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ORIGINAL ARTICLE

Physicochemical and biological properties of chitosan derivatives with varying molecular weight produced by chemical depolymerization

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Abstract

Chitosan derivatives with lower Mw were prepared by chemical depolymerization using hydrochloric acid. This modification improves functional and biological properties of shrimp chitosan and facilitates its utilizations. The obtained chitosan depolymerization products (CDP) were characterized in terms of molecular weight (Mw), degrees of acetylation (DA) and polymerization (DP), solubility, viscosity, crystallinity, and FTIR spectroscopy. High Mw-CDP (from 94.10 to 396.46 kDa) and low Mw-CDP (< 4.4 kDa), with a DP up to 7, were withdrawn at different hydrolysis times. It is clearly demonstrated that the viscosity, the DA, and the crystallinity decreased upon depolymerization, especially in low Mw-CDP, while solubility in water and acetic acid was highly improved. FTIR analysis showed similar spectra of chitosan and CDP. The antibacterial, antifungal, and antioxidant properties of CDP were investigated and demonstrated that they were related to its Mw. Indeed, as compared to chitosan, high Mw derivatives, especially C120, possess higher antibacterial and antifungal potentials. While, low Mw-CDP, especially H120, exhibited the highest antioxidant properties. Interestingly, the used chemical depolymerization process seems to be an efficient, simple, and easy method to produce bioactive chitosan derivatives with attractive characteristics to be applied in an industrial scale, especially as functional-food components.

Keywords Chitosan depolymerization · Chemical hydrolysis · Physicochemical characteristics · Antimicrobial activity · Antioxidant properties · Functional-food components

1 Introduction

Chitosan is one of the most abundant renewable polysaccharides which is prepared from chitin by deacetylation and composed of β-1,4-linked glucosamine with various degrees of N-acetylated residues [1, 2]. This polymer is attracting a wide attention due to its biodegradability and non-toxicity that make it suitable for use in a wide variety of applications, such as biomedical, pharmacological, agricultural, and biotechnological industries [3–7]. However, its poor solubility in water and various aqueous solutions restricts many of its potential applications [8, 9].

Recently, chitosan depolymerization products (CDP) have attracted much more interest, because they are not only more soluble in aqueous solutions and possess lower molecular weight (Mw), but also exhibit interesting biological activities, such as antitumor, antifungal, and antibacterial properties, as well as immune-enhancing effects on animal health [2, 4, 10, 11]. Although some reports mention that the biological potential of CDP depends on their structure,

Highlights
- Chitosans with varying molecular weight were prepared by chemical depolymerization.
- Physiochemical characteristics of chitosan derivatives were determined.
- Antimicrobial and antioxidant potentials of chitosan derivatives were evaluated.

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especially their Mw and degrees of acetylation (DA) and polymerization (DP) [2, 12, 13].

Chitosan derivatives have been synthesized by several technological approaches, such as physical, enzymatic, and chemical methods. Physical approaches use the microwave and ultrasound energy for chitosan hydrolysis, but they are not often used commercially because of their energy-intensive nature [14, 15]. Chito-oligomers preparation can be achieved by enzymatic hydrolysis using specific enzymes [8, 16–18]; however, this method is limited due to the high cost and not availability of chitosanases. The hydrolysis of chitosan was further studied using different other commercial non-specific enzymes, such as chitinase [19, 20], lysozyme, papain, cellulose [21], and lipase [22], while these enzymes were added at high concentrations.

To overcome these limitations, chitosan hydrolysis by acids, mainly acetic acid [10], nitrous acid [23], phosphoric acid, hydrogen fluoride [9], and hydrochloric acid (HCl) [12, 24] has been evaluated as simple, easy, and practical method. Industrially, acid hydrolysis with HCl is preferred, since it is relatively simple, easy, and practical and gives a high yield [24, 25]. It is previously reported that HCl not only breaks the glycosidic bonds in chitosan chains but also hydrolyzes the N-acetyl amide groups to give chitosan chains with lower Mw and DA. Indeed, the acid concentration, the reaction time, and the used temperature are considered among the most crucial factors of the acid hydrolysis process [10, 24].

In the present study, chitosan derivatives (CDP) with varying characteristics were prepared by chemical hydrolysis of shrimp chitosan using HCl (6 M). The antimicrobial effect of the obtained CDP, compared to chitosan, was investigated against seven pathogenic bacteria and two fungi, which were mostly putrefactive microorganisms in food and aquatic preservation. Moreover, their antioxidant potential through different mechanisms was also studied.

2 Materials and methods

2.1 Reagents and chemicals

D-glucosamine hydrochloride and N-acetyl-glucosamine, the monomer unit of chitosan structure, were from Sigma Chemical Co. (USA). All other chemicals and reagents were of analytical grade. Shrimp chitosan was prepared as described in our previous study [26] and used as substrate for the chemical production of varying Mw chitosan derivatives. Prior to use, shrimp shells (SS) were washed thoroughly with tap water to remove contaminants, desiccated at room temperature, and then milled to powder in a Moulinex® blender. For chitin extraction, minerals, associated to SS, were firstly removed by chemical demineralization using three successive 0.5 M HCl baths at a ratio of 1:10 (w/v) at 4 °C with stirring at 30 rpm for 30 min. In a second step, chemical deproteinization of the demineralized material was carried out under standard autoclaving conditions at 121 °C using 10% (w/v) NaOH. The deacetylation of the obtained chitin was performed by treatment with 12.5 M NaOH at a ratio of 1:10 (w/v) for 4 h at 140 °C. After filtration, the residue was washed with distilled water until neutral pH was reached, and finally dried at 45 °C overnight to obtain shrimp chitosan.

2.2 Chitosan hydrolysis process

Chitosan depolymerization products (CDP) were prepared by acid hydrolysis of shrimp chitosan using hydrochloric acid (HCl) as described by Li et al. [12] with some modifications. Chitosan (20 g) was added to 600 ml of HCl solution (6 M). The obtained mixture was heated in a 70 °C bath under stirring for 2 h. Samples were periodically withdrawn at regular time intervals (30, 60, 90, and 120 min), adjusted to pH 8.0 with concentrated NaOH and centrifuged at 8000 × *g* during 30 mn.

The insoluble parts, obtained at 30, 60, 90, and 120 min, were freeze-dried and referred as high Mw-CDP: C30, C60, C90, and C120, respectively.

The soluble parts were concentrated on a rotary evaporator to remove HCl. Then, in order to remove salts, these compounds, considered as low Mw-CDP, were desalted on a size exclusion chromatography Sephadex G-25 column (100 × 13 mm). The column was eluted with ultrapure water with a flow of 0.5 ml/min. Elution was monitored spectrophotometrically by measuring the absorbance under UV light (OD 260 nm) and the reducing sugar content (SR mg/g chitosan), by the modified dinitrosalicylic acid (DNS) method using glucosamine as standard [25], in each collected tube (of 5 ml). The elution profiles of OD 260 nm and SR were graphed as function of the volume as depicted in Fig. S1. To collect the desalted fractions, the first 20 tubes, which correspond to the death volume, were discarded while the rest was collected according to OD 260 nm and SR results as follows: H30 (tubes from 21 to 110), H60 (tubes from 21 to 103), H90 (tubes from 21 to 107), and H120 (tubes from 21 to 75), obtained at 30, 60, 90, and 120 min, respectively. After G25, the collected fractions were freeze-dried and referred as low Mw-CDP.

2.3 Physicochemical characterization of chitosan and CDP

2.3.1 Molecular weight determination by steric exclusion chromatography (SEC-HPLC)

SEC-HPLC was performed with Waters 625 LC System Pump using an Ultrahydrogel column (I.D = 7.8 mm,

l = 300 mm) thermostated at 35 °C and connected to a Waters 2414 differential refractometer. Samples were dissolved in 0.15 M ammonium acetate/0.2 M acetic acid buffer (pH 4.5), at a concentration of 0.2% (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 molecular weights (Mw) of the different samples were obtained from the SEC profiles by extrapolation in a calibration curve using different known Mw dextrans as standards.

2.3.2 Mass spectrometric analysis (MALDI-TOF MS) of low Mw-CDP

MALDI-TOF MS was performed using an Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a Smart Beam TM ND YAG-laser (355 nm) in the positive ion mode. For ionization, 2,5-dihydroxybenzoic acid was used as matrix. All spectra were obtained in reflection mode with an acceleration voltage of 25 kV, a reflector voltage of 26 kV, and pulsed ion extraction of 40 ns in positive ion mode. The acquisition range was m/z 50–4000. The data were obtained by taking the average value of 500 laser shots, with the lowest laser energy necessary to obtain sufficient signal-to-noise ratios [2].

2.3.3 Measurement of the degree of acetylation

The potentiometric determination of the degree of acetylation (DA) was carried out following the first derivative UV spectrophotometric method given by Muzzarelli et al. [27]. UV measurements were carried out in a spectrophotometer in the wavelength range 190–240 nm. A calibration curve of N-acetyl-glucosamine (10–40 ppm) and samples, at a concentration of 0.1% (w/v), were prepared in 0.01 M acetic acid.

2.3.4 Functional properties evaluation

The intrinsic viscosity [η] of chitosan and its derivatives was studied according to Rinaudo et al. [28] using a semi-automatic Ubbelohde viscometer at 25 (±0.2) °C. Different concentrations (1.0–5.0 mg/ml) were prepared in an acetic acid (0.3 M)/sodium acetate (0.2 M) buffer (pH 4.5) (five determinations per concentration). The [η] (ml/g) was estimated by extrapolation and averaging from the Huggins equation:

$$\eta \mathrm{sp}/C = [\eta] + [\eta]^2 \times k_{\mathrm{H}} \times C$$

where ηsp/C is the reduced viscosity (ml/g); k_{H} is the Huggins' coefficient and C is the sample concentration (g/ml).

The solubility in water and acetic acid (1%) was performed according to the method of Fernandez-Kim [29]. The solubility of the sample was determined and calculated using the following equation:

$$\mathrm{Solubility}(\%) = \frac{(W_1 - W_2)}{(W_1 - W_0)} \times 100$$

where W_1 and W_2 are the initial and final tube weights containing sample, respectively, and W_0 is the initial tube mass.

2.3.5 X-ray diffraction measurements (XRD)

XRD patterns were performed using an X-ray diffractometer (Philips X′Pert SW) equipped with a copper anode. Samples were continuously scanned from 0 to 50° (2θ) at 45 kV and 40 mA. The crystallinity index (CrI) was calculated as described by Focher et al. [30]:

$$\mathrm{CrI}(\%) = \frac{(I_{110} - I_{\mathrm{am}})}{I_{110}} \times 100$$

where I_{110} is the crystallinity diffraction intensity at $2\theta = 20°$ and I_{am} is the amorphous diffraction intensity at $2\theta = 16°$.

2.3.6 Fourier-transform infrared spectroscopy analysis (FTIR)

FTIR spectra were recorded at 25 °C, in the spectral range frequencies of 500–4000 cm^{-1} and at a resolution of 4 cm^{-1}, on a Performer Spectra Tech spectrometer (Agilent Technologies, Carry 630 series) linked to an attenuated total reflectance accessory with a diamond crystal. Calibration was done using background spectrum recorded from the clean and empty cell.

2.4 Biological properties evaluation

2.4.1 Antibacterial activity

The antibacterial activity of chitosan and CDP was tested, using two methods, against three Gram-negative bacteria: *Salmonella enterica* (ATCC 14,028), *Pseudomonas aeruginosa* (ATCC 49,189), and *Enterobacter* sp and four Gram-positive strains: *Staphylococcus aureus* (ATCC 6538), *Micrococcus luteus* (ATCC 14,110), *Listeria monocytogenes* (ATCC 19,117), and *Bacillus cereus* (ATCC 11,778).

The agar well diffusion method was performed, as described by Vanden Berghe & Vlietinck [31]. Chitosan and CDP were dissolved in 0.1% acetic acid at a final concentration of 50 mg/ml and sterilized by filtration through

a 0.22-µm Nylon membrane filter. Firstly, Petri plates containing Mueller–Hinton agar were inoculated with bacterial solutions containing 10^6 CFU/ml of each bacterium suspension. Then, a hole with a diameter of 6 mm was punched in the agar aseptically using a sterile Pasteur pipette. A volume of 60 µl of each sample was introduced into previously cute wells. Acetic acid (0.1%), used to dissolve the tested samples, and gentamicin were used as negative and positive controls, respectively, to determine the sensitivity of each bacterial strain. The obtained agar plates were incubated at 4 °C for 2 h then placed at 37 °C for 24 h. Finally, the antagonistic effect of each sample was determined by measuring the diameter (including well diameter of 6 mm) of the growth inhibition zone around the wells.

Furthermore, the minimum inhibitory concentration (MIC) of chitosan and CDP, defined as the lowest sample concentration that inhibited the visible growth of tested bacteria after 24 h, was determined by liquid growth inhibition assay in a sterile 96-well microplates assay system according to Farag et al. [32]. The minimum bactericide concentration (MBC), which determines the sufficient sample concentration to destroy 99.99% of the microorganism population, was also studied. MIC and MBC values are expressed in % (or in g/100 ml).

2.4.2 Antifungal activity

The antifungal activity of chitosan and its derivatives was evaluated against two fungi: *Fusarium solani* and *Rhizoctonia solani*, using the agar well diffusion technique, as described by Vanden Berghe & Vlietinck [31]. Culture suspension, containing a concentration of 10^8 CFU/ml of each tested fungi, was spread over the malt-extract agar medium plates. Then, 60 µl of each tested samples, chitosan and CDP at a concentration of 50 mg/ml dissolved in acetic acid (0.1%), were delivered into wells, previously cut in the agar. Acetic acid (0.1%) and cycloheximide were used as negative and positive controls, respectively. The diameter of the clear growth inhibition zone around each well (including well diameter of 6 mm) was measured, after incubation of the obtained agar plates firstly at 4 °C during 2 h then at 30 °C for 72 h, and reported as the antifungal activity.

2.4.3 Antioxidant activity

DPPH and ABTS radical scavenging potentials The ability of chitosan and its derivatives to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was estimated using the method described by Bersuder et al. [33].

The capacity of chitosan and CDP to quench the long-lived 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) species was also studied according to the method of Re et al. [34]. The DPPH and $ABTS^+$ scavenging activities were expressed as the 50% effective concentration (IC_{50}) (mg/ml). Butylated hydroxyl-anisole (BHA) was used as a positive control.

Reducing power activity The ability of BHA, chitosan, and its derivatives to reduce iron (III) was evaluated according to the method described by Yildirim et al. [35]. Higher absorbance of the reaction mixture showed higher reducing power. The reducing power activity was reported as the effective concentration which allows to obtain an absorbance of 0.5.

Total antioxidant assay The total antioxidant ability of chitosan and CDP to reduce Mo (VI) to Mo (V) and to form a phosphate/Mo (V) complex at acidic pH was tested as reported by Prieto et al. [36]. The total antioxidant activity was reported as α-tocopherol equivalents and BHA was used as a positive control.

2.5 Statistical analysis

All experiments were carried out in triplicate, and average values with standard deviation errors are reported. Mean separation and significance were analyzed using the SPSS software package ver. 17.0 professional edition (SPSS, Inc., Chicago, IL, USA) using ANOVA analysis with Duncan post hoc testes. Differences were considered significant at $p < 0.05$.

3 Results and discussion

3.1 Preparation of CDP by acid depolymerization of chitosan

Among depolymerization methods, acid hydrolysis of chitosan has emerged as a convenient method, especially when owing industrial-scale production. Industrially, acid hydrolysis is preferred, since it is relatively simple and practical and gives a high yield [9, 24]. Indeed, hydrolytic degradation of a polymer involves diffusion of the active medium into the polymer and reactions involving chemically unstable bonds. Both main chains (D-glucopyranoside linkages) and side chains (N-acetyl) in chitosan are susceptible to acid hydrolysis [37]. In this study, different chitosan derivatives (CDP) were produced from shrimp chitosan (Mw: 1244.70 kDa, DA: 7.60%) by chemical depolymerization with HCl, at different time intervals (30, 60, 90, and 120 min), confirming the efficiency of such conditions in the hydrolysis of chitosan glycosidic and the N-acetyl bonds. As well, the energy required to break the two linkages is available at the used temperature (70 °C).

3.2 Physicochemical characterization of CDP

The Mw reduction of samples withdrawn during chitosan hydrolysis was followed by SEC-HPLC analysis, and the obtained average Mw values are given in Table 1. The chromatograms of SEC-HPLC (Fig. 1) showed that the peak of the elution curve of the original chitosan (Mw = 1244.70 kDa) shifted toward longer retention time as the Mw of CDP decreased evidencing depolymerization. Results revealed that insoluble parts obtained after chitosan hydrolysis and recovered at different incubation times, 30, 60, 90, and 120 min, exhibited an average Mw of 396.46, 170.65, 109.63, and 94.10 kDa for C30, C60, C90, and C120, respectively. These compounds were referred as high Mw-CDP. Whereas, SEC-HPLC analysis of the desalted soluble parts showed that in all cases, the retention times were out of the dextran calibration curve showing a Mw lower than 4.4 kDa, these samples were considered as low Mw-CDP. Such decrease in the Mw of CDP, as compared to initial chitosan, can give clear cut evidence of the hydrolysis of chitosan under the used reaction conditions, particularly at higher reaction time (120 min). Similarly, Kasaai et al. [37] reported that the Mw of the resultant chitosan polymers decreases with increasing reaction time. This chitosan depolymerization process mechanism involves the attachment of a proton (H^+) to the glycoside linkage (protonation), followed by scission of large macromolecules into smaller ones [37]. In the same context, Li et al. [38] reported a marked decrease in the Mw of chitosan (DA = 14.1%) from 230 to 90 kDa in 12 h of hydrolysis with 1.5 M HCl using an induced electric field and temperature variation between 25 and 45 °C. In another study using chitosan (Mw: 200 kDa, DA: 10%) hydrolyzed with HCl 2 M for 12 h, chitooligosaccharides (COS) of 2 kDa were obtained [24].

In order to evaluate the oligomer composition of low Mw-CDP, samples were analyzed by MALDI-TOF MS and identified as sodium adducts $[M+Na]^+$. The assigned structure of each sample is given in Table 2. It appeared that

Table 1 Average molecular weight (Mw), intrinsic viscosity, degree of acetylation (DA), acetic acid (AS) and water (WS) solubilities, and crystallinity index (CrI) of chitosan and its derivatives obtained by chemical hydrolysis of shrimp chitosan

Sample	Mw (kDa)	Viscosity (dl/g)	DA (%)	AS (%)	WS (%)	CrI (%)
Chitosan	1244.70	7.81 ± 0.21^{A}	7.60 ± 0.54^{A}	75.89 ± 0.83^{D}	15.09 ± 0.06^{F}	74.40
C30	396.46	2.07 ± 0.02^{B}	7.50 ± 0.60^{A}	96.52 ± 0.26^{C}	18.04 ± 0.03^{E}	45.25
C60	170.65	1.44 ± 0.04^{C}	6.66 ± 0.04^{AB}	98.20 ± 0.97^{B}	18.46 ± 0.03^{D}	ND
C90	109.63	1.12 ± 0.00^{C}	6.63 ± 0.04^{AB}	99.65 ± 0.19^{A}	19.59 ± 0.08^{C}	ND
C120	94.10	0.66 ± 0.01^{D}	5.94 ± 0.15^{B}	99.75 ± 0.15^{A}	21.07 ± 0.16^{B}	25.76
H30	< 4.4	UD	4.32 ± 0.06^{C}	100 ± 0.00^{A}	100 ± 0.00^{A}	00.00
H60	< 4.4	UD	5.00 ± 0.19^{C}	100 ± 0.00^{A}	100 ± 0.00^{A}	00.00
H90	< 4.4	UD	4.93 ± 0.01^{C}	100 ± 0.00^{A}	100 ± 0.00^{A}	00.00
H120	< 4.4	UD	4.76 ± 0.11^{C}	100 ± 0.00^{A}	100 ± 0.00^{A}	00.00

Note: Values are means ± standard deviation ($n = 3$). *UD* indicates data were undetected, *ND* indicates data were undetermined, Means with different letters (A–F) and within a column indicate significant difference ($p < 0.05$)

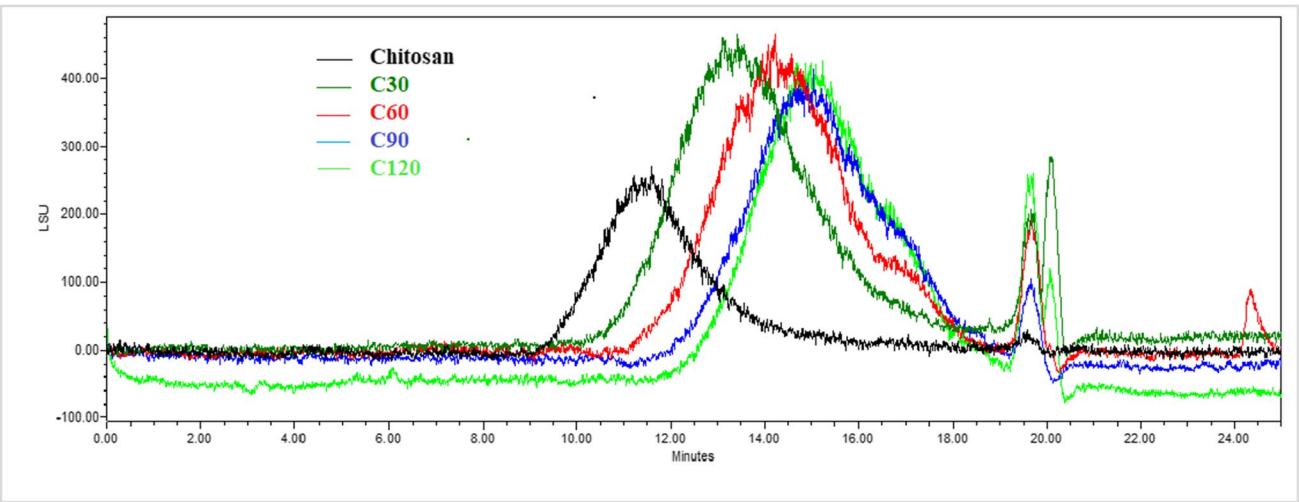

Fig. 1 SEC-HPLC chromatograms of chitosan and its derivatives (C30, C60, C90, and C120) obtained by chitosan depolymerization

Table 2 Ion composition (%) of desalted low Mw-CDP (H30, H60, H90 and H120) prepared by chemical hydrolysis of shrimp chitosan at different hydrolysis times (30, 60, 90, and 120 min), analyzed by MALDI-TOF MS

m/z $[M+Na]^+$	Ion composition		Intensity			
			H30	H60	H90	H120
363.14	**DP 2**	$(GlcN)_2$	15	12	12	10
405.15	**DP 2**	(GlcN)–GlcNac	14	14	11	13
447.16	**DP 2**	$(GlcNac)_2$	29	15	10	
524.21	**DP 3**	$(GlcN)_3$	27	24	13	22
566.22	**DP 3**	$(GlcN)_2$–GlcNac	15	18	33	23
608.23	**DP 3**	GlcN–$(GlcNac)_2$	46	59	57	53
650.24	**DP 3**	$(GlcNac)_3$	37	48	44	20
685.27	**DP 4**	$(GlcN)_4$	18	17	22	19
727.27	**DP 4**	$(GlcN)_3$–GlcNac	21	49	26	14
769.29	**DP 4**	$(GlcN)_2$–$(GlcNac)_2$	11	40	22	15
811.3	**DP 4**	GlcN–$(GlcNac)_3$	13	81	13	11
853.31	**DP 4**	$(GlcNac)_4$	17	27		21
846.34	**DP 5**	$(GlcN)_5$	16	17	17	
888.35	**DP 5**	$(GlcN)_4$–$(GlcNac)_1$	16			18
930.36	**DP 5**	$(GlcN)_3$–$(GlcNac)_2$	10			
972.37	**DP 5**	$(GlcN)_2$–$(GlcNac)_3$	14			
1056.39	**DP 5**	$(GlcNac)_5$	17	10		
1007.41	**DP 6**	$(GlcN)_6$	18	17	15	10
1049.42	**DP 6**	$(GlcN)_5$–$(GlcNac)_1$	10			
1091.43	**DP 6**	$(GlcN)_4$–$(GlcNac)_2$	10			
1175.45	**DP 6**	$(GlcN)_2$–$(GlcNac)_4$	13			
1217.46	**DP 6**	$(GlcN)_1$–$(GlcNac)_5$	14			
1210.49	**DP 7**	$(GlcN)_6$–$(GlcNac)_1$	10			

Note: *DP* indicates the degree of depolymerization. Only peaks corresponding to oligosaccharides are listed; matrix peaks and unidentified components are not shown

during hydrolysis, the O-glycosidic and the N-acetyl linkages between chitosan residues are chemically hydrolyzed, leading to a mixture of acetylated (GlcNac-oligomers) (A) and deacetylated chito-oligomers (GlcN-oligomers) (D) with DP up to 7. This pattern could be related to a random distribution of the acetyl substituents in the chitosan used for hydrolysis [25]. Similarly, Vårum et al. [39] reported that during acid hydrolysis of chitosan using HCl, not only the O-glycosidic linkage between residues (depolymerization) but also the N-acetyl linkage (deacetylation) can be hydrolyzed, mainly using higher acid concentrations.

After 2 h of chitosan chemical hydrolysis, the yielded products were a mixture of hetero-chito-oligomers with DP 2, 3, 4, and 5 containing $(GlcN)_2$ (D2), (GlcN)-GlcNac (D1A1), $(GlcN)_3$ (D3), $(GlcN)_2$–GlcNac (D2A1), GlcN–$(GlcNac)_2$ (D1A2), $(GlcNac)_3$ (A3), $(GlcN)_4$ (D4), $(GlcN)_3$–GlcNac (D3A1), $(GlcN)_2$–$(GlcNac)_2$ (D2A2), GlcN–$(GlcNac)_3$ (D1A3), $(GlcNac)_4$ (A4), $(GlcN)_4$ $(GlcNac)_1$ (D4A1), and $(GlcN)_6$ (D6) as the major components. In the same context, Li et al. [12] reported that COS with DP ranging from 2 to 12 can be obtained from chitosan (Mw: 658 kDa, DA: 18%), hydrolyzed using HCl (6 M) at the same conditions (70 °C, for 2 h). Moreover, Gonçalves et al. [40] stated that the use of HCl is effective in the depolymerization and deacetylation of chitosan making possible to obtain trimer, pentamer, hexamer, and heptamer-decamer COS fractions.

The degree of acetylation (DA) of chitosan and its derivatives was further determined and results are depicted in Table 1. It appeared that there is no significant difference in DA between chitosan and high Mw-CDP ($p > 0.05$), except of C120 in which the DA slightly decreased ($p < 0.05$). However, all low Mw-CDP showed a significant decrease in the DA values as compared to chitosan and high Mw-CDP. The decrease of the DA in chitosan derivatives obtained by acid hydrolysis was previously reported [24].

Moreover, the hydrolysis of chitosan was highlighted by the significant decrease in the intrinsic viscosity of chitosan (7.81 ± 0.21 dl/g) as compared to CDP, which is correlated with the decrease in Mw ($p < 0.05$). Results, illustrated in Table 1, showed that the decrease of the viscosity of high Mw-CDP was more pronounced during the first 30 min (2.07 dl/g), followed by a slow decrease during the rest of the reaction (0.66 ± 0.01 dl/g for C120). While, low Mw-CDP are not viscous.

In addition, solubility in acetic acid (1%) and water of chitosan and CDP was studied. Results illustrated in Table 1 showed that, as expected, the samples solubility increases

with the Mw decrease. Contrarily to chitosan, high Mw-CDP are soluble in acetic acid (1%), while, they exhibited a poor water solubility (< 22%) (Table 1). However, low Mw-CDP were totally soluble in acetic acid 1% and water (100%). The reason for the increased solubility of chitosan derivatives was the destruction of intra macromolecular and inter chain hydrogen bonds, which alters the secondary structure of chitosan, decreasing its crystallinity and unfolding its molecular chains [1].

Besides, the acid degradation of chitosan was accompanied by a decrease in the crystallinity. The x-ray diffraction patterns of chitosan and high Mw-CDP (C30 and C120) are illustrated in Fig. 2. Chitosan exhibited two crystalline peaks at $2\theta = 10$ and 20°, which corresponded to its hydrated polymorphe structure, with a crystallinity index (CrI) of 74.4% (Table 1). However, after chemical hydrolysis, the crystallinity decreased in C30 and C120, showing a less intense peak at $2\theta = 20°$, while the peak at $2\theta = 10°$ disappeared, suggesting their amorphous structure. The CrI of C30 and C120 were 45.25 and 25.76%, respectively (Table 1). Low Mw-CDP are not crystalline (data not shown).

The physicochemical properties of chitosan and CDP (C30, C120, H30, and H120) were also studied by FTIR analysis, showing similar spectra and confirming the conservation of the basic structure of chitosan during hydrolysis. FTIR spectra (Fig. 3) showed characteristic absorption bands at 3370 and 3296 cm^{-1} attributed to the stretching vibration of –OH and NH groups, respectively. The small peak at around 2875 cm^{-1} was ascribed as CH_2 and CH_3 groups. The signal observed at 1650 cm^{-1} was characteristic of the amide I band (C = O in the NH-$COCH_3$ group). At around 1562 cm^{-1}, appeared the band of amine (NH). The peak at around 1420 cm^{-1} was characteristic to the C–H bending vibration of CH_2 groups. Similar FTIR spectra of chitosan and its derivatives were obtained by Affes et al. [26] and Li et al. [41].

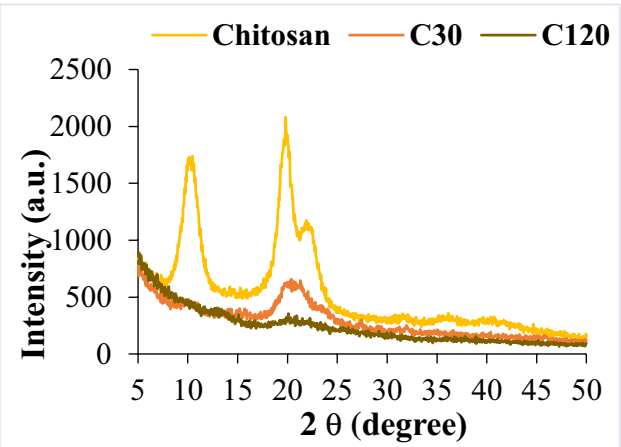

Fig. 2 X-ray diffractograms of chitosan and its derivatives (C30 and C120)

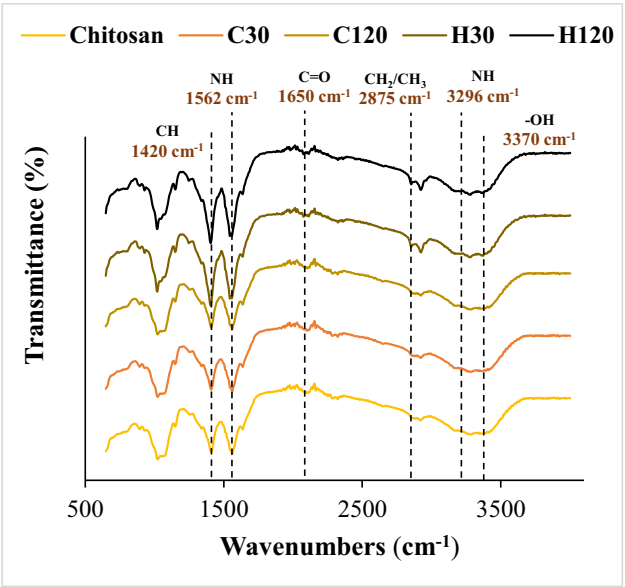

Fig. 3 FTIR spectra of chitosan and its derivatives (C30, C120, H30, and H120) obtained after 30 min and 120 min

3.3 Biological properties evaluation

3.3.1 Antibacterial potential

The antibacterial potential of chitosan and its derivatives was firstly performed using the agar well diffusion method. Results reported in Table 3 showed that chitosan and all CDP inhibited the growth of the seven studied pathogenic bacteria, with varying diameters of inhibition and with higher inhibitory potential against Gram (−) than Gram (+) strains. Moreover, the resistance of the bacterial strains tested toward chitosan and its derivatives decreased in the following order: *B. cereus* > *M. luteus* > *L. monocytogenes* > *S. aureus* > *E.* sp > *S. enterica* > *P. aeruginosa*. Furthermore, high Mw-CDP, especially C120 (Mw = 94.10 kDa, DA = 5.94%), possess significantly higher diameters of growth inhibition comparing to chitosan and low Mw-CDP ($p < 0.05$). For the strains *S. enterica*, *P. aeruginosa*, and *L. monocytogenes*, C120 showed similar antagonistic effect than that of the positive control gentamycin ($p > 0.05$).

Subsequently, the MIC and MBC were determined to compare the antibacterial potential of these compounds. As depicted in A and B in Table 4, the MIC and MBC values fluctuate from 0.0012 to 0.039% and from 0.01 to 0.3125%, respectively, against Gram (−) strains and from 0.0048 to 0.3125% and 0.019 to 1.875%, respectively, toward Gram (+) strains, confirming the better inhibitory potential against the Gram (−) bacteria obtained with agar diffusion method. Similarly, among the tested samples, C120 exhibited the lowest MIC and MBC values, leading to the highest

Table 3 Antimicrobial potential of chitosan and CDP samples, through agar diffusion method, against various bacteria and fungi at 50 mg/ml

Strain sample	Diameter of inhibition zone (mm)								
	Gram (−) bacteria			Gram (+) bacteria				Fungi	
	Salmonella enterica	*Pseudomonas aeruginosa*	*Enterobacter* sp	*Staphylococcus aureus*	*Micrococcus luteus*	*Listeria monocytogenes*	*Bacillus cereus*	*Rhizoctonia solani*	*Fusarium solani*
Positive control	18^{A}	18^{A}	26^{A}	25^{A}	19^{A}	16^{A}	22^{A}	22^{A}	23^{A}
Chitosan	12.3 ± 0.4^{G}	13.3 ± 0.4^{EF}	12.2 ± 0.3^{EF}	11 ± 0.7^{F}	11.4 ± 0.5^{EF}	11.4 ± 0.6^{C}	10.5 ± 0.7^{D}	13.5 ± 0.7^{E}	13.3 ± 0.4^{C}
C30	14.2 ± 0.2^{DE}	14.6 ± 0.5^{DE}	13.3 ± 0.5^{DE}	13.2 ± 0.3^{CD}	12.5 ± 0.5^{DE}	12.5 ± 0.2^{C}	12.5 ± 0.5^{C}	14.9 ± 0.2^{D}	15.0 ± 0.5^{B}
C60	15.2 ± 0.5^{CD}	15.5 ± 0.5^{CD}	14.5 ± 0.5^{CD}	13.8 ± 0.2^{C}	13.7 ± 0.3^{CD}	14.3 ± 0.2^{B}	13.8 ± 0.2^{B}	15.6 ± 0.5^{CD}	16.5 ± 0.5^{B}
C90	16.5 ± 0.5^{BC}	16.2 ± 0.7^{BC}	15.5 ± 0.5^{BC}	14.2 ± 0.2^{C}	14.8 ± 0.5^{BC}	15.5 ± 0.3^{A}	14.5 ± 0.8^{B}	16.5 ± 0.5^{BC}	17.4 ± 0.7^{AB}
C120	17.7 ± 0.5^{AB}	17.6 ± 0.2^{AB}	16.3 ± 0.5^{B}	15.5 ± 0.3^{B}	15.5 ± 0.5^{B}	15.7 ± 0.3^{A}	15.0 ± 0.5^{B}	17.3 ± 0.5^{B}	18.5 ± 0.2^{AB}
H30	13.8 ± 0.6^{DEF}	13.2 ± 0.7^{EF}	12.2 ± 0.4^{EF}	12.3 ± 0.3^{DE}	11.4 ± 0.5^{EF}	11.5 ± 0.2^{C}	10.8 ± 0.2^{D}	12.8 ± 0.6^{EF}	13.2 ± 0.7^{BC}
H60	13.5 ± 0.2^{EFG}	13.0 ± 0.3^{F}	12.0 ± 0.3^{EF}	12.0 ± 0.4^{AB}	10.7 ± 0.2^{FG}	10.2 ± 0.4^{D}	10.6 ± 0.2^{D}	12.5 ± 0.2^{EF}	13.0 ± 0.3^{BC}
H90	12.5 ± 0.5^{FG}	12.7 ± 0.5^{F}	11.8 ± 0.2^{F}	11.8 ± 0.2^{AB}	10.2 ± 0.3^{FG}	10.2 ± 0.5^{D}	10.2 ± 0.2^{D}	12.2 ± 0.5^{EF}	12.7 ± 0.5^{BC}
H120	12.4 ± 0.3^{FG}	12.5 ± 0.2^{F}	11.4 ± 0.7^{F}	11.6 ± 0.4^{AB}	10.0 ± 0.2^{G}	10.0 ± 0.3^{D}	9.5 ± 0.3^{D}	11.7 ± 0.3^{F}	12.3 ± 0.2^{BC}

Note: Diameter well: 6 mm. Acetic acid 0.1% was used as negative control (pH 5); Gentamycin and cycloheximide were used as positive control. Values are means ± standard deviation ($n = 3$). Means with different superscripts (A–G) within a column indicate significant difference ($p < 0.05$)

Table 4 Minimum inhibitory (MIC) (A) and bactericide (MBC) (B) concentrations (w/v, %) of chitosan and CDP samples, determined by liquid growth inhibition assay, against seven pathogenic bacteria

(A)		Gram −		Gram +			
Bacteria	*Salmonella enterica*	*Pseudomonas aeruginosa*	*Enterobacter* **sp**	*Staphylococcus aureus*	*Micrococcus luteus*	*Listeria monocytogenes*	*Bacillus cereus*
Chitosan	0.019	0.019	0.039	0.078	0.078	0.156	0.156
C30	0.01	0.0048	0.01	0.019	0.039	0.039	0.039
C60	0.01	0.0048	0.0048	0.019	0.019	0.039	0.039
C90	0.048	0.0024	0.0048	0.01	0.019	0.019	0.019
C120	0.0024	0.0012	0.0048	0.01	0.01	0.019	0.019
H30	0.019	0.01	0.019	0.019	0.078	0.039	0.078
H60	0.019	0.019	0.039	0.039	0.156	0.078	0.156
H90	0.019	0.019	0.039	0.078	0.156	0.156	0.3125
H120	0.039	0.039	0.039	0.078	0.3125	0.156	0.3125
(B)		Gram −		Gram +			
Chitosan	0.156	0.3125	0.625	0.625	1.25	0.625	0.3125
C30	0.156	0.078	0.078	0.156	0.625	0.625	0.3125
C60	0.078	0.039	0.039	0.078	0.3125	0.3125	0.3125
C90	0.039	0.019	0.019	0.039	0.156	0.156	0.156
C120	0.019	0.01	0.01	0.019	0.156	0.156	0.078
H30	0.156	0.078	0.078	0.3125	0.625	1.25	0.625
H60	0.3125	0.078	0.156	0.3125	1.25	1.25	0.625
H90	0.3125	0.078	0.156	0.625	1.25	1.875	1.25
H120	0.3125	0.156	0.3125	1.25	1.875	1.875	1.875

Note: MIC and MBC values are expressed in %

antibacterial potential, as compared to chitosan, the other high Mw-CDP (C120 > C90 > C60 > C30) and low Mw-CDP (H120 < H90 < H60 < H30). Furthermore, the higher inhibitory potential of this sample was obtained toward *P. aeruginosa*, *S. enterica*, *E.* sp, followed by *S. aureus*. Furthermore, MBC values demonstrate a bacteriostatic effect

that all the samples against the seven tested bacteria with a ration MBC/MIC ≥ 4.

To sum up, both tested methods of antibacterial activity evaluation demonstrated that the characteristics of chitosan and its derivatives, mainly their Mw, along with the type of the pathogenic bacterium are crucial factors affecting their antibacterial potential. Interestingly, CDP with Mw ranging from 94.10 to 396.46 kDa, particularly C120 (Mw = 94.10 kDa), showed higher antibacterial effect than native chitosan showing very high Mw (1244.70 kDa) and CDP (H30, H60, H90, and H120) with very low Mw (< 4.4 kDa). The best antibacterial potential of high Mw-CDP was probably attributed to the formation of films in the bacterial cell wall that inhibit the absorption of nutrients by the microorganisms [40]. Another explanation of the variation of the antibacterial potential between varying Mw-CDP is that the DP is critical for activity [42]. Indeed, Asli et al. [43] showed that oligosaccharides of at least 6 units provide better antibacterial activity than DP 2 and 3. Moreover, Uchida et al. [44] observed that chitosan oligomer I composed of mainly components of tetramer, pentamer, and hexamer possessed antimicrobial effect, while chitosan oligomer II consisted of mainly trimer and tetramer showed no activity. In our study, low Mw-CDP contained COS with low DP (up to 7) and probably glucosamine units (not detected by MALDI-TOF) that can explain the difference on their lower antibacterial effect as compared to high Mw-CDP.

Moreover, owing to its higher Mw, chitosan possesses high viscosity and low solubility, which result in decrease of its inhibitory activity. However, chitosan derivatives with lower Mw and viscosity and higher solubility are advantageous as antimicrobial agents compared to native chitosan [16, 18]. Similarly to our results, Sánchez et al. [2] demonstrated that COS ranging from 6 to 17 kDa showed higher antibacterial activity than oligomers with Mw around 1 kDa and that this effect was more pronounced against the Gram (−) bacteria *E. coli* than the Gram (+) strain *L. monocytogenes*. Furthermore, Li et al. [45] stated that chitosan with Mw of 50 kDa showed higher antibacterial potential toward *E. coli* than chitooligomers with lower (3 kDa) and higher Mw (1000 kDa). Furthermore, Jeon et al. [46] illustrated that Mw of chitosan is an important factor governing the inhibition of Gram (−) and Gram (+) bacteria and that it should be higher than or around 10 kDa for effective inhibition. Uchida et al. [44] reported that chitosan hydrolysate, slightly hydrolyzed with chitosanase, was more active as an antibacterial agent than were native chitosan and chitosan oligomers. Ueno et al. [47] reported that COS with an average Mw < 2.2 kDa, was not capable of suppressing the bacterial growth, but COS with Mw around 5.5 kDa suppressed the growth in a dose-dependent manner.

Molecular mechanism for antibacterial activity of COS is not fully-known but the presence of primary amino groups ($-NH^{3+}$ residues) in their structure directly affected their antibacterial potential. COS cause the death of microbial cell by altering the permeability of cell membrane, which is a vital structure of protecting the release of cell constituents and controlling the entry of materials into the cell from the environment [48]. Although, the antibacterial activities of chitosan and its derivatives were demonstrated to be correlated to their positive charge, thus enabling their binding to the bacterial cell wall through ionic interaction. This adsorption leads to their penetration into the DNA and blocking of RNA transcription, thereby inhibiting its growth [48, 49]. Hamdi et al. demonstrated that the importance of the positive charge density is reflected by the DA of chitosans [50]. The greater negative charges on the cell surface of Gram (−) strains could explain their higher sensitivity to CDP than Gram (+) bacteria.

Therefore, according to our results and to the literature, it can be concluded that chitosan derivatives show antibacterial activity which seems to be highly dependent on their chain length, DP, and DA, along with the bacterial type. As well, high Mw-CDP, ranging from 94.10 to 396.46 kDa, have been deliberated to develop as novel antimicrobial agents to use in food, agriculture, and medicine.

3.3.2 Antifungal activity

The antifungal potential of chitosan and CDP was further studied using agar well diffusion method. Results depicted in Table 3 showed that the tested compounds were most effective against *Fusarium solani* than *Rhizoctonia solani*. Similarly to the antibacterial activity, the same correlation between antifungal potential and chitosan Mw was observed. Indeed, C120 (Mw = 94.10 kDa) exhibited the highest antagonistic effect toward the two fungi, compared to chitosan, as well as the other high and low Mw-CDP. In the same context, Affes et al. [16] and Rahman et al. [51] demonstrated that medium Mw-CDP were significantly more effective than the native chitosan and low Mw-CDP. The exact mechanism of the antifungal activity of chitosan is not well-known, and various hypotheses were proposed, depending on the particular type of fungus and the molecular characteristics of chitosans (Mw, DA, and DP) [1, 12, 17, 47].

3.3.3 Antioxidant activity

The DPPH and ABTS radical-scavenging activities, reducing power, and total antioxidant activity of CDP were studied and compared to the chitosan and BHA. DPPH and ABTS radical scavenging activities were reported as the effective concentration needed to reduce DPPH and ABTS radicals by 50% (IC50). The reducing power was reported as the concentration needed to obtain an absorbance of 0.5 (DO

Table 5 IC_{50} values of DPPH and $ABTS^+$ radicals-scavenging, reducing power ($OD_{700\,nm} = 0.5$), and total antioxidant activity values of chitosan and CDP (5 mg/ml)

Antioxidant	DPPH IC_{50} (mg/ml)	$ABTS^+$ IC_{50} (mg/ml)	Reducing power ($OD_{700\,nm} = 0.5$)	Total antioxidant activity (α-tocopherol (μmol/ml))
BHA	0.26^{A}	0.06^{A}	0.10^{A}	272.28 ± 0.00^{A}
Chitosan	3.07^{G}	1.61^{G}	6.22^{I}	80.46 ± 1.28^{I}
C30	2.69^{F}	0.52^{F}	5.22^{H}	114.57 ± 1.02^{H}
C60	1.82^{E}	0.28^{E}	2.36^{G}	137.19 ± 2.80^{G}
C90	1.35^{E}	0.28^{E}	1.87^{F}	140.05 ± 1.55^{FG}
C120	1.33^{DE}	0.26^{DE}	1.85^{F}	143.33 ± 0.97^{F}
H30	0.85^{CD}	0.24^{CD}	1.65^{E}	170.12 ± 2.35^{E}
H60	0.71^{BCD}	0.23^{BCD}	0.99^{D}	179.23 ± 2.45^{D}
H90	0.63^{BC}	0.21^{BC}	0.95^{C}	231.52 ± 0.77^{C}
H120	0.60	0.20^{B}	0.87^{B}	240.24 ± 1.75^{B}

Note: Values are means ± standard deviation ($n = 3$). Means with different letters (A–I) and within a column indicate significant difference ($p < 0.05$)

$_{700\,nm} = 0.5$), and the total antioxidant potential was measured at a concentration of 5 mg/ml.

Results illustrated in Table 5 showed that, the antioxidant potential of CDP was significantly higher than that of BHA but lower than that of chitosan. Moreover, the Mw reduction of CDP resulted in increased antioxidant activity. Indeed, H120 exhibited the highest antioxidant potential (0.60, 0.20, 0.87, and 240.24 ± 1.75 for DPPH and ABTS radical-scavenging activities, reducing power, and total antioxidant activity, respectively) than the other varying Mw-CDP ($p < 0.05$).

Similar results, in vitro, were reported by Zhou et al. [20], who stated that chitosan oligomers obtained by chitosan hydrolysis using ChiEn3 chitinase exhibited better DPPH scavenging activity than the undigested chitosan. Furthermore, Laokuldilok et al. [21] observed that COS with Mw of 5.1 kDa possess better DPPH radical activity than chitosan and COS with 14.3 and 4.1 kDa. As well, the increase of the antioxidant activity toward ABTS of low Mw-CDP, in comparison with chitosan and high Mw-CDP, was in accordance with those reported by Affes et al. [16] and Chang et al. [52]. Furthermore, Huang et al. [53] and Laokuldilok et al. [21] demonstrated that lower Mw chitosan derivatives showed greater reducing power capacity.

The lowest antioxidant potential of high Mw-CDP was mainly due to their higher inter and intramolecular bonding and compact structure which restrict the chance of exposure of their amine groups [1].

4 Conclusion

Current attention on chitosan focuses to its derivatives because of their advantages, including its reduced Mw and water-solubility. In this work, we showed that the chitosan chemical depolymerization, during 2 h using HCl, is a simple and practical method to produce high and low Mw-CDP at different hydrolysis times. Chitosan derivatives with reduced Mw, viscosity, and crystallinity and higher solubility, as compared to the native chitosan, were produced. These obtained products have been demonstrated to possess better antibacterial, antifungal, and antioxidant activities than chitosan, which were influenced by their Mw. Interestingly, such depolymerization process may be considered as a promising method for the preparation of effective varying Mw chitosan derivatives to be used as functional food additives and natural food preservatives for food-processing applications.

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Declarations

Competing interests The authors declare no competing interests.

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