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Preparation and characteristic properties of 5-methyl pyrrolidinone chitosan

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The proper selection of preparative conditions intended to favor lactam formation — mainly high chitosan and borohydride concentrations, final pH higher than 5 and slow borohydride delivery rate — allowed the preparation of 5-methyl pyrrolidinone chitosan from levulinic acid in aqueous solution. The modified chitosan was characterized by ^{1}H-NMR, ^{13}C-NMR and FTIR spectroscopy. In the ^{1}H-NMR spectra, the doublet at δ = 1·22–1·25 ppm, typical for methyl pyrrolidinone, was the major feature, while in the ^{13}C-NMR spectra signals for CH, CH_2 and CH_3 confirmed the lactam ring formation, which was revealed by the band at 1730 cm^{-1} in the FTIR spectra. The novel chitosan was water-soluble over an extended pH range.

INTRODUCTION

The biological significance of modified chitosans in wound management involves their biodegradability by lysozyme. The insolubility of most modified chitosans in the physiological pH range makes work on such applications difficult, and explains the paucity of studies on the use of modified chitosans as wound-dressing materials. For instance, modified chitosans in the form of monosubstituted amides carrying carboxyl functions and obtained from organic anhydrides are insoluble (Muzzarelli *et al.*, 1986), as are non-functionalized amides such as *N*-stearoyl and *N*-decanoyl chitosans (Hirano & Tokura, 1982). Chitosans carrying sugar moieties yield gels (Yalpani & Hall, 1984), all of the *N*-alkyl chitosans studied (secondary amines) are gels (Muzzarelli *et al.*, 1983), and *N*-alkylidene chitosans (6–12 carbon atoms) are insoluble (Kurita, 1988).

On the other hand, the introduction of certain novel functions deprives chitosan of certain desirable characteristics; for example, inorganic esters of chitosan, such as sulfate esters, have no film-forming and bacteriostatic capacity (Muzzarelli *et al.*, 1984).

One of the few water-soluble chitosans so far described is *N*-carboxybutyl chitosan obtained from levulinic acid (Muzzarelli *et al.*, 1989); levulinic acid is formed by the acid-catalyzed degradation of hexoses via the intermediary of hydroxymethyl furfural (Conner, 1989). Literature concerning levulinic acid reports the capacity of levulinic acid to react with amines and produce lactams, with formation of the pyrrolidinone ring (Frank *et al.*, 1955; Leonard, 1956; Kitano *et al.*, 1975; Shilling, 1965); however, methyl pyrrolidinone chitosan has not been synthesized until recently (Muzzarelli, 1990).

Aspects which make the presence of the pyrrolidinone function desirable in medical materials arise mainly from information on poly(vinylpyrrolidinone), a widely used product, and are summarized below.

The ring of proline and hydroxyproline found in pyrrolidinone is the same as that which comprises the monomeric units of gelatin; in polymers it is deprived of the hydrogen atom on the ring nitrogen. Monomeric units carrying a pyrrolidinone moiety are, therefore, unable to form hydrogen links, and pyrrolidinone polymers should be superior to gelatin in behavior. Poly(vinylpyrrolidinone) exists, in fact, as a viscous solution (not a gel) and imparts hydrophilicity (Ling, 1984). It is known that poly(vinylpyrrolidinone) does not produce inflammation when applied to the cornea of the rabbit and it is biocompatible (Gebelein & Carraher, 1985). It is also a filmogenic substance used to reinforce membranes. A coating imparts biocompatibility, thus it has been tried on patients in association with various drugs (Chiellini & Giusti, 1983). Water-

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alcohol mixtures are solvents for poly(vinylpyrrolidinone) when it is used as a binder of pigments in liners, mascara, lipsticks and other cosmetics, and in shampoos. It is also an ingredient of hairsprays. Pyroglutamic acid and its salts and esters which contain a pyrrolidinone ring are widely used in cosmetics (Proserpio, 1985).

The purpose of the present work was therefore to produce 5-methyl pyrrolidinone chitosan, a substituted chitosan where the nitrogen atom is simultaneously part of the methyl pyrrolidinone moiety and of the glucosamine repeating unit.

MATERIALS AND METHODS

Materials

Crab chitosan was supplied by Protan (Redmond, WA, USA); its average molecular weight was 384 500 + 13 500 Da and its degree of deacetylation 0·82 (it was from the stock analyzed by Muzzarelli *et al.*, 1987). For some preparations, shrimp chitosan supplied by Merck Clevenot (Nogent-sur-Marne, France) was used, with certified average molecular weight 450 000 Da and degree of deacetylation 0·90–0·95. Sieved fractions were used (180–350 μm). Levulinic acid was purchased from Aldrich, Milwaukee, WI, USA, and borohydride was from Chemtec, Milan, Italy.

Methods

Levulinic acid was determined in ethyl ether by spectrophotometry at 275 nm with a Kontron 810 spectrophotometer. Unreacted levulinic acid could be extracted from aqueous chitosan levulinate solutions, and its concentration measured after the distribution coefficient had been determined. Extraction of levulinic acid was a slow process, strongly dependent on stirring mode and could be performed satisfactorily with adequate mechanical stirring. From aqueous solutions (2 ml) extracted with ether (10 ml) at 20°C under mechanical stirring (5 min) the distribution coefficient K was 1·82 (water/ether). The molar extinction coefficient for levulinic acid in ether was found to be e = 18·145 liters mol^{-1} cm^{-1} at 275 nm.

For the determination of levulinic acid or its reduction products after treatment with sodium borohydride, it was verified experimentally that the UV-vis spectra for the ether extracts were not substantially different from that of authentic levulinic acid, when ether was removed and the soluble compounds measured in water. A reference curve (equation Y = 0·0078 + 0·1744 X) was therefore drawn using levulinic acid previously titrated by alkalimetry. For levulinic acid concentrations of 0·5, 1·0, 2·0, 3·0 and 4·0 g kg^{-1}, readings at 266·5 nm gave the following absorbance values: 0·0931, 0·1781, 0·3632, 0·5372 and 0·6990, respectively. It was also determined experimentally that five ether extractions were necessary to remove the whole extractable amount of levulinic acid from an aqueous sample containing chitosan.

Levulinic acid was also determined with hydrazine by spectrophotometry, according to Shilling and Hunter (1965). This method is based on the formation of cyclic hydrazide hydrazone with absorption at 242 nm. A 50% solution of hydrazine hydroxide was mixed with a cold equimolar HCl (20%) solution and a portion (4 ml) of the resulting salt solution was added to the analyte (0·5–1·0 g levulinic acid), diluted to 10 ml, heated for 1 h at 85°C, diluted 1:100, and three drops of concentrated HCl were added. The apparent molar absorption coefficient calculated for levulinic acid was ε = 7500 liters mol^{-1} cm^{-1} at 242 nm.

Infrared spectra were recorded with a Nicolet model 215 FTIR spectrometer on thin films obtained by evaporating chitosan solutions carefully at 35°C and pH 5·2–6·0. The ^{1}H-NMR spectra were obtained in D_2O with a Varian (Leini, Italy) Gemini 200 spectrometer at 200 MHz and 20°C, while the ^{13}C-NMR spectra were obtained with a Brucker (Karlsruhe, Germany) CXP-300 spectrometer at 75 MHz. The chemical shifts were calculated with respect to TMS. Elemental analyses were done with an instrument manufactured by Carlo Erba (Milan, Italy). Lyophilization was done with a B. Braun (Frankfurt, Germany) model Beta freeze-drier set at −20°C for freezing and at +30°C for drying.

RESULTS AND DISCUSSION

Upon introduction of levulinic acid into a stirred suspension of chitosan powder in water at 20°C, the pH dropped to values in the range 3·7–4·5 depending on the levulinic acid concentration (30–120 mmol kg^{-1}) for a given concentration of chitosan (30 mmol kg^{-1}); complete dissolution and pH stabilization was observed 40–60 min after mixing.

The dissolution of chitosan is certainly due to protonation by levulinic acid; however, at these pH values, ketimine formation is expected to take place (Muzzarelli *et al.*, 1989). The free levulinic acid concentration drop, measured by one-step ether extraction for various initial concentrations, is illustrated in Fig. 1, where the two straight lines, relevant to chitosan concentrations 6·5 and 13·0 g kg^{-1}, have slopes close to 45° and intercept the abscissa at two distinct levulinic acid concentration values: 0·015 and 0·030 mol kg^{-1}, respectively. The amount of levulinic acid reacted was therefore *c.* 0·3 mol per mol chitosan. This means that a minor part of the levulinic acid was bonded to chitosan and the rest was free and extractable. The inflection of both curves at high initial levulinic acid concentrations meant that at those concentrations a larger amount of

levulinic acid reacts with a given amount of chitosan.

These results were confirmed by measurements on solutions submitted to dialysis against distilled water in tubes with a cutoff of 12 000 Da (chitosan concentration 6·5 g kg^{-1}, i.e. 0·045 mol kg^{-1}, and levulinic acid 0·045 mol kg^{-1}). The rate of levulinic acid removal dropped rapidly in the course of the dialysis as illustrated in Fig. 2, and tended towards its constant value of 0·012 mol kg^{-1}, which coincides with the value of the bonded levulinic acid as determined by the hydrazine method. Thus, chitosan combined with levulinic acid to form a salt and/or a ketimine in the molar ratio from 1·0 to 0·27–0·30, at the said concentrations. At the end of the dialysis, the chitosan solutions were perfectly clear and more dilute. Chitosan, therefore, surprisingly required less than one-third of the stoichiometric quantity of levulinic acid to produce a soluble salt or ketimine that was indifferent to ether extraction but could be destroyed by hydrazine hydrochloride.

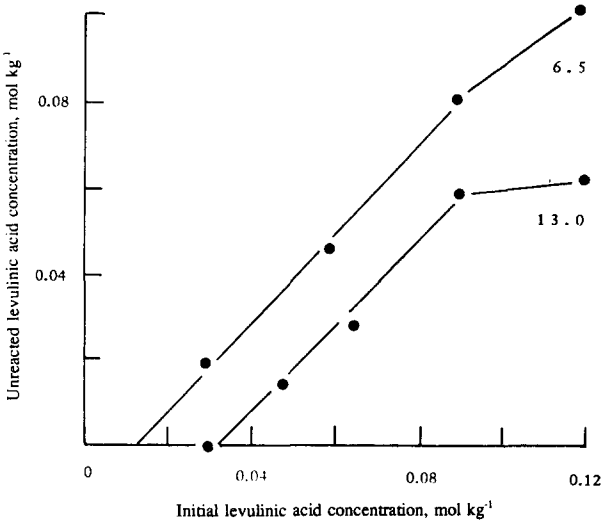

Fig. 1. Ketimine formation between levulinic acid and chitosan, at two chitosan concentrations (6·5 and 13·0 g kg^{-1}). Concentration of unreacted ether-extractable levulinic acid as a function of the levulinic acid initial concentration (g kg^{-1}). The inflection of both curves, more marked for the higher chitosan concentration, indicates the favorable action of high concentrations on the ketimine formation.

After the sodium borohydride addition, the residual levulinic acid and/or reduction products were determined by spectrophotometry on the aqueous solutions obtained from the evaporated and pooled ether fractions for samples at two chitosan concentrations (9·0 and 18·0 g kg^{-1}) and two levulinic acid concentrations. As indicated in Table 1, the calculated uptakes were 57·9–62·0% for the higher and 40·1–42·2% for the lower levulinic acid concentrations. For the same molar ratio (1:1), therefore, a much higher uptake was obtained (57·9% instead of 40·1%).

These data indicated that concentrations of both reagents are key parameters for the control of the levulinic acid uptake by chitosan during hydrogenation. This becomes evident from the plot in Fig. 3: the same uptake (60%) can be obtained with chitosan at 9·0 g kg^{-1} and an initial molar ratio of 1·5, as with chitosan at 18·0 g kg^{-1} and an initial molar ratio of 1·0. Of course, the latter conditions are more favorable for the preparation of the modified chitosan, and, by acting on them, the reaction with sodium borohydride could be driven in such a way as to have a high degree of substitution and lactam formation.

In fact, the ^{1}H-NMR spectra for hydrogenated, dialyzed and freeze-dried samples showed some novel interesting features (compared with chitosan spectra reported by Domard *et al.*, 1987, and by Hirai *et al.*, 1991, with levulinic acid spectra reported by Sunjic *et al.*, 1984, and with hydroxypropyl chitosan spectra reported by Maresch *et al.*, 1989). First of all, the twin signals at 1·113 and 1·144, typical of methyl pyrrolidinone, became prominent with the adoption of the preparation conditions indicated in Table 1; that is, high chitosan concentration, close to 20 g kg^{-1}, depending on viscosity; higher pH value during the reduction step; higher sodium borohydride concentration and slow sodium borohydride delivery rate. A typical set of preparative conditions were the following: chitosan powder (9 g) suspended in water (500 ml) was stirred overnight, then levulinic acid (9·7 g) was added and stirring continued for 1 h to final pH 4·35; sodium borohydride aqueous

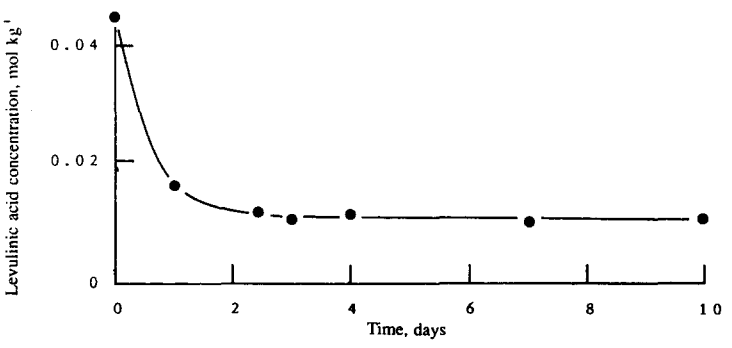

Fig. 2. Rate of levulinic acid removal in the course of dialysis against water (asymptote 0·012 mol kg^{-1}), for 10 days, cutoff 12 000 Da. Three days suffice for the removal of unreacted levulinic acid.

Table 1. Determination of the degree of substitution of samples prepared from Protan chitosan (degree of deacetylation 86·5%), by extraction with diethyl ether and spectrophotometric analysis on water at 266·5 nm

	Preparation			
	I	II	III	IV
Chitosan (g)	4·5	4·5	9·0	9·0
Water (ml)	375	375	500	500
Levulinic acid (g)	4·064	2·709	5·419	4·335
Molar ratio levulinic acid/chitosan	1·5:1·0	1·0:1·0	1·0:1·0	0·8:1·0
Added water (ml)	125	125	0	0
$NaBH_4$ reduction	to pH 5·5	to pH 5·5	to pH 5·8	to pH 5·8
Corrected absorption reading	0·1741	0·1203	0·1598	0·1506
'Levulinic acid' (g/liter^{-1})	0·954	0·645	0·872	0·819
'Levulinic acid' in 10 g	0·048	0·032		
'Levulinic acid' in 500 g sample	2·384	1·613	2·179	2·047
Levulinic acid reacted (g)	1·680	1·097	3·240	2·288
Levulinic acid reacted (mol)	0·0145	0·0094	0·279	0·0197
Glucosamine (mol)	0·0233	0·0233	0·0467	0·0467
Degree of substitution (%)	62·0	40·1	57·9	42·2

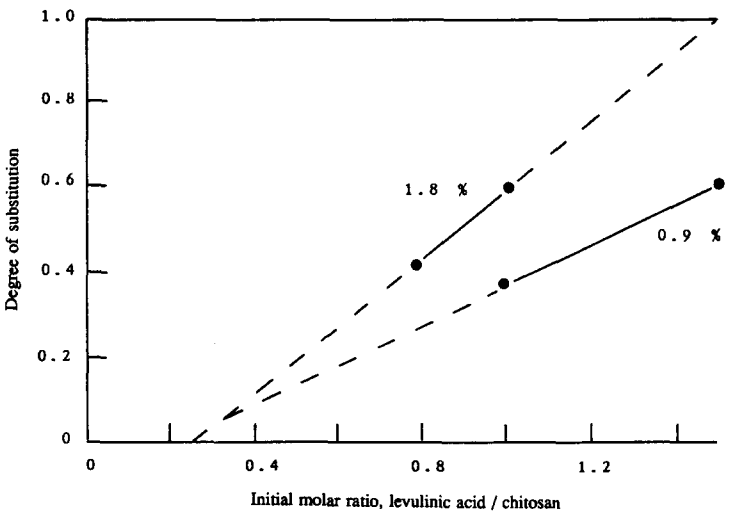

Fig. 3. Dependence of the degree of substitution on the molar ratio between levulinic acid and chitosan for samples submitted to hydrogenation with sodium borohydride, at two initial chitosan concentrations (9 and 18 g kg^{-1}). The chitosan concentration seems to be an important parameter controlling the degree of substitution. In practice, the same degree of substitution (0·60) can be achieved with 1·0:1·0 molar ratio provided that the chitosan concentration is 18 g kg^{-1}. Extrapolation of the upper curve would indicate that fully substituted chitosans can be obtained when choosing the highest values for both parameters. Extrapolation to zero confirms that molar ratios higher than *c.* 0·3 are required for the modification of chitosan under hydrogenation: molar ratio 0·3 is also the one required to keep the chitosan salt/ketimine in solution, as shown in Fig. 1.

solution (20 ml, 70 g liter^{-1}) was delivered over a 3-h period to final pH 5·6. The product was dialyzed against distilled water and freeze-dried. The elemental analysis for the parent chitosan gave the following results: C, 41·16805; H, 6·627528; N, 7·885385 from which the following formula was calculated: $C_{6\cdot08827}H_{11\cdot08827}N_1O_{4\cdot044136}\cdot 0\cdot81127H_2O$, indicating degree of deacetylation 0·9558 and water content 8·23% in substantial agreement with the data declared by the producer. The methyl pyrrolidinone chitosan obtained from said chitosan yielded the following results: C, 41·77475; H, 6·387395; N, 5·06982 from which the following formula was calculated: $C_{9\cdot60898}H_{17\cdot50729}N_1O_{8\cdot07585}\cdot 3\cdot07797H_2O$, indicating degrees of substitution 70·4143 and water content 20·07% in substantial agreement with data shown in Fig. 3. The ^{1}H-NMR data relevant to such preparations are given in Table 2 and Fig. 4. The spectra for authentic levulinic acid and 5-methyl pyrrolidinone were found to be in agreement with those in the literature (Pouchert, 1983).

From the data in Table 2, it is possible to see that the methyl pyrrolidinone moiety is formed upon reduction and the relevant signals are present in the reaction product and in the purified final product. Solvent

Table 2. ^{1}H-NMR chemical shifts (ppm) of the reactants (levulinic acid and chitosan), the products (ketimine and methyl pyrrolidinone chitosan) and authentic 5-methyl pyrrolidinone

Levulinic acid	Chitosan	5-Methyl pyrrolidinone	Ketimine	5-Methyl pyrrolidinone chitosan			Assignment[a]
				Dialyzed	Dialyzed and extracted		
					Neutral	Alkaline	
					1·02		
		1·113		1·11	1·22	1·18	
		1·144		1·14	1·25	1·20	
		1·55			1·76	1·70	
	1·983		1·983	1·983	2·02	1·90	Methyl
2·176			2·139				(acetamido)
		2·20		2·17	2·35	2·20	
2·55 t			2·30 t	2·37 t	2·50 t	2·38	
2·80 t			2·67 t	2·72 t	2·82 t		
		3·70 q					
	3·18		3–5	3–5	3–5	3–5	H-2
	3·72		broad	broad	broad	broad	H-6
	3·88						H-3, H-4, H-5
	4·87						H-1

[a] For assignments, see text and references, in particular Naggi *et al.* (1986), Sunjic *et al.* (1984), Pouchert (1983). t, triplet; q, quartet.

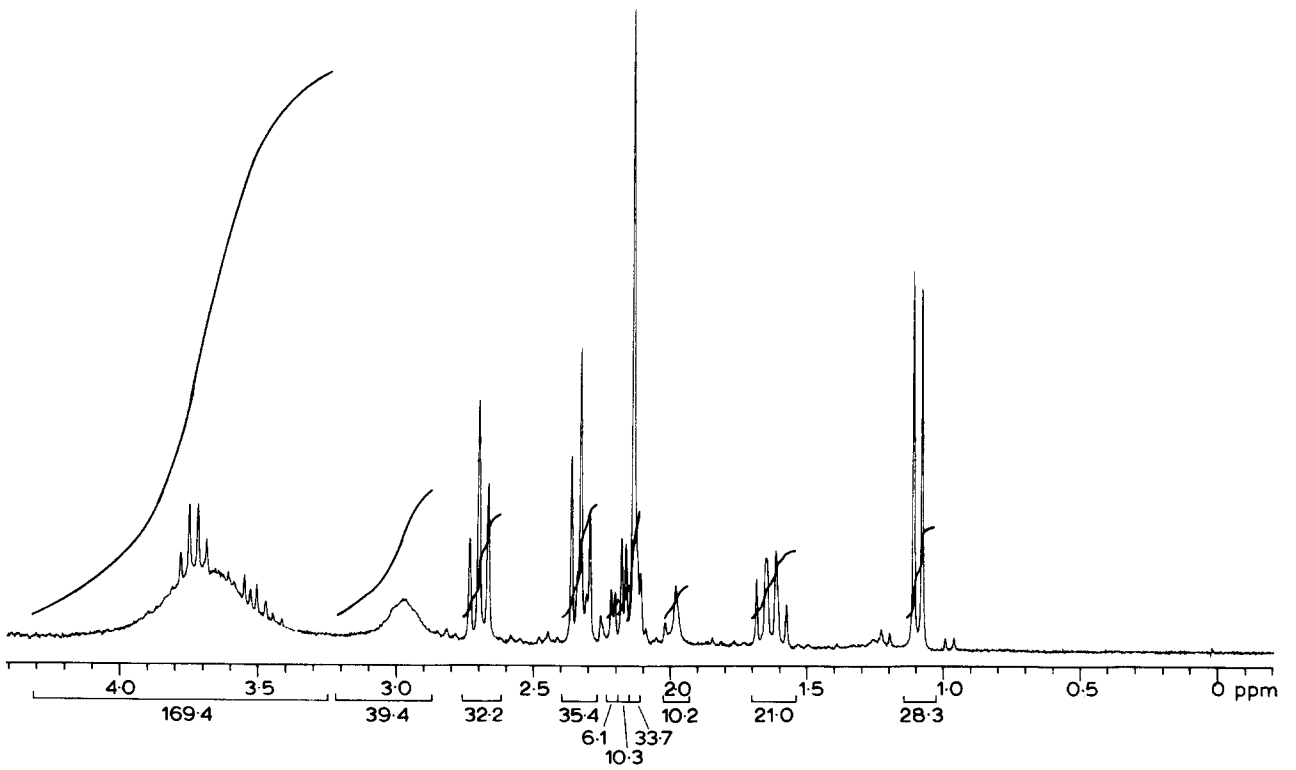

Fig. 4. ^{1}H-NMR spectrum (200 MHz) for methyl pyrrolidinone chitosan after dialysis.

extraction with dimethylsulfoxide or with ether indicated that some hydroxypentanoic acid and other low molecular weight compounds from levulinic acid were present in the crude preparation and could be removed.

The purified and freeze-dried polymer, redissolved in deuterated water, showed the prominent doublet at 1·22–1·25 ppm, typical for the methyl pyrrolidinone substituent. Other features of the methyl pyrrolidinone ring were present in the spectra, such as the quartet centered at 3·70 ppm, as well as the other signals at 2·2 ppm, thus confirming the high degree of substitution. Signals for levulinate were also present; remarkably, they were altered in the spectrum for methyl pyrroli-

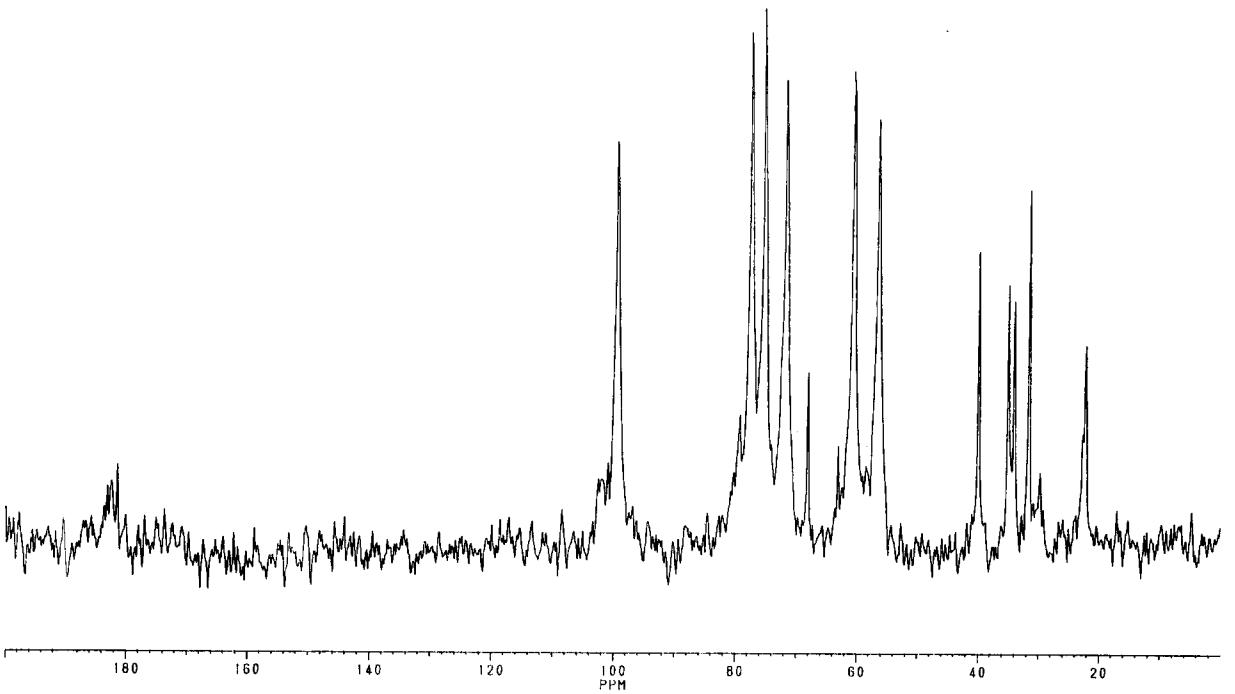

Fig. 5. ^{13}C-NMR spectrum for methyl pyrrolidinone chitosan after dialysis.

dinone chitosan in alkaline solution. It would seem that the salt formed by levulinic acid with the unmodified glucosamine units is particularly stable.

According to the ^{13}C-NMR spectra, upon reaction with sodium borohydride cyclization of the carboxybutyl group took place and two isomers formed. For each atom of the 5-methyl pyrrolidinone ring, two distinct signals were present in the spectrum, one for each isomer. Assignments in the spectra (Fig. 5) were the following: CH, 63·07 and 68·17; CH_2, 31·89, 34·29, 35·16, 39·45, 39·70 and 40·02; CH_3, 22·89, 22·22 (acetamido) and 29·75. The signals for levulinic acid were reported by Sunjic *et al.* (1984) to be: α-CH_2, 27·9, β-CH_2, 32·9, CH_3 29·6 and CO 177·3. Remarkable shifts took place in ring formation. These data are in agreement with those reported by Brant (1981) for poly(vinylpyrrolidone). The signal for the carboxyl group was hardly noticeable, its height being less than twice the background noise, whilst it was clearly measurable in *N*-carboxybutyl chitosan. Different ratios between the methylene signals also revealed a different chemical environment in the side-chain compared with *N*-carboxybutyl chitosan.

Using FTIR spectrometry, bands at 1400 cm^{-1} for methylene and methyl groups, at 1730 for the lactam and at 1700 for the amide carbonyl group revealed the altered structure of the methyl pyrrolidinone chitosan; moreover, the free amine band at 1590 was depressed (Fig. 6). A comparison of the spectrum in Fig. 6 with the one for *N*-carboxymethyl chitosan (Muzzarelli *et al.*, 1982), which is certainly exempt of cyclic side structures, showed that one of the major differences was in the region 1300–1500 cm^{-1}, indicative of the contribution

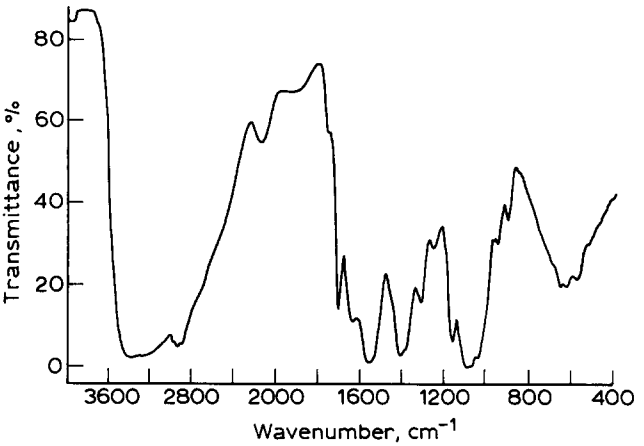

Fig. 6. FTIR spectrum for methyl pyrrolidinone chitosan, after dialysis, in the form of a thin film.

of the lactam in the methyl pyrrolidinone chitosan; the sharp band at 1730 cm^{-1} in the methyl pyrrolidinone chitosan (Fig. 6) was the main feature, compared to *N*-carboxybutyl chitosan (figure 2 in Muzzarelli *et al.*, 1989; see also authentic methyl pyrrolidinone spectrum in Pouchert, 1981).

In conclusion, the proper choice of experimental conditions allows the production of methyl pyrrolidinone chitosan, which is in general accompanied by anionic species from levulinic acid, which are relatively difficult to remove. Lyophilized methyl pyrrolidinone chitosan is spontaneously soluble in distilled water and gelifies with biological fluids. It is susceptible to the hydrolytic action exerted by lysozyme even when it is obtained from highly deacetylated chitosans

(Muzzarelli, 1992) and has been successfully tested for wound management in dental surgery (Muzzarelli *et al.*, in press) and other fields (Muzzarelli *et al.*, in preparation).

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