plates. The results represent the mean ± SD of three experiments in triplicate ( $p < 0.05$ ).

The 3T3 cells were used as control to assess the toxicity of the com-



**Figure 1:** Chromatograms of hydrolyzed chitosan formed by the incubation of chitosan with the crude enzyme extract during 20 minutes.



**Figure 2:** Cytotoxic assessment of the effect of the oligomer mixture in HepG2 (■), HeLa (●) and 3T3 (◆) cells.

pound. When these cells were treated with the hydrolysate containing the chitosan oligomers, no alteration in cell proliferation was detected. Thus, the tumor cells (HepG2 and HeLa) were treated with concentrations that were non-toxic to normal cells.

The HepG2 cells, when treated with the chitoooligosaccharides at a concentration of 0.2 mg/mL, induced cell viability of around 20% and at higher concentrations, it remained practically unaltered. These results corroborate literature data, in that chitoooligosaccharides did not show any activity against Hep3B cells [14,15].

In the HeLa cells, diminished cell viability was directly proportional to the increase in concentration, where cell viability decreased by 60% at a concentration of 0.5 mg/mL. Similar results were found by Jeon and Kim [16], where chitoooligosaccharides inhibited uterine tumors in rats by 73.6%. The modified chitoooligosaccharides used in HeLa cells showed an IC<sub>50</sub> value of 0.45 mg/mL [14] and similar results were found in this study, where IC<sub>50</sub> was 0.67 mg/mL. Kim and Rajapakse [12] observed that a mixture of chitosan oligomers from tetramer to pentamer could inhibit tumor cell growth in rats. It was observed

that HeLa cells were more sensitive than the other ones showing that the biological functions of chitoooligosaccharides depend not only on the degree of polymerization, but also on its molecular weight [12-16].

## Conclusion

According to the results obtained, chitoooligosaccharides may reduce the viability of HeLa tumor cells. The effect of the antitumor activity of chitoooligosaccharides is mainly due to stimulation in the immune system, increasing *in vivo* macrophage activity. However, according to our findings, chitosan oligomers act directly on HeLa tumor cells, decreasing cell proliferation and viability. Moreover, it is interesting to observe the non-toxicity of the compound in 3T3 fibroblast cells.

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