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Solid-state characterization of chitosans derived from lobster chitin

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Abstract

Two samples of chitosan (CH1 and CH2) of different molecular weights and degrees of deacetylation were prepared from lobster chitin under two different processes. Solid-state properties of CH1 and CH2 were characterized and compared with four commercial chitosans prepared from crab and fresh shrimp shells. Infrared spectroscopy (IR), solid-state CP-MAS ^{13}C NMR, powder X-ray diffraction and differential scanning calorimetric techniques were used to characterize the molecular structure and solid-state properties of the materials. Changes in the crystallinity and polymorphic forms of CH1 and CH2 were attributable to the different process conditions used. The differences in crystallinity were confirmed by powder X-ray diffraction data. The methods of preparation of CH1 and CH2 did not significantly influence the bulk, tap and true densities of the bulk material, but they affected the flow properties of CH1 and CH2. In conclusion, the physicochemical properties of the present chitosans prepared from lobster chitin (CH1 and CH2) are comparable with those of commercial chitosan materials of crab or shrimp shell origin.

Keywords: Chitin; Chitosan; Lobster; Deacetylation; Molecular weight; Solid-state properties; Polymorphism

1. Introduction

Chitin is one of the most abundant natural amino polysaccharide. The main commercial sources of chitin are the shell wastes of shrimp, lobster, krill and crab. Worldwide, millions of tons of chitin are harvested annually (Rha, Rodriguez-Sanchez, & Kienzle-Sterzer, 1984; Roberts, 1992). Chitin is partially deacetylated, usually by alkaline N-deacetylation using industrial processes to produce a variety of polymers (Brugnerotto et al., 2001). When the degree of deacetylation (DD) is 75% or higher, the product is called chitosan (Li, Revol, & Marchessault, 1997). It becomes water-soluble due to the protonation of the $-NH_2$ groups of the glucosamine unit. The production of chitosan from crustacean shells, waste of food industry, is economically feasible (Ravi Kumar, 2000). A lot of chitin-rich food waste is discarded, especially in Asian countries, where people have seafood-rich diets (Ball, 2002). Therefore, the efforts to convert those wastes into a useful product are rational and important.

Chitosan is the N-deacetylated derivative of chitin, although this N-deacetylation is almost never complete. In the last 10 years, chitosan has received much attention because of its extraordinary properties and for its inexpensive and abundant resources (Harish Prashanth, Kittur, & Tharanathan, 2002). Chitosan is biodegradable, biocompatible and non-toxic. The incomplete characterization of chitosans and the variability of commercial chitosans have discouraged the pharmaceutical industry from adopting it as a pharmaceutical excipient or formulation component. The heterogeneity of these chitosans mainly results from the sources of chitin and relatively uncontrolled commercial processing of native chitin involving both N-deacetylation and depolymerization (Rege & Block, 1999; Rege, Garmise, & Block, 2003).

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The characterization of chitin and chitosan derived from crab and shrimp shells has been largely discussed in the literature (Harish Prashanth et al., 2002; Heux, Brugnerotto, Desbrières, Versali, & Rianudo, 2000; Kittur, Haris Prashanth, Udaya Sankar, & Tharanathan, 2002; Saito & Tabeta, 1987). The determinations of the DA as well as its molecular weight (MW) are the two fundamental physical properties of these polymers. In the present study, samples of chitosan from lobster chitin were prepared under two different processes and conditions. Hence, the aim of this study was to characterize and compare the physicochemical properties of those chitosan samples with four commercial chitosans prepared from crab and shrimp shells.

2. Materials and methods

2.1. Materials

Chitosans derived from lobster chitin, i.e. chitosan 1 (CH1) and chitosan 2 (CH2), were prepared industrially in a Cuban enterprise by N-deacetylation of lobster chitin. Chitin was suspended in an alkali solution, 45% NaOH, at 130 °C for 30 min to obtain CH1, whereas CH2 was obtained with 49% NaOH at 130 °C for 30 min. The solids were filtered, intensively washed with distilled water until nearly neutral pH was obtained and dried in vacuum at 40 °C.

Four commercially available chitosan samples derived from crab or fresh shrimp shells varying in MW and degree of deacetylation (% DD) were used as reference materials for CH1 and CH2. High molecular weight chitosan ((HMW), 79.0% DD), medium (MMW, 81.4% DD), and low (LMW, 85.0% DD) MW chitosans (Aldrich Chemical Company Inc., USA) and Primex chitosan (85.6% DD) (Primex Ingredients ASA, Norway) were used. All other reagents and solvents used were of analytical grade.

2.2. Physicochemical material characterization

2.2.1. Molecular weight determination

The viscosity average MW of chitosans was calculated from the classical Mark–Houwink relationship,

$$[\eta] = K_m(\mathrm{MW})^{\alpha}$$

where $[\eta]$, intrinsic viscosity; K_m, 1.81×10^{-3} and α, 0.93 (Ravi Kumar, 2000). The values of constants K_m and α have been determined in 0.1 M acetic acid and 0.2 M sodium chloride solution. Six polymer solutions of known concentrations of CH1 and CH2 were prepared. Relative viscosity was measured in triplicate using an Ubbelohde viscometer kept in a constant-temperature bath at 25 ± 0.1 °C.

2.2.2. Viscosity

Viscosity measurements of solutions of CH1 and CH2 1% in 1% acetic acid were performed on a Brookfield digital viscometer (Model DV-II+, Stoughton, USA) with a LV Spindle Set number 1 at room temperature (25.0 ± 0.1 °C).

2.2.3. Infrared spectroscopy

IR spectra were obtained using the Nicolet 60 SX Fourier Transform Infrared Spectrometer under dry air at room temperature and KBr pellets. Approximately 150 mg of KBr and 2 mg of chitosan powder (particle size $\leq$125 μm) were blended with an agate mortar and pestle for 5 min. The sample pellets were prepared at a pressure of 9 tons for 2 min. The disk was conditioned in an oven at 80 °C for 48 h before analysis.

The IR spectrum was then recorded in the frequency range of 4000–400 cm^{-1}. The absorbances at 1655 cm^{-1} (amide I band), a measure of the *N*-acetyl group content, and 3450 cm^{-1} (hydroxyl band) were determined using the baseline proposed by Baxter, Dillon, Taylor, and Roberts (1992) and the modifications reported by Domszy and Roberts (1985). Three chitosan samples of known DD (HMW, MMW and LMW) were selected for generating the standard curve, and the relationship of absorbance ratio to DD was utilised to determine the DD of unknown chitosans (Fig. 2). Chitosan samples were analysed by calculating their IR absorbance ratios in conjunction with the standard curve in order to determine the corresponding DD, following the procedure described by Sabnis and Block (1997).

2.2.4. Solid-state CP-MAS ^{13}C NMR

The NMR experiments were performed on a Varian Unity Inova spectrometer operating at 300 MHz for ^{1}H frequency, using the combined techniques of proton dipolar decoupling (DD), magic angle spinning (MAS) and cross-polarization (CP). The contact time was 1 ms, the acquisition time 51.2 ms and the recycle delay 4 s. The proton pulse width was 6 μs and 18 kHz spectral window was used. A typical number of 2000 scans was acquired for each spectrum. The chemical shifts were externally referenced by setting the methyl resonance of hexamethylbenzene (HMB) to 17.3 ppm. The samples were contained in a SiN_4 cylindrical rotor which was spun at 5 kHz during measurements.

The degree of acetylation (DA) of chitosan was calculated from the relative intensities of the resonance of the ring carbon ($I_{C_1}, I_{C_2}, I_{C_3}, I_{C_4}, I_{C_5}, I_{C_6}$) and methyl carbon ($I_{CH_3}$) obtained from ^{13}C NMR spectra (Ottoy, Varum, & Smidsord, 1996) by the following equation:

$$\mathrm{DA} = \frac{I_{CH_3}}{I_{C_1} + I_{C_2} + I_{C_3} + I_{C_4} + I_{C_5} + I_{C_6}/6}$$

2.2.5. Powder X-ray diffraction

X-ray diffraction patterns on powders (before and after milling) were obtained by using a variable temperature X-ray diffractometer (D8 Advance Bruker AXS GmbH, Karlsruhe, Germany) (VT-XRPD). The VT-XRPD experiments were performed in symmetrical reflection mode with Cu K_α radiation (1.54 Å) using Göbel Mirror bent gradient multilayer optics. The scattered intensities were measured with

a scintillation counter. The angular range was from 5 to 40° with steps of 0.2°, and the measuring time was 10 s/step. The estimation of the crystallinity was based on the assumption that the experimental XRPD intensity curve is a linear combination of intensities of the crystalline and amorphous component. The crystallinities of the samples were estimated by fitting the intensities of the crystalline and amorphous component to the experimental intensity curve. The diffraction pattern of the totally amorphous ground HMW sample was used as the amorphous model intensity curve and the crystalline one consisted only of the diffraction peaks. The crystallinities were calculated as the ratio of the integrals of the intensities of the crystalline component and the sample studied. The samples of chitosan were subjected to size reduction using a Planetary Mono Mill pulverisette 6 (Fritsch GmbH, Germany).

2.2.6. Differential scanning calorimetry (DSC)

DSC thermograms of chitosan powders were measured using a differential scanning calorimeter (DSC 821^e, Mettler Toledo AG, Schwerzenbach, Switzerland). Samples of 2–3 mg were sealed in an aluminium pan. In this method, the pans were probably not hermetically sealed. A nitrogen purge with a flow rate of 80 ml/min was used in the furnace. The scans were obtained by first heating to 190 °C, cooling to 25 °C and a second heating to 400 °C at a rate of 10 °C/min in order to estimate the glass transition temperature. Each run was performed in triplicate.

2.2.7. Thermogravimetric analysis (TGA)

TGA thermograms of chitosan powders were measured using a thermogravimetric analyser (TGA/SDTA 851^e, Mettler Toledo AG, Schwerzenbach, Switzerland). A nitrogen purge of 50 ml/min was used in the furnace. The sample size of 5 mg was accurately weighed into an aluminium pan. The measurements were obtained at 25–250 °C at a heating rate of 10 °C/min and the weight loss was calculated from three determinations.

2.2.8. Physical powder properties

Bulk, tap and true densities of the powders were determined by the method of European Pharmacopoeia (2002). A standardized tapped density tester (Erweka SVM1, Erweka GmbH, Heusenstamm, Germany) was employed. The volume occupied by the powder was recorded and the bulk density was calculated. For calculating the tapped density the volume occupied after 1250 taps was used. Each sample was measured in triplicate. The true density of materials was measured using a pycnometer (Micrometrics, Model 1305, Norcroos, GA) and helium as an inert gas. The results are averages of three determinations. The Carr index and Hausner ratio were calculated from the tap, bulk and true densities (Wells & Aulton, 1998). The experimental data was analyzed in accordance with the analysis of variance (ANOVA). When a statistically significant difference ($p < 0.05$) was obtained, a Tukey HSD test was performed.

3. Results and discussion

3.1. Dependence of viscosity on the molecular weight

Viscometry is a simple and rapid method for the determination of MW, which requires the determination of constants through correlation of $[\eta]$ with MW (Roberts & Domszy, 1982) The MW of CH1 (309,000 g/mol) was slightly higher than that of CH2 (290,000 g/mol). As expected, increasing molar mass corresponds to increasing intrinsic viscosity ($[\eta]_{CH1} = 231$ ml/g and $[\eta]_{CH2} = 218$ ml/g). This could be attributed to the alkali concentration used in the process. With the increase of the alkaline strength the content of the acetyl group decreases and the nitrogen content increases. Also, the lower viscosity of CH2 compared to CH1 suggests a decrease of MW.

3.2. Degrees of deacetylation and acetylation

The infrared spectra of all chitosan powders (Fig. 1) exhibited broad peaks assigned to OH stretching, indicating intermolecular hydrogen bonding of chitosan molecules. The absence of sharp absorption around 3500 cm^{-1} in all samples indicated that there are no free OH groups. As expected, N-deacetylation is associated with progressive weakening of the band occurring at 1655 cm^{-1} (amide I). A C=O stretching (amide I) peak near the 1655 cm^{-1} and a NH bending (amide II) peak near the 1590 cm^{-1} regions were observed.

The DDs of the samples CH1 and CH2 were obtained from the standard curve that has the following form: $DD = 87.8 - [3(A_{1655}/A_{3450})]$ ($r^2 = 0.987$) (Fig. 2). Using extrapolation, the DD values for CH1 and CH2 were roughly 86–89% which show a high degree of deacetylation.

The IR spectroscopic method, commonly used for the estimation of chitosan DDs, has a number of advantages because it is relatively fast and does not require dissolution

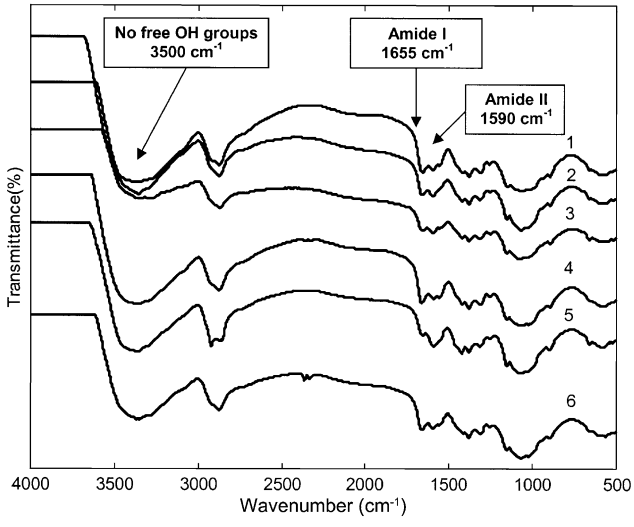

Fig. 1. Transmission infrared spectra of chitosan samples: (1) CH1, (2) CH2, (3) Primex, (4) MMW, (5) LMW, (6) HMW.

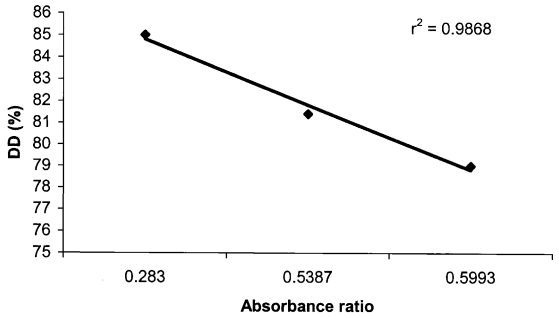

Fig. 2. Standard curve for determination of % DD of CH1 and CH2.

of the sample in an aqueous solvent. However, the sample preparation, the type of instrument used and the experimental conditions may influence the sample analysis (Khan, 2002). Furthermore, since the presence of free water may interfere with the analysis by contributing towards the intensity of the hydroxyl band, the chitosan samples were dried in an oven at 80 °C for 48 h.

Different methods have been applied to determine the acetyl content of chitosan, including infrared spectroscopy (Domszy & Roberts, 1985; Miya, Iwamoto, Yashikawa, & Mima, 1980; Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996), solid-state NMR spectroscopy (Heux et al., 2000; Raymond, Morin, & Marchessault, 1993; Saito & Tabeta, 1987), ultraviolet spectrometry (Muzzarelli & Rochetti, 1985), ^{1}H liquid-state NMR (Hirai, Odani, & Nakajima, 1991; Varum, Anthonsen, Grasdalen, & Smidsord, 1991) and elemental analysis (Roberts, 1992). Solid-state ^{13}C NMR appears to be the most reliable for the evaluation of the acetyl content (Heux et al., 2000). It does not need the solubilization of the polymer but needs a high level of purification of the samples studied. ^{13}C CP-MAS NMR spectra of the chitosan samples are shown in Fig. 3. Corresponding chemical shifts and DA values appear in Tables 1 and 2, respectively.

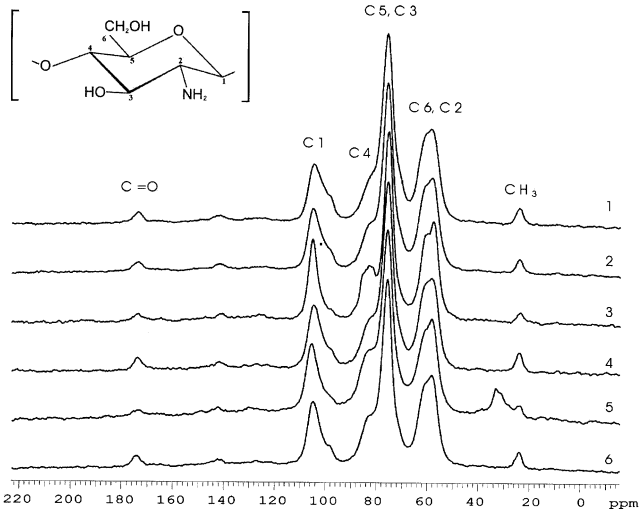

Fig. 3. CP-MAS ^{13}C NMR spectra of chitosan samples: (1) CH1, (2) CH2, (3) Primex, (4) MMW, (5) LMW, (6) HMW.

Table 1
^{13}C chemical shift values (ppm) for chitosan from various sources

Sample	C=O	C_1	C_3/C_5	C_2	CH_3
HMW	173.7	104.8	75.7	58.3	24.3
MMW	174.0	104.5	75.5	58.4	24.0
LMW	174.1	105.4	75.7	58.2	23.6
Primex	173.4	105.0	75.3	57.7	23.7
CH1	173.7	104.8	75.9	58.9	24.0
CH2	173.5	105.2	75.8	58.1	24.0

The methyl and carbonyl signals, associated with the monomeric form of chitin, were detectable in the polymeric chain of all the chitosan samples, showing incomplete deacetylation of the original chitin. The sample of LMW shows undesired signals at 33 ppm due to impurities (proteins and/or lipids) which were not removed adequately.

Solid state CP-MAS ^{13}C NMR is known to be very sensitive to changes in the local structure. The chemical shifts of C-1 and C-4 carbon in 1,4-linked carbohydrates are believed to be highly sensitive to any conformational change at the glycosidic linkage (Tanner, Chanzy, Vincendon, Roux, & Gaill, 1990). As shown in Fig. 3, the C-1 ^{13}C NMRsignal of CH1 is a single peak and the C-4 signal appears as a shoulder which is resolved best for the Primex sample. According to these results, chitosans derived from lobster chitin are similar to those of the commercial samples evaluated.

The dependence of the DD values on the type of analytical methods (Baxter et al., 1992; Khan, 2002) and method of purification (Sabnis & Block, 1997) has been reported in the literature. As such, it can be concluded that the values of DD depend on the analytical method employed. This should be noticed when comparisons of chitin and chitosans of different sources are made.

3.3. Polymorphism

Figs. 4 and 5 show the powder X-ray diffraction patterns of chitosan samples. All chitosan powders showed diffraction peaks at approximately 10° (2θ) and 20° (2θ) before milling (Fig. 4). All types of chitosan powders were partly crystalline (Table 3).

Table 2
Properties of chitosans from various sources

Sample	Viscosity (mPa)	Degree of deacetylation (% DD)	Degree of acetylation (DA) ^{13}C NMR
HMW	1.77[a]	79.0[b]	0.11
MMW	286.0[a]	81.4[b]	0.15
LMW	53.0[a]	85.0[b]	0.02
Primex	59.0[a]	85.6[c]	0.08
CH1	10.2	86–89[d]	0.13
CH2	9.6	86–89[d]	0.10

[a] Suppliers' data.
[b] Determined by colorimetric assay (reference method).
[c] Determined by potentiometric titration.
[d] Determined by IR.

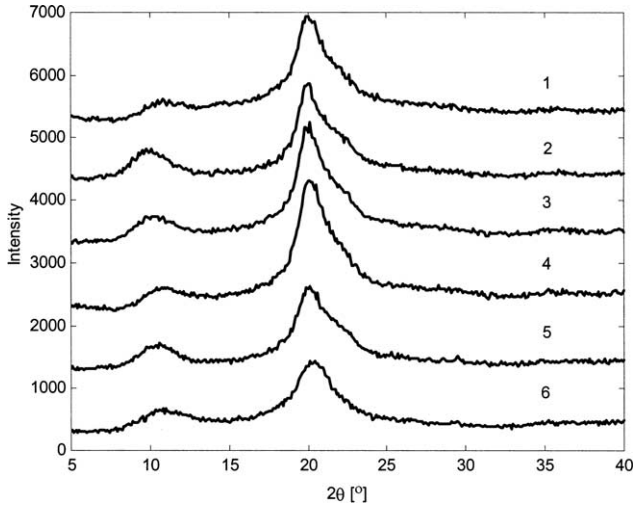

Fig. 4. X-ray diffraction patterns of chitosan samples before milling: (1) Primex, (2) HMW, (3) MMW, (4) LMW, (5) CH2, (6) CH1.

Six polymorphs have been proposed for chitosan: 'tendon chitosan' (Clark & Smith, 1936), 'annealed' (Saito & Tabeta, 1987), '1-2', 'L-2' (Saito & Tabeta, 1987), 'form I' and 'form II' (Samuels, 1981). The X-ray powder pattern of CH1 showed that it is polymorph 'L-2' and differed from the other chitosans studied. The two peaks having lattice angles of 10.8 and 20.4° corresponds to the respective equatorial (100) and (020) reflections of the 'L-2' polymorph of chitosan. XRPD diffraction of the CH2, HMW, MMW, LMW and Primex of chitosan coincides with tendon chitosan: the reflections corresponded to the equatorial (200), (020) and (220) reflections of the tendon chitosan.

As shown in Fig. 5, when the chitosan samples were milled, halo diffraction patterns were observed, indicating an amorphous state of the powders (Table 3). However, the reflections at approximately 12° (2θ) and 20.2° (2θ) were observed at low intensity in LMW chitosan.

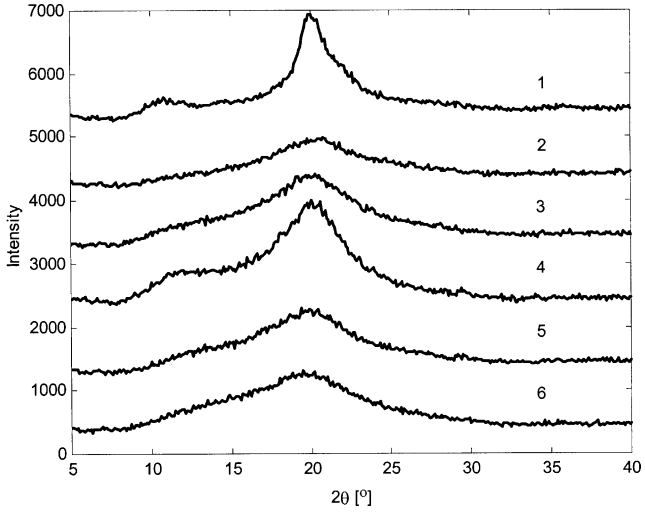

Fig. 5. X-ray diffraction patterns of chitosan samples after milling: (1) Primex, (2) HMW, (3) MMW, (4) LMW, (5) CH2, (6) CH1.

Table 3
Crystallinities of chitosans from various sources

Sample	Crystallinity (% ± 10)	
	No milling	Milling
HMW	43	0
MMW	50	0
LMW	49	43
Primex[a]	–	46
CH1	39	0
CH2	45	0

[a] Milled sample.

3.4. Thermal behaviour of chitosan powders

The thermal data of the chitosan samples are listed in Table 4. During the first heating run, a broad endothermic peak, centred at 130–150 °C was observed. According to TGA analyses, the changes in weight loss were attributed to dehydration, which is in agreement with the DSC results. Therefore, these results support the view that water evaporation occurred during the first DSC scan. The differences in the position and shape of the endotherm peak indicate differences in the water holding capacity and strength of water–polymer interaction (Kittur et al., 2002; Sakurai, Maegawa, & Takahashi, 2000). CH2 showed higher ΔH values than CH1, indicating a high DD value; i.e. it has more hydrophilic centres (amine groups) in the polysaccharide chain to bind more water molecules and to increase the content of bound water. The commercial chitosans do not show clear differences. The fact that the SD was increased indicates that the samples were less homogeneous. In addition, chitosan is not a completely crystalline polymer, so differences in the mobility of water molecules could be expected.

As reported by Sakurai et al. (2000), two cycles of heating and cooling runs were performed to eliminate the effect of moisture. Careful examination of the second heating run allows estimation of the glass transition temperature of the samples. The results of the second heating run obtained in the present study are shown in Table 4. Sakurai et al. employed chitosan films (chitosan with 96% of DD) and assigned a value of 205 °C to the glass transition temperature. In the present study, chitosan samples as a powder form and with a lower % of DD were used. Different properties such as crystallinity, amount of water, degree of deacetylation and OH- or amine-groups in the chain of the macromolecule, can be associated with glass transition and its variability. The second thermal change registered for the chitosan samples in the present study was an exothermic decomposition peak with onset at 280–300 °C.

3.5. Particle and powder properties

The physical properties of the chitosan samples are listed in Table 5. The Carr index and Hausner ratio widely used in characterising flow properties of pharmaceutical excipients,

Table 4
Thermal transitions of chitosan samples

Sample	DSC/first heating (endotherm)			DSC/second heating (glass transition)		TGA weight loss (%)
	T_o (°C)	T_p (°C)	ΔH (J/g)	T_o (°C)	Midpoint (°C)	
HMW	121±11	142±6	78±22	117±7	129±11	9.91±0.03
MMW	130±6	135±5	70±5	130±29	133±18	8.53±0.03
LMW	136±1	139±1	61±3	105±16	113±7	7.64±0.07
Primex	139±9	142±8	69±20	107±9	121±12	8.30±0.01
CH1	146±2	147±2	64±2	116±12	139±4	7.29±0.21
CH2	127±3	133±2	92±9	112±5	130±4	10.66±0.09

T_o, onset temperature; T_p, peak temperature; ΔH, enthalpy; mean±SD; $n=3$.

Table 5
Physical properties of chitosans

Sample	Bulk density (g/cm^3)	Tap density (g/cm^3)	True density (g/cm^3)	Packing fraction	Carr's index (%)	Hausner ratio
HMW	0.223±0.012^a	0.246±0.016^a	1.446±0.007^a	0.157^a	9.5^a	1.10^a
MMW	0.180±0.017^b	0.206±0.019^b	1.426±0.007^{a,b}	0.125^b	12.7^a	1.14^b
LMW	0.311±0.014^c	0.351±0.019^c	1.434±0.000^a	0.226^c	9.2^a	1.10^a
CH1	0.195±0.007^b	0.211±0.005^b	1.407±0.004^c	0.139^{a,b}	7.8^b	1.08^a
CH2	0.186±0.002^b	0.216±0.002^b	1.413±0.004^{b,c}	0.131^b	14.1^c	1.16^b

Letters a–d illustrate the statistical difference ($p<0.05$) of the results based on ANOVA and Tukey tests. For example in bulk density code 'a' means that HMW is not statistically equivalent with any of the others. In the same column, MMW, CH1 and CH2 all having code 'b' are not statistically differing from each other.

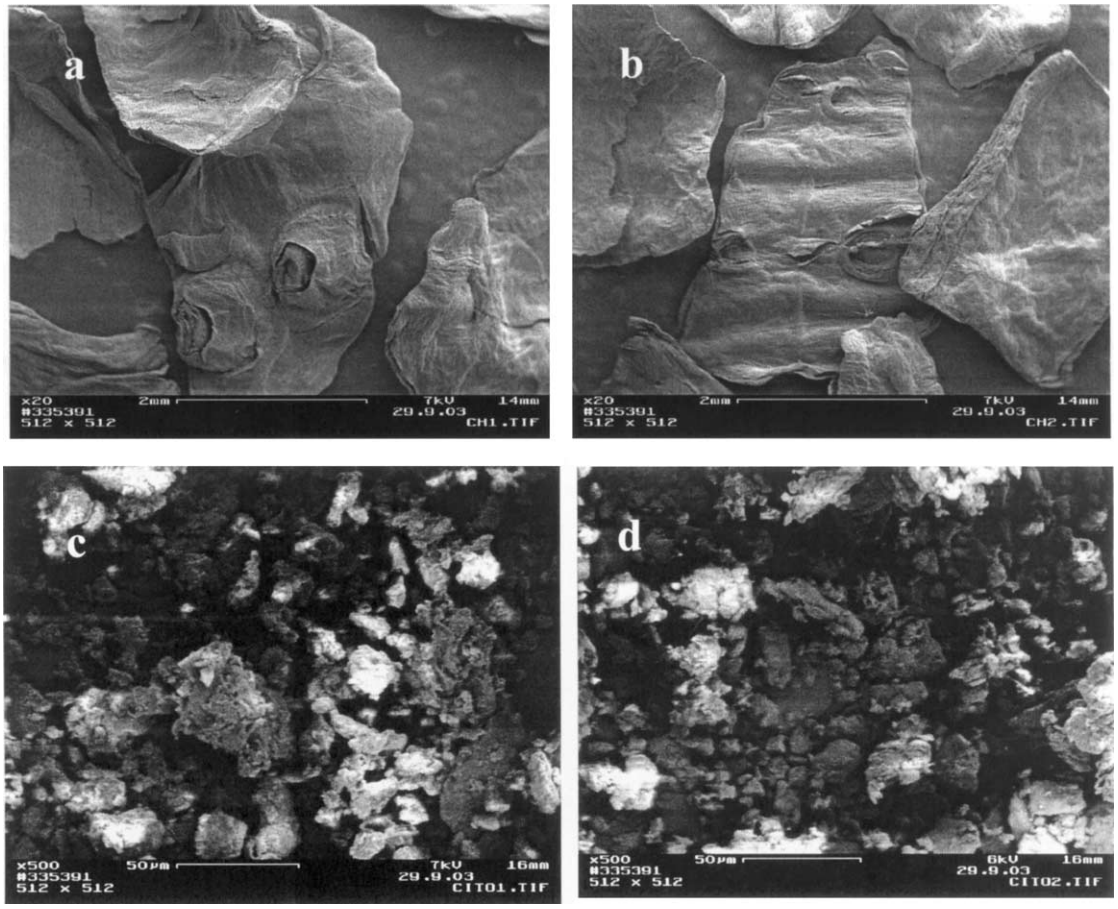

Fig. 6. Scanning electron micrographs (SEMs) on (a) native unmilled CH1, (b) native unmilled CH2, (c) milled CH1 and (d) milled CH2.

indicated that the present chitosan powders had an excellent or good flowability. Table 5 provides evidence of the variability encountered in the chitosans. The significant differences ($p < 0.05$) are supported by the results of the Tukey HSD test. There were no significant differences in the bulk, tap and true densities and the packing properties ($p > 0.05$) of CH1 and CH2, whereas the differences in flow properties were significant ($p < 0.05$).

The scanning electron micrographs (SEMs) of both native and milled CH1 and CH2 are shown in Fig. 6. The chitosans prepared from lobster chitin consisted of amorphous particles of rather irregular size and shape. The particle sizes of native unmilled and milled chitosans (CH1 and CH2) were 2000 and 20 μm, respectively.

4. Conclusions

The properties of chitosans are very much dependent on the degree of deacetylation, which depends on the synthesis of chitosan as well as the source of the starting material (i.e. chitin). Powder X-ray diffraction permitted the analyses of the polymorphs of chitosan. The synthesis process does not significantly influence the bulk, tap and true densities and packing properties of chitosan powders of lobster origin. The method of synthesis significantly affects the flow properties of the present materials. The chitosans (CH1 and CH2) prepared by N-deacetylation from lobster chitin have physico-chemical properties that are similar to the commercial chitosans of crab or shrimp shell origin.

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