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6-Oxychitins, novel hyaluronan-like regiospecifically carboxylated chitins

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Abstract

Crustacean chitins and fungal chitin–glucan complexes are subjected to regiospecific oxidation at C-6 with NaOCl in the presence of Tempo® and NaBr at 25°C in aqueous solution. The resulting products have anionic character and are fully soluble over the entire pH range; they lend themselves to metal chelation, polyelectrolyte complex formation with a number of biopolymers including chitosan, and to microsphere and bead formation. 6-Oxychitin coagulates papain, lysozyme and other hydrolases active on chitosans. 6-Oxychitins of fungal and animal origins, in the form of free acids, salts and esters, might find use as surrogates of hyaluronans and of bacterial antigens in medical and health care products. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Chemical modifications of chitin are often made in order to obtain water-soluble products (Muzzarelli & Peter, 1997). Examples are the deacetylation that yields chitosan, the reacetylation of the latter that yields reacetylated chitin, and the 6-O-carboxymethylation that yields 6-O-carboxymethylchitin (Muzzarelli, 1977; Yalpani, 1985). These processes are carried out in the presence of excess quantities of NaOH and require separation of the biopolymers from large quantities of salts and unreacted NaOH. The reacetylation step usually requires hydroalcoholic media in large proportions.

The oxidation of chitosan proposed by Horton and Just (1973) including the protection of the amino group as perchlorate salt, precipitation with excess perchlorate, suspension in acetic acid and oxidation at C-6 with chromic anhydride, has been applied occasionally (Lillo & Matsuhiro, 1997). Of course this chemistry would have a deep environmental impact, should biomasses be treated.

Painter (1977) and Cesaro, Delben, Painter and Paoletti (1985) proposed an oxidation of cellulose and amylose based on the use of N_2O_4, but this was poorly selective, dangerous and accompanied by uncontrolled degradation of the polysaccharides.

However, there are indications that the C-6 regiospecific oxidation to a carboxyl group can be achieved under milder conditions with the aid of catalysts (Ma & Bobbit, 1991; Miyazawa, Endo, Shiihashi & Okawara, 1985; Anelli, Biffi, Montanari & Quici, 1987). Difficulties were faced with insoluble polysaccharides (DeNooy, Besemer & van Bekkum, 1995), which required more drastic reaction conditions (Chang & Robyt, 1996).

In the case of fungal chitin, the situation is complicated by the presence of glucans, which further contribute to the insolubility of the polysaccharide (Muzzarelli, Illari, Tarsi, Dubini & Xia, 1994). Chitosan can be extracted with boiling acetic acid, but again the chemistry of this process does not lend itself to scaling-up, in spite of the large availability of fungal biomasses from industrial fermentations.

The present work was undertaken with the intention of defining the mildest experimental conditions at which animal and fungal chitins can be chemically oxidized regiospecifically to yield novel biopolymers soluble over an extended pH range, that might be proposed as surrogates of hyaluronan, as derivatives similar to antigens, and as commodities for industrial use (Muzzarelli, 1997).

2. Experimental

2.1. Chitins

The experiments were performed with chitins of different origins, namely lobster chitin donated by Prof. Ofelia Bilbao-Revoredo, Cuba (Muzzarelli et al., 1994a); *Crangon*

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crangon chitin (Muzzarelli, Ilari & Tomasetti, 1994; Focher, Naggi, Torri, Cosani & Terbojevich, 1992), and crab chitin from Katakura Chikkarin Co., Japan. These animal chitins were derivatized without any preliminary chemical treatment. Fungal samples were obtained from fresh cultures of *Absidia coerulea* (Muzzarelli et al., 1994b), and from dried mycelia of *Absidia orchidis* (Jaworska & Szewczyk, 1997). These materials were previously treated with 0.5 M NaOH solution in order to inactivate the fungus and lower the pigment content.

2.2. Chemicals

The stable nitroxyl radical 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo®, Aldrich, Milano) was used as a catalyst, together with NaBr, to regiospecifically oxidize chitin with either a 4% or a 13% NaOCl solution (Aldrich, Milano). All chemicals were reagent grade. The enzyme preparations were the following: papain from *Carica papaya*, recombinant lipase B from *Candida antarctica*, wheat germ lipase, and amylase from human saliva (Fluka, Milano).

2.3. Instrumental analytical characterization of 6-oxychitin

X-ray diffraction spectra were obtained by using a vertical powder diffractometer; the source was a rotating anode generator Rigaku Denki RU-300 and Ni filtered CuKα radiation ($\lambda = 0.154$ nm) was used.

Metal ions were determined by atomic absorption spectrometry with a Perkin–Elmer 2380 spectrometer.

The infrared spectra were recorded with a Nicolet 20-SX FT-IR spectrometer (DTGS detector) equipped with a Spectra Tech. Multiple Internal Reflectance (DRIFT) accessory for measurements in the solid state (Muzzarelli et al., 1998).

A Philips SEM 505 scanning electron microscope was also used: the samples were prepared after cutting them to expose the inner part, fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 h, dehydrated and submitted to critical point drying and gold coated; gold coated samples showed no difference, thus excluding artifact formation.

NMR spectra were recorded with a Bruker CXP-300 (75 MHz) spectrometer at a concentration of 100 mg/0.6 ml D_2O.

The molecular weight determinations were made by gel permeation chromatography with pullulan standards. 6-Oxychitin samples were eluted from TSK 30 + 40 columns, with 0.125 M Na_2SO_4 phosphate buffer, 0.02% NaN_3, at a flow-rate of 0.6 ml/min.

2.4. Equivalent weight determinations

Oxychitin (25 mg) was dissolved in water (20 ml) and loaded to an Amberlite IR 120 H^+ column (1.0×12.0 cm^2). The elution was made with water (80 ml) and the eluate was titrated to the equivalent point with 0.1 M NaOH.

2.5. Spectrophotometric determinations of chitosans

The concentration of chitosan in solution was determined with the anionic reactive azo dye Cibacron Brilliant Red 3B-A (C.I. 18105, Reactive Red 4). The absorbance values were measured at 575 nm with a Beckman DV 640 visible spectrophotometer (Muzzarelli, 1998).

3. Results and discussion

Preparations of crustacean 6-oxychitins were made from commercial chitin powders, and from freshly caught scampi *Nephrops norvegicus* and *Munida intermedia*.

Preparation from commercial chitin powders. Technical grade crustacean chitin powder was either soaked in warm water (50°C) for 4 h, or soaked in water (20°C) in the presence of 0.02% NaN_3 (to prevent microbial growth) for several days (14 d for lobster chitin); pH reached a value of 5.3–6.0 (swollen chitin). This treatment was absolutely necessary in order to avoid large portions of insolubles. As an alternative, the oven-dried chitin powder was dissolved in DMAc-LiCl 5% and reprecipitated with water as amorphous chitin (Focher et al., 1992).

Preparation from Munida intermedia. Frozen entire animals (100 g) were minced in a VaryBlendor (−5°C, 3 min), boiled with NaOH (0.1 M, 400 g) for 1 h, washed to neutrality, treated with HCl (0.5 M, 400 g) for 15 min at 20°C, and washed to neutrality. The yield of wet chitin was 21 g, corresponding to 4.02 g of dry chitin.

Preparation from scampi heads. Frozen heads (100 g) were minced in a VaryBlendor (−5°C, 3 min), boiled with NaOH (0.1 M, 400 g) for 1 h, washed to neutrality, treated with HCl (0.5 M, 400 g) for 15 min at 20°C, and washed to neutrality. An additional HCl treatment (400 g, 2 M) was also done at 20°C for 50 min. The yield of wet chitin was 18 g, corresponding to 3.60 g of dry chitin.

Regiospecific oxidation of chitin. Tempo® (12 mg) and NaBr (0.4 g) were added to the aqueous suspension of chitin (either swollen or amorphous, 1 g dry weight + 50 g water), followed by NaOCl (24 ml, 4%). Immediately after the introduction of the latter, the pH was adjusted to 10.8 and kept at this value for 30 min with the aid of 0.5 M NaOH. The amount of NaOH (11 ml) delivered to the lobster chitin was close to the stoichiometric quantity (calculated 9.8 ml, 0.5 M) requested to neutralize the newly formed carboxyl groups, one per each repeating unit. The suspension became a clear, yellow solution within this time period and was kept under stirring for up to 120 min; at the end of this time period the pH was 9.3–9.7. A small quantity of undissolved particles was observed in the freshly prepared chitin specimens: these particles turned out to be partially calcified from the IR analysis.

The reaction could be optionally quenched with ethanol (2.5 ml) and the pH neutralized with drops of 4 M HCl. The solution was subjected to dialysis for at least two changes of

Table 1
Experimental conditions for the preparation of 6-oxychitin

Chemical	Conditions A Preferred	Conditions B	Conditions C
Chitin, wet	1 g, dry weight	1 g, dry weight	1 g, dry weight
Water	50 ml	28 ml	28 ml
Tempo®	12 mg	12 mg	12 mg
NaBr	0.4 g	0.4 g	0.7 g
NaOCl	24 ml, 4.0%	24 ml, 5.0%	24 ml, 13.0%

demineralized water for 24 h, or desalted by other means. It was finally freeze-dried to yield a white powder. Slightly different conditions were also tested, including those under B and C in Table 1.

For the conditions shown in Table 1, the rate of NaOH consumption was measured both in the presence and absence of Tempo®. The data in Fig. 1 show that some base was consumed even in the absence of Tempo®, particularly when oxidation was more drastic (conditions C), due to partial non-regiospecific oxidation; secondary alcohol groups were possibly oxidized. Under the preferred conditions (A in Table 1; Fig. 1), the catalyzed reaction approached completion within 1 h, and the random oxidation (lower curve) was kept to a minimum. Under the other conditions B and C, oxidation took place even in the absence of Tempo®, and hence was not regiospecific.

3.1. Preparation of fungal 6-oxychitin

The fungal chitin–glucan complex had to be liberated from the biomass and had to be submitted to physical pretreatments such as the dispersion with the aid of an emulsifier and washing with detergents.

Biomasses of *Absidia* were treated with alkali in order to remove pigments and proteins, extensively washed with water, and carboxylated according to the protocol mentioned before. The 6-oxychitin–glucuronan complex obtained from the *A. coerulea* material was insoluble in HCl but soluble in aqueous solutions, including NaOH and calcium acetate solutions.

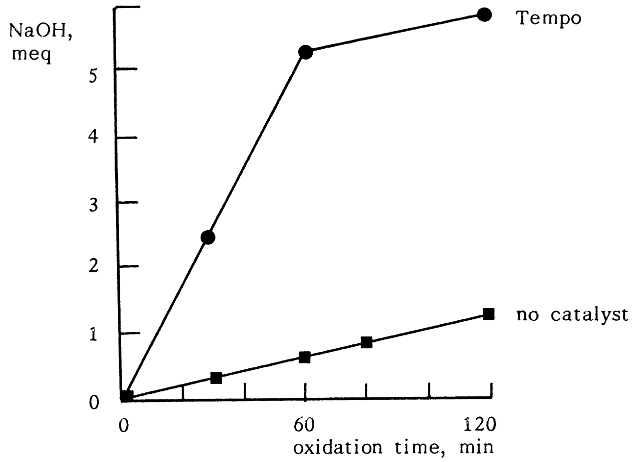

Fig. 1. Rate of NaOH consumption in the presence (●) and in the absence (■) of Tempo®, under conditions A (see Table 1).

The polysaccharide obtained from *A. orchidis* was darker, but as soon as it was submitted to dialysis, the oxidized material became colorless: before freeze-drying it was filtered through Whatman Multigrade GMF 150 glass microfiber filters. The freeze-dried material was fully soluble in water. The overall yield was ca. 25%.

3.2. Instrumental characterization

The oxidation reaction adopted for the present work was straigthforward, simple and easy to carry out in aqueous media at room temperature. It required pre-swelling of the chitin in water. When the pre-swelling was omitted, up to one-third of the chitin remained undissolved.

The 6-oxychitin sodium salt obtained from lobster chitin was a white powder, highly soluble in water in the pH range 1–13; it was also soluble in water–ethanol mixtures (7:3) and in phosphate buffers. The 6-oxychitin sodium salt was, however, easy to store because it was not deliquescent. The overall yield was higher than 90% (freeze-dried water-soluble polysaccharide).

The 6-oxychitin sodium salt can be viewed as a polyuronate obtained from chitin: a polyanion, identifiable as 2-acetamido-2-deoxy-glucuronan sodium salt, endowed with enhanced chelating ability and polyelectrolyte complex formation capacity.

The freeze-dried 6-oxychitin was not totally amorphous, weak diffraction lines were present at 9.26, 19.26 and 26.2 2θ values, which correspond to those of the parent chitin. The infrared spectrum showed intense bands at 1618 and 1415 cm^{-1}, assigned to the carboxylate group, partially overlapping the 1656 and 1552 cm^{-1} bands typical for chitin (Fig. 2).

At the electron microscope, the freeze-dried 6-oxychitin appeared as an expanded material, possessing a large surface, but fragile and easily crushable upon compression, as testified by the fractured parts visible in Fig. 3. These observations were in agreement with the behaviour of the freeze-dried mats, amenable to fine powders by moderate compression.

For the 6-oxychitin obtained under the preferred conditions (A), the N/C ratio was in good agreement with the calculated values for 6-oxychitin having a degree of substitution 1, as shown in Table 2. Conditions A provided equivalent weight values closer to the calculated oxychitin values than conditions C. The data for conditions B and C indicated that the polysaccharides had degrees of carboxylation higher than 1.0, presumably because of oxidative ring opening and oxidation of the anomeric carbon at the chain end. The NMR spectrum for the animal 6-oxychitin obtained under the preferred conditions A, in Fig. 4, showed signals typical of the methyl group, the C-2, C-1 and the amide carbonyl very close to the data in the literature, while the signals for C-3, C-4 and C-5 were at lower values than for chitin. The C-6 signal was no longer detected at 62 ppm,

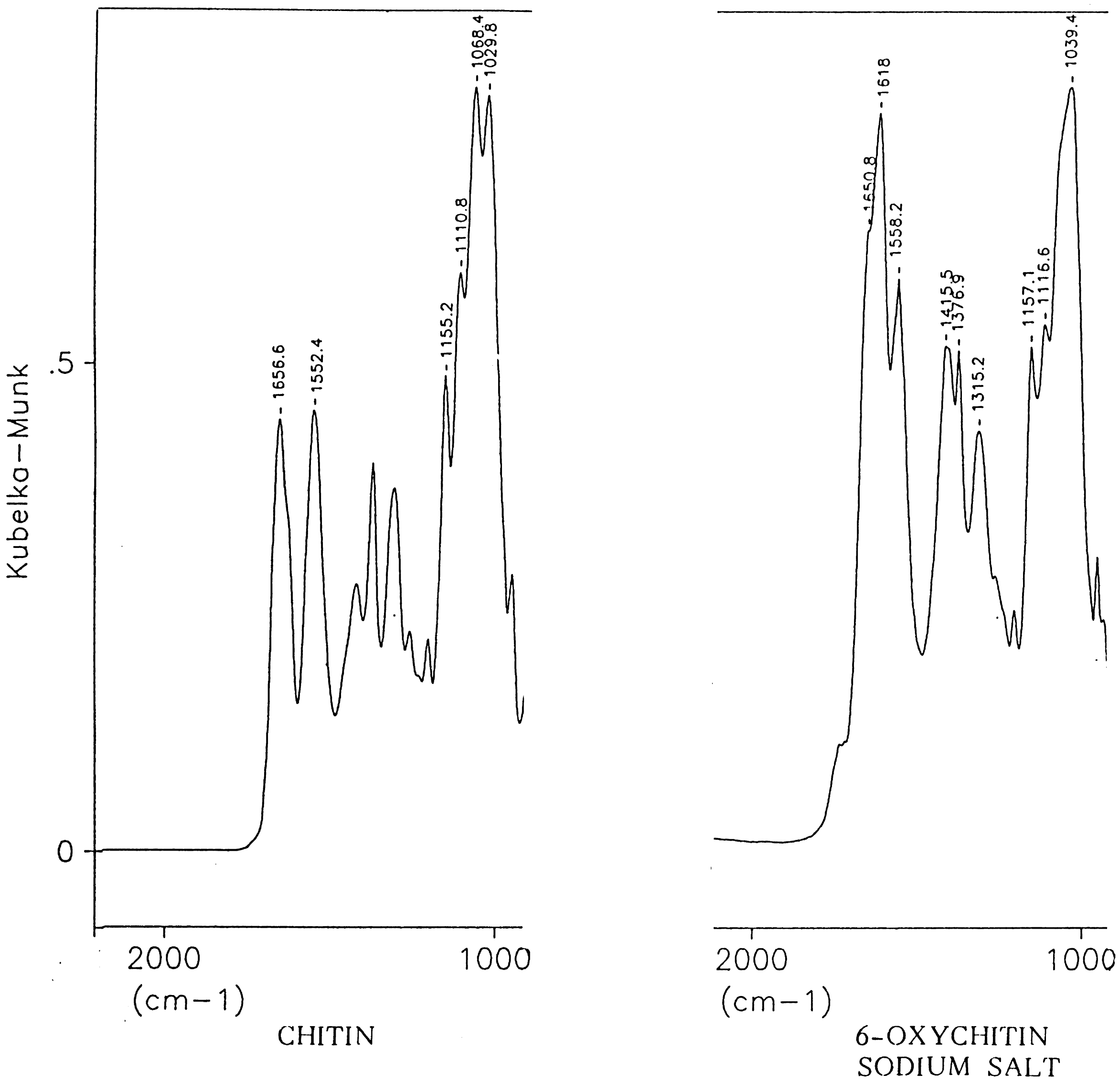

Fig. 2. Infrared spectra of lobster chitin, 6-oxychitin, and 6-oxychitin ethyl ester.

and a novel peak appeared at 178 ppm, indicative of the regiospecific oxidation.

Gel permeation chromatography showed that for all samples the M_w values were close to 10 kDa, while the M_n values ranged from 3200 to 5200 (Table 2). The elution curves were superimposable for all samples.

Based on the overall yields, a certain loss of sugars took place during dialysis, but the average molecular weight was sharply above the cutoff values of the dialysis tubings (3200 Da), even thought the depolymerization extent was high.

3.3. Coagulation of proteins, and polyelectrolyte complex formation

Several hydrolases were tested in order to verify their capacity to depolymerize 6-oxychitin, but immediate coagulation of proteins took place upon mixing with 6-oxychitin solutions. This was verified for papain, lipases of various origins, hen egg white lysozyme, and human amylase.

Being a polyanion, 6-oxychitin formed insoluble polyelectrolyte complexes with polycations such as chitosan, DEAE-dextran, DEAE-cellulose and polylysine. The complex chitosan + 6-oxychitin is of particular interest, because it provides microcapsules and microspheres including two polyelectrolytes of opposite charges, both derived from chitin. Upon mixing a 6-oxychitin sodium salt solution (5%, 10 g) with a chitosan lactate solution (1%), the polyelectrolyte complex formed at any pH value, preferably at pH 3.8, a value usually observed in these self-buffered systems.

The spectrophotometric determinations of unreacted chitosan in 6-oxychitin solutions where chitosan was introduced in excess quantities, indicated that the complex formed with

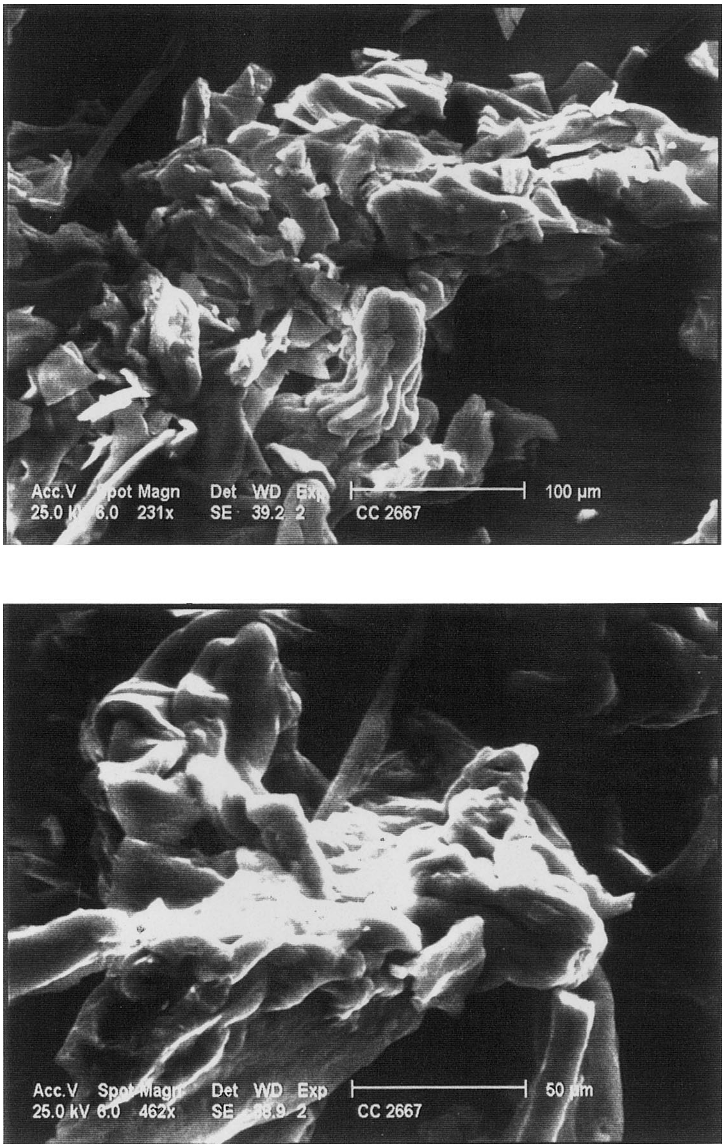

Fig. 3. Scanning electron microscopy of freeze-dried lobster 6-oxychitin sodium salt, providing evidence of its fragility. Inorganic content in the ratios Na 94.7%, K 1.0%, Ca 4.2%.

Table 2
Elemental analysis, gel permeation chromatography and equivalent weight (EW) data for animal 6-oxychitin sodium salts obtained under experimental conditions A and C

Sample	C	N	N/C	EW	M_w	M_n
Lobster, A[a]	30.71	3.95	0.128	283	9900	5200
Lobster, A[a]	30.73	4.15	0.135	239		
Lobster, A[a]	30.71	4.44	0.144	239	7300	3700
Crangon, A	30.24	4.37	0.144			
Crangon, C	30.46	3.48	0.114	180	9200	4100
Munida, A	29.51	3.32	0.112	203	6800	4000
Crangon chitin	45.41	6.29	0.138	–		
6-Oxychitin, calc.	40.16	5.86	0.146	239	–	–

[a] Three distinct preparations

a molar ratio 1:1. When treated with KNO_3 or $NaNO_3$, these complexes dissolved due to high ionic strength.

Microcapsules were prepared by dropping through a needle a chitosan lactate solution into a 6-oxychitin solution. Due to the different densities and viscosities, the chitosan drops passed through the 6-oxychitin solution where they reacted with the latter, and sedimented as spherical microcapsules.

3.4. Filmogenic properties

Chitosan + 6-oxychitin film (double face). Chitosan (0.76 g) was dissolved in acetic acid (1%, 100 ml). Portions of this solution (10 g each) were kept at 50°C for 6 h, in order to prepare chitosan films. 6-Oxychitin sodium salt solution (1% in water, 7.6 g) was put into the same petri

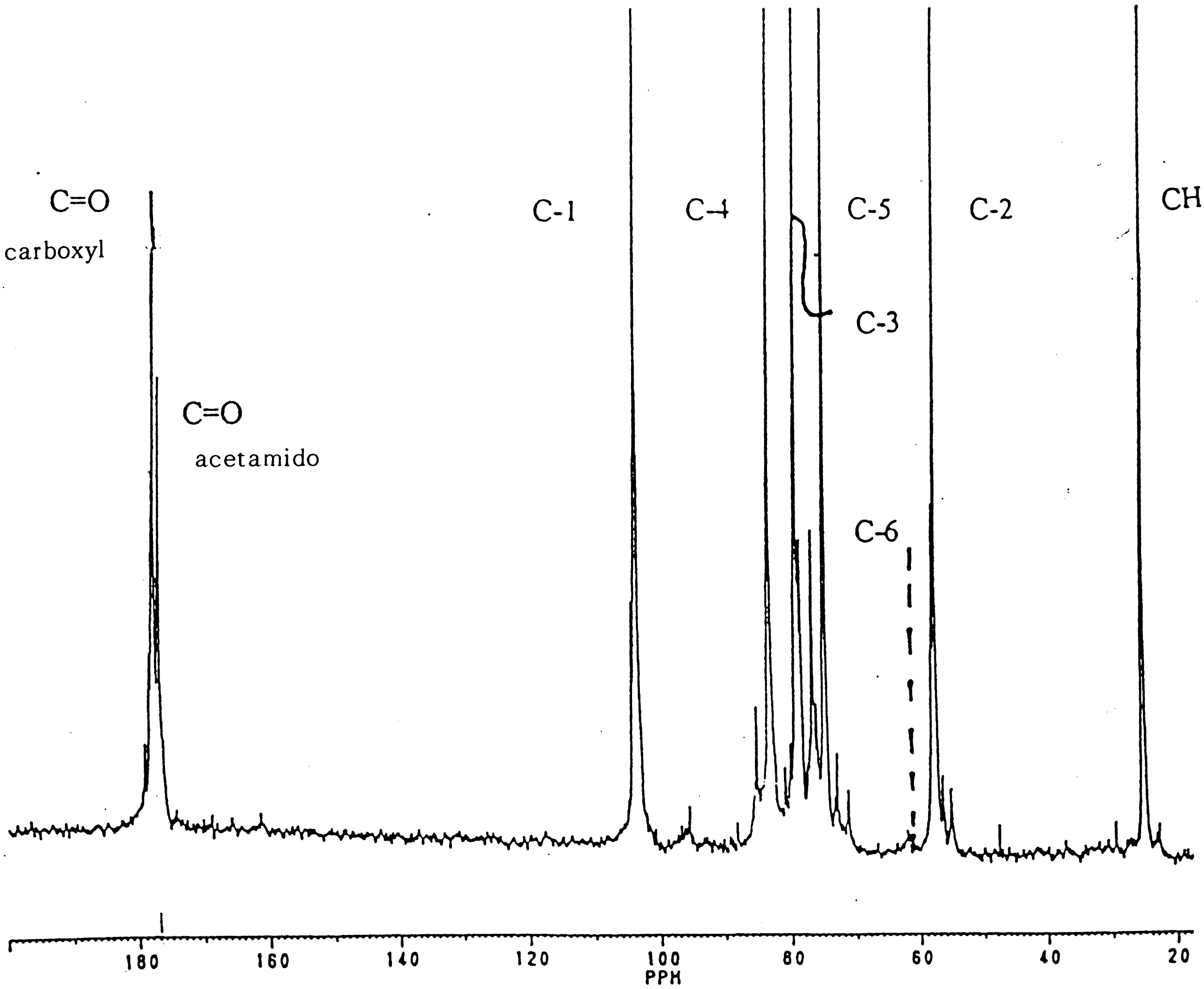

Fig. 4. ^{13}C NMR spectrum recorded on *Crangon crangon* 6-oxychitin sodium salt, showing the absence of the chitin C-6 signal (–––) and the presence of the new C=O signal (carboxyl group) at 178 ppm.

dish, over the adhering chitosan film, and dried (6 h at 50°C). In this way, the double face film chitosan + 6-oxychitin was obtained: one side was chitosan, while the other was 6-oxychitin. For this preparation, it is important that the chitosan film is prepared first.

Chitosan + 6-oxychitin soluble preparation. Whilst 6-oxychitin has modest filmogenic properties due to its low molecular weight, films can be manufactured upon mixing a chitosan solution with a 6-oxychitin solution, both in 4 M HCl. Under these conditions, no precipitation took place, not even during the dialysis of the mixture, presumably because an ordered polyelectrolyte complex was formed. Once dialyzed, these solutions, whose pH spontaneously adjusts itself at 5.5, are highly filmogenic, and yield transparent and mechanically resistant films.

3.5. 6-Oxychitin alkyl esters

To 6-oxychitin sodium salt (300 mg), suspended in absolute ethanol, methanol or iso-propanol (15 ml), concentrated HCl was added (three drops) and kept at 25°C for 7 d. After centrifugation, the products were washed with ether, and then submitted to 0.1 mbar depression for several hours. The IR spectrum showed a new band at 1741 cm^{-1}, typical of both the ester carboxylate group and the free carboxyl group (Fig. 2).

3.6. Metal-ion chelation

Upon mixing the 6-oxychitin solution with transition metal ion solutions, colored chelate like the insoluble chelates of ferric ion (yellow) or Pb(II) (white), and the soluble chelates of Cu(II) (green), or Ni(II) (green) were formed. Upon slow evaporation, the Cu chelate crystallized as large blue crystals, while the Ni chelate yielded a gel that retained water and did not dry at room temperature.

4. Conclusions

The regiospecific oxidation of chitins of various origins at

C-6 is feasible with NaOCl in the presence of Tempo® and NaBr. Pre-swelling of chitin is a requisite that simplifies substantially the preparation. Chitin is extensively oxidized and partially depolymerized, to yield 6-oxychitin sodium salt, a low molecular weight compound fully soluble over the entire pH range, whose characteristics are presented for the first time in this article. In the light of the data presented here, animal and microbial chitins are amenable to soluble polyanionic forms.

The average molecular weight obtained after chemical treatment of animal chitins was close to 10 kDa, with comparable polydispersity; the gel permeation chromatography curves were superimposable for all samples studied. The moderate molecular weight value was an additional aspect of versatility, considering the current trend towards low molecular weight chitosans for various applications, and did not represent a problem during freeze-drying.

For the exploitation of chitin, the preparation of 6-oxychitin is a valid alternative in the preparation of chitosan because of the inherent advantageous characteristics of 6-oxychitin, that is soluble at acidic, neutral and alkaline pH values, whereas chitosans are soluble at $\mathrm{pH} < 6$ only. 6-Oxychitin is prepared in a simpler, faster and more environmentally friendly way than chitosan.

The preparation of the esters of 6-oxychitin will be studied in more detail; it seems that it permits to depress the anionic character and to obtain gels of modified chitin that might prove interesting as surrogates of hyaluronan esters in cosmetics, medical items and drug carriers.

The chitin–glucan complex of fungal origin can be derivatized in one step at C-6 of both chitin and glucan moieties, so that both polysaccharides contribute to the solubility and preparation yield.

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