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Influence of the physico-chemical characteristics of chito-oligosaccharides (COS) on antioxidant activity

Marian Mengíbar[a], Inmaculada Mateos-Aparicio[b], Beatriz Miralles[c], Ángeles Heras[a,*]

[a] Instituto de Estudios Biofuncionales, Dpto. Química Física II, Facultad de Farmacia, Universidad Complutense de Madrid, Pso. Juan XXIII, no. 1, 28040 Madrid, Spain
[b] Departamento de Nutrición y Bromatología II, Universidad Complutense de Madrid, Plaza Ramón y Cajal s/n, 28040 Madrid, Spain
[c] Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), C/ Nicolás Cabrera no. 9, Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain

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ABSTRACT

Chito-oligosaccharides (COS) are being used as important functional materials for many applications due to their bioactivities. The aim of this research has been to assess the relationship between the physico-chemical characteristics, average molecular weight (Mw), acetylation degree (DA), polymerization degree (DP) and specially sequence composition determined by MALDI-TOF MS and the antioxidant properties of COS. These oligosaccharides were obtained by enzymatic depolymerization with chitosanase and lysozyme using a specific chitosan and its reacetylated product. The COS fraction below 5 kDa obtained from chitosanase depolymerization showed the highest capacity to scavenge DPPH radicals and to reduce Fe^{3+}. A correlation was found between the relative amount of molecules with a given A/D (acetylated *vs* deacetylated units) ratio within the COS and their antioxidant activity, which could be used to predict the antioxidant behavior of a fraction of chito-oligosaccharides.

1. Introduction

Chitosan is a natural biopolymer with a great variety of agricultural, medical, pharmaceutical and food applications. This fact makes chitosan an interesting multifunctional polymer. However, the low solubility of chitosan and the high viscosity of its solution due to its polycationic character and its high molecular weight constitute disadvantages for many biological and technological applications. The production of chito-oligosaccharides (COS) is an effective way to improve the solubility of chitosan and to decrease the viscosity of its solution enhancing the potential of these COS as functional materials for many applications. COS are being used due to their antimicrobial activity, antioxidant capacity, antitumor activity, inmuno-moduladory effect and wound healing (Mengíbar, Ganan et al., 2011; Xia, Liu, Zhang, & Chen, 2011). These properties allow to consider COS as potential novel functional materials. The known influence of the physico-chemical characteristics of COS on their biological activity makes worthwhile the production of well-defined oligosaccharides in order to determine the relationship between their bioactivities and these characteristics more effectively (Barroca-Aubry, Pernet-Poil-Chevrier, Domard, & Trombotto, 2010). The biological activities of COS are related to the N-acetylation degree (DA), which is the molar fraction of N-acetyl glucosamine (GlcNAc) units in the oligomer chain, the N-acetylation pattern (PA) which indicates the intramolecular distribution of N-acetyl groups along the oligomer chain, and also molecular size that has generally been described as the depolymerization degree (DP) and the average molecular weight (Mw). Due to the different criteria used in previous studies to determine the molecular size, DA and PA, the comparison of the biological effects of COS is quite difficult, and it explains the disagreement on COS activities reported by different authors (Chen, Zhu, Li, Guo, & Ling, 2010).

During years the development of *in vitro* antioxidant activity of chitosan and chito-oligosaccharides (Ai, Wang, Xia, Chen, & Lei, 2012; Je, Park, & Kim, 2004; Li et al., 2012; Park, Je, & Kim, 2004; Sun, Zhou, Xie, & Mao, 2007; Tomida et al., 2009; Xie, Xu, & Liu, 2001) has received much attention. This is a promising characteristic to create added value products for food preservation or even functional foods. It seems that the molecular weight of chitosan is one of the most important factors affecting its antioxidant activity, but there is no clear explanation for the observed radical scavenging abilities in terms of its structural properties. The increase in antioxidant activity has been attributed to a decrease

* Corresponding author at: Instituto de Estudios Biofuncionales, Universidad Complutense de Madrid, Paseo Juan XXIII, no. 1, 28040 Madrid, Spain.
Tel.: +34 913943284; fax: +34 913943284.
E-mail address: aheras@farm.ucm.es (Á. Heras).

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in Mw (Feng, Du, Li, Hu, & Kennedy, 2008; Park, Je, & Kim, 2003b; Sun et al., 2007; Tomida et al., 2009). Recently, Huang, Zhao, Hu, Mao, and Mei (2012) have shown that the antioxidant activity was poorer for high molecular weight samples, being the most active the lowest molecular weight chitosan (963 g/mol). Je et al. (2004) found a higher activity in COS with Mw ($1–5 \times 10^3$ g/mol) compared to COS with Mw ($5–10 \times 10^3$ g/mol) and COS with Mw below 1×10^3 g/mol. Regarding DA, the same authors reported increasing antioxidant activity as the DA value decreased.

However, up to now, the influence of DA on the development of antioxidant activity has not been studied in depth and is not very clear. The aim of this research has been to assess the relationship between the molecular characteristics and the antioxidant properties of the products obtained by enzymatic depolymerization. Chitosanase and lysozyme were used to depolymerize two chitosans with different DA values to take into account the influence of the enzyme and the parent chitosan on the type of COS produced.

2. Experimental

2.1. Chitosan samples

Commercial chitosan ChitoClear® fg 90 from fresh North Atlantic shrimp shells (*Pandalus borealis*) (Primex, Iceland) was purified by dissolution in 0.5 M acetic acid, filtrated through 250–160 μm, 100–40 μm and 16–10 μm sintered-glass funnel and precipitated with 10% (v/v) NaOH (pH 8). The neutralized precipitate was dried in an oven at 50 °C and labelled as Chitosan A (CHT A, DA 14%, M_w 180×10^3 g/mol). This chitosan was dissolved in 0.5 M acetic acid at 1% (w/v) and 1,2-propanodiol was added until reaching 0.5% (w/v) concentration. Acetic anhydride was added for reacetylation and the resulting solution was kept stirring during 90 min at room temperature. The reacetylated chitosan was precipitated with 30% (w/v) ammonia solution, neutralized with (70, 80, 90 and 100%, v/v) ethanol solution and dried at 50 °C. It was labelled as Chitosan B (CHT B, DA 35%, M_w 261×10^3 g/mol). The DA (Muzzarelli, Rocchetti, Stanic, & Weckx, 1997) and the weight average molecular weight (M_w) were measured in these chitosans.

2.2. Enzymatic depolymerization

Lysozyme from hen egg white (EC 3.2.1.17) and chitosanase from *Streptomyces griseus* (EC 3.2.1.132) were purchased from Sigma–Aldrich, St. Louis, MO, USA. All other reagents were of analytical grade. Both chitosans were dissolved in 0.1 M acetate buffer and the enzyme solution (0.627 mg/ml, pH 4.5 for lysozyme and 3.48×10^{-3} mg/ml, pH 5.7 for chitosanase) was added to initiate the reaction after adjusting the pH. Depolymerization was carried out at 37 °C in a Lab Therm LT-X orbital shaker (Thermo Fisher Scientific Inc, Waltham, MA, USA) at 100 rpm during 6 days for lysozyme and 4 days for chitosanase for complete depolymerization (Galed, Miralles, Mengíbar, & Heras, 2007).

Separation of the products was carried out with a Vivaflow 200 tangential ultrafiltration system (Sartorius-Stedim Biotech, Goettingen, Germany) with polyestersulfone (PES) membranes with Mw of 30, 10 and 5×10^3 g/mol. Fractions F30, F10-30, F5-10 and F5 were separated for each chitosan. They were subsequently dialyzed against distilled water with 12–14, 7 and 3.5×10^3 g/mol pore size membranes (Medicell International Ltd, London, UK) depending on the size of the obtained fraction until complete salt elimination. Thus, F30 was dialysed using membranes with $12–14 \times 10^3$ g/mol of pore size, F10-30 with 7×10^3 g/mol membranes and F5-10 and F5 with 3.5×10^3 g/mol membranes. The dialysed solutions were freeze-dried.

2.3. Characterization of the depolymerized products

SEC-HPLC was performed in Waters 625 LC System pump with an Ultrahydrogel (Waters, i.d = 7.8 mm, *l* = 300 mm) column thermostated at 35 °C. Waters 2414 differential refractometer and Evaporative Light Scattering (ELS Waters 2424 Milford, MA, USA) were connected online. A 0.15 M ammonium acetate/0.2 M acetic acid buffer (pH 4.5) was used as eluent. Samples were dissolved in the buffer (0.05%, w/v), filtered through a 0.45 μm pore size membrane (Millipore Corporation, Beverly, MA, USA) before injection of aliquots of 20 μL. The flow rate was 0.6 mL/min. The M_w of the different fractions were obtained from the SEC profiles by extrapolation in a calibration curve using different known M_w chitosans as standards.

The DA of COS was determined by the first derivative UV-spectrophotometric method proposed by Muzzarelli et al. (1997). UV measurements were carried out in a spectrophotometer in the wavelength range 190–240 nm. A calibration curve of N-acetylglucosamine (10–40 ppm) in 0.01 M acetic acid was prepared. Samples at a 0.1% (w/v) concentration were dissolved in 0.01 M acetic acid.

For NMR spectroscopy, samples were dissolved at a 1% (w/v) concentration in D_2O/DCl and placed into 5 mm NMR tubes. All chemical shifts were determined relative to internal TSP (sodium 3-(trimethylsilyl)-propionate-d_4). Measurements were performed on AMX 500 Bruker spectrometer (Bruker, Ettinglen, Germany). For ^{1}H NMR experiments the conditions used were actual pulse repetition time 2.5 s and number of scans 128.

MALDI-TOF MS of the COS fractions was carried out using a Bruker Ultraflex (Bruker Daltonik, Bremen, Germany) in positive ion mode. Samples were dissolved in water:methanol mixtures. For ionization 2,5-Dihydroxybenzoic (DHB) acid was used as matrix.

2.4. Determination of in vitro antioxidant activity

The α,α-diphenyl-β-picryhydrazyl (DPPH) radical-scavenging activity of COS was assayed by the method proposed by Chen, Tsai, Huang, and Chen (2009) with slight modifications. Sample solution (250 μL) at 1% (w/v) in distilled water was mixed with 1 mL of methanolic DPPH solution (100 μM). Distilled water was used as a control. The mixture was shaken and kept at room temperature in the dark. After 60 min, the absorbance was measured at 517 nm. The DPPH radical scavenging activity (%) was calculated by the following equation:

$$\text{Scavenging activity}\,(\%) = 1 - \frac{\text{Abs sample}}{\text{Abs control}} \times 100$$

where Abs control is the absorbance of the control and Abs sample is the absorbance of samples.

The FRAP (ferric reducing antioxidant power) assay was carried out according to the method proposed by Benzie and Strain (1996) and modified by Pulido, Bravo, and Saura-Calixto (2000) using a water-soluble vitamin E analogue, trolox, as standard. This method is based on the direct reduction of a Fe (III)-2,4,6-tripyridyl-s-triazine complex (Fe(III)-TPTZ) to the Fe(II)-TPTZ form. A volume of 30 μL of each sample in distilled water at 1% (w/v) was mixed with 90 μL of water and 900 μL of TPTZ reagent, and the absorbance was measured at 595 nm after 30 min.

2.5. Statistical analysis

Differences in the antioxidant activity data were tested for significance by one-way ANOVA with Duncan post hoc tests using Statgraphics Plus 5.1 (Warrenton, VA, USA). Differences at $p < 0.05$ were considered to be significant.

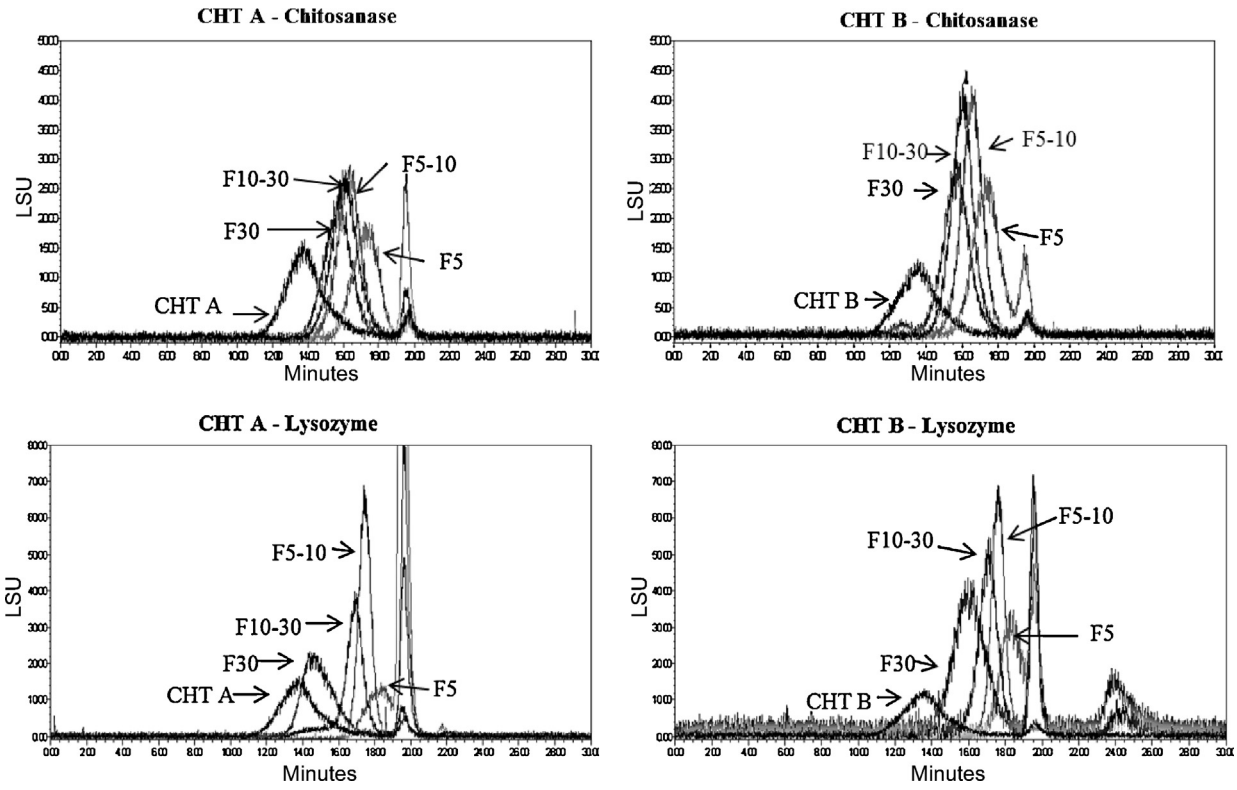

Fig. 1. SEC-HPLC chromatograms of COS from CHT A and CHT B depolymerized with chitosanase and lysozyme.

3. Results and discussion

3.1. Enzymatic depolymerization and characterization of the depolymerized products

3.1.1. Physico-chemical characterization

The effect of depolymerization was followed by size exclusion chromatography (SEC-HPLC). The peak of the elution curve of the original chitosan shifted towards longer retention time as the molecular weight of COS decreased evidencing depolymerization (Fig. 1). The molecular weight distribution was less polydispersed in the lower size fractions. Table 1 presents the average M_w calculated for each fraction. In the COS derived from lysozyme depolymerization of CHT A, higher M_w products remained in F30, whereas the other fractions presented considerably lower M_w. The overlapping of this chitosan and the F30 peak is patent in the chromatogram (Fig. 1). On the contrary, higher hydrolysis of COS F30 is observed in the case of CHT B. It has been reported that at the beginning of depoymerization reacetylated chitosans such as CHT B, present higher susceptibility to lysozyme hydrolysis as compared to chitosans with lower DA (Verheul et al., 2009). Therefore, the DA of the original chitosan was determinant for its degradation by lysozyme.

On the other hand, the enzymatic degradation with chitosanase was similar on CHT A and B. At the beginning of depolymerization, this enzyme has preference for distributed sequences of six glucosamine residues, probably easier to find with a chitosan with low DA (Fukamizo, Honda, Goto, Boucher, & Brzezinski, 1995). However the random distribution of acetylated and deacetylated units in both chitosans could contribute to a similar hydrolysis by this specific enzyme. The calculated M_w of the COS obtained did not always coincide with the molecular weight cut off range of the ultrafiltration membranes. This is not unexpected since, these are membranes designed for proteins and they may not work in the same way when a polymer as chitosan is used. The hydrodynamic volume of a chitosan sample of similar molecular weight is much larger due to its polycationic character (Chen et al., 2010). Furthermore, the high viscosity of chitosan solutions could produce partial obstruction of the membranes (Kim & Rajapakse, 2005).

The DA values of the different fractions (Table 1) obtained from CHT A and CHT B with both enzymes were higher than the DA of the original chitosan, except in COS F5 from CHT A obtained with chitosanase. This increase could be related with the particular microstructure of these oligosaccharides as they have different DP and DA (Vishu Kumar et al., 2005). Lysozyme hydrolyzes AA–AA and AA–AD links, and does not generate many AD terminal units. Thus, the higher the DA, the lower the yield of AD terminals, which would pass on to the smallest fractions when this enzyme is used to depolymerize. This effect is clearly observed in the case of CHT B. This fact also should influence the depolymerization with

Table 1
Average molecular weight (M_w) (g/mol) and acetylation degree (DA) values (%) for fractions of COS of CHT A and CHT B depolymerized with lysozyme and chitosanase determined by SEC and UV-spectroscopy, respectively.

Sample	M_w ($\times 10^{-3}$ g/mol)		DA (%)	
	Chitosanase	Lysozyme	Chitosanase	Lysozyme
CHT A	180 ± 3.6		14 ± 0.8	
CHT A F 30	28 ± 1.9	61 ± 2.9	23 ± 3.5	20 ± 1.5
CHT A F 10–30	18 ± 1.2	4 ± 0.5	18 ± 1.6	18 ± 2.7
CHT A F 5–10	9 ± 2.7	1 ± 0.2	17 ± 1.6	37 ± 1.8
CHT A F 5	3 ± 0.3	0.6 ± 0.2	8 ± 0.7	39 ± 1.2
CHT B	261 ± 8.5		35 ± 2.5	
CHT B F 30	28 ± 1.3	22 ± 2.5	47 ± 2.0	43 ± 1.0
CHT B F 10–30	19 ± 1	3 ± 0.1	46 ± 1.5	38 ± 1.5
CHT B F 5–10	9 ± 0.7	1 ± 0.1	38 ± 1.7	47 ± 1.7
CHT B F 5	2 ± 0.05	0.2 ± 0.05	37 ± 0.7	50 ± 1.4

Values are means ± standard deviation ($n = 3$).

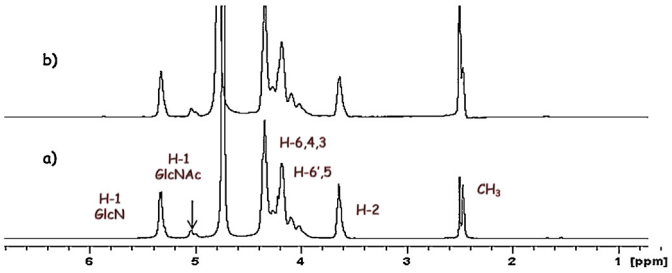

Fig. 2. ^{1}H NMR spectrum: (a) CHT A and (b) COS F 10–30 CHT A depolymerized with chitosanase.

chitosanase in the same way. However, the smaller the size of the polymer chain, the easier the breaking-off of D-D links, which explains the lower DA of COS F5 from CHT A with respect to the other fractions.

3.1.2. Structural analysis

The structure of the different fractions of COS was checked by ^{1}H NMR (Fig. 2). It is known that the α-anomeric reducing end protons of the hexosamine ring appear at 5.43 ppm (D-unit) and 5.19 ppm (A-unit) (Ishiguro, Yoshie, Sakurai, & Inoue, 1992), while internal D- and A-units resonate at 4.9 and 4.6 ppm, respectively (Vårum, Antohonsen, Grasdalen, & Smidsrød, 1991). In the present study the signals corresponding to the internal units were masked with the water chemical shifts. However, the corresponding signals of H-1 in the reducing end of the GlcN and GlcNAc units appear at 5.33 and 5.03 ppm, respectively, which slightly differs from the values found in literature using other experimental conditions. The proton signals H (1–6) and these H-1 reducing end signals showed the preservation of chitosan structure in COS. The similar intensity signals between COS and original chitosan showed a high purity in these fractions probably coming from the dialysis process used here. Also a correspondence was observed between lower DA values determined by first derivative method and the high intensity of H-1 GlcN internal units signals as is observed in COS 10–30 from CHT A depolymerized with chitosanase (Fig. 2).

3.1.3. Mass spectrometric characterization

The products of the enzymatic reactions were analyzed by MALDI-TOF-MS. The chito-oligosacharides composition is listed in the supplementary data. In the case of F30 no spectra were detected because of the high molecular weight and the difficulty to ionize these fractions. The DP of the fractions and the composition calculated as ratio of acetylated *vs* deacetylated (A/D) units in the sequences were determined (Table 2). The DP values ranged from 3 to 24 and decreased with Mw, showing, as described above, a lack of matching with the declared Mw cut off range of the ultrafiltration membranes. Higher content of acetylated units in the products resulting from the lysozyme depolymerization (CHT A and CHT B) and from chitosanase depolymerization of CHT B and therefore higher A/D values were detected compared to the chitosanase depolymerization products obtained from CHT A. These last fractions presented a different N-acetylation pattern with series of fully deacetylated oligomers of GlcN as well as mono-di, and triacetylated sequences with the molecular composition $D_{n-m}A_m$, where $m = 0–3$ and n takes values between the minimum and the maximum of DP data. In some cases, the composition was not in accordance with the determinations of Mw and DA from Table 1. The SEC and UV-spectroscopy are performed as average values and show limitations as is revealed by the mass spectroscopic analysis.

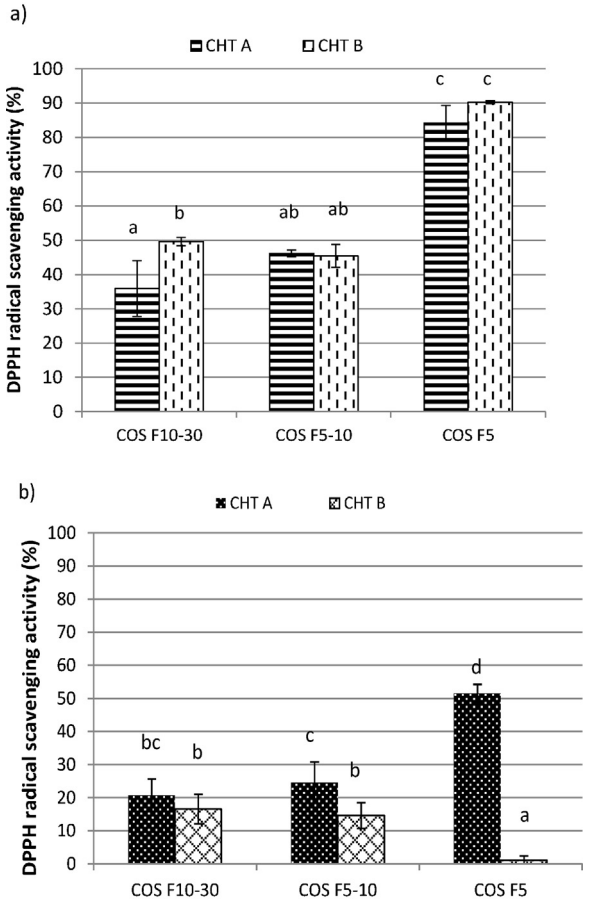

Fig. 3. DPPH radical scavenging activity (%) of COS fractions obtained from CHT A and CHT B depolymerized with: (a) chitosanase and (b) lysozyme. Values are means ± standard deviation. Significant differences between average values ($n = 3$) in different samples by one-way ANOVA (Duncan test). The different letters (a, b, c and d) indicate significant difference ($p < 0.05$) between different fractions.

3.2. In vitro antioxidant activity and its relationship with COS studied characteristics

Fig. 3 shows DPPH radical scavenging activity for CHT A and CHT B COS. Radical scavenging activity increased significantly ($p < 0.05$) in COS F5 except in CHT B F5 obtained with lysozyme. Original chitosans and their COS F30 did not show antioxidant activity. In chitosan depolymerized with chitosanase, in spite of the difference in the DA values (Table 1), no significant ($p < 0.05$) differences in activity were found regarding the parent chitosan except in F10–30 (Fig. 3a). Lysozyme CHT A and CHT B COS (Fig. 3b) presented significantly ($p < 0.05$) lower radical scavenging activity compared to the COS obtained with chitosanase. Scavenging activity of lysozyme COS from CHT A increased when M_w decreased, reaching 50% in the case of COS F5. On the contrary, a trend to decrease was noted in CHT B COS and negligible activity was detected for CHT B COS F5.

Fig. 4 shows the reducing power of Fe^{3+} (FRAP) of CHT A and CHT B COS. A similar trend to the one found in DPPH scavenging activity was observed. The highest reducing capacity was found with COS F5 from chitosanase and significantly ($p < 0.05$) lower activity was found in lysozyme products. There were no significant differences between COS with regard to the parent CHT except in lysozyme COS F5 (Fig. 4b). Lysozyme COS F5 from CHT A presented higher activity than COS F10–30 and COS F5–10 from chitosanase. However, the activity found in lysozyme COS from CHT B was similarly low in all fractions.

Table 2
COS composition determined by MALDI-TOF MS spectrometry.

Sample	DP range		A/D complete range		% Sequences with A/D values					
	Chitosanase	Lysozyme	Chitosanase	Lysozyme	0–1 Chitosanase	1–2	>2	0–1 Lysozyme	1–2	>2
CHT A F 10–30	3–17	4–17	0–2	0–4	96	4	0	44	43	13
CHT A F 5–10	3–24	3–18	0–0.5	0–4	100	0	0	44	42	14
CHT A F 5	3–7	3–15	0–2	0–5	91	9	0	26	47	27
CHT B F 10–30	3–21	3–20	0–2.5	0–4	81	18	1	48	43	9
CHT B F 5–10	3–21	3–18	0.06–2	0–4	69	31	0	30	47	23
CHT B F 5	3–12	1–12	0–4	0–6	73	22	5	22	33	44

The increased activity in lower Mw fractions is in agreement with the results obtained by Tomida et al. (2009) and Huang et al. (2012) who also found increasing antioxidant activity with M_w decrease. It has been reported that while low molecular weight chitosans are non-compact structures, high molecular weight chitosans present packed structures with stronger intramolecular hydrogen bonds, decreasing the ability of hydroxyl and amine groups to react with different radicals (Feng et al., 2008; Sayas-Barberá et al., 2011; Sun et al., 2007; Xing, Liu et al., 2005; Yang, Shu, Shao, Xu, & Gu, 2006; Yen, Yang, & Mau, 2008; Zhong et al., 2007). The different DA of the fractions had little influence on the scavenging activity. This is opposed to the results found by Je et al. (2004) who showed that low DA COS (10%) yielded higher DPPH neutralizing activity, hydroxyl and superoxide radicals. However, Jung and Zhao (2012) found that DPPH radical scavenging in β-chitosan was not significantly affected by DA, and neither did Yen et al. (2008), who obtained chitosan from crab shells using different deacetylation times and checked that the samples were not effective in scavenging DPPH radicals.

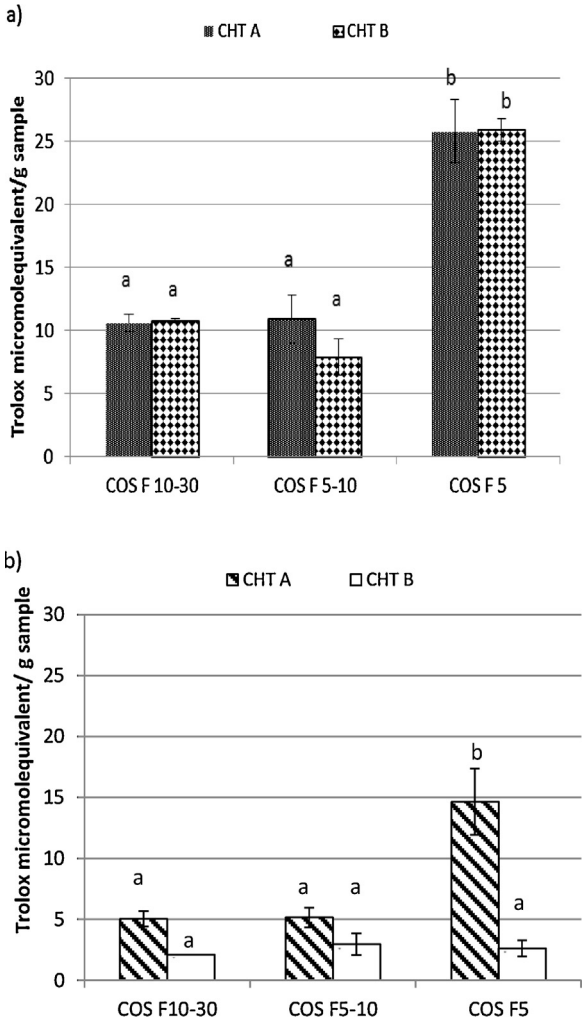

Fig. 4. Reducing power expressed as Trolox micromolequivalent/g sample of COS fractions obtained from CHT A and CHT B depolymerized with: (a) chitosanase and (b) lysozyme. Values are means ± standard deviation. Significant differences between average values ($n = 3$) in different samples by one-way ANOVA (Duncan test). The different letters (a, b, c and d) indicate significant difference ($p < 0.05$) between different fractions.

Some researchers suggest a proton donation or electron transference from the amino groups as antioxidant mechanism. In addition, it could be due to the proton donation from hydroxyl groups linked to C-2, C-3 and C-6 in the glucopyranose ring (Je et al., 2004; Kim & Rajapakse, 2005; Park, Je, & Kim, 2003a; Park, Je, & Kim, 2003b; Xing, Yu et al., 2005). Chen et al. (2009) describe the DPPH scavenging activity as the capacity of low molecular weight chitosan to donate an electron through hydroxyl and amine groups to the DPPH unpaired electron. So, a DPPH ion would be formed which depends on the combined effects of the different sizes of the electron-cloud density and the different accessibility between DPPH and low molecular weight chitosan.

On the other hand, some authors have reported an increase of the reducing power with the decrease in Mw (Feng et al., 2008; Huang et al., 2012; Tomida et al., 2009; Xing, Yu et al., 2005) but they did not consider the effect of the number of acetylated units. Recently, Li et al. (2012) have also shown that reducing power was higher in COS with low DP ($DP \leq 3$) and Jung and Zhao (2012) did not find influence of DA on this capacity. The FRAP of COS could be related with the electron donor capacity and the ionic transition from Fe^{3+} to Fe^{2+} being the mechanism of accessibility of the groups and donation of electrons similar to the scavenging activity described above.

According to our results, in general the effect of low molecular weight COS on the easy reaction of hydroxyl and amine groups with different DPPH radicals and with the capacity to done electrons and reduce Fe^{3+} is clear. However, this trend was not observed in lysozyme COS from CHTB. Some more characteristics could be influencing the development of this activity. Lower A/D values seemed to coincide with a better antioxidant activity, but the overlapping between ranges did not allow to find a clear correlation. In order to establish a closer relationship, the percentages of sequences with an A/D value in the ranges 0–1, 1–2 or higher than 2 were calculated (Table 2). The combination of these percentages was found useful to characterize the fractions. All the chitosanase products showed the highest percentage of sequences in the 0–1 A/D range. The differences in DPPH radical scavenging and reducing power antioxidant activity in these products are, therefore, attributed to the Mw. Regarding the lysozyme products, the comparison of the same Mw fractions indicated that the lower activity encountered could match up with the shift in the A/D percentages. Interestingly, in these lysozyme fractions similar percentages were found for 0–1 and 1–2 ranges together with the lower amount of sequences with A/D values over 2 in fractions with similar activity (COS F10–30). In contrast, in the F5–10 and F5 groups, where statistically

differences were found between CHT fractions (except in the reducing power of F5–10), the decrease in activity correlated well with an increase of products in the 1–2 and >2 groups. In the case of the COS F5 from CHTB, the high percentage (44%) reached by the >2 group seemed to be responsible for the dramatic decrease in activity compared to CHTA. The potent antioxidant activity in fractions with low A/D values could be linked to a higher number of deacetylated units present in these fractions and therefore to a higher availability of amine groups for proton donation or electron transference as antioxidant mechanism. On the other hand, the high content of acetylated units can increase the hydrophobic interactions producing aggregation (Sorlier, Viton, & Domard, 2002) and decrease the solubility. Therefore the detailed knowledge about the sample molecular composition and the analysis of the A/D values has permitted to explain the antioxidant activity from the molecular point of view and could be used to predict the lack of activity of a COS fraction.

4. Conclusions

This study has revealed that the antioxidant activity shown by the chitosanase products is better compared to the lysozyme products. Thus, fraction COS F5 coming from chitosanase depolymerization presents around 90% scavenging DPPH radicals and reduced Fe^{3+} at 25 micromolequivalent/g sample. Therefore, the low molecular weight in COS favors the mechanism to develop the antioxidant activity. However it is not the only factor to consider. An important relationship between composition expressed as ratio of acetylated *vs* deacetylated units in the COS sequences and antioxidant activity has been established. The COS fractions which present sequences with a majority (over 70%) of A/D ratio values between 0 and 1 in their units shows the best antioxidant activity. On the other hand, COS fractions where at least 40% A/D values are over 2 can be predicted as non antioxidant.

It would be interesting in further studies to deepen in the study of the intramolecular distribution pattern to know the architecture of the COS more accurately and to explain the antioxidant mechanism. Anyway, it is important to highlight the development of antioxidant activity of these COS from the point of view of their potential use as food preservative taking into account the actual recognition of ChitoClear® as GRAS substance and since the antimicrobial activity of these COS is known (Mengíbar, Ganan et al., 2011; Mengíbar, Mateos-Aparicio et al., 2011). These COS could be considered as potential antioxidants and may be a starting point in the development of valuable food additives.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.05.035.

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