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Preparation of chitooligosaccharides with degree of polymerization higher than 6 by acid or enzymatic degradation of chitosan

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Abstract

Chitosan was depolymerized either by HCl hydrolysis or enzymatic degradation with a commercial preparation Pectinex Ultra Spl. The chitooligosaccharides released by both methods were selectively precipitated in methanol solutions and characterized using MALDI-TOF mass spectrometry. Differences between the two methods were detected and concerned the degrees of polymerization of the fragments produced and their acetylation. The enzymatic method yielded shorter fragments with a higher proportion of fully deacetylated chitooligomers. Conversely, acid hydrolysis of the starting chitosan resulted in fragments with degrees of polymerization up to sixteen and more monoacetylated residues than with the enzymatic procedure.

Keywords: Chitooligosaccharides; Enzyme technology; MALDI-TOF-MS; Enzymatic hydrolysis; Acid hydrolysis; Viscosity

1. Introduction

Chitosan is a linear heteropolysaccharide composed of β-1,4-linked-D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) in varying proportions. This polysaccharide is a derivative of chitin, one of the most abundant natural amino polysaccharide extracted from the exoskeleton of crustaceans and insect, from fungal cell walls, etc. These substances have a wide variety of applications in the biomedical, pharmacological, agricultural and biotechnological industries [1,2]. Therefore, recent studies on chitosan have attracted interest in converting it to more soluble chitooligosaccharides, which possess a number of interesting biological activities, such as antibacterial, antifungal [3] and antitumor [4] properties as well as immunoenhancing effects [5] on animal health. Chitosan oligosaccharides also have been shown to induce various plant defense-related cellular responses [6–8] and possess by themselves antimicrobial properties [9] against a wide spectrum of phytopathogens.

The biological activity of chitooligosaccharides is known to depend on their structure [8]. Although some reports mention a size-dependent biological activity of chitosan oligomers, larger oligomers being more potent, most studies do not consider soluble chitooligosaccharides with degrees of polymerization (DPs) higher than 6 and some methods used to estimate these DPs are even ambiguous.

Different protocols have been developed to prepare chitooligosaccharides, among which acid or enzymatic depolymerization of chitosan are most frequently used. Short oligosaccharides with DPs up to 6 were mainly studied, partly because of the heterogeneous composition of chitosan hydrolysates and partly because of analytical limitations to isolate and identify chitooligosaccharides with DPs higher than 6.

Chitosanolysis achieved by acids can be carried out mainly using HCl [10,11] or HNO_2 [12]. These are very simple methods with good yield but they do not lend themselves to easy control and the removal of strong acid and byproducts of concomitant browning reactions is difficult. A further disadvantage of HNO_2 use is the structural modification of the end products it can provoke. The mechanism of this

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reaction involves a deamination of a 2-amino-2-deoxy-D-glucopyranose forming 2,5-anhydro-D-mannose at the new reducing end.

Enzymatic methods are specific and easier to control but their commercial use is limited due to cost and availability of chitosanases. To overcome these limitations, chitosan hydrolysis by food grade enzymes has been evaluated. Non-specific activity of various enzymes on chitosan has been reported and includes proteases [13], hemicellulases [14–16], lipases [17] and pectinases [18,19]. These studies focused mainly on factors affecting the enzymatic depolymerization, its kinetic and the characterization of low molecular weight chitosans obtained.

In this study, we prepared and characterized defined chitooligosaccharide mixtures enriched in oligomers with DPs >6. Chitosan was degraded either by acid hydrolysis or enzymatically using a commercial pectinase and a selective precipitation of the degradation products was evaluated using MALDI-TOF mass spectrometry. To our knowledge, this is the first detailed report on the production of chitooligosaccharides using a non-purified commercial enzyme preparation.

2. Materials

Chitin from Cuban lobster was supplied by Mario Muñoz Pharmaceutical Laboratories (Cuba) and used to prepare chitosan under heterogeneous conditions following the methodology described by Cabrera et al. [20]. Commercially available Pectinex Ultra Spl (Novozymes A/S, Bagsvaerd, Denmark) was used without further purification.

2.1. Charcaterization of the chitosan

The potentiometric determination of the degree of acetylation was carried out following the method given by Muzzarelli [21]. Chitosan was dissolved in an excess of acid and titrated with 0.1 M NaOH. The volume of base used between the two inflexion points observed on the titration curve corresponded to the acid consumed for the salification of amine groups and allowed the determination of the degree of acetylation of the chitosan.

The viscosity average molecular weight (M_v) determination of chitosan was performed using a Ubbelohde capillary viscometer ($Ø = 0.5$ mm) at 25 °C. The solvent was 0.3 M acetic acid/0.2 M sodium acetate and the average viscometric molecular weight was calculated using the classical Mark–Houwink equation; $[\eta] = K_m M_v^a$, where $[\eta]$ is the intrinsic viscosity $K_m = 3.5 \times 10^4$, $a = 0.76$.

2.2. Hydrolysis of chitosan by Pectinex Ultra Spl

Chitosan at a 10.0 g/L concentration was dissolved by overnight shaking at room temperature in 0.175 M acetate buffer pH 5.5. This chitosan solution (90 mL) was mixed with 10 mL of Pectinex Ultra Spl. The reaction mixture was incubated at 37 °C for 24 h.

Viscosity measurements—The viscosity of an aliquot (0.4 mL) of the reaction mixture was measured using a micro falling ball viscometer (Thermo Haake Inc., USA). Time courses of relative ($\eta_{rel} = t/t_0$) and specific $\eta_{sp} = (t - t_0/t_0)$ viscosities were determined (t: flow time of the reaction mixture; t_0: flow time of buffer).

Reducing sugars and glucosamine analysis—At appropriate time intervals, aliquots of the reaction mixture were boiled for 10 min to terminate the reaction. Unhydrolyzed chitosan was precipitated by adjusting the pH to 9.0 with 0.1 M NaOH, and the amounts of reducing sugars and glucosamine in the supernatant were determined by Schales' modified method [22] and Ride and Drysdale [23] method, respectively, using D-glucosamine as standard.

2.3. Acid hydrolysis of chitosan

Chitosan (2 g) was dissolved in dilute acetic acid and dried under vacuum until the content became a gelatinous paste. 100 mL of concentrated HCl (37%) was added and the suspension heated for 30 min at 72 °C under stirring. The reaction was stopped by immersion in an ice bath. Most of the solvent and HCl was evaporated under vacuum; the residue was then resuspended in water and the solution evaporated, those last two operations being repeated twice. The residue was finally dissolved in water and the solution brought to pH 6.5 by addition of concentrated 10 M NaOH.

2.4. Selective fractionation of chitosan oligosaccharides in methanol/water

The neutralized chitosan hydrolysates were precipitated with final methanol concentrations of 70, 80 and 90% (v/v). Each precipitate was centrifuged, washed with its corresponding methanol solution and vacuum dried. The supernatants were concentrated under reduced pressure.

2.5. TLC of chitooligosaccharides

The chitooligomers were separated by TLC on silica gel plates (MERCK 60. GF-254) using *n*-propanol: water: concentrated ammonia 7:2:1 (v:v:v) as solvent. Spots were visualised by charring with 10% H_2SO_4 in ethanol and each spot was identified by comparison with authentic samples.

2.6. Mass spectrometry

An amount of 0.5 μL of the sample solution was mixed on the target with 2 μL of a solution of 2,5-dihydroxybenzoic acid as a matrix (15 mg/mL) in 30% aqueous ethanol. Mass spectra were recorded on a Bruker Ultraflex (Bruker Daltonik, Bremen, Germany) in the positive ion mode. A nitrogen laser (337 nm, 3 ns pulse width, 3 Hz) was used. All spectra were measured in the reflector mode using external calibration.

3. Results and discussion

3.1. Chitosan oligomers obtained by acid hydrolysis

The molecular weight (M_w) of the chitosan used was found to be 82.9 kDa by viscosimetry as deduced from an intrinsic viscosity [η] of 416 mL/g and its DA was 12% as determined by titration. These parameters are in close agreement with previously published data for other chitosan obtained by similar ways [20]. For depolymerization, a low DA chitosan was submitted to acid hydrolysis following essentially the procedure described by Domard and Cartier [10] but the reaction time was limited to 30 min in order to increase the proportion of high molecular weight oligomers.

To reduce the proportion of low DP oligomers in the hydrolysis mixture, a selective precipitation in methanol solutions was used. By MALDI-TOF analysis of the soluble phase, it appeared that chitooligomers with DP up to 11 were soluble in 70 and 80% methanol and up to DP 8 only in 90% methanol (Fig. 1). In the 90% methanol precipitate, chitooligosaccharide yield amounted to 17% of the initial chitosan under the current conditions. The higher DP chitooligomers were precipitated as a white powder, leaving in the methanol solution the brown byproducts generated during the acid hydrolysis.

Since MALDI-TOF analysis is limited to molecular weights higher than 500 Da due to interference of the matrix signals, methanol soluble chitooligosaccharides were also analysed by silica TLC. Fig. 2 shows that methanol soluble chitooligosaccharides comprise oligomers with DPs up to 6. Higher DPs could not be separated by this method.

Tenuous little spots were observed between spots corresponding to fully deacetylated chitosan oligosaccharides. We speculate that these tiny spots correspond to partially acetylated chitooligosaccharides. This is supported by the MALDI-TOF-MS observation of monoacetylated chitooligosaccharides in the samples.

The MALDI-TOF-MS of the chitosan fractions precipitated by 90% methanol from the acid hydrolysate is shown in Fig. 3. Oligomers of DPs up to 16 were identified, which is clearly higher than what was found in the supernatant. Letzel et al. [24] showed that separation of chitosan hydrolysates by gel permeation chromatography allows the MALDI-TOF detection of chitooligosaccharides with DPs up to 50.

This fraction contained chitooligosaccharides (GlcN-oligomers) and several of their partial *N*-acetylated form (Table 1). During acid hydrolysis, not only the *O*-glycosidic linkage between residues but also the *N*-acetyl linkage can be hydrolyzed, even if the rate of hydrolysis in concentrated acid is about ten times higher than the rate of deacetylation [25]. These last authors also showed a high specificity in the acid reaction of the different glycosidic linkages of partially *N*-acetylated chitosans: the rate of acid hydrolysis of GlcN–GlcN and GlcN–GlcNAc is lower than the rate of cleavage of GlcNAc–GlcNAc and GlcNAc–GlcN, explaining why new reducing ends are dominated by acetylated

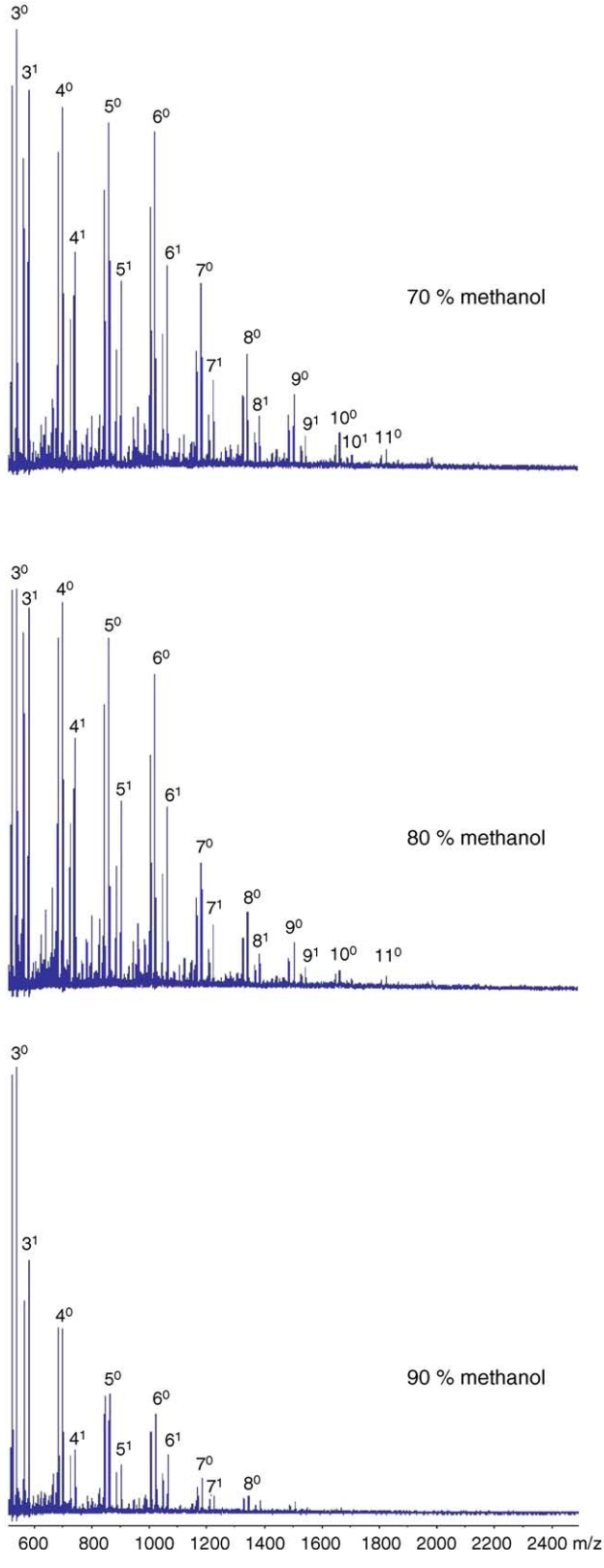

Fig. 1. MALDI-TOF-MS of methanol soluble chitooligosaccharides obtained by acid hydrolysis of the chitosan. Identified peaks are labeled as dp^{Ac}, where dp indicates degree of polymerization and Ac, the number of acetyl group.

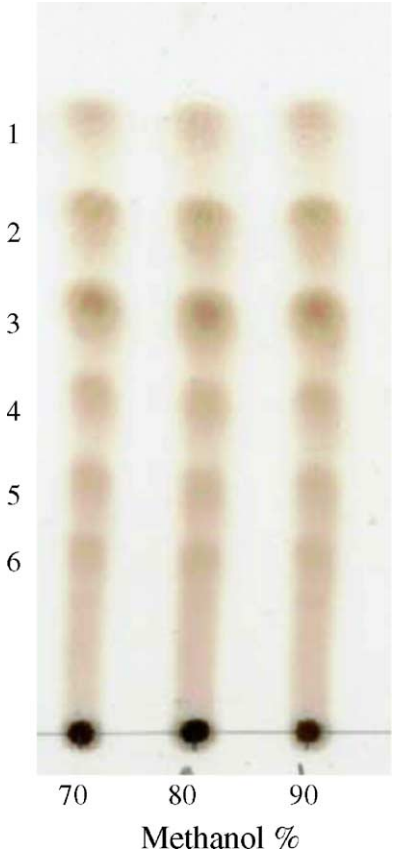

Fig. 2. Thin layer chromatography analysis of methanol soluble products resulting from chitosan hydrolysis by HCl. TLC performed on silica gel plate in a solvent system composed of *n*-propanol–water–ammonia water (7:2:1, v/v/v). The plate was developed by spraying ethanol containing 10% sulfuric acid. To the left of the TLC, the degree of polymerization of corresponding chitooligosaccharides is shown.

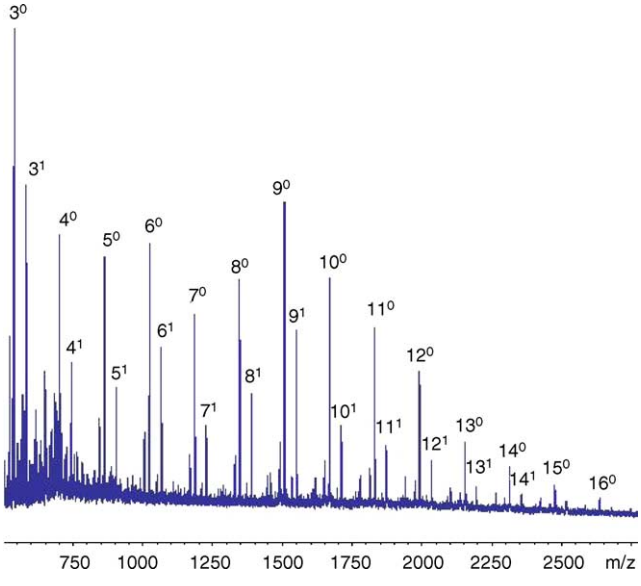

Fig. 3. MALDI-TOF-MS of 90% methanol insoluble fraction of chitooligomers obtained by a 30 min acid hydrolysis of chitosan. Identified peaks are labeled as dp^{Ac}, where dp indicates degree of polymerization and Ac, the number of acetyl group.

Table 1
Assigned ion composition of MALDI-TOF-MS spectra of chitooligosaccharides prepared by 30 min of acid hydrolysis and isolated by selective precipitation in 90% methanol

m/z	Types	Ion composition
524.2	$[M + Na]^+$	$(GlcN)_3$
540.1	$[M + K]^+$	
566.2	$[M + Na]^+$	$(GlcN)_2$–GlcNAc
582.1	$[M + K]^+$	
684.9	$[M + Na]^+$	$(GlcN)_4$
701.2	$[M + K]^+$	
743.2	$[M + K]^+$	$(GlcN)_3$–GlcNAc
846.3	$[M + Na]^+$	$(GlcN)_5$
862.3	$[M + K]^+$	
904.3	$[M + K]^+$	$(GlcN)_4$–GlcNAc
1007.4	$[M + Na]^+$	$(GlcN)_6$
1023.4	$[M + K]^+$	
1065.4	$[M + K]^+$	$(GlcN)_5$–GlcNAc
1168.4	$[M + Na]^+$	$(GlcN)_7$
1184.4	$[M + K]^+$	
1226.4	$[M + K] +$	$(GlcN)_6$–GlcNAc
1329.5	$[M + Na]^+$	$(GlcN)_8$
1345.5	$[M + K]^+$	
1387.5	$[M + K]^+$	$(GlcN)_7$–GlcNAc
1490.5	$[M + Na]^+$	$(GlcN)_9$
1506.5	$[M + K]^+$	
1548.5	$[M + K]^+$	$(GlcN)_8$–GlcNAc
1651.6	$[M + Na]^+$	$(GlcN)_{10}$
1667.6	$[M + K]^+$	
1709.6	$[M + K]^+$	$(GlcN)_9$–GlcNAc
1812.7	$[M + Na]^+$	$(GlcN)_{11}$
1828.7	$[M + K]^+$	
1870.7	$[M + K]^+$	$(GlcN)_{10}$–GlcNAc
1973.7	$[M + Na]^+$	$(GlcN)_{12}$
1989.7	$[M + K]^+$	
2031.8	$[M + K]^+$	$(GlcN)_{11}$–GlcNAc
2150.8	$[M + K]^+$	$(GlcN)_{13}$
2192.9	$[M + K]^+$	$(GlcN)_{12}$–GlcNAc
2311.9	$[M + K]^+$	$(GlcN)_{14}$
2473.0	$[M + K]^+$	
2634.1	$[M + K]^+$	$(GlcN)_{16}$

units. The analysis of our MS data show that the acid digests contained a wide distribution of both fully deacetylated and monoacetylated chitooligosaccharides. This pattern could be related to a random distribution of the acetyl substituents in the chitosan used for hydrolysis.

From these results, acid hydrolysis of chitosan combined with a selective methanol precipitation appears to be a quick and simple method to obtain good yields of chitooligosaccharides with DPs up to 16 and little low molecular weight oligomers.

3.2. Enzymatic degradation of chitosan

Chitosan was incubated with Pectinex Ultra Spl at 37 °C and pH 5.5 under constant stirring. The optimal pH and temperature were found to be between 5.0 and 6.0 and 40 and 50 °C, respectively. However, the reaction was carried out at 37 °C since the enzyme lost activity at its optimum

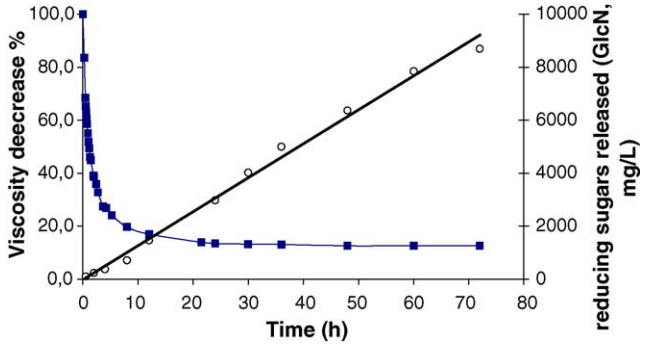

Fig. 4. Time course of enzymatic chitosan hydrolysis. Viscosity decrease (■) of the enzyme–chitosan mixture and reducing sugars content (◯) of the supernatant after alkaline precipitation as a function of hydrolysis time.

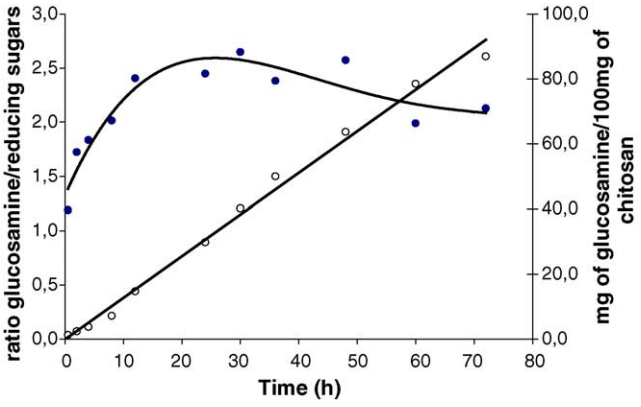

Fig. 5. Glucosamine content (◯) and glucosamine/reducing sugars ratio (●) in the supernatant after alkaline precipitation of the reaction mixture as a function of enzymatic hydrolysis time.

temperature range (data not shown). The optimum pH range for chitosanase activity of Pectinex Ultra Spl was in the range of reported values for its chitinolytic activity [26] and higher than those of chitosanase activity for the pectinase isozyme purified from *Aspergillus niger* [27].

As chitosans are relatively rigid linear polysaccharides, any change of their viscosity reflects modification of their degree of polymerization. The enzymatic degradation was therefore followed by measuring the viscosity of the reaction

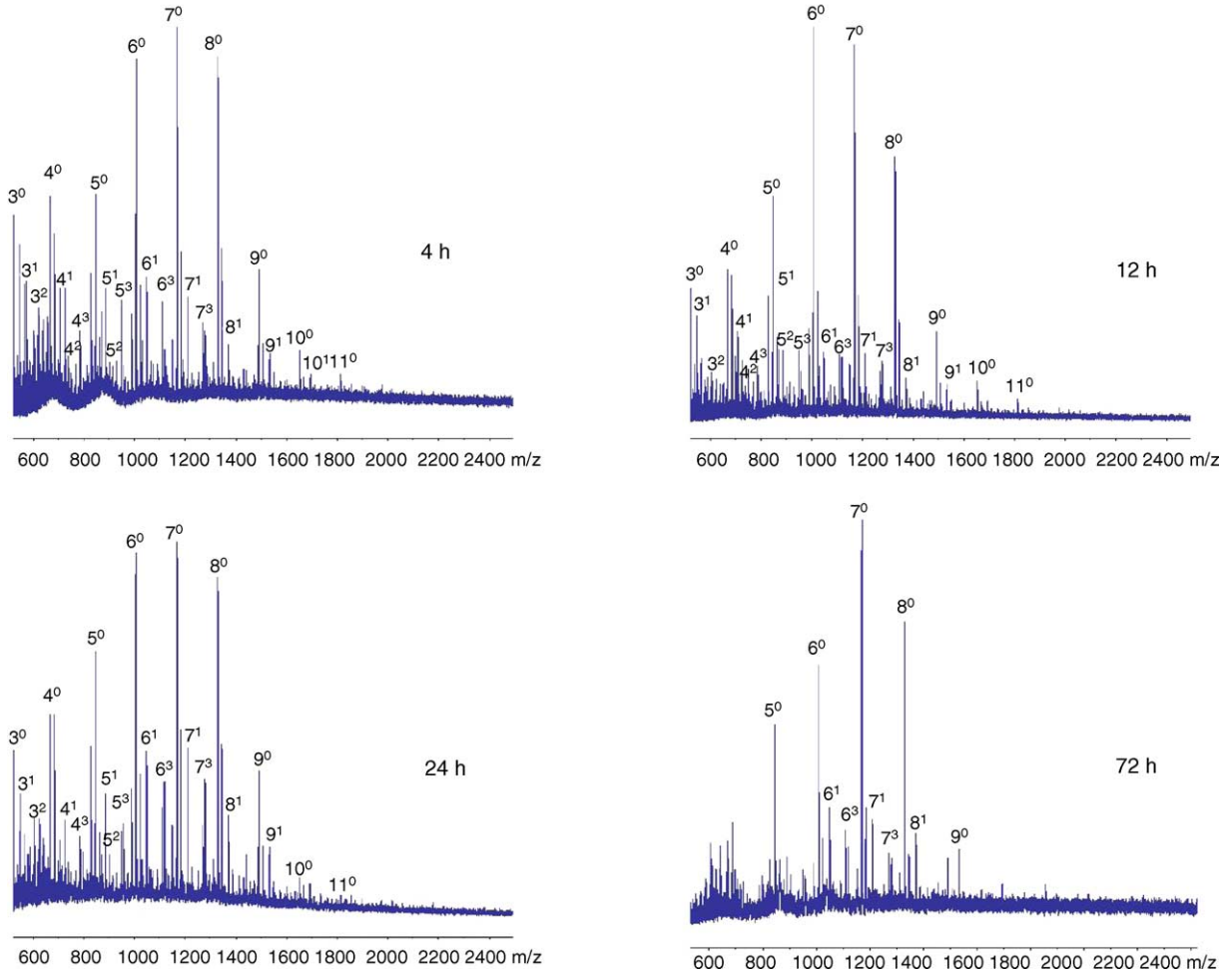

Fig. 6. MALDI-TOF-MS of the chitooligosaccharides oligomers mixtures obtained during the chitosan hydrolysis with Pectinex Ultra Spl. The proportion of low molecular weigth oligomers was reduced by precipitation in 90% MeOH. Identified peaks are labeled as dp^{Ac}, where dp indicate degree of polymerization and Ac, the number of acetyl group. The hydrolysis time is labeled in each spectrum.

mixture and determining reducing sugar and glucosamine contents in the hydrolyzed chitosan fraction as a function of time (Fig. 4). The enzyme treatment resulted in a fast and substantial loss of viscosity of the solution during the initial 5 h. A 50% decrease of viscosity within the first 60 min was associated with a low reducing sugar and glucosamine content in the degraded fraction, suggesting an endo mode of action of the Pectinex Ultra Spl preparation on chitosan.

The amount of degraded chitosan measured as glucosamine in the hydrolyzed fraction gradually increased during the enzymatic hydrolysis (Fig. 5). However, the glucosamine to reducing sugar ratio did not increase very much in solution and tended to stabilize at value of about 3 after 1 day of enzymatic hydrolysis. This behavior suggests a low and relatively similar degree of polymerization of chitooligosaccharides released during the enzymatic degradation.

In order to confirm this, the composition of the hydrolyzed fractions produced by the enzymes was analyzed by MALDI-TOF mass spectrometry. Since we are interested in longer oligomers, most of the shorter DPs were eliminated with the supernatant of a methanol selective precipitation. MALDI-MS analysis obtained in positive-ion mode of 90% methanol insoluble chitooligosaccharides in the Pectinex Ultra Spl digests after 4, 12, 24 and 72 h are shown on Fig. 6. More information about the assigned structure of each signal at different times of hydrolysis is given in Table 2. Chitosan fragments were detected mainly as sodium and/or potassium adduct ions and, under the conditions used, only chitooligosaccharides up to 12 mers were observed.

Enzymatically produced oligosaccharides with DPs >11 have been obtained from chitosans: Zhang and coworkers [15] using MALDI-TOF-MS detected chitooligosaccharides with DPs between 3 and 17 released by a mixture of cellulase, alpha amylase, and proteinase. They found that the peak intensities of non-acetylated fragments and of the mono-, di- or tri-acetylated forms of the same fragments were all similar.

It is very clear from Fig. 6 that the strongest *m*/*z* signals originate from oligomers of DPs 6–8 and that the *m*/*z* intensity profiles of these enzymatic digests do not change significantly over time. This confirms that the Pectinex mixture constantly releases short fragments of homogeneous size, which is to be compared to the MS spectra obtained from the acid hydrolyzed chitosan precipitate (Fig. 3) that revealed the presence of much longer chitooligomers. Another characteristic of the Pectinex enzymes is the presence in the digestion products of fragments with one and/or three acetyl residues. The MALDI-TOF analysis could not reveal the position of the acetyl substituents, but in all cases the most abundant peak did still correspond to fully desacetylated oligomers.

From the comparison of the MS spectra, it seems that there were less acetylated oligomers in the enzymatically prepared samples than in the acid hydrolyzed ones. Since no chitin acetylesterase activity could be detected in Pectinex Ultra Spl (unpublished results), this suggests that the enzymatic preparation preferentially attacks close to acetylated residues on chitosan chains. This is in agreement with previous observations showing an increased degrading activity of Pectinex enzymes with an increased degree of acetylation of the chitosan sample [28].

Table 2
Assigned ion composition of MALDI-TOF-MS spectra of chitooligosaccharides with degree of polymerization higher than 6 prepared by enzymatic hydrolysis for the indicated time and fractionated by selective precipitation in 90% methanol

m/*z*	Ion composition	Types	Hydrolysis time (h)							
			2	4	8	12	16	24	48	72
1007.4	$(GlcN)_6$	$[M+Na]^+$	X	X	X	X	X	X	X	X
1023.4		$[M+K]^+$		X	X	X	X	X	X	X
1049.9	$(GlcN)_5$–GlcNAc	$[M+Na]^+$		X	X	X	X	X	X	X
1065.4		$[M+K]^+$			X				X	
1109.9	$(GlcN)_3$–$(GlcNAc)_3$	$[M+H]^+$		X	X	X	X	X	X	X
1168.4	$(GlcN)_7$	$[M+Na]^+$	X	X	X	X	X	X	X	X
1184.4		$[M+K]^+$	X	X	X	X	X	X	X	X
1210.7	$(GlcN)_6$–GlcNAc	$[M+Na]^+$		X	X	X	X	X	X	X
1226.4		$[M+K]^+$			X			X	X	
1270.7	$(GlcN)_4$–$(GlcNAc)_3$	$[M+H]^+$		X	X	X	X	X	X	X
1329.5	$(GlcN)_8$	$[M+Na]^+$	X	X	X	X	X	X	X	X
1345.5		$[M+K]^+$	X	X	X	X	X	X	X	X
1371.8	$(GlcN)_7$–GlcNAc	$[M+Na]^+$		X	X	X	X	X	X	X
1387.5		$[M+K]^+$			X		X	X	X	
1490.5	$(GlcN)_9$	$[M+Na]^+$	X	X	X	X	X	X	X	X
1506.5		$[M+K]^+$		X	X	X	X	X	X	
1532.8	$(GlcN)_8$–GlcNAc	$[M+Na]^+$		X	X	X	X	X	X	X
1548.5		$[M+K]^+$			X				X	
1651.6	$(GlcN)_{10}$	$[M+Na]^+$	X	X	X	X	X	X		
1667.6		$[M+K]^+$			X					
1812.7	$(GlcN)_{11}$	$[M+Na]^+$	X	X	X		X	X		
1973.7	$(GlcN)_{12}$	$[M+Na]^+$	X		X					

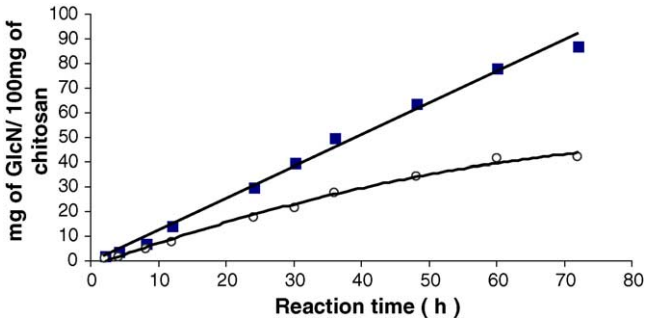

Fig. 7. Yield (as glucosamine content) of hydrolyzed chitosan fraction (■) and its 90% methanol insoluble fraction (○) as a function of the enzymatic reaction time.

The yields of hydrolyzed chitosan and of its 90% methanol insoluble fraction are shown on Fig. 7 as a function of the enzymatic reaction time. Both curves increased linearly over most of the experiment but the yield of the 90% methanol insoluble chitooligosaccharides reached a plateau after 60 h. The MALDI-TOF analysis showed us that no oligomer with DP >11 was still present after a 24 h hydrolysis. A 24 h enzymatic hydrolysis using Pectinex Ultra spl thus appears to result in a good compromise between oligomer size obtained (between 6 and 11) and yield (17% at 24 h).

In conclusion, the present study shows that a mixture of chitooligosaccharides enriched in oligomers with DPs higher than 6 can be easily produced from chitosan either by acid or by enzymatic degradation using a commercial pectinase. In both cases, mainly a mixture of fully desacetylated or monoacetylated forms of each chitooligosaccharide was obtained but the acid degradation produced higher DP fragments that could be readily precipitated in 90% methanol. The biological activity of these mixtures as elicitors of plants defense reactions is under study.

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References

[1] G.J. Brine, P.A. Sandford, J.P. Zikakis, Advances in Chitin and Chitosan, Elsevier Applied Science, London, 1992.
[2] N.V. Majeti, R. Kumar, A review of chitin and chitosan applications, React. Funct. Polym. 46 (2000) 1–27.
[3] Y.J. Jeon, P.J. Park, S.K. Kim, Antimicrobial effect of chitooligosaccharides produced by bioreactor, Carbohyd. Polym. 44 (2001) 71–76.
[4] A. Tokoro, N. Tatewaki, K. Suzuki, T. Mikami, S. Suzuki, Growth-inhibitory effect of hexa-*N*-acetylchitohexaose and chitohexaose, Chem. Pharm. B 36 (1988) 784–790.
[5] S. Hirano, M. Iwata, K. Yamanaka, H. Tanaka, T. Toda, H. Inui, Enhancement of serum lysozyme activity by injecting a mixture of chitosan oligosaccharides intravenously in rabbits, Agr. Biol. Chem. 55 (1991) 2623–2625.
[6] C.A. Ryan, Oligosaccharide signalling in plants, Annu. Rev. Cell Biol. 3 (1987) 295–317.
[7] M.S. Barber, R.E. Bertram, J.P. Ride, Chitin oligosaccharides elicit lignification in wounded wheat leaves, Physiol. Mol. Plant P 34 (1989) 3–12.
[8] M.G. Hahn, Microbial elicitors and their receptors in plants, Annu. Rev. Phytopathol. 34 (1996) 387–412.
[9] D.F. Kendra, L.A. Hadwiger, Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisum sativum*, Exp. Mycol. 8 (1984) 276–281.
[10] A. Domard, N. Cartier, Glucosamine oligomers: 1. Preparation and characterization, Int. J. Biol. Macromol. 11 (1989) 297–302.
[11] Y. Kikkawa, T. Kawada, L. Furukawa, T. Sakuno, A convenient preparation method of chitooligosaccharides by acid hydrolysis, J. Facul. Agr., Tottori University 26 (1990) 9–17.
[12] K. Tommeraas, K.M. Varum, B.E. Christensen, O. Smidrod, Preparation and characterization of oligosaccharides produced by nitrous acid depolymerisation of chitosans, Carbohyd. Res. 333 (2001) 137–144.
[13] A.B.V. Kumar, R.N. Tharanathan, A comparative study on depolymerization of chitosan by proteolytic enzymes, Carbohyd. Polym. (2004) 1–9.
[14] H. Zhang, S.H. Neau, In vitro degradation of chitosan by a commercial enzyme preparation: effect of molecular weight and degree of deacetylation, Biomaterials 22 (2001) 1653–1658.
[15] H. Zhang, Y. Du, X. Yu, M. Mitsutomi, S. Aiba, Preparation of chitooligosaccharides from chitosan by a complex enzyme, Carbohyd. Res. 320 (1999) 257–260.
[16] A.B.V. Kumar, R.G. Varadaraj, R.G. Lalitha, R.N. Tharanathan, Low molecular weight chitosans: preparation with the aid of papain and characterization, Biochim. Biophys. A 1670 (2004) 137–146.
[17] R.A.A. Muzzarelli, X. Wenshui, M. Tomasetti, P. Ilari, Depolymerization of chitosan and substituted chitosans with the aid of a wheat germ lipase preparation, Enz. Microb. Technol. 17 (6) (1995) 541–545.
[18] Y. Shin-ya, M.-Y. Lee, H. Hinode, T. Kajiuchi, Effects of *N*-acetylation degree on *N*-acetylated chitosan hydrolysis with commercial available and modified pectinases, Biochem. Eng. J. 7 (2001) 85–88.
[19] F. Kittur, A.B.V. Kumar, L.R. Gowda, R.N. Tharanathan, Chitosanolysis by pectinase isozyme of *Aspergillus niger*—a non-specific activity, Carbohyd. Polym. 53 (2003) 191–196.
[20] G. Cabrera, G. Cárdena, E. Taboada, J. Alderete, P. Casals, P. Neyra, Synthesis and characterization of new phosphorylated derivatives of chitin and chitosan. Insecticide properties, In: L. Mattoso, A. Frollini Leao, (Eds.), Proceedings of the Third International Symposium on Natural Polymers and Composites-ISNaPol/2000 and the Workshop on Progress in production and processing of Cellulosic Fibres and Natural Polymers, Embrapa Instrumentacao Agropecuaria, Brasil, 2000, pp. 119–126.
[21] R.A.A. Muzzarelli, Chitosan-based dietary foods, Carbohyd. Polym. 29 (1996) 309–316.
[22] O. Schales, S. Schales, A simple method for the determination of glucose in blood, Arch. Biochem. Biophys. 8 (1945) 285–292.
[23] J.P. Ride, R.B. Drysdale, A rapid method for the chemical estimation of filamentous fungi in plant tissue, Physiol. Pathol. 2 (1972) 7–15.

[24] M.C. Letzel, B. Synstad, V.G.H. Eijsink, J. Peter-Katalini, M.G. Peter, Libraries of chito-oligosaccharides of mixed acetylation patterns and their interactions with chitinases, Adv. Chitin Sci. 4 (2000) 545–552.
[25] K.M. Varum, M.H. Ottoy, O. Smidsrod, Acid hydrolysis of chitosans, Carbohyd. Polym. 46 (2001) 89–98.
[26] L. Roy, M. Sardar, M.N. Gupta, Hydrolysis of chitin by Pectinex^{TM}, Enz. Microb. Technol. 32 (2003) 582–588.
[27] F.S. Kittur, A.B.V. Kumar, R.N. Tharanathan, Low molecular weight chitosans-preparation by depolymerization with *Aspergillus niger* pectinase, and characterization, Carbohyd. Res. 338 (2003) 1283–1290.
[28] I. Hernández, Estudio de la hidrólisis de la quitosana con preparados enzimáticos comerciales, MSc Thesis, Instituto Nacional de Ciencias Agrícolas, La Habana, Cuba, 2005.