Carbohydrate Polymers **19** (1992) 17–23

Chelating ability and enzymatic hydrolysis of water-soluble chitosans

F. Delben, S. Stefancich

Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Via Valerio 38, I-34127 Trieste, Italy

R.A.A. Muzzarelli*

Faculty of Medicine, University of Ancona, Via Ranieri 67, I-60100 Ancona, Italy

(Received 8 January 1992; accepted 15 January 1992)

A novel water-soluble chitosan (NHMF-chitosan) was synthesized from crab chitosan and 5-hydroxymethyl-2-furfural, an amply available derivative of sucrose. The chelating ability of NHMF-chitosan and *N*-carboxybutyl chitosan in dilute aqueous solutions was studied by spectrophotometric and calorimetric methods. Both carbohydrate polymers were found to chelate Cu(II), the binding constants, K_B, being 9×10^4 l.mol^{-1} for NHMF-chitosan and $2{\cdot}2 \times 10^3$ for *N*-carboxybutyl chitosan; the latter was also able to chelate Pb(II), but no enthalpy variations were observed for other divalent cations. Chelation was strongly influenced by pH, and, to some extent, by temperature. Circular dichroism (cd) measurements revealed a positive rather than negative band at 225 nm, indicative of chirality change for NHMF-chitosan, which was found, however, to be a substrate for lysozyme.

INTRODUCTION

In recent years, the ability of chitosan to chelate metal ions has been extensively studied. Quantitative determinations of the metals bound to the polysaccharides and hypotheses about the binding sites were made. In the case of hydrophilic chitosan derivatives, the binding ability of the polymer could be enhanced thanks to increased solubility of the polysaccharide, presence of certain functions such as the amino and carboxyl groups, and depressed tendency to establish hydrogen bonds. The metal ion chelating ability was extensively studied for *N*-carboxymethyl chitosan (Muzzarelli *et al.*, 1982, 1985, 1989*a*; Delben *et al.*, 1989; Delben & Muzzarelli, 1989; Dobetti & Delben, 1992) and for glutamate glucan, a chitosan carrying covalently linked glutamate moieties (Muzzarelli & Zattoni, 1986; Chiessi *et al.*, 1991).

The chemical and biochemical properties of *N*-carboxybutyl chitosan were assessed, including analytical data (Muzzarelli *et al.*, 1989*b*), antibacterial behavior (Muzzarelli *et al.*, 1990) biological significance in wound management (Biagini *et al.*, 1991), and functionality in cosmetic formulations (Muzzarelli *et al.*, 1991). Other interesting water-soluble chitosans can be obtained from 5-hydroxymethyl-2-furfural, a sugar derivative prepared via fructose from sucrose or inulin (Straathof *et al.*, 1987; Schiweck, 1990; Rapp, 1991), or glucosyloxymethyl furfural, a crystalline product obtainable from isomaltulose in 70% yield. Advantages in using the said substituted furfurals would be higher degrees of substitution for the modified chitosans, and higher reaction yields, in view of the reactivity of such aldehydes.

The preparation of NHMF-chitosan from 5-hydroxymethyl-2-furfural and chitosan, and the chelating ability of both NHMF-chitosan and *N*-carboxybutyl chitosan are presented in this paper, together with preliminary data on their biodegradability.

MATERIALS AND METHODS

Materials

Salts employed in the binding measurements were Ventron (Munchen, Germany) products; their aqueous solutions were titrated with EDTA. All other reagents

*To whom correspondence should be addressed.

Carbohydrate Polymers 0144-8617/92/$05.00

were analytical grade. Deionized, doubly distilled water was used for dialysis and preparations. Measurements were carried out on freshly prepared solutions. Chitosans were supplied by Katakura Chikkarin, (Tokyo, Japan) and Protan, (Redmond, WA, USA) and were from *Chionoecets opilio* crab and King crab, respectively; both had degree of deacetylation 0·85. The 5-hydroxymethyl-2-furfural was kindly donated by Sudzucker AG, Grunstadt, Germany.

Instruments and methods

The calorimetric measurements were performed with an LKB (Copenhagen, Denmark) 10700-2 batch-type twin microcalorimeter equipped with gold cells. In each experiment, 2 ml of polymer solution (6 mM) and a proper small amount (40–50 μl) of metal perchlorate solution (60 mM) were put in the reaction cell, while in the reference cell an equal volume of water replaced the perchlorate solution. Therefore, the heat reading was already corrected by the polymer dilution enthalpy, the heat of dilution of metal perchlorate being small and negligible. Electric calibration was performed. The dichroic spectra were recorded from 200 to 320 nm with a Jasco (Tokyo, Japan) J500-A dichrograph equipped with a Jasco DP 500-N data processor, or with a Jasco J600 dichrograph, connected to a PC-plotter system. Four spectra were currently combined for each determination. The UV absorption spectra relevant to chelation were collected with a Varian Cary 2200 spectrophotometer (Mulgrave, Australia). The twin cells method, which permits the chelate spectrum to be recorded with no interferences from other absorbing species, was currently employed (Dobetti & Delben, 1992). The infrared spectra were recorded with a Perkin-Elmer (Norwalk, CN, USA) 299-B spectrometer (KBr pellets or polymer films). The amount of water in the lyophilized polysaccharides was determined with a Mettler DL 18 apparatus, according to the Karl Fisher method. Hen egg white lysozyme supplied by Calbiochem (San Diego, CA, USA) was used to test the biodegradability of the modified chitosans, under the same experimental conditions described elsewhere (Muzzarelli, 1992); a rotational viscometer Haake (Karlsruhe, Germany) Rotovisco RV 20-M5 was used in conjunction with the computer program Rotation Haake, and was set to make continuous measurements at shear rate 200 s^{-1}.

EXPERIMENTAL

Preparation of the modified chitosans

The novel water-soluble chitosan here indicated as NHMF-chitosan was synthesized from crab chitosan by dissolving the chitosan powder with acetic acid (10 g/liter) and adding 5-hydroxymethyl-2-furfural (1·0 molar ratio to free amine) under reducing conditions obtained with the progressive introduction of sodium borohydride solution (10 g/liter). The resulting solution (pH 6·0) was extensively dialysed against distilled water (3 days) and submitted to lyophilization (freezing at −20°C and freeze-drying at +30°C). The lyophilized material containing less than 2% water, was redissolved in distilled water to make up solutions (usually 13 g/liter) for analytical purposes.

N-Carboxybutyl chitosan was prepared and characterized as previously published (Muzzarelli *et al.*, 1989*b*). Solutions were made up from freshly freeze-dried material.

Characterization of NHMF-chitosan

The NHMF-chitosan solutions were found to be slightly less viscous than the corresponding parent chitosan solutions: the flow curves were comparable in shape. The filmogenic ability of chitosan was preserved in the NHMF-chitosan.

Evidence of the chemical modification was provided by the appearance of sharp bands at 810 and 1510 cm^{-1} in the infrared spectrum, assigned to alkene carbons; these bands were prominent in the authentic 5-hydroxymethyl-2-furfural spectrum.

Alkalimetric measurements were carried out under nitrogen with 0·1 M sodium hydroxide solution on NHMF-chitosan (0·25 g) dissolved in hydrochloric acid (10 ml, 0·3 M) and further diluted with water (30 ml). The pK of the pure NHMF-chitosan was found to be 5·0, sensibly lower than 6·3 for the parent chitosan. As a point of difference from certain modified chitosans, the alkalimetric curve had just two inflection points and was therefore easy to read; the alkalimetric curve for *N*-Carboxybutyl chitosan was disturbed by a number of inflection points of doubtful meaning, preventing accurate reading of pK.

The UV spectrum of NHMF-chitosan showed a typical band (absent in *N*-carboxybutyl chitosan) at 278 nm. The height of the band was proportional to the polymer concentration over an extended concentration range (0·5–2·5 g/liter) and provided a means for a rapid quantitative determination of NHMF-chitosan in solutions.

The most remarkable feature of the CD spectrum for NHMF-chitosan was that the sign of the band at 225 nm, typical for the polysaccharide, was positive rather than negative as usually found for the other chitosan derivatives and chitosan itself; this means that the derivatization operated with 5-hydroxymethyl furfural was able to change the chirality of the chitosan backbone (Fig. 1).

The degree of substitution could be determined by quantitating the 5-hydroxymethyl-2-furfural with the

aid of 2-thiobarbituric acid, by spectrophotometry at 443 nm (Rosenberg *et al.*, 1979). For the preparative conditions adopted, the degree of substitution was found to be 0·3.

Metal ion chelation by NHMF-chitosan

The ability of NHMF-chitosan in binding Cu(II) and Pb(II) was tested by UV absorption and CD measurements. The CD spectra of the NHMF-chitosan–Cu(II) system, reported in Fig. 1, showed two bands centered at 247 and 291 nm, respectively, these values being very similar to those for other modified chitosans. While Pb(II) did not modify at all the UV-vis spectra, Cu(II) induced the formation of a band which resembled the charge transfer band observed in the cases of *N*-carboxymethyl chitosan–Cu(II) and *N*-carboxybutyl chitosan–Cu(II) systems. In the present case, however, the complex absorption band was clearly broader and constituted by two peaks of similar height (Fig. 2).

From the UV and CD spectra, the binding constants, K_B, were calculated, according to Dobetti and Delben (1992), to be 7×10^4 and $1{\cdot}1 \times 10^5$ liters mol^{-1}, respectively (average 9×10^4). These values were surprisingly high in view of the absence of carboxyl groups on the 5-hydroxymethyl-2-furfural. Measurements showed that Cu(II), whose affinity for the polycarboxylates is known to be in general very high, can be also chelated by polysaccharides bearing side chains having primary alcohol groups and deprived of carboxyl groups.

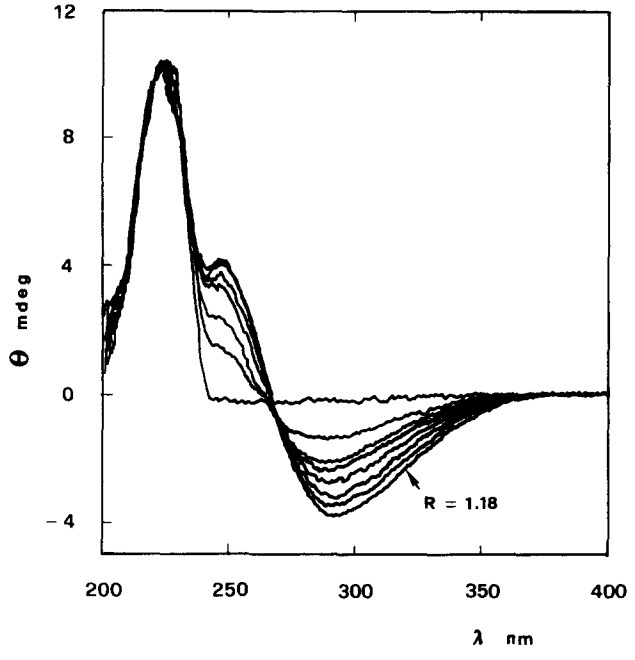


Fig. 1. Circular dichroism spectra of NHMF-chitosan in water at 25°C recorded upon addition of increasing amounts of copper perchlorate. *R* is the metal-to-polymer molar concentration ratio. Polymer concentration, $4{\cdot}16 \times 10^{-4}$ M; path length, 1 cm.

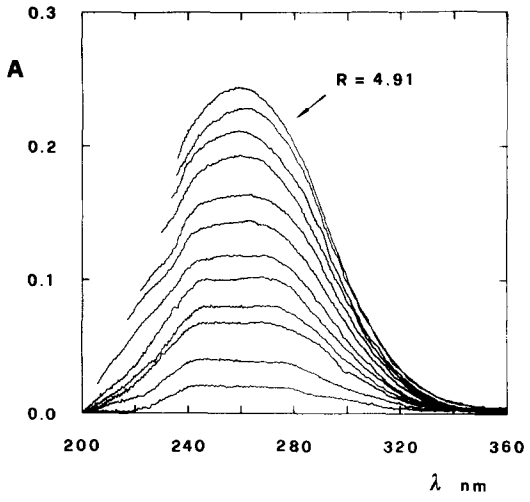


Fig. 2. Ultraviolet absorption spectra of NHMF-chitosan in water recorded upon addition of increasing amounts of copper perchlorate. *R* is the metal-to-polymer molar concentration ratio. Polymer concentration, $4{\cdot}16 \times 10^{-4}$ M; path length, 1 cm.

Metal ion chelation by *N*-carboxybutyl chitosan

UV absorption and CD measurements showed that Cu(II) interacts with *N*-carboxybutyl chitosan (Figs 3 and 4). Both techniques indicated that a complex with charge transfer from the ligands to the metal ions was formed. The values of the binding constants, K_B,

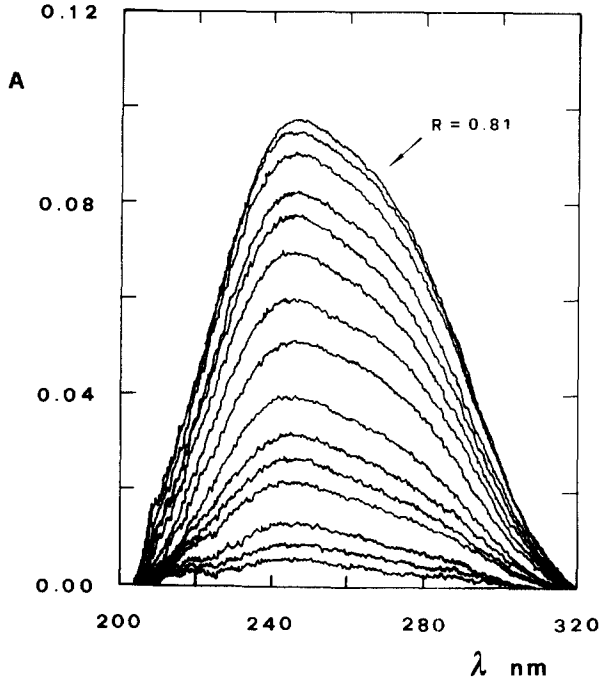


Fig. 3. Ultraviolet absorption spectra of *N*-carboxybutyl chitosan in water recorded upon addition of increasing amounts of copper perchlorate. *R* is the metal-to-polymer molar concentration ratio. Polymer concentration, $4{\cdot}56 \times 10^{-4}$ M; path length, 1 cm.

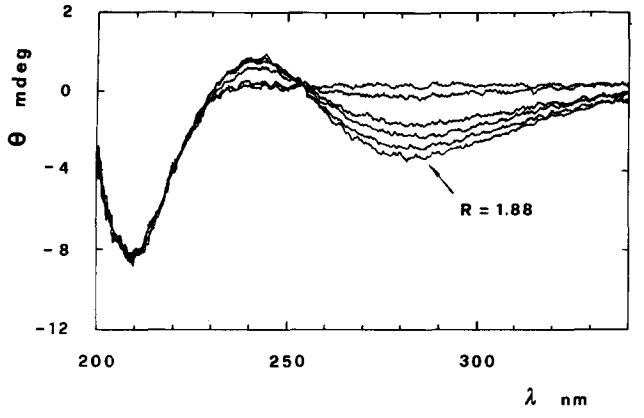


Fig. 4. Circular dichroism spectra of *N*-carboxybutyl chitosan in water, recorded upon addition of increasing amounts of copper perchlorate. *R* is the metal-to-polymer molar concentration ratio. Polymer concentration, $4{\cdot}56 \times 10^{-4}$ M; path length, 1 cm.

indicated that the interaction was much stronger in the case of *N*-carboxymethyl chitosan ($K_B = 2{\cdot}2 \times 10^3$ liters mol^{-1} in this case). Whether this difference in binding ability was due to the different chemical structure in the polysaccharides or to the different degree of substitution (0·58 instead of 0·28) is a matter of speculation.

In the case of *N*-carboxybutyl chitosan-Pb(II), the charge transfer band was evidenced by absorbance measurements only. In the CD measurements, in fact, the strong absorbance for the free Pb(II) hindered the use of the spectra. The absorption spectra of the *N*-carboxybutyl chitosan-Pb(II) system are reported in Fig. 5. Both absorption and CD spectra were recorded at 25 and 61°C, for *N*-carboxybutyl chitosan-Cu(II) and *N*-carboxybutyl chitosan-Pb(II) systems.

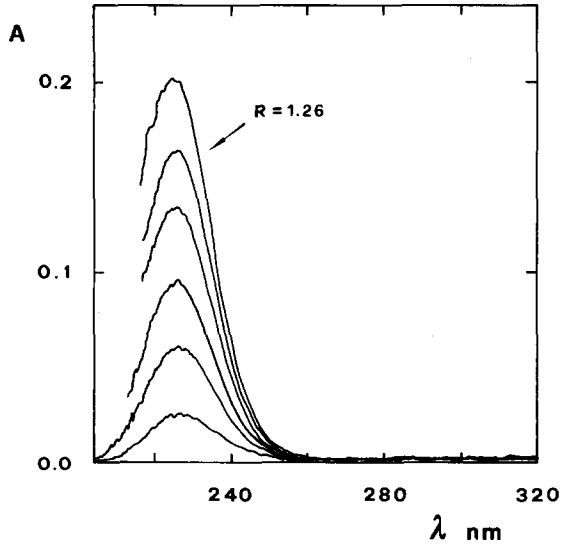


Fig. 5. Ultraviolet absorption spectra of *N*-carboxybutyl chitosan in water, recorded upon addition of increasing amounts of lead perchlorate. *R* is the metal-to-polymer molar concentration ratio. Polymer concentration, $4{\cdot}56 \times 10^{-4}$ M; path length, 1 cm.

Temperature had no influence on the binding of lead by the polysaccharide, whilst increasing temperature increased the charge transfer bands in the case of the *N*-carboxybutyl chitosan-Cu(II) system. This can tentatively be interpreted in terms of enhanced mobility of the polymer chain due to thermal motion. In fact, while Pb(II) ions were bound by carboxyl groups only, Cu(II) supposedly interacted simultaneously with amino and carboxyl groups; in the case of a more flexible chain, for thermal reasons the latter interaction should have been enhanced.

The pH value strongly affected both absorption and CD bands. The trends of molar absorption with pH for the *N*-carboxybutyl chitosan-Cu(II) and the *N*-carboxybutyl chitosan-Pb(II) systems, at constant R and λ values, are reported in Fig. 6, where *R* is the cation-to-polymer molar ratio. In comparison to *N*-carboxymethyl chitosan, two differences were detectable: (1) with *N*-carboxybutyl chitosan, the heights of the charge transfer bands were much higher with Cu(II) than with Pb(II), whilst they were similar in the case of *N*-carboxymethyl chitosan; (2) the charge transfer band of the *N*-carboxybutyl chitosan-Pb(II) system progressively decreased with decreasing pH values, being still evident at low pH values; with *N*-carboxymethyl chitosan it disappeared at pH 4·7.

Typical CD spectra of the *N*-carboxybutyl chitosan-Cu(II) system, recorded at constant *R* and at different

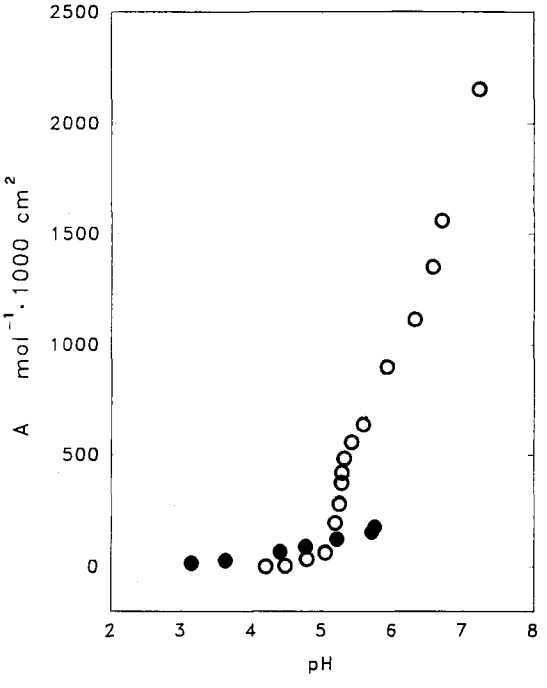


Fig. 6. Molar absorbance change of the maximum of the charge transfer bands as a function of pH for the *N*-carboxybutyl chitosan-Cu(II) (open symbols) and *N*-carboxybutyl chitosan-Pb(II) (full symbols). Aqueous systems at 25°C. λ = 246 and 226 nm, respectively. Metal-to-polymer molar concentration ratios, 1·43 and 0·55, respectively.

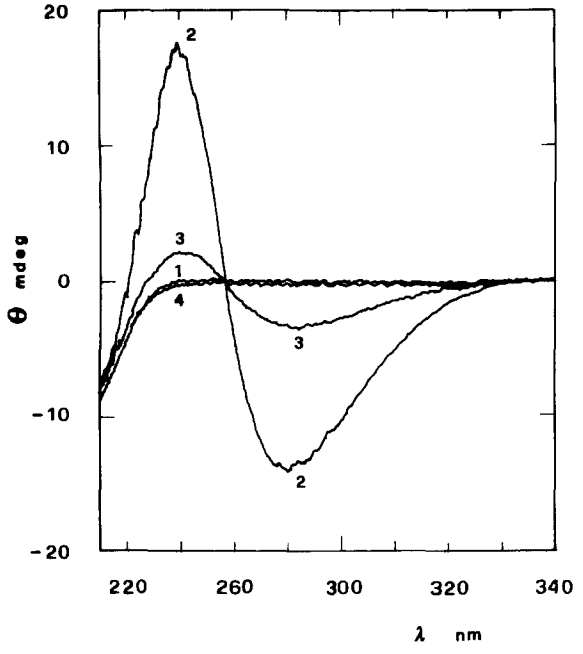


Fig. 7. Circular dichroism spectra of *N*-carboxybutyl chitosan in water at 25°C, in the absence (1) and in the presence of copper perchlorate, measured at different pH values: 6·77 (2), 5·24 (3) and 4·53 (4). Polymer concentration, $4{\cdot}56 \times 10^{-4}$; *R*, metal-to-polymer concentration ratio, 1·43, path length, 1 cm.

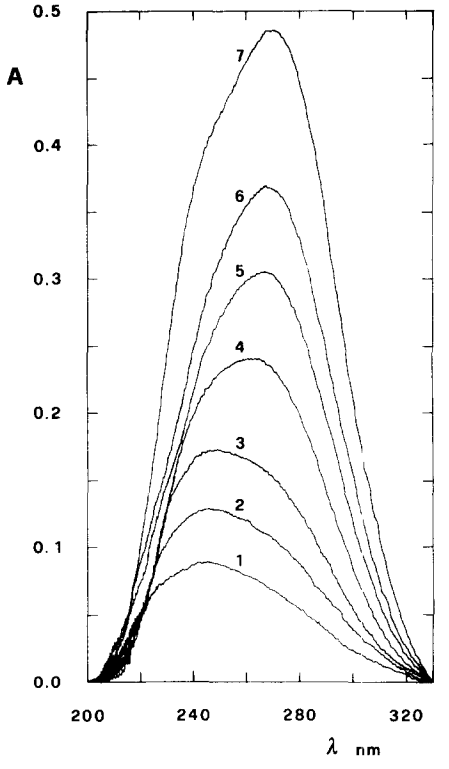


Fig. 8. Ultraviolet absorption spectra of the *N*-carboxybutyl chitosan-Cu(II) aqueous system at 25°C as a function of pH. pH values, 5·18 (1), 5·24 (2), 5·27 (3), 5·31 (4), 5·41 (5), 5·58 (6) and 5·90 (7). Polymer concentration, $4{\cdot}56 \times 10^{-4}$ M; *R*, metal-to-polymer concentration ratio 1·43, path length, 1 cm.

pH values, are reported in Fig. 7. The corresponding absorption spectra, recorded with the twin cells method, are in Fig. 8. The absorption complex charge transfer band, clearly formed by at least two peaks, appreciably varied in shape and height with varying pH, the peak at higher wavelengths becoming more prominent at higher pH values. This seems to confirm our interpretation of the nature of these peaks: we suggested that the peak centered around 270 nm derives from electron transfer from the amino nitrogen to the metal ion (Dobetti & Delben, 1992). Higher pH values, which increase the fraction of free amino groups, should favor the complexation of Cu(II) by nitrogen atoms rather than carboxyl groups.

On the other hand, CD spectra show height variation with pH but not shape alteration, which should mean that pH affects the intensity but not the mode of binding. This contradiction can be resolved by two different, but not conflicting, explanations. The first one is that only carboxyl groups are able to give an inner-sphere complex with Cu(II), where the chelating polymer induces chirality in the ion orbitals, while the interaction between nitrogen atoms and Cu(II) is characterized by a minor electron overlapping. The second tentative explanation is that the geometry of the polysaccharide-metal ion complex remains constant independently of the relative amount of the functional groups engaged in the binding. Also, the geometry could not affect at all the chirality of the polymer backbone. It is worth noting that the other modified chitosan studied here, in the presence of Cu(II), exhibits bands very similar to those of the *N*-carboxymethyl chitosan-Cu(II) and *N*-carboxybutyl chitosan-Cu(II) systems, thus supporting the latter hypothesis.

The enthalpy variations on mixing the polysaccharide and the metal perchlorate solutions at pH 6, were zero with Pb(II), Ni(II), Co(II), Cd(II) and Ca(II). In the case of Cu(II), the behavior illustrated in Fig. 9

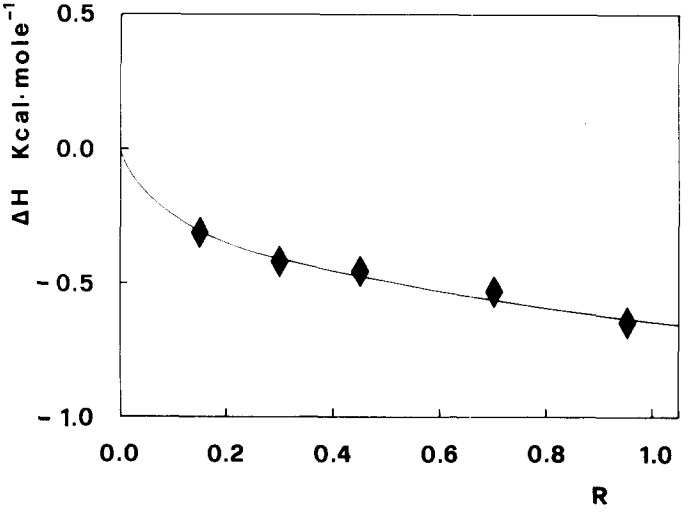
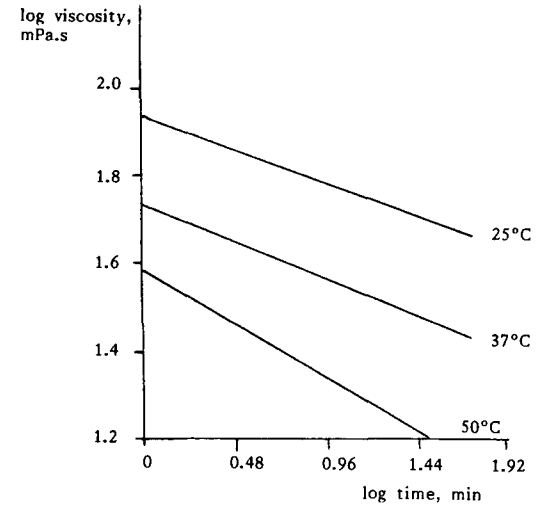


Fig. 9. Enthalpy change recorded upon addition of copper perchlorate to *N*-carboxybutyl chitosan in water at 25°C.

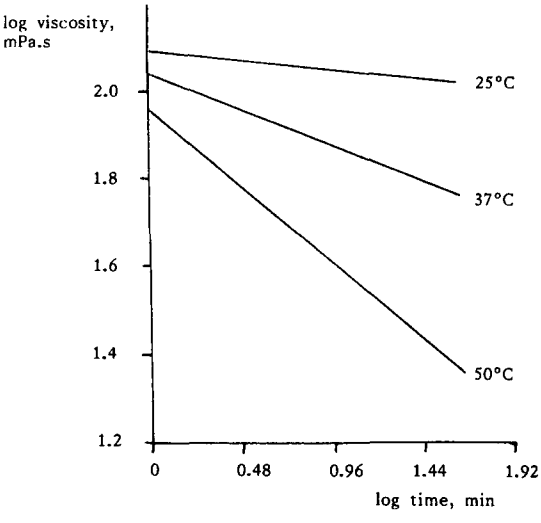
was found; the plot in this figure is very similar to that reported for a chitosan with degree of acetylation 0·42 and *N*-carboxymethyl chitosan, although in the latter case the value of the enthalpy variation was much more negative (Delben *et al.*, 1989). As suggested for *N*-carboxymethyl chitosan (Delben & Muzzarelli, 1989), the site of binding should be located in the carboxyl oxygen and the amine nitrogen.

Susceptibility to lysozyme

The CD data presented above, indicating so-far unobserved impressive alterations in the chirality of a modified chitosan, prompted us to verify the susceptibility of both the modified chitosans dealt with here to the hydrolytic action exerted by lysozyme. The most straightforward approach being the continuous monitoring of the viscosity of the lysozyme-containing polysaccharide solution, freshly prepared solutions (18 g liter^{-1}) were submitted to viscometric analysis for 50 min at three temperatures (25, 37 and 50°C). During this time, 400 points were plotted to record the viscosity decrease due to the lysozyme action. From the gradient and the position of the straight lines obtained in the double logarithmic plots (Fig. 10) for the 2–50-min time period, it can be observed, first of all, that the NHMF-chitosan solutions are more viscous than the corresponding *N*-carboxybutyl chitosan solutions. At 25°C, the gradient for the NHMF-chitosan curve indicates that lysozyme is less effective than on *N*-carboxybutyl chitosan, while increasing effectiveness is achieved at higher temperatures. Therefore, it would seem that the altered conformation of the polysaccharide alters significantly its availability as a substrate for lysozyme, but does not prevent biodegradation from taking place.



(a)



(b)

Fig. 10. Viscosity decrease as a function of contact time between modified chitosan and lysozyme. Double logarithmic plots at three temperatures: 25, 37 and 50°C. Data for the first contact minute are not included. Each curve contains 400 points. Freshly prepared solutions (18 g liter^{-1}) from freeze-dried modified chitosans: a, *N*-carboxybutyl chitosan; b, NHMF-chitosan.

CONCLUSIONS

Hydrophilic modified chitosans possess chelating ability for some metal ions; those studied here, namely *N*-carboxybutyl chitosan and NHMF-chitosan, are much less powerful chelating agents than *N*-carboxymethyl chitosan, however, because they do not appreciably interact with Ni(II), Co(II) and Cd(II). The flexible chain in *N*-carboxybutyl chitosan does not favor chelation, the pentaatomic ring of the *N*-carboxymethyl chitosan chelates being evidently much more favoured. The NHMF-chitosan is unable to chelate Pb(II), but efficiently chelates Cu(II). The metal binding for these modified chitosans is therefore specific and clearly related to the nature of the electron donor groups carried by the polyaminosaccharide. NHMF-Chitosan, whose side substituent carries furan rings and primary alcohol groups, has lower chelating ability than *N*-carboxybutyl chitosan where carboxyl groups are present. Both these modified chitosans exhibit limited chelating ability compared to *N*-carboxymethyl chitosan and glutamate glucan. The data on metal ion chelation are a contribution to the understanding of the mechanism of the antimicrobial action exerted by these substances. The surprising finding relevant to the altered structure for NHMF-chitosan could widen the field of non-medical uses of chitosans carrying furan rings.

ACKNOWLEDGEMENTS

This work was carried out with the financial contribution of Ministero per l'Università e la Ricerca

Scientifica e Tecnologica, Roma (Fondi Quaranta Percento), and Consiglio Nazionale delle Ricerche, Progetto Finalizzato Chimica Fine II, Roma. Some circular dichroism measurements were performed in the Technological Biopolymers Laboratory, POLYbiòs Research Center, Trieste, to which we express our thanks. Thanks are also due to Sudzucker AG, Grunstadt, Germany, for a gift of 5-hydroxymethyl-2-furfural.

REFERENCES

Biagini, G., Bertani, A., Muzzarelli, R.A.A., Damadei, A., Dibenedetto, G., Belligolli, A., Riccotti, G., Zucchini, C. & Rizzoli, C. (1991). Wound management with *N*-carboxybutyl chitosan. *Biomaterials,* **12,** 281–6.

Chiessi, E., Palleschi, A., Paradossi, G., Venanzi, M. & Pispisa, B. (1991). Conformational features and chelating ability of branched chain chitosan derivatives. *J. Chem. Res.,* **9,** 248–59.

Delben, F. & Muzzarelli, R.A.A. (1989). Thermodynamic study of the interaction of *N*-carboxymethyl chitosan with divalent metal ions. *Carbohydr. Polym.,* **11,** 221–32.

Delben, F., Muzzarelli, R.A.A. & Terbojevich, M. (1989). Thermodynamic study of the protonation and interaction with metal cations of three chitin derivatives. *Carbohydr. Polym.,* **11,** 205–20.

Dobetti, L. & Delben, F. (1992). Binding of metal cations by *N*-carboxymethyl chitosans in water. *Carbohydr. Polym.,* **18,** (in press).

Muzzarelli, R.A.A. (1992). The depolymerization of methylpyrrolidinone chitosan by lysozyme. *Carbohydr. Polym.,* **19,** 29–34, this issue.

Muzzarelli, R.A.A. & Zattoni, A. (1986). Glutamate glucan and aminogluconate glucan, new chelating polyampholytes obtained from chitosan. *Int. J. Biol. Macromol.,* **8,** 137–42.

Muzzarelli, R.A.A., Tanfani, F., Emanuelli, M. & Mariotti, S. (1982). *N*-(Carboxymethylidene) chitosans and *N*-(carboxymethyl) chitosans novel chelating polyampholytes obtained from chitosan glyoxylate. *Carbohydr. Res.,* **107,** 199–219.

Muzzarelli, R.A.A., Tanfani, F., Emanuelli, M. & Bolognini, L. (1985). Aspartate glucan, glycine glucan and serine glucan for the collection of cobalt and copper from solutions and brines. *Biotechnol. Bioengng.,* **27,** 1115–21.

Muzzarelli, R.A.A., Weckx, M., Filippini, O. & Sigon, F. (1989*a*). The removal of trace metal ions from industrial waters, nuclear effluents and drinking water with the aid of cross-linked *N*-carboxymethyl chitosan. *Carbohydr. Polym.,* **11,** 293–306.

Muzzarelli, R.A.A., Weckx, M., Filippini, O. & Lough, C. (1989*b*). Characteristic properties of *N*-carboxybutyl chitosan. *Carbohydr. Polym.,* **11,** 307–20.

Muzzarelli, R.A.A., Tarsi, R., Filippini, O., Giovannetti, E., Biagini, G. & Varaldo, P.E. (1990). Antimicrobial properties of *N*-carboxybutyl chitosan. *Antimicrob. Agents Chemother.,* **24,** 2019–23.

Muzzarelli, R.A.A., Weckx, M. & Bicchiega, V. (1991). *N*-Carboxybutyl chitosan as a wound dressing and a cosmetic ingredient. *Chem. Today,* **9**(4), 33–7.

Rapp, K.M. (1991). HMF, a 'petrochemical' intermediate for the production of polymers. Paper presented at Symp. Industrial Crops and Products, Maastricht.

Rosenberg, H., Modrak, J. B., Hassing, J. M., Al-Turk, W. A. & Stohs, S. J. (1979). Glycosylated collagen. *Biochem. Biophys. Res. Comm.,* **91,** 498–501.

Schiweck, H. (1990). New developments in the use of sucrose as an industrial bulk chemical. Paper presented at Conf. on Carbohydrates as Organic Raw Materials, Darmstadt.

Straathof, A.J.J., Vrolljk, J.M., Kleboom, A.P. & Van Bekkum, H. (1987). Application of microwave technology in the preparation of carbohydrate derivatives. Paper presented at 4th European Carbohydrate Symp., Darmstadt.