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Short communication

Low-molecular-weight chitosans: Preparation and characterization

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ABSTRACT

The chitosan depolymerization products prepared by enzymatic hydrolysis, oxidative reaction or microwave irradiation were characterized by FT-IR, MALDI TOF MS, solid state ^{13}C CP/MAS NMR and GPC. Enzymatic hydrolysis resulted in two fractions of low-molecular-weight (LMW) chitosans with M_w 34 kDa (3.9 wt%) and 14.6 kDa (8.5 wt%). In the oxidative reaction at the hydrogen peroxide and acetic acid molar ratio equal to 1.7, two LMW chitosans with M_w 9 and 10 kDa (the deacetylation degree 69% and 94%, respectively) were isolated with the total yield 86 wt%. In microwave irradiation, the M_w of chitosan (175.5 kDa) decreased at most in two times. Two chitosan fractions with M_w 80 and 120 kDa (total yield 70 wt%) were obtained. In all cases, LMW chitosans served as fortifiers for chitosan oligomers, which were retained with the former through multiple hydrogen bonds. Their content in LMW chitosans was roughly 20–30 wt%.

1. Introduction

The depolymerization products of chitosan (deacetylated derivative of natural linear polymer chitin) are usually a mixture of chitosan oligomers (COS) and low-molecular-weight (LMW) chitosans, which are differed in polymerization degree (PD). They consist of the statistically distributed glucosamine (GlcN) and acetylated glucosamine (GlcNAc) rings bound with each other through β-1,4-glycosidic bonds.

Owing to the presence of hydroxyl, amine and acetylated amine groups, chitosan, LMW chitosan and COS interact readily with various cell's receptors that triggers a cascade of interconnected reactions in living organisms resulting in anti-inflammatory (Fernandes et al., 2010), anti-cancerogenic (Huang, Mendis, Rajapakse & Kim, 2006), anti-diabetic (Ju et al., 2010), anti-microbial (Eun et al., 2010), anti-HIV-1 (Artan, Karadeniz, Karagozlu, Kim & Kim, 2010), anti-oxidant (Ngo, Lee, Kim & Kim, 2009), anti-angiogenic (Quan et al., 2009), neuroprotective (Gong, Gong, Gu, & Ding, 2009) and immunostimulative (Moon et al., 2007) effects.

In connection with broadening applications of chitosan oligomers and LMW chitosans as food (Friedman & Juneja, 2010; Jeon, Shahidi, & Kim, 2000) and diet (Muzzarelli, 1996) additives and as covering shells in microencapsulation of probiotics (Islam, Yun, Choi, & Cho, 2010), the standardization of chitosan depolymerization procedures for preparation of COS and LMW chitosans in preparative and industrial quantities are extremely actual task.

This study was aimed at characterization of chitosan depolymerization products by FT-IR, MALDI TOF MS, solid state ^{13}C CP/MAS NMR and GPC. The chitosan was depolymerized by enzymatic hydrolysis, oxidative reaction with hydrogen peroxide or microwave irradiation. Prior to analysis, the reaction media containing chitosan oligomers and LMW chitosans at different proportions were fractionated by the procedure developed by us, the details of which will be published elsewhere.

2. Experimental

2.1. Food chitosan

Commercial food-grade chitosans (Medicol, CR) with M_w 123.7 and 175.5 kDa and the deacetylation degree, DD 64.9% and 83.1%, respectively, were used.

2.2. Enzymatic hydrolysis

Enzymatic hydrolysis of chitosan was carried out with a mixture of pectinase from *Phizopus oryzae* (0.6 g, specific activity 0.42 U/mg), cellulase from *Aspergilus niger* (0.6 g, 0.32 U/mg) and papain (EC 3.4.22.2) from papaya latex (0.08 g, 0.67 U/mg), which was added to 4 g of a chitosan sample dispersed in 1 M phosphate buffer, pH 5.3 (200 ml). After 24 h hydrolysis at 39 °C, the reaction was stopped by

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acidifying and heating the hydrolysate for 3 min at 100 °C followed by centrifugation (40,000 rpm, 30 min, 25 °C). The fractionation of the obtained products was carried out by the membrane ultrafiltration.

2.3. Oxidative reaction

The oxidative depolymerization of chitosan (M_w 123.7 kDa, DD 64.9%) was carried out at the hydrogen peroxide acetic acid molar ratio 5.7; 2.8; 1.9 and 1.7. Chitosan (1 g in 100 ml of distilled water) was heated at 50 °C during 8 h; the reaction medium was cooled and the water-insoluble LMW chitosans were separated from water-soluble COS by precipitation with ammonium hydroxide followed by centrifugation at 6000 rpm for 30 min. After triple washing the precipitate with distilled water, it was fractionated. In each experiment, two fractions of LMW chitosans were obtained.

2.4. Microwave irradiation

Microwave irradiation was performed in a laboratory microwave reactor Plazmatronika (Plazmatronika a.s., Wroclaw, Poland) with a magnetic stirrer and an infrared reaction thermometer. An acidified chitosan solution (100 ml) in a 250 ml Erlenmeyer flask, equipped with a reflux condenser and a Teflon stirrer, was placed into the reactor cavity, which was cooled with cold water. The reaction temperature (98–100 °C) was maintained by applying the appropriate microwave intensity (magnetron output), equal mainly to 650 W or 390 W. The total time of microwave irradiation (including approximately 2–3 min to elevate the reaction temperature) was 10, 20, 30 or 60 min.

2.5. Determination of the weight average molecular weight (M_w) and the number average molecular weight (M_n)

Gel permeation chromatography (GPC) were performed using a system equipped with a high-pressure pump Deltachrom (Watrex, CZ), an autosampler Midas, two columns packed with 8 μm-beads of PL aquagel-OH MIXED separating in the range of molecular weights 10^2–10^7 g/mol, a laser light-scattering (LS) photometer DAWN-DSP-F (Wyatt Technology Corp., USA), a differential viscometer TDA 301 (Viscotek Corp., USA) and a refractive (RI) Shodex detector RI-71 (Showa Denko KK, Japan). The mobile phase was 0.15 M ammonium acetate adjusted to pH 4.24 with 0.2 M acetic acid. The injection-loop volume was 0.1 ml. The data were accumulated and processed using the Astra 4.70.07 and TriSEC 3.0 Software (Viscotek Corp., USA). Eluent and sample solutions (1–3 mg/ml) were filtered through 0.2 μm Millipore filters. The flow rate was maintained at 1.0 ml/min.

Table 1
Characteristics of LMW chitosans and chitosan oligomers obtained by enzymatic hydrolysis of chitosan followed by the subsequent ultrafiltration through a set of Millipore polyethersulfone membranes with cut-off 30, 10, 3, 1 and 0.5 kDa.

Sample	UF fraction	Content (%)	LMW chitosans		COS (Da)
			M_w (Da)	M_n (Da)	
E1	R-10	3.9	34,200	15,400	447–2873
E2	R-3	8.5	14,650	8144	447–1701
E3	R-1	3.2	Absent		447–1498
E4	R-0.5	30.3	Absent		447–1294
	P-0.5	54.2	Absent		447; 727

2.6. Determination of acetylation degree

Degree of acetylation (DA) values of LMW chitosans was determined by solid state ^{13}C cross polarization magic angle spinning NMR spectroscopy from the relative intensities of the resonances of the ring carbons (I_{C1}, I_{C2}, I_{C3}, I_{C4}, I_{C5}, I_{C6} and the methyl-carbon I_{CH_3} from the following equation (Duarte, Ferreira, & Rocha, 2001):

$$DA = \frac{I_{CH_3}}{(I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6})/6}$$

1D solid-state NMR spectra were measured using a Bruker Avance 500 NMR spectrometer. Magic angle spinning (MAS) frequency was 10 kHz. The lyophilized samples of LMW chitosans were placed into the ZrO_2 rotors and stored under silica-gel to prevent rehydration. Amplitude modulated cross-polarization (CP) with duration 1 ms was used to obtain ^{13}C CP/MAS NMR spectra. The ^{13}C scale was calibrated with glycine as external standard (176.03 ppm – low-field carbonyl signal).

2.7. Matrix-assisted laser-desorption ionization time-of-flight (MALDI TOF) mass spectrometry

A powder of lyophilized COS was dissolved in Q-water (10 mg/ml). Aqueous solution of the matrix (2,5-dihydroxybenzoic acid – DHB, 20 mg/ml) was added to a COS solution at the sample/matrix ratio 1/4 (v/v). 1 μL of the mixture was deposited onto the stainless steel target and dried at ambient atmosphere. Crystallization of the matrix occurred spontaneously. MALDI-TOF mass spectra were recorded on a Bruker Reflex III (Bruker Daltonik, Bremen, Germany) in the positive ion mode. For ionization, a nitrogen laser (337 nm, 3 ns pulse width, 3 Hz) was used. All spectra were measured in the reflector mode using external calibration. The laser was aimed at the whole area of a sample to optimize the mass spectra, in which monoisotopic peaks were labeled.

Table 2
Characteristics of LMW chitosans and chitosan oligomers prepared by oxidative depolymerization of chitosan.

Exp	Sample	H_2O_2/AA (mmol/mmol)	M_w (kDa)	M_n (kDa)	M_w/M_n	Content (g)	DD (%)	Yield (%)
	COS		0.5–1.7		–	0.29	–	36
1	H1	5.7	6.61	3.49	1.9	0.18	79.6	64
	H2		7.89	5.26	1.5	0.17	87.0	
	COS		0.5–1.7		–	0.17	–	22
2	H3	2.8	7.70	4.46	1.7	0.37	82.2	78
	H4		7.80	4.60	1.7	0.24	92.4	
	COS		0.5–2.1		–	0.12	–	18
3	H5	1.9	9.29	5.91	1.6	0.41	73.1	82
	H6		9.70	6.56	1.5	0.29	92.3	
	COS		0.5–2.1		–	0.09	–	14
4	H7	1.7	9.03	4.45	1.5	0.43	69.3	86
	H8		9.97	6.83	2.0	0.34	93.6	

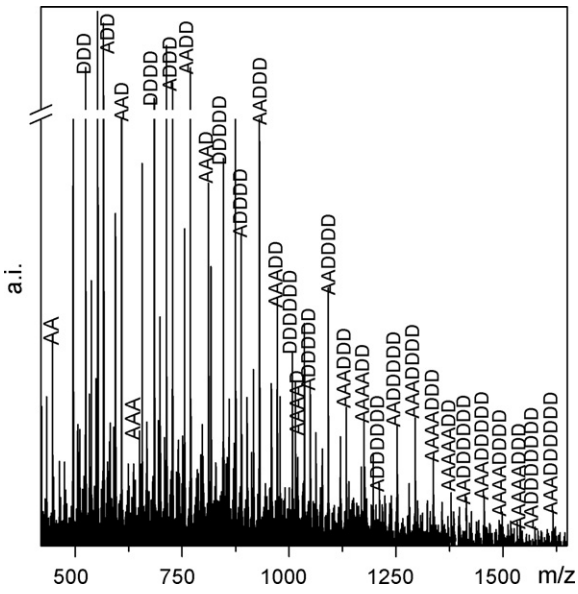

Fig. 1. MALDI TOF mass spectra of chitosan oligomers (sample E3 in Table 1) prepared by enzymatic hydrolysis of chitosan.

2.8. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectra of LMW chitosans were recorded using a spectrophotometer Perkin-Elmer Paragon 1000PC and Attenuated total reflection technique Specac MKII Golden Gate Single Reflection ATR system with a diamond crystal; the incidence angle was 45°. The 16 scan-spectra with 4 cm^{-1}-resolution were recorded in the range of wavenumbers: 4400–450 cm^{-1} and evaluated using Spectrum 2.00 software.

3. Results and discussion

The enzymatic depolymerization of chitosan (Table 1) resulted in preparation of LMW chitosans with M_w 34 kDa and 14.6 kDa (3.9 wt% and 8.5 wt%, respectively). The content of COS (dimers – octamers) and (dimers – tetramers) was 33% and 54%, respectively.

In oxidative depolymerization (Table 2), the content of COS (dimers – decamers) decreased from 36% to 14% with decreasing the H_2O_2/acetic acid (AA) molar ratio from 5.7 to 1.7. In each experiment, two fractions of LMW chitosans (e.g. H1 and H2) were obtained. The fractions (H1, H3, H5 and H7) had somewhat lower both M_w and deacetylated degree values compared with (H2, H4, H6 and H8) ones. The M_w values and the total yield of both fractions of LMW chitosans increased with decreasing the H_2O_2 and AA molar ratio.

In microwave irradiation, two fractions of chitosans, the M_w of which was only 19–55% lower than that of the initial chitosan (175.5 kDa), were obtained. Their total yield did not exceed 63–76%.

In each depolymerization procedure, the chitosan oligomers with different PD were usually formed. A typical MALDI TOF mass spectrum of COS with PD from 2 to 9 is shown in Fig. 1.

The main differences in FT-IR spectra of depolymerized chitosans and chitosan itself (Fig. 2) were observed in the range of the wavenumbers 1800–800 cm^{-1}. The new two dominant bands with high intensity at 1542 cm^{-1} and 1402 cm^{-1} can be seen in the spectrum of H1 sample. These bands can be assigned to the protonated amine groups ($-NH_3^+ {}^-OOC-$) (Fernandez-Saiz, Ocio, & Lagaron, 2006). Their intensity was considerably lower in the spectrum of H2 sample.

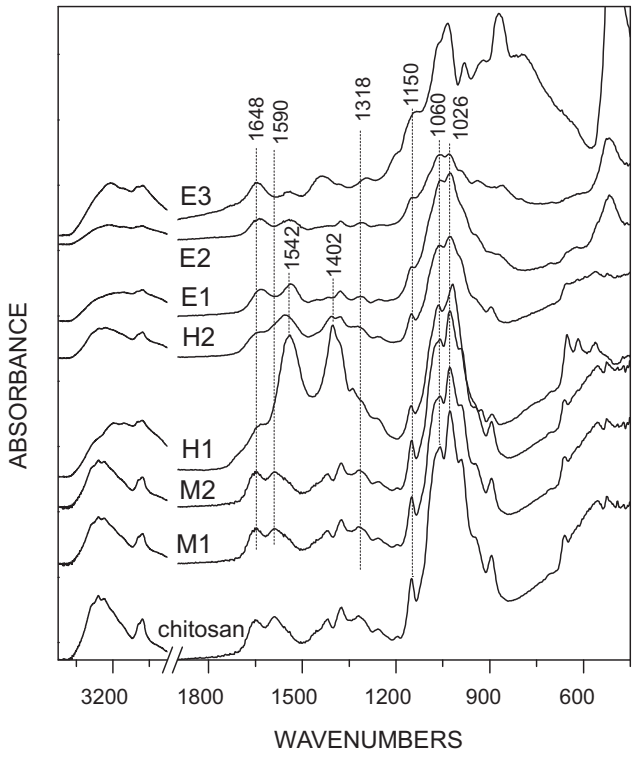

Fig. 2. FT-IR spectra of native chitosan and LMW chitosans. Chitosan – native chitosan with M_w 123.7 kDa; samples E1, E2 are LMW chitosans and sample E3 is chitosan oligomers prepared by enzymatic hydrolysis; samples H1 and H2 are LMW chitosans prepared by oxidative reaction; samples M_1 and M_2 are chitosans depolymerized by microwave irradiation (see Tables 1, 2 and 3, respectively).

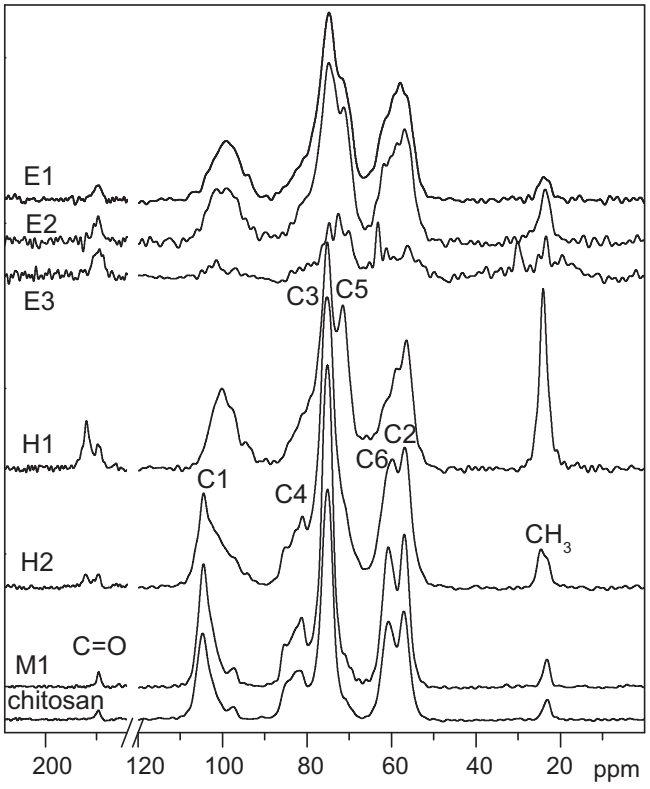

Fig. 3. ^{13}C CP/MAS NMR spectra of LMW chitosans. For details see caption to Fig. 2.

Table 3
Characteristics of chitosans depolymerized by microwave irradiation.

Exp.	Sample	Irradiation		M_w (kDa)	M_n	M_w/M_n	Content (g)	$M_i/(M_i+M_j)$[a] (%)
		Time (min)	Intensity (W)					
1	M_1	10	650	79.2	29.7	2.7	0.42	72
	M_2			118.2	52.9	2.2	0.30	
2	M_3	20	650	98.5	46.8	2.1	0.44	76
	M_4			109.9	57.8	1.9	0.32	
3	M_5	30	390	103.2	35.4	3.0	0.35	68
	M_6			142.2	41.8	3.4	0.33	
4	M_7	60	390	99.7	34.5	2.9	0.23	63
	M_8			116.9	55.2	2.1	0.40	

[a] M_i and M_j are the contents of chitosans in fractions i and j, respectively. For example, in the experiment 1, M_1 and M_2 correspond to chitosan content in fractions 1 and 2, respectively.

Multiplicity of carbonyl group signal in ^{13}C CP/MAS NMR spectra of all samples including the native chitosan can be attributed to polymorphic molecular heterogeneity (Fig. 3). The growth in intensity of CH_3 signals in H1 and H2 spectra can be explained by the presence of acetic acid molecules protonating the amine groups, the quantity of which increased due to the deacetylation of GlcNA units. The DD values were higher for H1 (69–80%) and H2 (87–94%) samples than that for chitosan used (64%).

The evaluation of M_w and M_n of prepared samples has shown (Fig. 4A–D) that the peaks of LMW chitosans (2 or 2x) were accom-

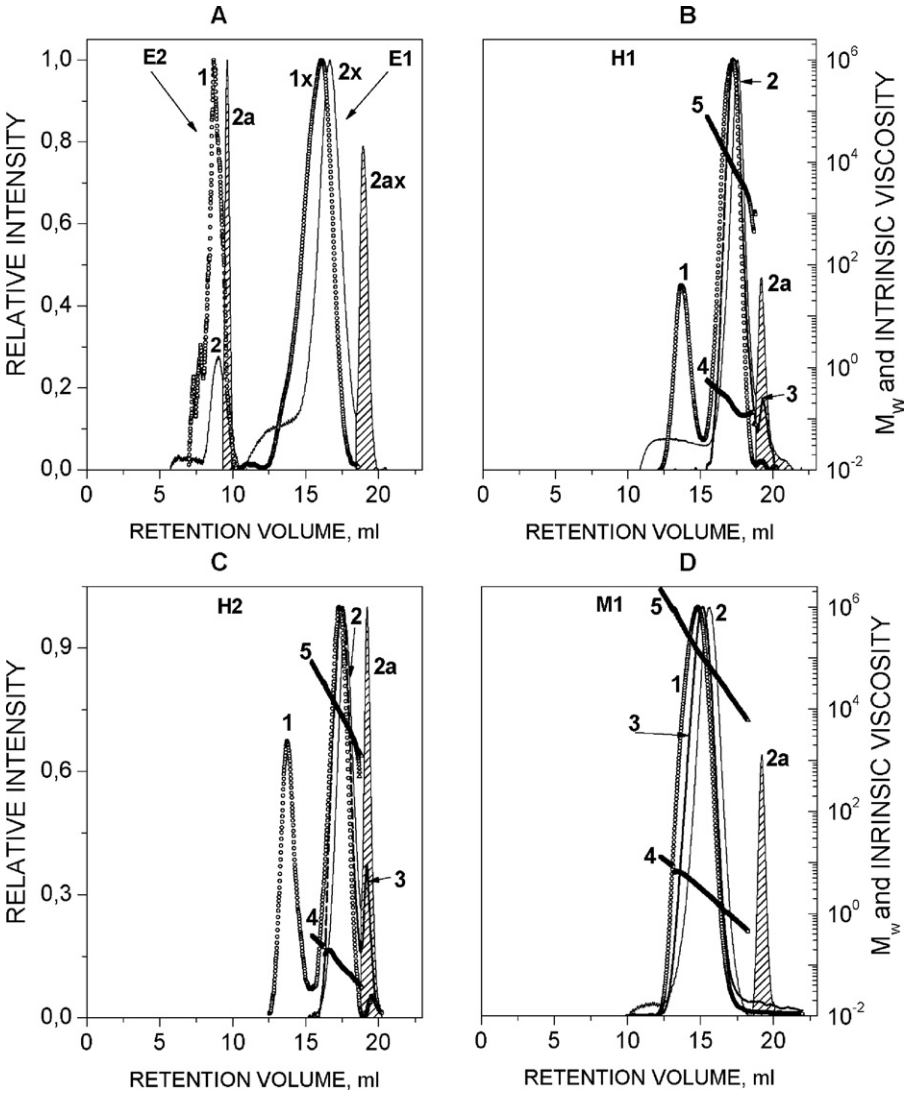

Fig. 4. GPC of LMW chitosans prepared by: (A) enzymatic hydrolysis (samples E1 and E2); (B and C) oxidative depolymerization (samples H1 and H2, respectively) and (D) microwave irradiation (sample M_1). Curves at chromatograms correspond to: 1, 1x light scattering; 2, 2x and 2a, 2ax concentration of LMW chitosan and chitosan oligomers, respectively; 3, pressure drop; 4, viscosity; 5, molecular weight.

panied by peaks of COS (2a or 2ax) due to multiple hydrogen bonds between polysaccharide units. This impeded their complete separation. The LMW chitosans obtained by the oxidative depolymerization tended to aggregation that was reflected in appearance of two peaks on the curves of the dependences of light scattering – the retention volume (Fig. 4B and C). In chromatograms of chitosan products depolymerizied by microwave irradiation (Fig. 4D), the aggregation of polysaccharide chains was negligible. However, the only chitosan peak was accompanied by the peak of COS as well. It seems that preparation of LMW chitosans absolutely free from any traces of chitosan oligomers is rather difficult task.

4. Conclusions

It should be concluded that each examined chitosan depolymerization method has both merits and demerits. Therefore, the choice of the preferable chitosan fragmentation procedure has to be dictated by the pursued aims. It is reasonable to use the enzymatic hydrolysis if chitosan oligomers with low polymerization degree (2–10) have to be prepared. The chemical degradation seems to be more preferable in large scale preparations if LMW chitosans with M_w 5–15 kDa are required. In contrast to the oxidative depolymerization, the microwave irradiation did not practically change the deacetylation degree of chitosans but it is very time-consuming for of the preparative preparation of LMW chitosans with the desired molecular weight.

The analysis of physicochemical properties of the chitosan depolymerization products has shown that the complete information about their structural and chemical changes, molecular weight and proportion of COS and LMW chitosans in the samples as well as about interactions between the chitosan fragments can be obtained by using the solid state ^{13}C CP/MAS NMR, MALDI TOF mass spectrometry, FT-IR and GPC.

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