Carbohydrate Polymers 84 (2011) 844–848

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Antibacterial activity of products of depolymerization of chitosans with lysozyme and chitosanase against *Campylobacter jejuni*

M. Mengíbar[a], M. Ganan[b], B. Miralles[a,b], A.V. Carrascosa[b], A.J. Martínez-Rodriguez[b], M.G. Peter[c], A. Heras[a,*]

[a] Instituto de Estudios Biofuncionales/Dpto. Química Física II, Facultad de Farmacia, Universidad Complutense de Madrid, Paseo Juan XXIII, 1, 28040 Madrid, Spain
[b] Instituto de Fermentaciones Industriales, CSIC, Dpto. Caracterización de Alimentos, C/Juan de la Cierva, 3, 28006 Madrid, Spain
[c] Institut für Chemie, Universität Potsdam, Karl-Liebknecht-Str. 25, D-14476 Potsdam, Germany

ARTICLE INFO

Article history:
Received 20 October 2009
Received in revised form 16 March 2010
Accepted 22 April 2010
Available online 29 April 2010

Keywords:
Campylobacter jejuni
Chitooligosaccharides
Chitosanase
Lysozyme
Depolymerization

ABSTRACT

Chitosan has several biological properties useful for the food industry, but the most attractive is its potential use as a food preservative of natural origin due to its antimicrobial activity against a wide range of food-borne microorganisms. Among food-borne pathogens, *Campylobacter jejuni* and related species are recognised as the most common causes of bacterial food-borne diarrhoeal disease throughout the world. Recently, it has been demonstrated that campylobacters are highly sensitive to chitosan. Even though chitosan is known to have important functional activities, poor solubility makes them difficult to use in food and biomedical applications. Unlike chitosan, the low viscosity and good solubility of chitosan oligosaccharides (COS) make them especially attractive in an important number of useful applications. In the present work, the effect of different COS on *C. jejuni* was investigated. Variables such as the physicochemical characteristics of chitosan and the enzyme used in COS preparation were studied. The COS had been fractioned using ultrafiltration membranes and each fraction was characterized regarding its F_A and molecular weight distribution. It has been demonstrated that the biological properties of COS on *Campylobacter* depend on the composition of the fraction analysed. COS prepared by enzymatic hydrolysis with chitosanase were more active against *Campylobacter* that lysozyme-derived COS, and this behaviour seems to be related with the acetylation of the chains. On the other hand, the 10–30 kDa fraction was the most active COS fraction, independently of the enzyme used for the hydrolysis. These results have shown that COS could be useful as antimicrobial in the control of *C. jejuni*.

1. Introduction

Low molecular weight chitosan (LMWC) is attracting increasing attention, since this group of polymers has potentially important biological activities, such as induction of chemotactic migration of polymorphonuclear cells, fat-binding, antithrombotic activity, antitumor activity, stimulating plant growth and antimicrobial activity (Kim & Rajapakse, 2005). Among these properties, one of the most attractive is its potential use as a food preservative of natural origin due to its antimicrobial activity against a wide range of food-borne microorganisms (Devlieghere, Vermeulen, & Debevere, 2004). Chitooligosaccharides (COS) can be prepared by chemical, physical and enzymatic hydrolysis of chitosan, among which, the enzyme hydrolysis receives more attention for its mild reaction conditions. The antimicrobial activity of COS is dependent on both, degree of acetylation (F_A) and molecular weight (M_w) (No, Park, Lee, & Meyers, 2002).

Campylobacter jejuni and related species are recognised as the most common causes of bacterial food-borne diarrhoeal disease throughout the world (Skovgaard, 2007). Although *Campylobacter* can colonize a variety of warm-blooded animals asymptomatically, this pathogen can also cause disease in humans, producing a self-limiting gastroenteritis (Lastovica, 2006). Among the sources of infection, the handling and/or consumption of poultry meat is considered a significant risk for human infection (Corry & Atabay, 2001), although an unknown number of human illnesses may be a consequence of infections associated with pet animals or surface waters (Acke et al., 2006; Richardson et al., 2007). For all these reasons, new potential antibacterial compounds for controlling *Campylobacter* are necessary. In a recent work, we demonstrate the sensitivity of *Campylobacters* to chitosans within a range of MW from 643 to 120 kDa (Ganan, Carrascosa, & Martínez-Rodríguez, 2009). However, the antimicrobial capacity of chitosan oligosaccharides on *C. jejuni* is unknown. The aim of this study was to prepare hetero-chitooligosaccharides with two enzymes of differ-

* Corresponding author. Tel.: +34 913943284; fax: +34 913943284.
E-mail address: aheras@farm.ucm.es (A. Heras).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.04.042

ent specificities for chitosan and to study the effect of M_w and F_A on their antibacterial activity against *C. jejuni*.

2. Experimental

2.1. Materials and preparation of chitosan oligosaccharides

Chitosan (F_A 0.09, M_w 210 kDa) was from Primex ehf, Siglufjørdur, Iceland. Chitosan (F_A 0.25, M_w 294 kDa) was prepared by deacetylation of snow crab chitin (Galed, Diaz, Goycoolea, & Heras, 2008). The F_A was determined by UV-spectroscopy (Tan, Khor, Tan, & Wong, 1998) and the average M_w by GPC (Domard & Rinaudo, 1984). 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 Chemie (Taufkirchen, Germany). All other reagents were of analytical grade. For preparation of chitooligosaccharides, chitosan (500 mg) was dissolved in 100 ml of acetate buffer 0.1 M. Enzyme solution (62.7×10^{-2} 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 pH adjusting (Galed, Miralles, Mengíbar, & Heras, 2007). The reaction mixtures were incubated at 37 °C in a shaking water bath at 100 rpm.

Samples were withdrawn at 0, 2, 72, 74 (addition of fresh enzyme), and 144 h (end time) from reaction mixtures with lysozyme, and at 0, 1, 24, 48, 72 and 96 h (end time) from reactions with chitosanase. Viscosity measurements were made in a semi-automatic Ubbelohde viscometer. Ultrafiltration of the samples at the end time was performed in a stirred cell with cellulose acetate membrane disks of 30, 10 and 3 kDa cut-off (Millipore Corporation, MA, USA). The ultrafiltrate fractions were spray dried using a Mini Spray Dryer B-290 (Buchi, Switzerland).

SEC-HPLC was performed by means of an IsoChrom LC pump (Spectra Physics, USA) connected to a protein pack 200 SW glass column packed with TSK gel 6000 PW. A Waters 410 differential refractometer and a multiangle laser-light scattering detector operating at 632.8 nm (Wyatt Dawn DSP) were connected online. A 0.15 M ammonium acetate/0.2 M acetic acid buffer (pH 4.5) was used as an eluent. Samples were dissolved in the buffer (5 mg/ml), filtered through a 0.45 μm pore size membrane (Millipore) before injection of aliquots of 120 μL. MALDI-TOF MS of the LMWC fractions was carried out using a Bruker Reflex II (Bruker Daltonik, Bremen, Germany) in the positive ion mode. For ionisation, a nitrogen laser (337 nm, 3 ns pulse width, 3 Hz) was used. 2,5-Dihydroxybenzoic acid was used as a matrix.

2.2. Antimicrobial activity of chitosan and chitosan oligomers against C. jejuni

The strain *C. jejuni* LP1 used in this work was a clinical isolate provided by Hospital La Paz, Madrid. It was stored at −70 °C in Microbank vials (Pro-Lab Diagnostics, Neston, UK). Liquid growth medium for *Campylobacter* consisted of Brucella Broth (BB) (Bencton, Dickinson and Company, Le Pont de Claix, France). The agar plating medium consisted of Müeller Hinton Agar (MHA) supplemented with 5% defibrinated sheep blood (MHB) (Biomedics, Madrid, Spain). Cultures of *C. jejuni* were prepared as follows: a frozen bead was inoculated into 50 ml BB contained in a 100 ml flask. This culture was incubated at 38 °C on a shaking platform at 150 rpm for 48 h, under microaerobic conditions: 85% (v/v) nitrogen, 10% (v/v) carbon dioxide, 5% (v/v) oxygen maintained using a Variable Atmosphere Incubator (VAIN) (MACS-VA500, Don Whitley Scientific, Shipley, UK). The cells were diluted 1:100 into 50 ml of fresh BB and grown for 18 h, until the late exponential phase.

The antimicrobial activity of chitosan and chitooligosaccharides was investigated against *C. jejuni*. One millilitre of bacterial culture in exponential phase (10^6 CFU/mL) was mixed with 1 ml of chitosan stock solution (1%) diluted with PBS to final concentration of 0.05% (final pH 7) and inoculated onto 24-multiwell plates (Sarstedt, Nürnberg, Germany). The same protocol was used for both, chitosans and fractions. Controls using PBS instead of chitosan were done. Samples were incubated during 24 h following the procedure described above. After incubation, serial decimal dilutions were prepared in PBS (Lonza, Verviers, Belgium) pH 7 and 20 μl volumes were spread onto fresh plates of Mueller Hinton Agar supplemented with 5% defibrinated sheep blood (MHB) (Biomedics, Madrid, Spain). The number of colony-forming units (CFU) was assessed after plates had been incubated for 48 h. Results were expressed as % of growth inhibition of *C. jejuni* respect to control without chitosan/oligosaccharides.

2.3. Statistical analysis

The analysis of variance was performed by SPSS 14.0 for Windows, version 16.0.1; this analysis was followed by the Student–Newman–Keuls post hoc test, when appropriate ($p < 0.05$).

3. Results and discussion

The course of degradation of these chitosan samples was conveniently studied by viscosity measurement. Fig. 1 shows the specific viscosity of the samples as a function of reaction time. The activity of lysozyme on both chitosans resulted in similar pattern (Fig. 1A). The intrinsic viscosity of the samples decreased dramatically in the early reaction stage, which can be attributed to an endo-type degradation process. Although the addition of fresh lysozyme has been frequently recommended, its addition after 72 h did not cause any improvement in the viscosity decrease. Chitosan (F_A 0.25) reached a lower viscosity than chitosan (F_A 0.09). This can be explained by the higher F_A of chitosan (0.25) which is close to optimal F_A (0.3) reported for lysozyme action on chitosan (Sashiwa, Saimoto,

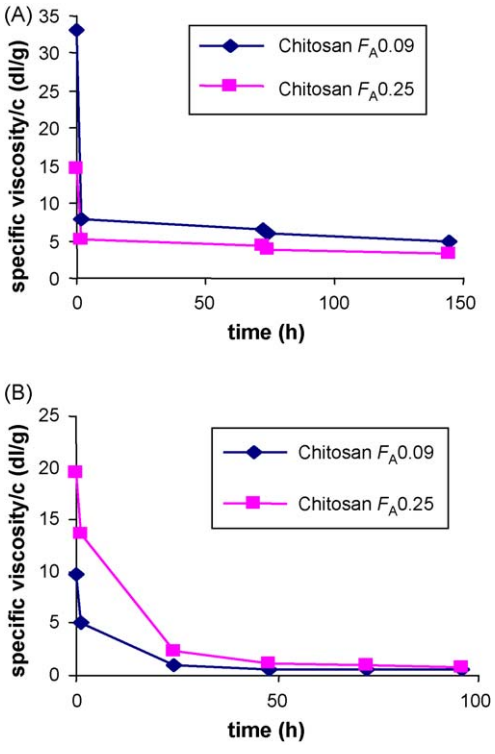

Fig. 1. Time course measurement of the specific viscosity of chitosan A and chitosan B depolymerized with (A) lysozyme and (B) chitosanase.

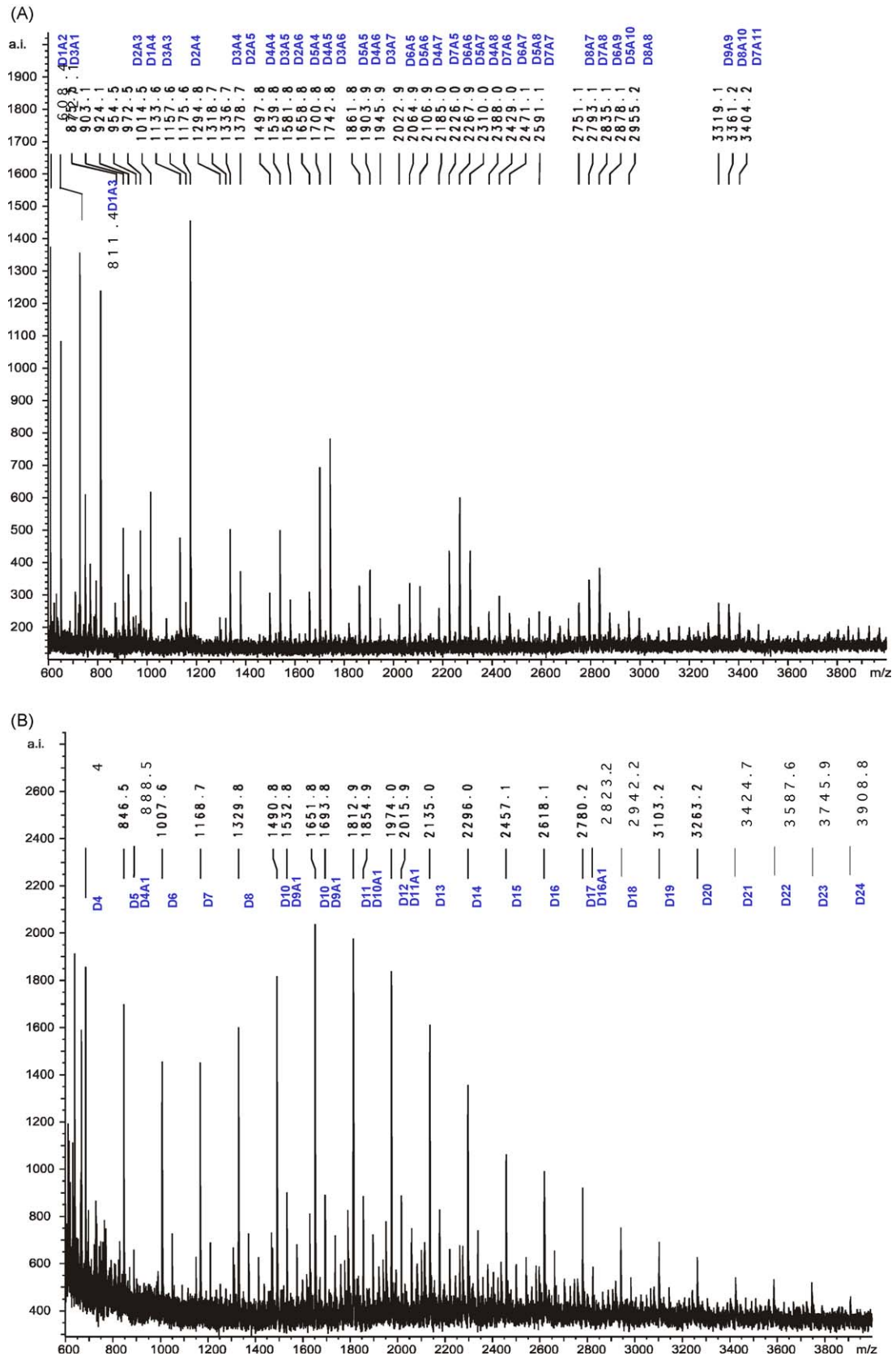

Fig. 2. MALDI-TOF MS of *F*10/3 obtained from chitosan (F_A 0.09) with (A) lysozyme and (B) chitosanase. Only some of the identified peaks are marked depolymerization of chitosan (F_A 0.25) with chitosanase.

Table 1
Molecular weight determined by SEC-HPLC of the original chitosan and $F \geq 30$ kDa after enzyme action (final time).

Chitosan	$F \geq 30$ after chitosanase action (kDa)	$F \geq 30$ after lysozyme action (kDa)
F_A 0.09, M_w 210	176	112
F_A 0.25, M_w 294	31	39

Shigemasa, Ogawa, & Tokura, 1990). A large viscosity decrease during the first hour is also observed with chitosanase (Fig. 1B). *Streptomyces* chitosanase has at least six monosaccharide binding subsites which showing a preference for GlcN (Fukamizo, Honda, Goto, Boucher, & Brzezinski, 1995). Due to the low F_A of both chitosans, the viscosity dropped to values close to 0.

COS produced from different chitosans or with different enzymes, even though they share a similar M_w, may have significantly different molecular size distributions. The products of the enzymatic hydrolysis were separated by ultrafiltration into fractions of $M_w \geq 30$ kDa ($F \geq 30$), 30–10 kDa ($F30/10$), and 10–3 kDa ($F10/3$). Also a fraction <3 kDa was obtained which could not be used for bioassays because of the low amount of the sample. In addition, this fraction contained the salts that came from pH adjustment and reaction buffer. SEC-HPLC of all samples after the ultrafiltration process showed a wide molecular distribution. We attribute the polydispersity to the characteristics of the native chitosans. Table 1 shows the M_w of the fractions $F \geq 30$. Higher elution volumes were found for chitosan (F_A 0.25) products. This can be explained by the way of deacetylation of the original chitosans: chitosan F_A 0.09, was prepared by homogenous deacetylation (random distribution of acetyl groups) while chitosan F_A 0.25 by heterogenous deacetylation (blockwise distribution). The conformation of the resulting oligomers of the $F \geq 30$ would be more extended in the first case, giving rise to a similar viscosity with a lower depolymerized chitosan.

MALDI-TOF MS of the LMWC fractions reveals pronounced differences between the chitooligosaccharides generated by the two enzymes, as shown for the depolymerization of chitosan (F_A 0.25) in Fig. 2. The product from the reaction with lysozyme is a mixture of hetero-chitooligosaccharides of DP 3–18, containing GlcN(GlcNAc)$_2$ (short notation: D_1A_2), D_3A_1, D_2A_4, D_3A_6, D_5A_7, D_7A_6, D_6A_9, and D_8A_{10} as the major components of each of DP 3, 4, 6, 9, 12, 13, 15, and 18, respectively (Fig. 2A and Supplement Table S1). According to Vårum, Holme, Izume, Stokke, and Smidsrød (1996), lysozyme hydrolyzes chitosan by cleavage of glycosidic bonds of the type –AA|AA– and –AA|AD–, whereas –AD|AA– and –DD|AA– are not, or only very slowly, susceptible to hydrolysis. The appearance of chitooligosaccharides of DP >6 would mean in this case that those do not contain any sequence that could be susceptible to hydrolysis, e.g. the sequence of D_7A_{11} must be composed mostly of alternating D and A units.

In contrast, the major products generated by hydrolysis of chitosan (F_A 0.25) with chitosanase are a series of fully deacetylated oligomers of GlcN as well as mono-, di-, and triacetylated homologs of the molecular composition ($D_{n-m}A_m$, $n = 4$–24, $m = 0$–3, $n + m < 25$) (Fig. 2B, see also Supplement Fig. S1 and Table S2). The appearance of the higher oligomers of GlcN, i.e. up to D_{24} indicates that glycosidic bonds of the type –A|D– and/or –D|A–, but not –D|D– are cleaved which is apparently in contradiction to the definition of the three classes of chitosanases (Fukamizo, Ohkawa, Ikeda, & Goto, 1994; Fukamizo et al., 2005), including a chitosanase from *S. griseus* HUT 6037 (Mitsutomi & Ohtakara, 1992), all of which cleave glycosidic bonds between two D residues. We assume that the persistence of the higher oligomers of GlcN is due to product inhibition or to a steady state of degradation and transglycosylation. Clearly, this item requires further investigations.

Table 2
Effect of chitosans and COS on the viable count of *C. jejuni*, sample concentration 0.05%. Data are expressed as % inhibition with respect to controls (±SD, $n = 3$).

Sample	% inhibition	
Chitosan (F_A 0.09)	51.1 ± 1.0	
Hydrolysis with	Lysozyme	Chitosanase
$F \geq 30$	52.4 ± 1.2	83.8 ± 1.2
$F30/10$	89.4 ± 0.0	99.6 ± 0.3
$F10/3$	0.0 ± 0.0	0.0 ± 0.0
Chitosan (F_A 0.25)	70.3 ± 3.8	
Hydrolysis with	Lysozyme	Chitosanase
$F \geq 30$	29.6 ± 3.7	73.0 ± 1.2
$F30/10$	63.2 ± 1.1	74.9 ± 0.4
$F10/3$	41.5 ± 4.3	60.1 ± 4.7

3.1. Antibacterial activity of chitosan oligomers against C. jejuni

Significant differences with regard to the antibacterial activity against *C. jejuni* were found with respect to the fractions prepared by enzymatic depolymerization with lysozyme and chitosanase (Table 2). As general behaviour, chitosan fractions prepared by enzymatic depolymerization with chitosanase inhibit stronger than those obtained using lysozyme. This behaviour seems to be related with the F_A, which is lower in oligomers obtained with chitosanase than in the oligomers produced using lysozyme, as shown in the mass spectra (Fig. 2). Currently, the number of primary amino groups is dependent on F_A and M_w of chitosan oligomers and it has been observed that the antibacterial activity tends to increase upon the increase in the deacetylation degree (DD) of COS (Tsai, Su, Chen, & Pan, 2002).

M_w of COS also influenced its antibacterial response. High molecular weight chitosan (HMWC) and medium molecular weight chitosan (MMWC) fractions, $F \geq 30$ and $F30/10$, obtained from lower acetylated chitosan (F_A 0.09) inhibit stronger than those obtained from chitosan (F_A 0.25), regardless of the enzyme used for depolymerization. Nearly complete inhibition is observed with the MMWC fraction obtained from chitosan (F_A 0.09) with chitosanase, being the most active of the fractions analysed. A relationship between the M_w of COS and their antimicrobial activity has been reported by several researchers (No et al., 2002). It has been shown that an M_w between 1 and 10 kDa is critical for inhibition of microorganisms and that the antimicrobial efficacy increases with the M_w (Jeon, Park, & Kim, 2001). Although there is not a general consensus about the most effective M_w, COS ranging in an average from 5 to 27 kDa are generally observed to be effective in suppressing bacterial growth (Gerasimenko, Avdienko, Bannikova, Zueva, & Verlamov, 2004). Partially hydrolyzed chitosan has been found to have a better antibacterial efficacy than chitosan submitted to extended hydrolysis (Uchida, Izume, & Ohtakara, 1989).

On the other hand, the low molecular weight chitosan (LMWC) fraction $F10/3$ from chitosan (F_A 0.09) does not show any inhibition, while it is a weak inhibitor when prepared from chitosan (F_A 0.25) with either enzyme. The explanation for this behaviour could be related with the size of the COS present in each fraction. COS with a polymerization degree (DP) of 30 ($M_w \sim 5000$) were found in the fraction corresponding to chitosan (F_A 0.25), while the fraction from chitosan (F_A 0.09) had oligosaccharides up to 18 DP ($M_w \sim 3000$) or 24 ($M_w \sim 3800$) depending on the enzyme used (Fig. 2). This is in accordance with previous works which consider that chitosans with an average M_w around 3 kDa could be not capable of suppressing the microbial growth (Kim & Rajapakse, 2005).

The analysis of the antibacterial response of the original chitosans showed that higher acetylated chitosan (F_A 0.25) inhibits stronger than chitosan (F_A 0.09). These results indicate that, in spite to its higher DD, other properties of chitosan (F_A 0.09) could interfere with the antibacterial activity against *C. jejuni*. For example,

it has been described by others that an excessive concentration of amino groups could promote a structure that involves cross-linking through strong intramolecular hydrogen bonds, being reduced the number of amino groups available to attach bacterial surfaces (Aranaz et al., 2009). This hypothesis and other possible explanations require further studies.

In conclusion, the results show that the *Streptomyces* chitosanase generates more deacetylated products that show higher antibacterial effect against *C. jejuni*. This antimicrobial effect is more pronounced for fractions with M_w between 10 and 30 kDa.

Acknowledgements

The strain of *C. jejuni* was kindly provided by Hospital La Paz, Madrid. This work was partially funded through Projects MAT2007-63753 of the Spanish Ministry of Education and CCG07-CSIC/AGR-2255 and AGL2009-07894 of the CSIC. BM thanks the Deutscher Akademischer Austauschdienst (DAAD) for a research scholarship at the University of Potsdam.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.04.042.

References

Acke, E., Whyte, P., Jones, B. R., McGill, K., Collins, J. D., & Fanning, S. (2006). Prevalence of thermophilic *Campylobacter* species in cats and dogs in two animal shelters in Ireland. *The Veterinary Record*, *158*, 51–54.

Aranaz, I., Mengibar, M., Harris, R., Paños, I., Miralles, B., Acosta, N., et al. (2009). Functional characterization of chitin and chitosan. *Current Chemical Biology*, *3*, 203–230.

Corry, J. E. L., & Atabay, H. I. (2001). Poultry as a source of *Campylobacter* and related organisms. *Journal of Applied Microbiology*, *90*, 96S–114S.

Devlieghere, F., Vermeulen, A., & Debevere, J. (2004). Chitosan: Antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiology*, *21*, 703–714.

Domard, A., & Rinaudo, M. (1984). Gel permeation on chromatography of cationic polymer on cationic porous silica gels. *Polymer Communications*, *25*, 55–58.

Fukamizo, T., Honda, Y., Goto, S., Boucher, I., & Brzezinski, R. (1995). Reaction mechanism of chitosanase from *Streptomyces* sp. N174. *The Biochemical Journal*, *311*(Pt. 2 SU), 377–383.

Fukamizo, T., Ohkawa, T., Ikeda, Y., & Goto, S. (1994). Specificity of chitosanase from *Bacillus pumilus*. *Biochimica et Biophysica Acta*, *1205*, 183–188.

Fukamizo, T., Yoshikawa, T., Katsumi, T., Amano, S., Saito, J.-I., Miki, K., et al. (2005). Substrate-binding mode of bacterial chitosanases. *Journal of Applied Glycoscience*, *52*, 183–189.

Galed, G., Diaz, E., Goycoolea, F., & Heras, A. (2008). Influence of *N*-deacetylation conditions on chitosan production from alpha-chitin. *Natural Products Communications*, *3*, 543–550.

Galed, G., Miralles, B., Mengíbar, M., & Heras, A. (2007). Characterisation of chitosan heterooligosaccharides fractions obtained by enzymatic depolymerization. In A. Domard, E. Guibal, & K. Varum (Eds.), *Advances in chitin science* (p. 467). Montpellier, France: European Chitin Society.

Ganan, M., Carrascosa, A. V., & Martínez-Rodríguez, A. J. (2009). Antimicrobial activity of chitosan against *Campylobacter* spp. and other microorganisms and its mechanism of action. *Journal of Food Protection*, *72*(8), 1735–1738.

Gerasimenko, D. V., Avdienko, I. D., Bannikova, G. E., Zueva, O. Y., & Verlamov, V. P. (2004). Antibacterial effects of water-soluble low-molecular-weight chitosans on different microorganisms. *Applied Biochemistry and Microbiology*, *40*, 253–257.

Jeon, Y. P., Park, P. J., & Kim, S. K. (2001). Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydrate Polymers*, *44*, 71–76.

Kim, S. K., & Rajapakse, N. (2005). Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydrate Polymers*, *62*, 357–368.

Lastovica, A. J. (2006). Emerging *Campylobacter* spp.: The tip of the iceberg. *Clinical Microbiology Newsletter*, *28*, 44–55.

Mitsutomi, M., & Ohtakara, A. (1992). Difference between microbial chitinase and chitosanase in the mode of action on partially *N*-acetylated chitosan. In C. J. Brine, P. A. Sandford, & J. P. Zikakis (Eds.), *Advances in chitin and chitosan* (pp. 304–313). London: Elsevier Applied Science.

No, H. K., Park, N. Y., Lee, S. H., & Meyers, S. P. (2002). Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology*, *74*, 65–72.

Richardson, G., Thomas, D. R., Smith, R. M. M., Nehaul, L., Ribeiro, C. D., Brown, A. G., et al. (2007). A community outbreak of *Campylobacter jejuni* infection from a chlorinated public water supply. *Epidemiology and Infection*, *135*, 1151–1158.

Sashiwa, H., Saimoto, H., Shigemasa, Y., Ogawa, R., & Tokura, S. (1990). *International Journal of Biological Macromolecules*, *12*, 296–1296.

Skovgaard, N. (2007). New trends in emerging pathogens. *International Journal of Food Microbiology*, *120*, 217–224.

Tan, S. C., Khor, E., Tan, T. K., & Wong, S. M. (1998). The degree of deacetylation of chitosan: Advocating the first derivative UV-spectrophotometry method of determination. *Talanta*, *45*, 713–719.

Tsai, G. J., Su, W. H., Chen, H. C., & Pan, C. L. (2002). Antimicrobial activity of shrimp chitin and chitosan from different treatments and applications of fish preservation. *Fisheries Science*, *68*, 170–177.

Uchida, Y., Izume, M., & Ohtakara, A. (1989). Preparation of chitosan oligomers with purified chitosanase and its application. In G. Skjåk-Bræk, T. Anthonsen, & P. Sandford (Eds.), *Chitin and chitosan: Sources, chemistry, biochemistry, physical properties and applications* (pp. 372–382). Barking, UK: Elsevier Applied Science.

Vårum, K. M., Holme, H. K., Izume, M., Stokke, B. T., & Smidsrød, O. (1996). Determination of enzymatic hydrolysis specificity of partially *N*-acetylated chitosans. *Biochimica et Biophysica Acta*, *1291*, 5–15.