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Enzymatic production of fully deacetylated chitooligosaccharides and their neuroprotective and anti-inflammatory properties

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Keywords

Chitooligosaccharides; Chitosan; Chitosanolytic enzymes; Bioactive oligosaccharides; Chitosanase; Neuroprotective substances.

ABSTRACT

Among several commercial enzymes screened for chitosanolytic activity, Neutrase 0.8L (a protease from *Bacillus amyloliquefaciens*) was selected in order to obtain a product enriched in deacetylated chitooligosaccharides (COS). The hydrolysis of different chitosans with this enzyme was followed by size exclusion chromatography (SEC-ELSD), mass spectrometry (ESI-Q-TOF), and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Neutrase 0.8L converted 10 g/L of various chitosans into mostly deacetylated oligosaccharides, yielding approximately 2.5 g/L of chitobiose, 4.5 g/L of chitotriose and 3 g/L of chitotetraose. We found out that the neutral protease was not responsible of the chitosanolytic activity in the extract, whilst it could participate in the deacetylating process. The synthesized COS were tested *in vitro* for their neuroprotective (towards human SH-S5Y5 neurons) and anti-inflammatory (in RAW macrophages) activities, and compared with other functional ingredients, namely fructooligosaccharides.

1. INTRODUCTION

Chitin, the second most abundant polymer in nature after cellulose, is formed by N-acetyl-glucosamine (GlcNAc) moieties linked by β(1→4) bonds. It is a cheap and available feedstock that can be obtained from crustacean shells (more than 10,000 tons per year) and fungi (Hamed et al. 2016); however, its applicability is limited by its low aqueous solubility. Total or partial deacetylation of chitin, by chemical or enzymatic methods, yields chitosan, a biodegradable and non-toxic biopolymer that is employed as drug carrier (Varshosaz et al. 2006), in bone repair (Jayakumar et al. 2010, Muzzarelli 2009), gene therapy (Jayakumar et al. 2010, Muzzarelli 2009) and as enzyme support (Sjoholm et al. 2009). Chitosan also exhibits antimicrobial, antifungal or anti-hypercholesterolemic activities (Aranaz et al. 2014, Xia et al. 2011), which are dependent on its molecular weight (MW) and deacetylation degree (DD). However, some applications of chitosan are limited by its high viscosity and low aqueous solubility at neutral pH.

Chitooligosaccharides (COS) are obtained by partial hydrolysis of chitosan and are formed by random GlcNAc and D-glucosamine (GlcN) units. COS present a degree of polymerization (DP) lower than 20 (MW ≤ 4000), are soluble in water and display antimicrobial, antioxidant, antiviral, antiangiogenic, antitumoral and prebiotic properties (Sanchez et al. 2017, Zou et al. 2016). Their properties strongly depend on the DP and DD, although the structure-function relationships are still quite unknown (Mateos-Aparicio et al. 2016, Mengibar et al. 2011, Mengibar et al. 2013, Xiong et al. 2009). For that reason, the source of chitosan and the hydrolytic method exert a significant influence on COS properties. Some studies suggested that COS might exert neuroprotective effect in rat cortical neurons against Cu^{2+}–induced cellular oxidative stress (Xu et al. 2010), as well as in glucose deprivation-induced cell apoptosis (Xu et al. 2011). Recently, the neuroprotective activity of COS with DP < 10 was assessed in a human neuronal cell line showing potential application in therapies against Alzheimer's disease (Huang et al. 2015).

The hydrolysis of chitosan can be achieved by physical, chemical or enzymatic methods. The two first require extreme reaction conditions and the composition of the final product is difficult to control (Yang et al. 2014). On the other hand, the use of enzymes involves milder conditions (moderate temperature, neutral or slightly acidic pH, atmospheric pressure, etc.) and a better reproducibility of the process.

Several chitosan-hydrolyzing enzymes have been reported, including chitosanases (EC 3.2.1.132, the most specific ones), chitinases (EC 3.2.2.14), exo-β-glucosaminidases (EC 3.2.1.165), exo-β-N-acetylglucosaminidases (EC 3.2.1.52) and chitin deacetylases (EC 3.5.1.41). Interestingly, chitosan is also susceptible to the attack of non-specific enzymes such as lysozyme, proteases, endoglucanases, pectinases and even lipases (Kittur et al. 2003, Thadathil et al. 2014). Chitosanases are endo-acting enzymes that belong to the GH5, GH8, GH46, GH75 and GH80 glycoside hydrolase (GH) families (CAZy database) and are classified into three subclasses depending on their specificity towards GlcN-GlcN and GlcNAc-GlcN linkages. Exo-β-glucosaminidases and exo-β-N-acetyl-hexosaminidases cleave GlcN and GlcNAc, respectively, from the non-reducing end of chitosan (Thadathil et al. 2014).

Although some chitosanases –mainly from *Bacillus sp.* (Choi et al. 2004)– have been isolated and cloned in heterologous microorganisms for overexpression (Pechsrichuang et al. 2013), further optimization is still required for their use in industry. Interestingly, some commercial enzyme preparations are able to hydrolyze chitosan (Pantaleone et al. 1992). Such activity is usually low, probably because

chitosanolytic activity represents a minor contribution to total enzyme activity in such extracts (Fu et al. 2003).

In this work we describe the screening of chitosanolytic activity in a series of commercial enzymes, followed by the identification and quantification (by the combined use of chromatographic and mass spectrometry techniques) of the COS synthesized by a neutral protease preparation. The neuroprotective and anti-inflammatory activities of the synthesized COS were further assessed.

2. MATERIALS AND METHODS

2.1. Enzymes and chemicals

Rapidase TF and Klerzyme 150 were kindly donated by DSM (Heerlen, NL). Pectinex Ultra SP-L, Neutrase 0.8L, NovoShape, Ultraflo L, Shearzyme 2X, Pentopan Mono Conc. BG, Flavourzyme and Alcalase were gracefully donated by Novozymes (Bagsvaerd, Denmark). β-Glucanase from *Bacillus amyloliquefaciens* (E-CELBA) was acquired from Megazyme (Wicklow, Ireland). Chitosan CHIT100 (100-300 kDa, DD ≥ 90%) and CHIT600 (600-800 kDa, DD ≥ 90%) were acquired from Acros Organics (Thermo Fischer Scientific Inc., Waltham, MA). Chitosan QS1 (98 kDa, 81% DD) and QS2 (31 kDa, 77% DD) were produced by InFiQuS. COS standard (MW ≤ 2000, DD ≥ 90%) was purchased from Qingdao BZ Oligo Biotech Co. Ltd. (China). D-Glucosamine (GlcN), N-acetyl-glucosamine (GlcNAc) and papain were purchased from Sigma-Aldrich (St. Louis, MO). 1-Kestose (GF2), 1^F-fructofuranosyl-fructose (GF4), chitobiose, chitotriose, chitotetraose, and chitopentaose were purchased from Carbosynth Ltd. (Berkshire, UK). All other reagents were of the highest purity grade.

2.2. Activity assay

Chitosanolytic activity was determined by detection of reducing sugars with a modified 3,5-dinitrosalicylic acid (DNS) method (Ghazi et al. 2007). Prior to the assay, low-molecular-weight contaminants in the enzyme samples were removed with a PD-10 desalting column (GE Healthcare, Uppsala, Sweden). Reactions were performed in 1.5 mL centrifuge tubes by addition of 200 µL of enzyme to 800 µL of 1% (w/v) chitosan CHIT100 dissolved in 50 mM sodium acetate buffer (pH 5.0). Tubes were incubated at 50°C and 1,400 rpm in a Thermo Shaker TS-100 (Boeco, Hamburg, Germany) and reactions were stopped by addition of 0.25 M NaOH in a 1/1 (v/v) ratio. The addition of NaOH also caused the precipitation of the remaining polysaccharide, which was removed by centrifugation at 5,000x g for 10 min. The quantification of reducing sugars in the supernatant was carried out by the DNS method in a 96-well microplate with a calibration curve of D-glucosamine. One unit of activity (U) corresponded to the release of one µmol of reducing sugars per minute.

2.3. SDS-PAGE and zymogram

Proteins were visualized by electrophoresis in denaturing conditions in 12% acrylamide gels. Samples were prepared as follows: 15 µL of commercial preparation conveniently diluted was mixed with 5 µL of 4x loading buffer with β-mercaptoethanol and heated for 10 min at 96°C. Gel was stained with ProtoStain Blue (National Diagnosis, USA) and bands were compared with molecular weight markers (Precision Plus Protein™ All Blue Pre-stained Protein Standards, BioRad, USA).

Chitosanolytic activity in gel was assayed by native polyacrylamide gel electrophoresis in 12% gels without SDS containing 0.1% (w/v) glycol chitosan following the Laemmli method (Laemmli 1970). After electrophoresis the gel was

soaked in 100 mM sodium acetate buffer (pH 5.0) with 1% (v/v) Triton X-100 and incubated for 2 h at 37°C. The gel was washed with distilled water and stained with Congo red (0.1%). The contrast was enhanced for the development of dark blue colour with the addition of 5% (v/v) acetic acid. Chitosanolytic activity was observed as a clear area against a dark blue background.

2.4. Analysis of chitosan hydrolysis by SEC-ELSD

To 960 µL of a 1% (w/v) chitosan solution in 50 mM sodium acetate buffer (pH 5.0), Neutrase (40 µL) was added. Reactions were incubated at 50°C in 1.5 mL centrifuge tubes at 900 rpm with orbital shaking. At different times, aliquots were taken, diluted with water, and filtered with 0.45 µm cellulose filters (Analisis Vinicos, Tomelloso, Spain). Samples were analyzed by size exclusion chromatography (SEC) using a ternary pump (Varian) and a PolySep-4000 column (7.8 x 300 mm, Phenomenex, Torrance, CA) coupled to an evaporative light scattering detector (ELSD 2000ES, Alltech). Mobile phase was 0.25 M ammonium acetate (pH 4.7) at 0.6 mL/min. ELSD conditions were set at 115°C and a nitrogen flow of 3.5 L/min.

2.5. Characterization and quantification of COS by HPAEC-PAD

Reactions were performed as described in Section 2.4. In this case, aliquots were mixed with 0.25 M NaOH in a 1/1 (v/v) ratio to stop the reaction and to precipitate the remaining polysaccharide, which was removed by centrifugation at 5,000x g for 10 min. The supernatant was diluted with water (2.5 mM NaOH final concentration) and analyzed by HPAEC-PAD on a Dionex ICS3000 system (Dionex, Thermo Fischer Scientific Inc., Waltham, MA) consisting of an SP gradient pump, an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode, and an autosampler (model AS-HV). All eluents were degassed by flushing with helium. A peculiar anion-exchange 4 × 250 mm Carbo-Pack PA-200 column (Dionex) connected to a 4 × 50 mm CarboPac PA-200 guard column was used at 30°C. Eluent preparation was performed with Milli-Q water and NaOH. The initial mobile phase was 4 mM NaOH at 0.3 mL/min for 30 min. Then, column was washed for 20 min at 0.5 mL/min with a solution containing 100 mM sodium acetate and 100 mM NaOH, and further equilibrated with 4 mM NaOH. The chromatograms were analyzed using Chromeleon software. The identification and quantification of the different carbohydrates was done on the basis of commercially available standards when available.

Deacetylated COS were produced at 50 mL-scale with 1% (w/v) chitosan CHIT600 in 50 mM ammonium acetate buffer (pH 5.0), and 10% (v/v) Neutrase 0.8L. COS were separated from the enzyme by ultrafiltration with a 10 kDa membrane in an Amicon® system, lyophilized and further dried in a desiccator with phosphorous pentoxide.

2.6. Mass spectrometry

The molecular weight of COS was assessed using a mass spectrometer with hybrid QTOF analyzer (model QSTAR, Pulsar i, AB Sciex). Reaction samples were analyzed by direct infusion and ionized by electrospray (with methanol as ionizing phase) in positive reflector mode.

2.7. Neuroprotective properties

The synthesized COS were assayed *in vitro* in cell cultures to determine their neuroprotective activity. SH-S5Y5 neurons were cultured in collagen-pretreated petri-dishes with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) medium supplemented with penicillin/streptomycin and 10% inactivated fetal bovine serum (iFBS). The assays were done in collagen-pretreated 96 well plates by seeding 2

x 10^4 neurons per well in a 100 µL volume and with 24 h of incubation before compound addition. The deacetylated COS were dissolved in DMSO and then added at different concentrations (0.02, 0.2 and 2 mg/ml) to determine compound toxicity. Final DMSO percentage in each cell was adjusted to 1% DMSO. Cell viability was evaluated 24 h after compound addition by mitochondrial MTT assay, according to manufacturer.

To analyze the neuroprotective effect, neurons were cultured and plated as described in the cell viability assay. COS dissolved in DMSO were added at three concentrations (0.02, 0.2 and 2 mg/mL) and incubated for 10 min before the addition of hydrogen peroxide (100 µM). Cell viability was evaluated 24 h after compound addition by mitochondrial MTT assay.

2.8. Anti-inflammatory properties

The deacetylated COS were also assayed *in vitro* in cell cultures to determine their anti-inflammatory activity. RAW 264.7 macrophages were cultured in DMEM high-glucose medium supplemented with penicillin/streptomycin and 10% iFBS. The assays were done in 96-well plates by seeding 2.5 x 10^4 macrophages per well in a 100 µL volume with 4 h of incubation time before compound addition. The deacetylated COS were dissolved in DMSO and then added at different concentrations (0.02, 0.2 and 2 mg/ml) to determine compound toxicity. Final DMSO percentage in each cell was adjusted to 1% DMSO. Cell viability was evaluated 24 h after compound addition by mitochondrial MTT assay.

The anti-inflammatory activity was assayed on macrophages that were cultured and plated as described for the viability assay. After 10 min incubation of the cells with the tested COS (at the three assayed concentrations), 100 ng/mL of lipopolysaccharide (LPS) was added. Cell viability was evaluated 24 hours after compound addition by mitochondrial MTT assay.

2.9. Statistical analysis

Data are expressed as means ± standard error (SE), with n=8. ANOVA on ranks and post-hoc Dunn's Method were used to find differences between groups. Statistical analysis was performed with SigmaPlot 13.0 and differences were considered significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Chitosanolytic activity screening

Based on previous works that report the presence of chitosanolytic activity in commercial enzyme preparations (Cabrera et al. 2005, Montilla et al. 2013, Pantaleone et al. 1992), we screened the hydrolytic activity towards chitosan CHIT100 (100-300 kDa, DD ≥ 90%) of a series of commercial enzymes whose stated activity was pectinase, cellulase (endo-1,4-β-D-glucanase), xylanase or protease (Table 1). Using the DNS assay based on the detection of released reducing sugars, the two more active preparations were Rapidase TF (a pectinase from *Aspergillus niger*) and Neutrase 0.8L (a protease from *Bacillus amyloliquefaciens*), which showed a moderate activity towards chitosan (0.47 ± 0.03 and 0.54 ± 0.03 U per mL, respectively). Two other pectinolytic preparations (Pectinex Ultra SP-L and Klerzyme 150) and a xylanase (Shearzyme 2X) displayed minor chitosanolytic activity.

In order to obtain a product enriched in deacetylated COS, and given that several proteases are able to hydrolyze the N-acetyl moieties present in chitosan (Li et al. 2005, Pan et al. 2016, Vishu Kumar et al. 2004), the proteolytic preparation Neutrase 0.8L

was selected for further experiments. To our knowledge, the presence of chitosanolytic activity in Neutrase 0.8L had not been described before. This enzymatic preparation was also assayed towards other chitosans with different MW and DD (Table 2). Interestingly, the activity was similar with all the substrates, despite the substantially differences in their deacetylation degree.

The protein profile of Neutrase 0.8L was analyzed by SDS-PAGE in denaturing conditions, and compared with an activity gel in native conditions. Figure 1 shows the presence of several proteins with MW < 75 kDa. The neutral protease of *B. amyloliquefaciens* is reported in the UniProt database (accession number: Q44677) to have a molecular mass of 56.7 kDa. This correlated well with the most intense band in the SDS-PAGE. The activity gel (Fig. 1) was not directly comparable with the SDS-PAGE gel because the behavior of proteins under native conditions is different than under denaturing conditions. However, observing the lytic band corresponding to the Neutrase 0.8L preparation (Fig 1, lane 2, white arrow), we concluded that the chitosanolytic activity did not correlate with the main band of the SDS-PAGE (the neutral protease).

3.2. Monitoring of chitosan hydrolysis by SEC-HPLC

The hydrolysis of chitosan catalyzed by Neutrase 0.8L was followed by size exclusion chromatography with evaporative light-scattering detection (SEC-ELSD). Figure 2 shows the progress of the reaction with chitosan CHIT600 as substrate. The high-molecular-weight (HMW) chitosan (peak *1*) disappeared after 24 h, which indicated efficient hydrolysis of the substrate. A new peak appeared (*2*) with the same retention time that a chitosan oligosaccharide standard (MW ≤ 2000). The monomers (GlcN and GlcNAc) and the buffer salts coeluted in peak *3*. However, the specific composition of the COS fraction cannot be inferred by this methodology. The reactions using chitosans of different DP and DD gave rise to similar SEC-HPLC profiles (data not shown). In conclusion, SEC-ELSD analyses confirmed the complete disappearance of the HMW chitosan after 24 h under the assayed conditions.

3.3. Identification of COS by mass spectrometry

Mass spectrometry (MS) techniques, such as LC-MS/MS (Kim et al. 2013) or MALDI-TOF (Montilla et al. 2013), are of great potential to identify (or to discard) the formation of certain COS in the reactions, especially combined with NMR spectroscopy (Mahata et al. 2014). Figure 3 illustrates the ESI-Q-TOF MS spectrum that resulted from the hydrolysis of chitosan CHIT600 with Neutrase 0.8L. MS spectra indicated that this enzymatic extract only formed deacetylated products, thus suggesting the presence of a deacetylating enzyme able to hydrolyze the remaining acetamido groups in the N-acetyl-glucosamine units. Even in the case of the chitosan with lower DD (QS2, 77%), all the identified products were deacetylated, except for a minor contribution of $(GlcN)_2$-GlcNAc (see Supplementary material). The deacetylating activity could be attributed to the protease since N-acetyl moieties resemble the peptide bonds; however, the presence of a contaminant chitin deacetylase activity in Neutrase 0.8L cannot be ruled out. These results are in agreement with those described for a commercial neutral protease from *Bacillus subtilis* (Li et al. 2005, Li et al. 2007); however, the authors postulated that the responsible of both chitosanolytic and deacetylating activities could be the protease itself. On the contrary, in our case, we observed that the neutral protease was not responsible of the chitosanolytic activity, whilst it could catalyze the deacetylating process.

In a recent work, we developed a dual reactor for COS production using the chitosanolytic activity present in another enzymatic preparation (BAN) of *B.*

amyloliquefaciens, whose main declared activity is α-amylase. In that case, a mixture of partially acetylated and deacetylated COS was obtained (Santos-Moriano et al. 2016a), which could be related with the absence of protease activity in such preparation.

3.4. Quantification of COS by HPAEC-PAD

Since COS properties are highly dependent on their charge (related to their DD) and size (DP), an accurate chemical characterization of the products derived from chitosan hydrolysis is truly necessary. A complex mixture of fully acetylated COS (containing only GlcN) and partially acetylated COS (paCOS, with variable composition of GlcN and GlcNAc) is commonly obtained. In addition, the low availability of COS standards, especially of paCOS, complicates the analysis. Methods to separate COS include size exclusion chromatography (SEC) (Song et al. 2014), hydrophilic interaction liquid chromatography (HILIC) and anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Xiong et al. 2009, van Munster et al. 2015). COS are poorly retained at alkaline pH on anion-exchange columns typically employed to separate carbohydrates, thus requiring unusual mobile phase conditions.

HPAEC-PAD was employed to identify (Fig. 4) and quantify (Fig. 5) the COS obtained by the hydrolysis of the four chitosans (CHIT100, CHIT600, QS1 and QS2) with Neutrase 0.8L. The four reactions were carried out under the same conditions and treated in the same way for their comparison. In order to avoid damage of the HPAEC-PAD column, any residual chitosan in the reaction mixtures (HMW chitosan, peak *1* in Figure 2) was removed by precipitation with 0.25 M NaOH (1:1 v/v) followed by centrifugation. The addition of alkali also allowed to inactivate the enzyme and to increase the sensitivity of the detector.

In a previous work, we demonstrated that a Carbo-Pack PA-200 column was appropriate for the separation of complex mixtures of COS in less than 30 min employing a diluted NaOH solution (4 mM) as mobile phase (Santos-Moriano et al. 2016b). This method represented a significant improvement compared with previous chromatographic protocols to separate deacetylated or partially acetylated COS (Xiong et al. 2009, Horsch et al. 1996, Lü et al. 2009). Since the ionic strength of the eluent is below the specifications of the amperometric detectors, the sensitivity of the method (and thus the quantification of the products) could be further improved by implementing a post-column delivery system with concentrated NaOH (van Munster et al. 2015).

With the aid of commercial standards, GlcN (peak *1*), $(GlcN)_2$ (*2*), $(GlcN)_3$ (*3*), $(GlcN)_4$ (*4*) and $(GlcN)_5$ (*5*) were identified by HPAEC-PAD (Fig. 4) and further quantified (Fig. 5). The order of elution with PA-200 columns usually correlates with the increasing degree of polymerization (DP), because more sugar moieties imply a higher negative charge to interact with the positive stationary phase. However, it is noteworthy that the retention time in the glucosamine series did not follow such order –e.g. $(GlcN)_3$ eluted between GlcN and $(GlcN)_2$–, probably due to the unusual eluting conditions (4 mM NaOH) and that the most acidic hydroxyl groups of glucose moieties (2-OH) are substituted by NH_2.

An additional problem is that alkaline mobile phases typically employed in HPAEC-PAD methods may cause epimerization of the N-acetyl-D-glucosamine moiety to N-acetyl-D-mannosamine (ManNAc) (Lee 1996), thus artificially increasing the number of analytes in the sample. We observed that the low concentration of NaOH (4 mM) used in our method did not promote epimerization of COS at least during the time of analysis (30 min).

In accordance with the SEC-HPLC study, chitosans CHIT100 and CHIT600 (initial concentration 10 g/L) were fully converted into COS in 24 h, yielding approximately

2.5 g/L of $(GlcN)_2$, 4.5 g/L of $(GlcN)_3$ and 3 g/L of $(GlcN)_4$. The HPAEC-PAD results also correlated well with data from MS analysis.

3.5. Biological activity of COS

The reaction of Neutrase with chitosan CHIT600 was scaled-up (50 mL) for the production of fully deacetylated COS to assess their biological properties. A mixture of 25% $(GlcN)_2$, 45% $(GlcN)_3$ and 30% $(GlcN)_4$ was obtained. The yield of the purification was 80%, and the protein content was almost negligible.

It has been postulated that deacetylation exerts a notable influence on the bioactivity of these molecules. Thus, the increased hypocholesterolemic activity of deacetylated derivatives could be attributed to the electrostatic attraction between charged amino groups and anionic bile salts and fatty acids (Xia et al. 2011). In contrast, partially acetylated COS exhibit better antibacterial activity towards *Escherichia coli* and *Listeria monocytogenes* than those fully deacetylated (Sanchez et al. 2017). In the present work, we studied the neuroprotective and anti-inflammatory activities of the deacetylated COS obtained with Neutrase 0.8L.The absence of the monomer GlcN in the reaction mixture was desirable, due to possible non-specific cytotoxic effects of this molecule (de Assis et al. 2012). COS were compared with two related fructooligosaccharides (FOS), whose biological activities are well established, in particular their use as prebiotics (Zambelli et al. 2016).

3.5.1. Neuroprotective activity

COS have been reported to protect primary cultures of rat hippocampal neurons against neurotoxicity induced by glutamate (Zhou et al. 2008) and Cu^{2+} (Xu et al. 2010). The mechanism of COS neuroprotection could be related with a decrease of intracellular reactive oxygen species (ROS), for example by complexing metal ions with the amino, hydroxyl and acetamido moieties present in COS. COS are also able to promote peripheral regeneration in rat model of sciatic nerve crush injury (Jiang et al. 2009), and have been even proposed as nutritional agents for Alzheimer's disease treatment (Huang et al. 2015). The neuroprotective activity of five fully deacetylated COS was recently compared by Jiang et al. (2014); they found that chitotriose induced the highest increase in Schwann cell survival.

In the present work, the neuroprotective activity of deacetylated COS produced by Neutrase 0.8L towards human SH-S5Y5 neurons was tested. First, viability of cells in the presence of COS at three concentrations (0.02, 0.2 and 2 mg/mL) was assayed. COS were not toxic for the cells (Fig. 6A). These concentrations were then tested for the neuroprotective activity in the presence of H_2O_2 (Fig. 6B). Values above 100% indicated neuroprotection. COS showed a dose-dependent behavior increasing cells viability after exposure to hydrogen peroxide (Fig. 6B). In particular, the activity was higher at the lowest assayed concentration (0.02 mg/mL), at which the neuroprotective effect was statistically significant. The effect was slightly better than the observed with fructooligosaccharide standards.

3.5.2. Anti-inflammatory activity

Chitosan oligosaccharides have been reported to exert an anti-inflammatory effect via the stimulus of tumor necrosis factor-α (TNF- α) in the LPS-induced inflammation in RAW cells (Yoon et al. 2007). The anti-inflammatory effect of COS is dose-dependent and molecular weight-dependent (Fernandes et al. 2010, Pangestuti et al. 2011), although the effect of the degree of acetylation remains less explored. Recently, COS were proposed as functional foods against inflammation (Azuma et al. 2015).

Anti-inflammatory activity of fully deacetylated COS produced by Neutrase 0.8L was tested in RAW 264.7 macrophages. First, viability of macrophages in the presence of COS was assayed: at the concentrations of 0.02, 0.2 and 2 mg/mL, COS were not toxic for the cells, and even increased significantly the viability of the cells at low concentrations (Fig. 7A). The same concentrations were tested for the anti-inflammatory activity measuring cell viability after inflammation caused by 100 ng/mL LPS. COS showed certain anti-inflammatory activity at the three concentrations assayed, although none of them was statistically significant (Fig. 7B). However, unlike the case of the neuroprotective activity, the effect was not greater than the obtained with other functional sugars such as fructooligosaccharides.

4. CONCLUSIONS

We found a commercial enzyme preparation approved for food applications (Neutrase 0.8L) that produced almost exclusively deacetylated COS from different chitosans. The reaction was very efficient and the DP of the synthesized COS varied from 2 to 4 (MW < 700). The process was scaled-up to produce a higher amount of fully deacetylated COS and assess their neuroprotective and anti-inflammatory activities. The COS mixture showed no toxicity on neurons and RAW cells, as well as a moderate biological activity, similar to that obtained with other functional food ingredients such as fructooligosaccharides.

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DECLARATION OF INTEREST

The authors report no declarations of interest.

SUPPORTING INFORMATION

Mass spectra of Neutrase 0.8L reactions with different chitosans (CHIT100, QS1 and QS2).

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FIGURE CAPTIONS

Figure 1. Protein electrophoresis of Neutrase 0.8L. MW: molecular-weight standards; Lane 1: SDS-PAGE; Lane 2: zymogram.

Figure 2. SEC-HPLC analysis of chitosan hydrolysis by Neutrase 0.8L at 0 h and 24 h**.** Reaction conditions: 1% (w/v) chitosan CHIT600, 20% (v/v) enzyme, 50 mM sodium

561 acetate buffer pH 5.0, 50°C. Peaks: (1) High-molecular-weight chitosan; (2) Chitosan
562 oligosaccharides; (3) GlcN, GlcNAc and buffer salts. The chromatogram of a
563 commercial COS mixture (MW ≤ 2000, DD ≥ 90%) is also shown.

564 **Figure 3**. ESI-Q-TOF mass spectrum of the reaction mixture after 24 h of chitosan
565 CHIT600 with Neutrase 0.8L. Reaction conditions: 1% (w/v) chitosan in 50 mM
566 sodium acetate buffer pH 5.0, 20% (v/v) enzyme, 50°C. Several $[M+H]^+$ and $[M+Na]^+$
567 peaks were identified.

568 **Figure 4**. HPAEC-PAD chromatograms of the chitooligosaccharides produced by
569 Neutrase 0.8L using different chitosans**.** Reaction conditions: 1% (w/v) chitosan, 20%
570 (v/v) enzyme, 50°C, 50 mM sodium acetate buffer pH 5.0, 24 h. HMW chitosans were
571 precipitated prior to the analysis. Identified peaks: (1) GlcN; (2) $(GlcN)_2$; (3) $(GlcN)_3$;
572 (4) $(GlcN)_4$; (5) $(GlcN)_5$.

573 **Figure 5.** Quantification by HPAEC-PAD of the major synthesized COS using different
574 chitosans. Reaction conditions: 1% (w/v) chitosan, 20% (v/v) enzyme, 50°C, 50 mM
575 sodium acetate buffer pH 5.0, 24 h.

576 **Figure 6.** *In vitro* analysis of neuroprotective activity of COS. **A:** Cell viability assays
577 on SH-SY5Y neuronal cells. **B:** Neuroprotective activity. Abbreviations: GF4: 1^F-
578 fructofuranosyl-nystose; GF2: 1-kestose; H_2O_2 (-): Viability in presence of DMSO;
579 H_2O_2 (+): Viability in presence of DMSO + H_2O_2. The data is expressed as mean ± SE
580 (n=8, $^*p < 0.05$ vs. H_2O_2 (+) group; $^\#p < 0.01$ vs. H_2O_2 (-) group).

581 **Figure 7.** *In vitro* analysis of anti-inflammatory activity of COS. **A:** Cell viability
582 assays on RAW 264.7 macrophages. **B:** Lipopolysaccharide (LPS) mitigation activity.
583 Abbreviations: GF4: 1^F-fructofuranosyl-nystose; GF2: 1-kestose; LPS (-): Blank; LPS
584 (+): 100 ng/mL of LPS. The data is expressed as mean ± SE [n=8, $^*p < 0.05$ vs. DMSO
585 group; $^\#p < 0.01$ vs. DMSO group (A)/LPS (-) group (B)].

Table 1. Screening of chitosanolytic activity in commercial enzyme preparations.

Enzyme	Supplier	Source	Declared activity	Chitosanase activity (U/mL) [a]
Pectinex Ultra SP-L	Novozymes	*Aspergillus aculeatus*	Pectinase	0.010 ± 0.004
Rapidase TF	DSM	*Aspergillus niger*	Pectinase/hemicellulose	0.470 ± 0.030
Klerzyme 150	DSM	*Aspergillus niger*	Pectinase	0.016 ± 0.005
NovoShape	Novozymes	*Aspergillus oryzae*	Pectin methyl esterase	–
E-CELBA	Megazyme	*Bacillus amyloliquefaciens*	β-Glucanase	–
Ultraflo L	Novozymes	*Humicola insolens*	β-Glucanase	–
Shearzyme 2X	Novozymes	*Aspergillus oryzae*	Xylanase	0.007 ± 0.005
Pentopan Mono Conc. BG	Novozymes	*Aspergillus oryzae*	Xylanase	–
Flavourzyme	Novozymes	*Aspergillus oryzae*	Protease	–
Papain	Sigma	*Carica papaya latex*	Acid protease	–
Neutrase 0.8 L	Novozymes	*Bacillus amyloliquefaciens*	Neutral protease	0.540 ± 0.030
Alcalase	Novozymes	*Bacillus licheniformis*	Alkaline protease	–

[a] Reaction conditions of DNS assay: 1% (w/v) chitosan CHIT100, 20% (v/v) enzyme, 50°C, 1400 rpm, pH 5.0.
(–) No measurable activity in DNS assay.

Table 2. Chitosanolytic activity of Neutrase 0.8L with different chitosans as substrates.

Chitosan	MW (kDa)[a]	DD (%)[b]	Activity (U/mL)[c]
CHIT600	600-800	> 90	0.67 ± 0.05
CHIT100	100-300	> 90	0.56 ± 0.07
QS1	98 [d]	81	0.50 ± 0.04
QS2	31 [d]	77	0.57 ± 0.04

[a] Average molecular weight of chitosan
[b] Deacetylation degree of chitosan
[c] Determined by the DNS method
[d] Determined by gel-permeation chromatography (GPC)

Figure 1

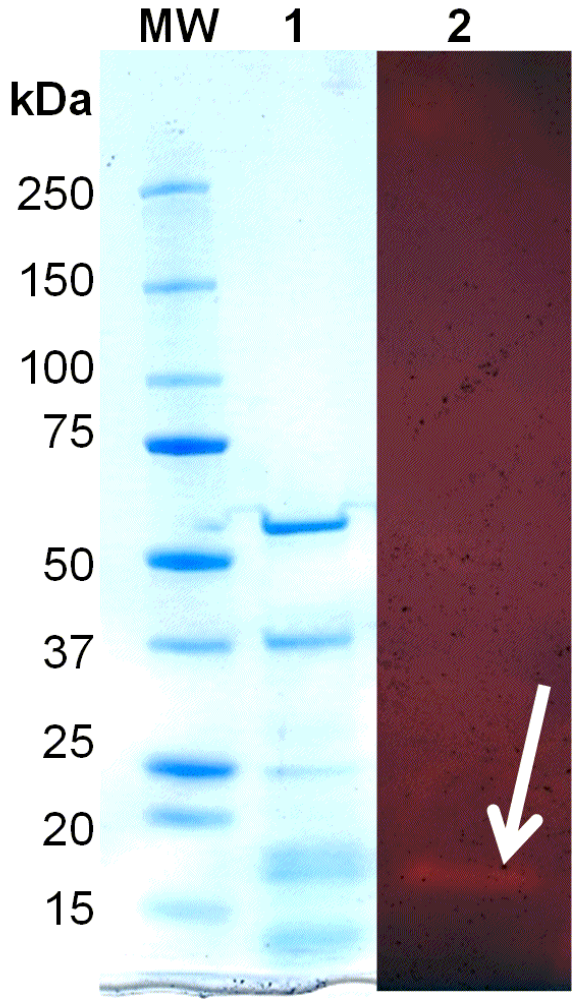


Figure 2

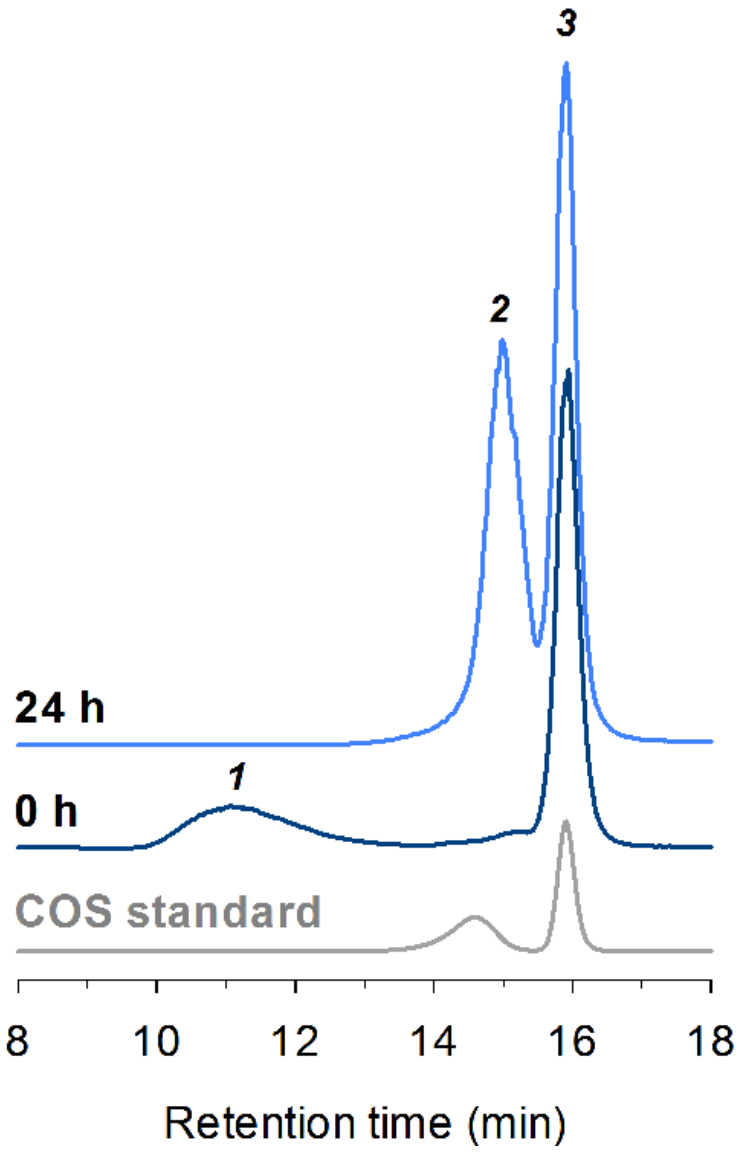


Figure 3

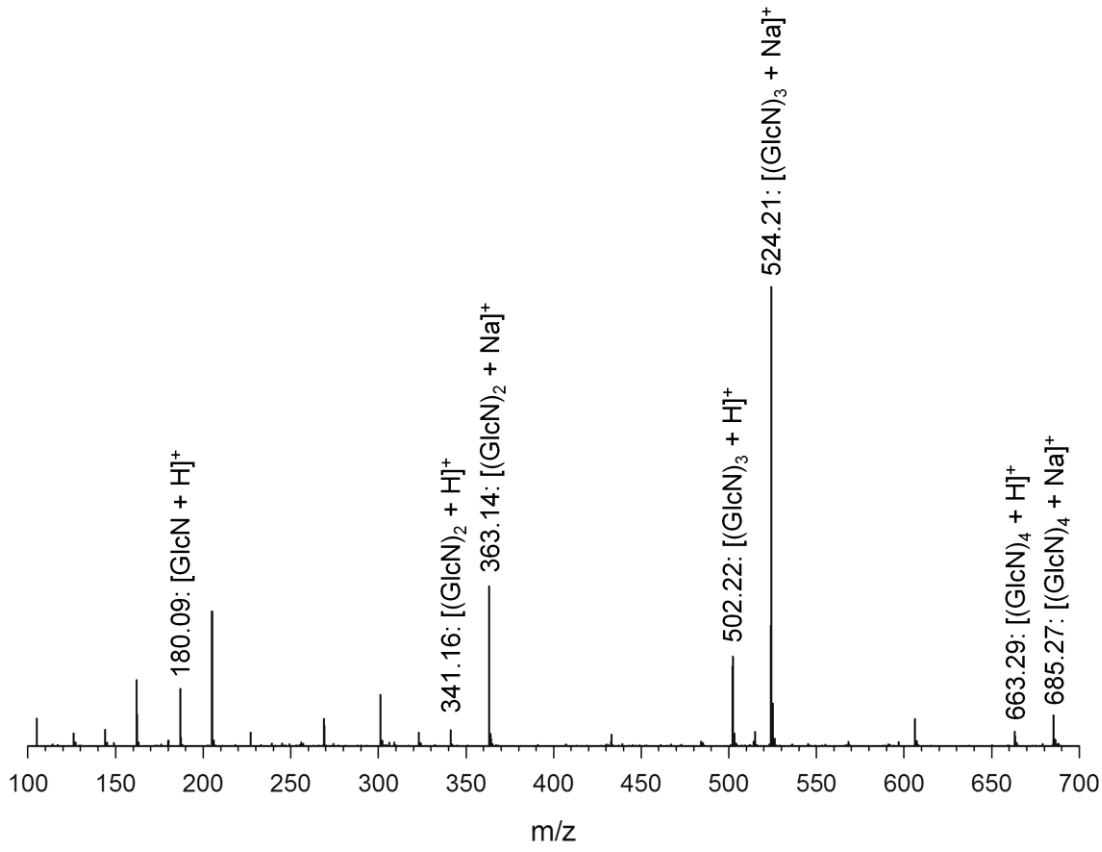

180.09: [GlcN + H]+
341.16: [(GlcN)2 + H]+
363.14: [(GlcN)2 + Na]+
502.22: [(GlcN)3 + H]+
524.21: [(GlcN)3 + Na]+
663.29: [(GlcN)4 + H]+
685.27: [(GlcN)4 + Na]+
100
150
200
250
300
350
400
450
500
550
600
650
700
m/z

Figure 4

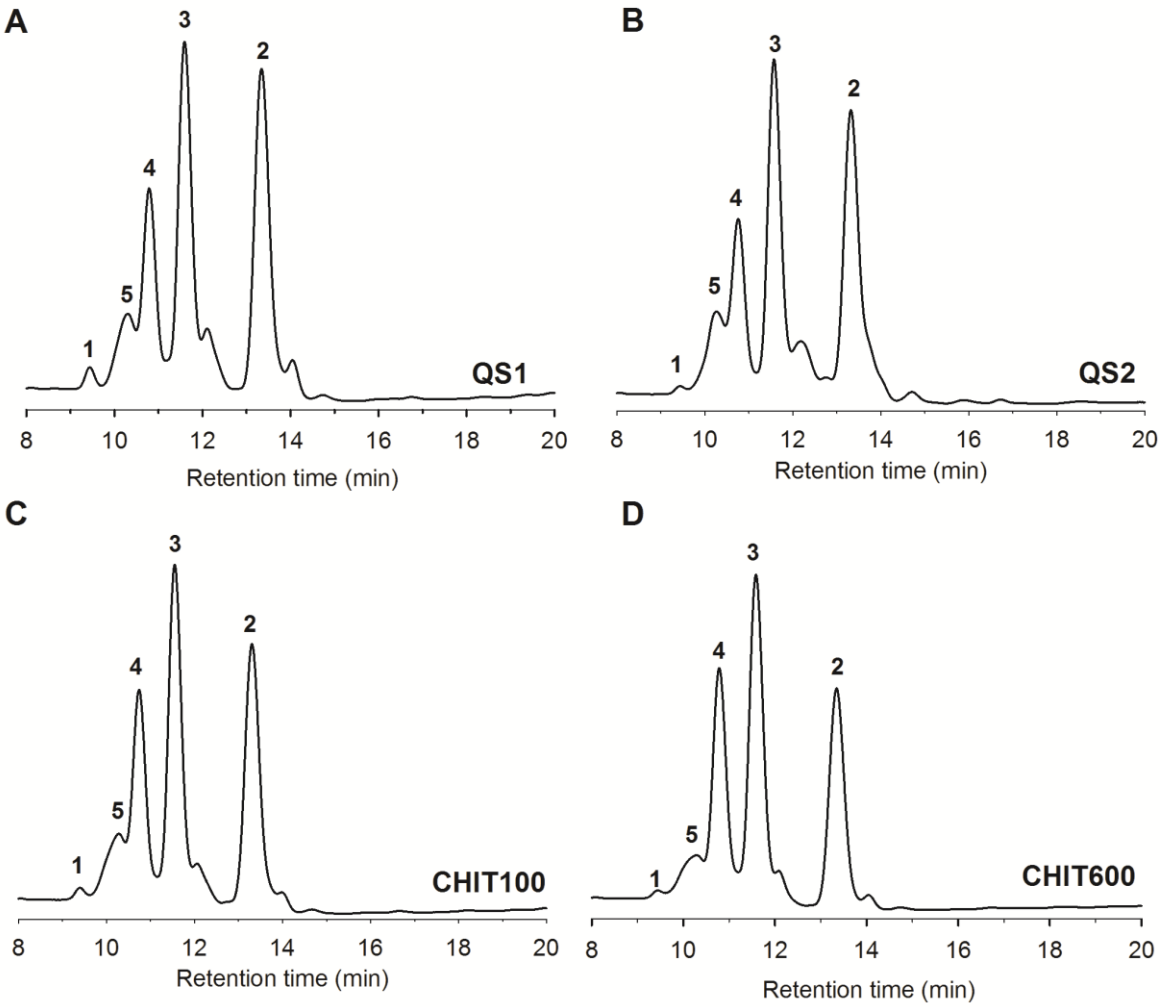

A
B
C
D
1
2
3
4
5
QS1
QS2
CHIT100
CHIT600
8
10
12
14
16
18
20
Retention time (min)

Figure 5

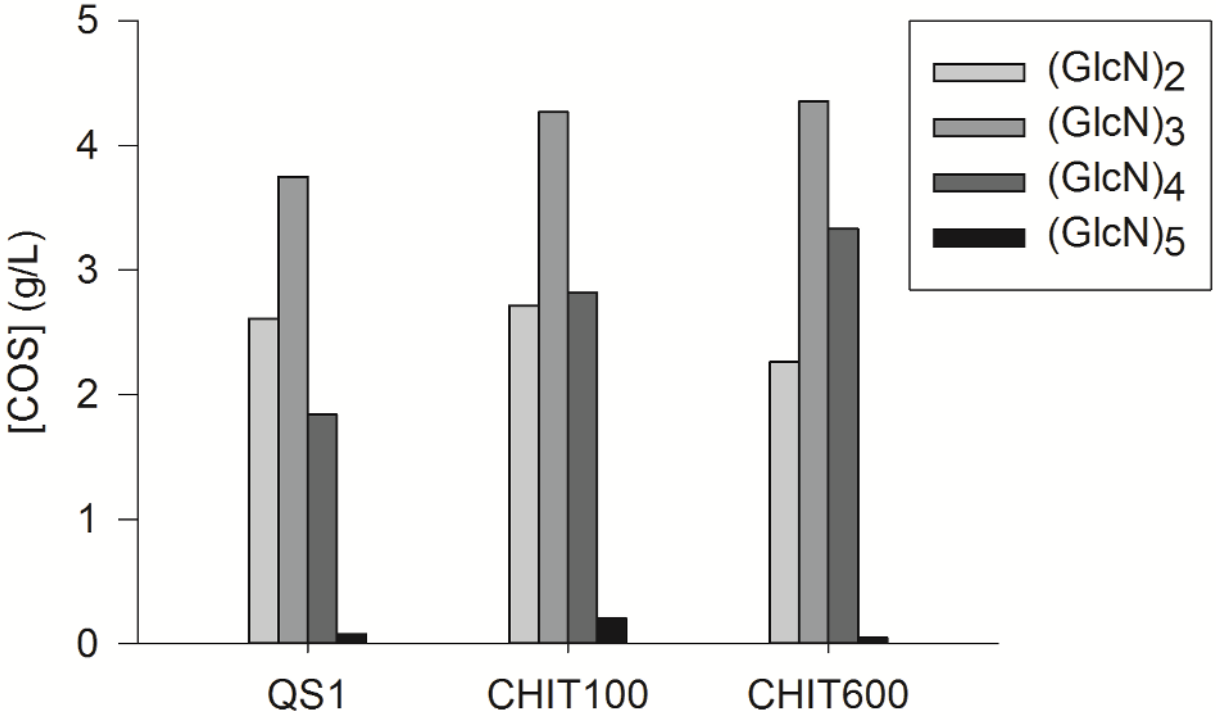


Figure 6

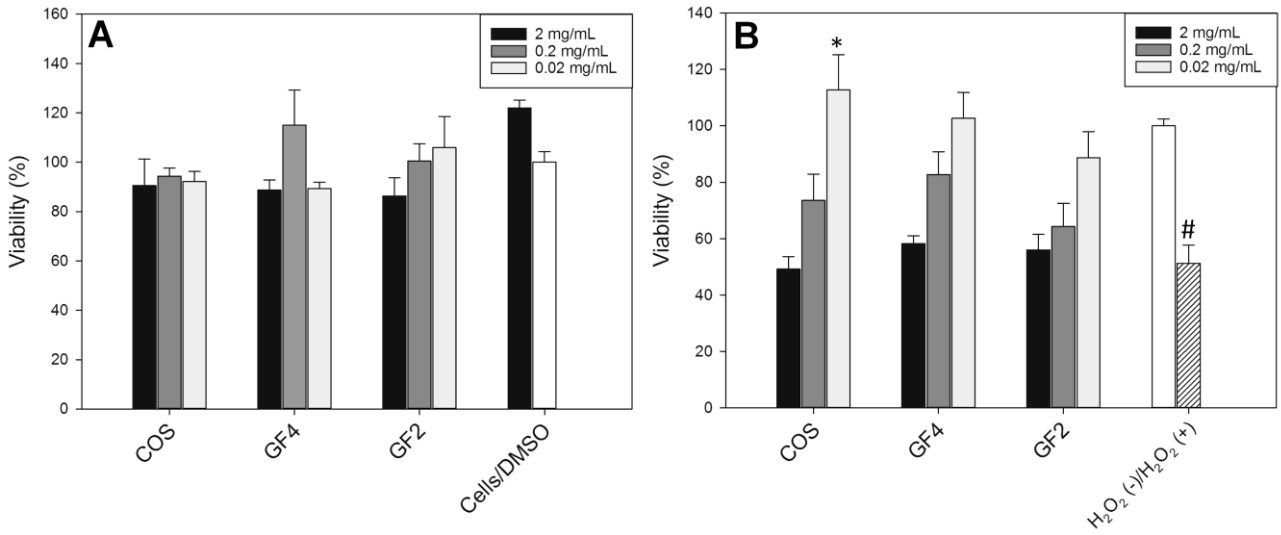
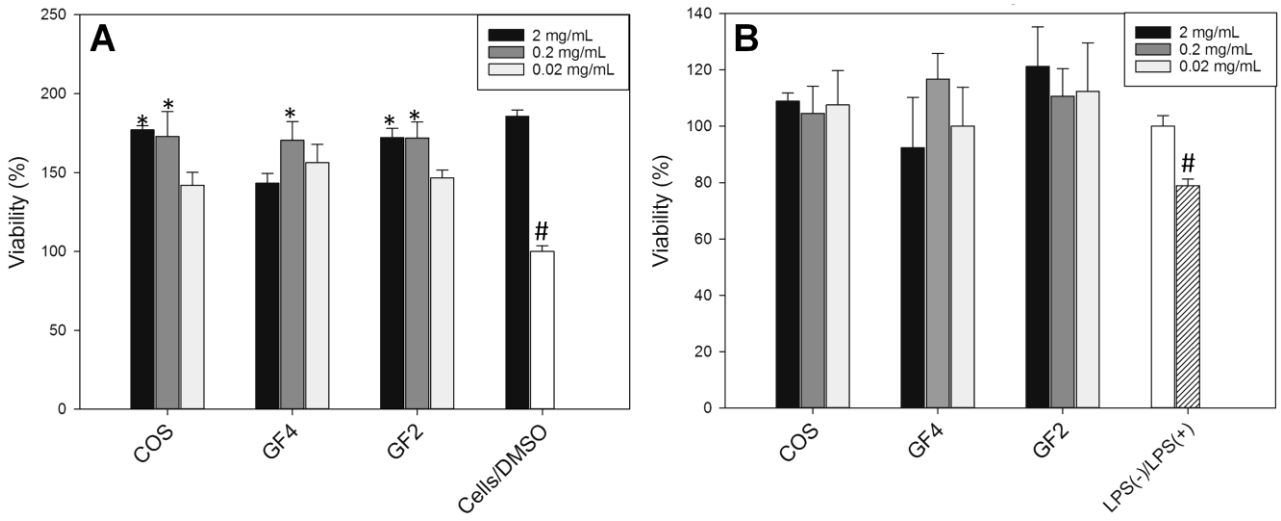

A
2 mg/mL
0.2 mg/mL
0.02 mg/mL
Viability (%)
0
20
40
60
80
100
120
140
160
COS
GF4
GF2
Cells/DMSO
B
2 mg/mL
0.2 mg/mL
0.02 mg/mL
Viability (%)
0
20
40
60
80
100
120
140
*
#
COS
GF4
GF2
H2O2 (-)/H2O2 (+)

Figure 7


A
2 mg/mL
0.2 mg/mL
0.02 mg/mL
Viability (%)
250
200
150
100
50
0
*
*
*
*
*
*
#
COS
GF4
GF2
Cells/DMSO
B
2 mg/mL
0.2 mg/mL
0.02 mg/mL
Viability (%)
140
120
100
80
60
40
20
0
#
COS
GF4
GF2
LPS(-)/LPS(+)