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Depolymerization of methyl pyrrolidinone chitosan by lysozyme

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Methyl pyrrolidinone chitosan is being tested as a wound dressing material endowed of biological significance. In order to clarify its fate and role when applied to a surgical wound, its hydrolytic depolymerization by hen egg white lysozyme was studied *in vitro*. Viscometric measurements at three temperatures (25, 37 and 50°C) and five methyl pyrrolidinone chitosan concentrations (9·0, 13·5, 18·0, 23·5 and 27·3 g litre^{-1}) permitted kinetic data to be obtained, including the Michaelis constant, 1×10^{-4} mol litre^{-1}. Linearity was observed when log K_M was plotted *versus* $1/T$. The viscosity decrease over the 50 min period of observation was a linear function of temperature, independent of the initial substrate concentration. It is concluded that methyl pyrrolidinone chitosan is susceptible to enzymatic hydrolysis and therefore it is an absorbable medical aid. Results support the hypothesis that depolymerized methyl pyrrolidinone chitosan *in vivo* would become available for tissue reconstruction.

INTRODUCTION

Current research on susceptibility of chitosans to depolymerization under the catalytic action exerted by lysozyme (Proctor & Cunningham, 1988) is confined to relationships between degree of deacetylation and rate of hydrolysis. Scanty information is available for modified chitosans.

A degree of deacetylation close to 0·80 was found to be optimal among those studied (0·2, 0·4, 0·8 and 1·0), and a facilitated formation of the enzyme-substrate complex, assisted by a balanced random presence of primary amino groups, was suggested to take place for the said degree of deacetylation (Hirano *et al.*, 1989). This is in agreement with data obtained by other authors by viscometric techniques, supported by molecular weight determinations, indicating that maximum susceptibility of chitosan to hydrolytic depolymerization in the presence of lysozyme occurs for the degree of deacetylation of 0·66 (Sashiwa *et al.*, 1990).

The same conclusion was reached in a simultaneous study (Yomota *et al.*, 1990) on the viscosity decrease for variously deacetylated chitosans; moreover, it was shown that chitosan membranes could be more or less effectively degraded depending upon pH and degree of deacetylation, with optimal values at pH 5–6 and degree of deacetylation 0·66–0·80.

More numerous works were done on chitin (Muzzarelli, 1977, 1980; Muzzarelli *et al.*, 1978); as far as modified chitins are concerned, O-carboxymethyl and O-dihydroxypropyl chitins in the form of fibers were exposed to lysozyme under controlled conditions (Tokura *et al.*, 1983). The improved accessibility for lysozyme, compared to plain chitin, was due to the higher hydrophilicity of the fiber surface.

Methyl pyrrolidinone chitosan belongs to the class of spontaneously water-soluble substituted chitosans possessing documented biological significance (Muzzarelli *et al.*, 1989; Biagini *et al.*, 1991*a*, *b*). Together with *N*-carboxybutyl chitosan and *N*-carboxymethyl chitosan, it is finding use as a biomedical material, in particular for wound dressing, drug delivery and cell culture. Methyl pyrrolidinone chitosan films and freeze-dried sponges gelify in contact with biological fluids and with water, and, while exerting favorable actions for ordered regeneration of connective tissues, are progressively absorbed via enzymatic hydrolysis.

The present investigation was undertaken in order to gain information on the susceptibility of methyl pyrrolidinone chitosan to hydrolytic degradation, which could help explain the clinical observations.

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Viscometry is a rapid and versatile technique for the examination of the effect of enzymes on chitosans, as already indicated by various studies on lysozyme (Sashiwa *et al.*, 1990; Yomota *et al.*, 1990) and chitinases (Ohtakara, 1988).

MATERIALS AND METHODS

Chemicals

Freshly prepared methyl pyrrolidinone chitosan was dialyzed against distilled water (3 changes, 72 h) by using Visking tubings (Technochimica Moderna, Perugia, Italy) with cutoff value 12 000 dalton, and then freeze-dried at 30°C. The original pH value of the MP was 5·30 ± 0·05 and remained as such after dissolution of the freeze-dried material. In the course of the enzymatic reaction it did not change. The cited literature and other works such as Nanjo *et al.* (1991) consistently indicate that a pH value between 5·0 and 6·2 is preferred for lysozyme solutions. Three batches of methyl pyrrolidinone chitosan were prepared and analyzed by FTIR, ^{1}H-NMR and ^{13}C-NMR spectroscopy (Muzzarelli, in preparation): no differences were detected between batches and their viscometric behavior was identical.

Enzyme

Hen egg-white lysozyme supplied by Calbiochem (San Diego, CA) was used, after dissolution in distilled water (5 mg ml^{-1}) and injected (1 ml) into the viscometer cup where the methyl pyrrolidinone chitosan solution (10 g) was already present at the desired temperature.

Instruments

The rotational viscometer Haake Rotovisco RV 20-M5 was computer-controlled (programme Rotation^{R}, Haake, Karlsruhe D), and was set to make continuous measurements at a shear rate of 200 s^{-1}, which is expected not to promote appreciable mechanical degradation of the macromolecules; this shear rate was reached in 0·1 min. The length of time for the measurements was 50 min and 400 readings were made automatically and elaborated into a plot of viscosity (mPa s) *versus* time (min). Preliminary measurements were also done in the absence of lysozyme on aliquots (11 g) of methyl pyrrolidinone solution. For all of these measurements, the double wall rotor NV was used.

Initial velocity measurements

The initial velocities of the hydrolytic reactions catalyzed by lysozyme were computed graphically based on the readings recorded automatically during the first 30 s of the viscometric measurements (interval 30–60 s after injection of lysozyme, start time). Such values were expressed in terms of viscosity decrements, (mPa s min^{-1}). During that time, the reaction rate could be considered equal to initial velocity because, in agreement with accepted criteria, the relevant viscosity decrease was only 3–10% of the viscosity of methyl pyrrolidinone chitosan in the absence of lysozyme. One should consider further that the percentage interval was possibly narrower, because after injection of lysozyme, the methyl pyrrolidinone chitosan solution suddenly became more viscous than the controls.

RESULTS AND DISCUSSION

It was preliminarily verified that the rheological behavior of methyl pyrrolidinone chitosan was such as to permit the correct use of the experimental data obtained in the presence of lysozyme. The computer-plotted rheological flow curves obtained at various concentrations and temperatures indicated Newtonian behavior. The superposition of the acceleration curve and deceleration curve represented the most favorable condition for the subsequent detection of enzymatic action through observations of the viscosity decrease.

Figures 1 and 2 show typical measurements of the viscosity decrease as a function of time for methyl pyrrolidinone chitosan at 23·5 g liter^{-1} at two temperatures (25 and 50°C). At the lower temperature, the viscosity decrease in 10 min was 18%, while at the higher temperature it was 120 − 40 = 80, corresponding to 33%. Over the entire 50-min period at 50°C, the

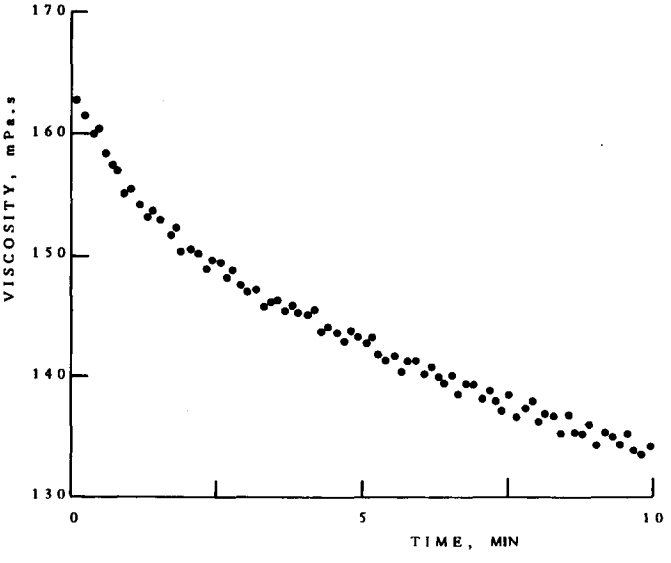

Fig. 1. Viscosity decrease in the period 0–10 min, for methyl pyrrolidinone chitosan at a concentration of 23·5 g liter^{-} at 25°C and pH 5·3. The viscosity decrease in 10 min is from 164 to 134 mPa s, i.e. 18%.

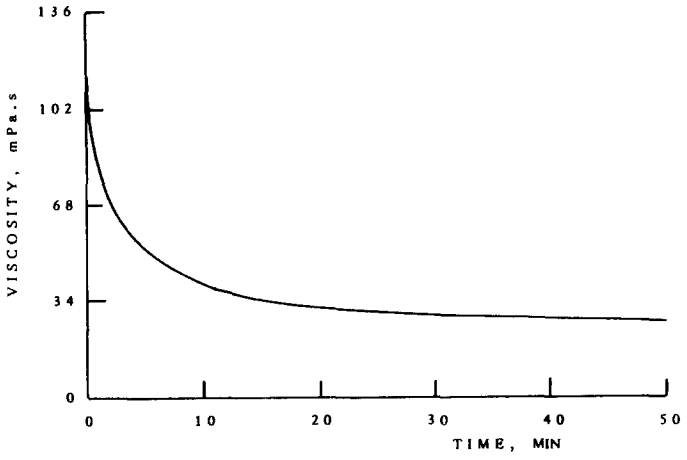

Fig. 2. Viscosity decrease in the period 0–50 min, for methyl pyrrolidinone chitosan at a concentration of 23·5 g liter^{-1} at 50°C and pH 5·3. The viscosity decrease in 10 min is from 120 to 40 mPa s i.e. 33%, while in 50 min it is from 120 to 30 mPa s, i.e. 75%.

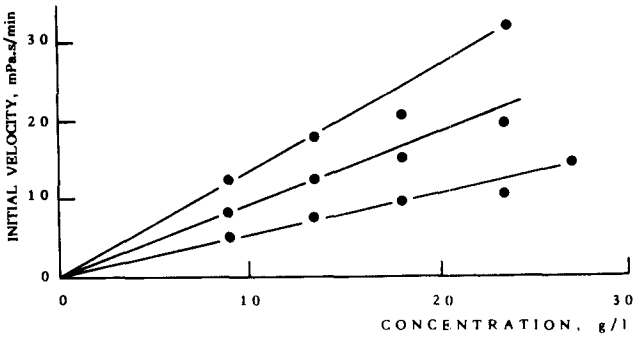

Fig. 3. Initial velocity, V_0, as a function of methyl pyrrolidinone chitosan concentration, at 25, 37 and 50°C and pH 5·3. The relevant numerical data are in Table 1.

viscosity decrease was as high as 75%. Initial velocity was calculated after electronically enlarging the initial one-minute part of the plot. For the three temperatures and the four concentrations studied, initial velocity curves are reported in Fig. 3. Due to the relatively low substrate concentration considered, straight lines were obtained; the numerical data relevant to Fig. 3 are in Table 1.

Lineweaver–Burk plots for the three temperatures studied are in Fig. 4 and the K_M and V_{max} values were graphically obtained from them by reading the intercepts on the $1/V_0$ and $1/[S_0]$ axes: these kinetic data are listed in Table 2. At 37°C the apparent polymer concentration decrease was 14·2 g liter^{-1}, and the Michaelis constant was 1×10^{-4} mol liter^{-1}.

The dependence of viscosity upon concentration in the absence of lysozyme at three temperatures is presented in Fig. 5. On a semilogarithmic plot, straight parallel lines describe such dependence and permit the velocity data expressed in viscosity *versus* time units to be transformed easily into more conventional concentration *versus* time units. The viscosity decrease was actually due to progressively lower average molecular weights of the modified polysaccharide; however, the contribution of small methyl pyrrolidinone chitosan to viscosity was negligible as indicated by the detection limits of the viscometric technique. Thus, what was recorded in practice was the variation of the concentration of large viscosity-building molecules. Results could therefore be expressed in terms of concentration decrease, even though the chemical concentration remained unchanged.

The logarithm of the Michaelis constant, log K_M, *versus* $1/T$ showed linearity (Fig. 6). Lysozyme exhibited remarkable affinity for methyl pyrrolidinone chitosan and, therefore, catalyzed its rapid hydrolytic depolymerization.

Table 1. Viscometric data relevant to the enzymatic hydrolysis of methyl pyrrolidinone chitosan in the presence of lysozyme, at pH 5·3; lysozyme concentration 0·454 g liter^{-1}

Methyl pyrrolidinone chitosan concentration (g liter^{-1})	Temperature T (°C)	Initial velocity V_0 (mPa s min^{-1})	$1/V_0$ (min mPa^{-1} s^{-1})
9·0	25	5	0·200
	37	7	0·143
	50	11	0·091
13·5	25	7	0·143
	37	12	0·083
	50	17	0·059
18·0	25	8	0·125
	37	14	0·071
	50	20	0·050
23·5	25	9[a]	0·111
	37	18	0·055
	50	27	0·037

[a]As shown in Fig. 1.

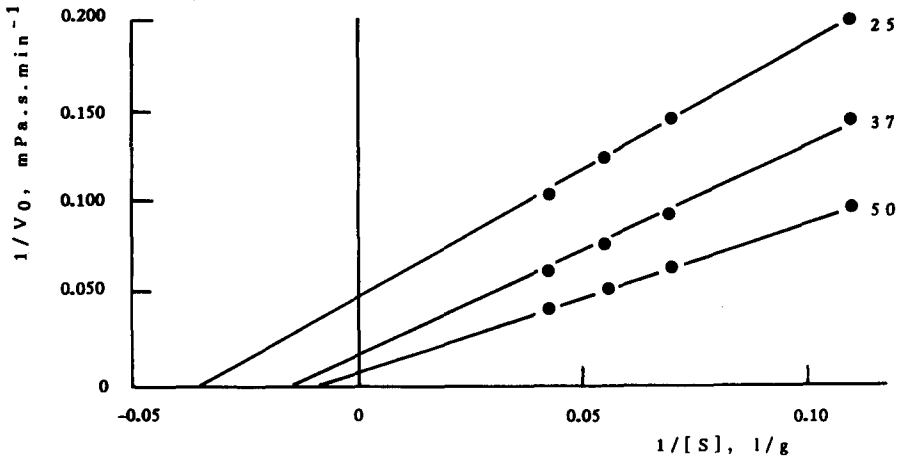

Fig. 4. Lineweaver-Burk plots for the enzymatic depolymerization of methyl pyrrolidinone chitosan at 25, 37 and 50°C and pH 5·3. The relevant kinetic data are in Table 2.

Table 2. Kinetic parameters obtained from the Lineweaver-Burk plots, relevant to the enzymatic hydrolysis of methyl pyrrolidinone chitosan in the presence of lysozyme, at pH 5·3; lysozyme concentration 0·454 g liter^{-1}

Temperature T (°C)	$1/V_0$ (min mPa^{-1} s^{-1})	Viscosity decrease V_{max} (mPa s min^{-1})	(g liter^{-1} min^{-1})	$-1/K_M$ (liter g^{-1})	Michaelis constant K_M (g liter^{-1})	(mol liter^{-1})[a]
25	0·048	20·8	3·0	0·034	29·4	$4·2 \times 10^{-5}$
37	0·015	66·7	14·2	0·014	71·4	$1·0 \times 10^{-4}$
50	0·008	125·0	20·2	0·007	142·8	$2·0 \times 10^{-4}$

[a]Average molecular weight of methyl pyrrolidinone chitosan, *c.* $7·10^5$ dalton.

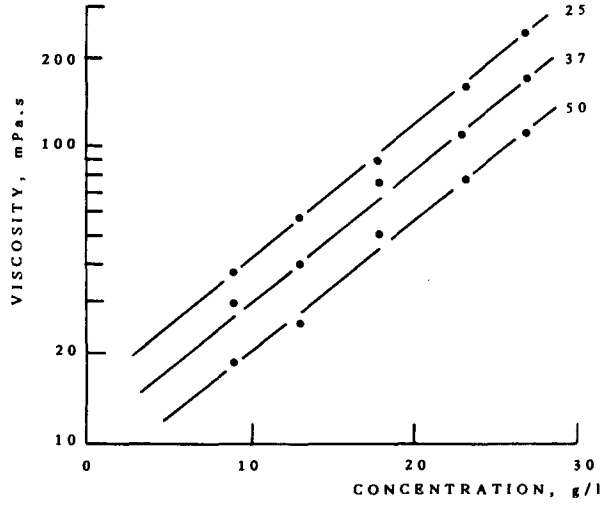

Fig. 5. Dependence of viscosity upon methyl pyrrolidinone chitosan concentration at 25, 37 and 50°C and pH 5·3.

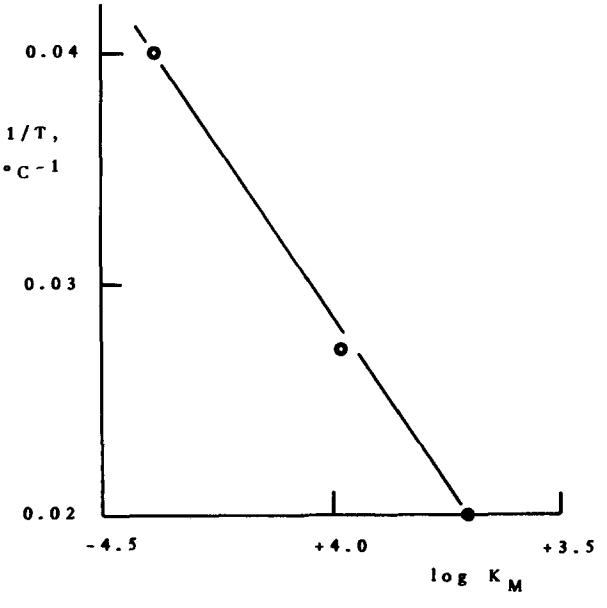

Fig. 6. Linear dependence of the Michaelis constant, K_M, upon $1/T$, for methyl pyrrolidinone chitosan at pH 5·3.

The subsequent course of the enzymatic hydrolysis of methyl pyrrolidinone chitosan was studied by plotting viscosity *versus* time, (mPa s min^{-1}), on a double logarithmic plot. In practice, straight lines were obtained for the time period 2-50 min, the viscosity decrement as a function of time being described in Fig. 7. Notwithstanding the methyl pyrrolidinone chitosan concentration, the viscosity decrease percentages were about the same for each temperature, that is *c.* 31% at 25°C, *c.* 48% at 37°C and *c.* 66% at 50°C (Table 3). When these data were plotted *versus* temperature, linearity was found (Fig. 8).

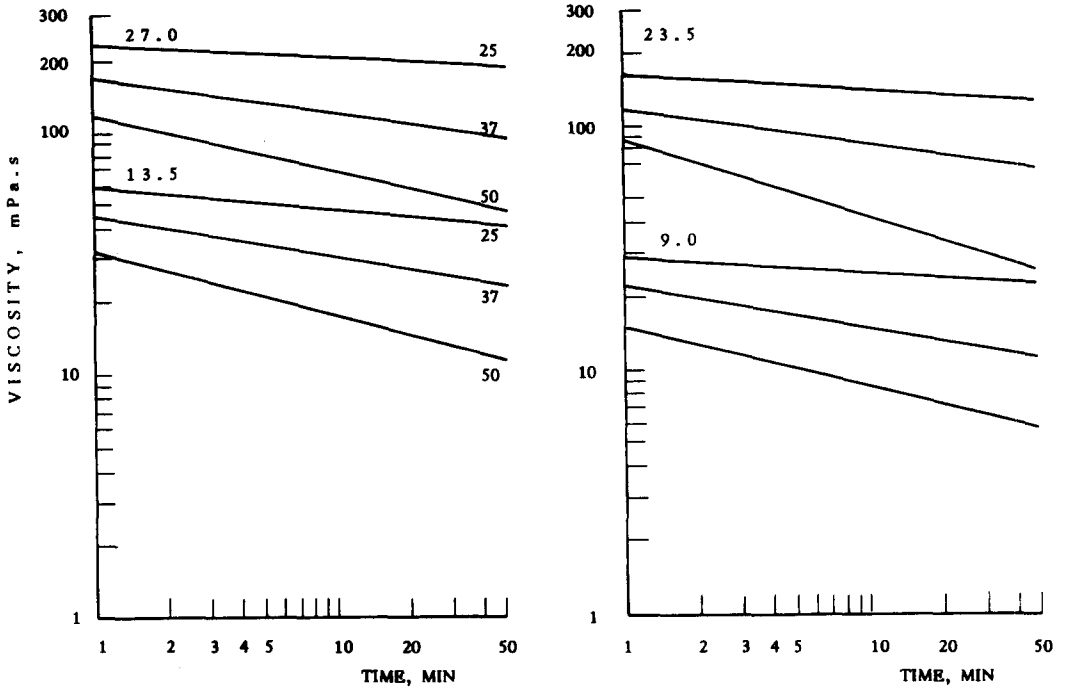

Fig. 7. Viscosity decrease in the course of the enzymatic hydrolysis (2–50 min period) for four concentrations (9·0, 13·5, 23·5 and 27·0 g liter^{-1}) and three temperatures 25, 37 and 50°C). The viscosity decrements are 31, 48 and 66% for the three temperatures studied, respectively.

Table 3. Viscosity decrease for solutions of methyl pyrrolidinone chitosan at pH 5·3 under the action of lysozyme, in the time period 2–50 min; lysozyme concentration 0·454 g liter^{-1}

Temperature T (°C)	Viscosity decrease in time period 2–50 min (mPa s)	Decrease in 49 min (%)
$[S_0]$ = 9 g liter^{-1}		
25	28 − 20 = 8	29
37	23 − 11 = 12	52
50	15 − 6 = 9	60
$[S_0]$ = 13·5 g liter^{-1}		
25	60 − 41 = 19	32
37	45 − 23 = 22	49
50	32 − 11 = 21	66
$[S_0]$ = 18·0 g liter^{-1}		
25	85 − 55 = 30	35
37	62 − 32 = 30	48
50	50 − 18 = 32	66
$[S_0]$ = 23·5 g liter^{-1}		
25	155 − 105 = 32	32
37	110 − 63 = 47	43
50	85 − 25 = 60	70

Over the short period of time considered in this study (50 min) one should not expect the polysaccharide to be depolymerized completely. It is known that full depolymerization of chitosan can be performed by the combined action of chitosanase and *N*-acetyl-β-D-glucosaminidase (Muzzarelli, 1992). However, the data here presented indicate that methyl pyrrolidinone chitosan is highly susceptible to enzymatic hydrolysis *in vitro* at physiological pH and temperature values.

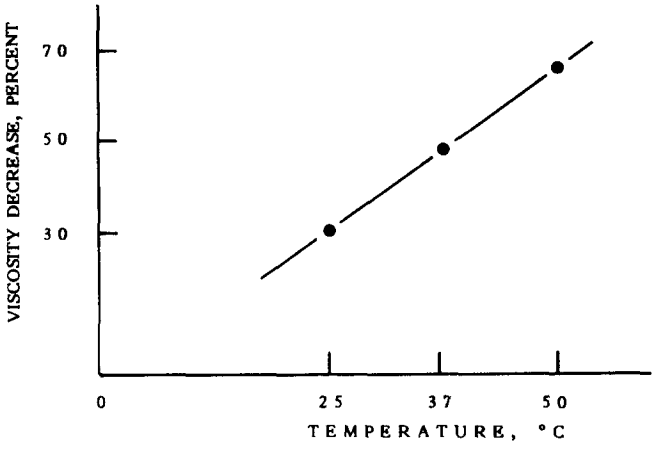

Fig. 8. Percent viscosity decrease as a function of temperature, for any methyl pyrrolidinone chitosan concentration at pH 5·3.

CONCLUSIONS

The kinetic data for methyl pyrrolidinone chitosan, a modified chitosan presently being tested for medical applications, indicate that it is particularly susceptible to hydrolytic depolymerization under experimental conditions very close to those adopted by other authors for plain chitosan. The pH values at which the reaction takes place with highest yields are not only the most favorable for the exertion of the catalytic activity but also correspond to physiological pH values.

The kinetic values here presented lie within intervals

where corresponding values for similar polysaccharides are found. Data support the hypothesis that methyl pyrrolidinone chitosan undergoes enzymatic degradation as soon as it is applied to a wound and therefore favor its use as a biocompatible and absorbable dressing material.

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