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Structural features of sulfated chitosans

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Chitosan sulfates prepared by different methods were analyzed by ^{13}C NMR spectroscopy. It was shown that the sulfation conditions of chitosan essentially affect the position and degree of substitution with sulfate in derivatives of chitosan. Sulfated products obtained under homogeneous conditions are characterized by more heterogeneity and they have to be considered as copolymers of chitosan 6-O-monosulfate and 3,6-O-disulfate, whereas those produced by semi-heterogeneous synthesis may be considered preferentially as chitosan 3,6-O-disulfate. Copyright © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

In general only the influence of molecular weight and/or substitution degree of sulfated polysaccharides on their biological activity is considered in the majority of works involving the anticoagulant or antiviral properties of these substances (Horton & Usui, 1978; Jurkiewicz *et al.*, 1989; Nakashima *et al.*, 1989).

Sulfation reactions of multi-functional polysaccharides are inevitably followed by the appearance of structural heterogeneity in polymer chains. When chitosan is sulfated, a structural variety of products is obtained, which may be related to the various reactivities of the three functional groups of the parent polymer, leading to different degrees of completion in the individual groups. On the one hand this gives rise to uncertainty but on the other hand some structures that emerge from random distribution of modified groups along the chain can reveal new features of biological functions.

In this connection, directed synthesis of similar heterogeneous structures is of great interest and, in any case, calls for attentive study of the preparation conditions of these compounds and correct identification of their structures.

Earlier works investigating the structural features of chitosan sulfates dealt with the consideration of the zwitterionic structure of chitosan 6-O-monosulfate relative to the pH-dependence of NMR signals in this substance (Naggi *et al.*, 1986; Terbojevich *et al.*, 1989; Gorbacheva *et al.*, 1991). These authors indicated the presence of molecular and structural heterogeneity in the chitosan sulfate obtained and connected this with a high content of acetyl groups or with an aftereffect of polymer degradation. In another work (Hirano *et al.*, 1991) three sulfated derivatives of chitosan were analyzed by ^{13}C NMR spectroscopy and the structures of the chitosan disulfates were confirmed.

The present paper describes the effect of preparation conditions of sulfated chitosans on their structural features by means of ^{13}C NMR spectroscopy.

MATERIALS AND METHODS

Materials

Chitosan from Antarctic krill (*Euphausia superba*), which has a degree of deacetylation of 0.89 and average molecular weights (M_v) of 12 000 Da and 230 000 Da, was used.

Preparation of solvent including chitosan
Chitosan was dissolved in 1% CH_3COOH, the solution was precipitated by 0.01 M NaOH and the precipitate obtained was washed with distilled water to neutrality and then washed several times with methanol and dimethylformamide (DMFA) for 12 h.

Preparation of sulfating complex
4.5 ml $HClSO_3$ or 7.0 ml oleum (30% sulfur trioxide) was added dropwise with stirring to 30 ml DMFA previously cooled at 0–4°C, then the reaction mixture

was stirred without cooling until the solution reached room temperature.

Preparation of chitosan solution in anhydrous solvent
Solutions of chitosan were made by dissolving the chitosan in a mixture of formamide–halogen organic acid (e.g. dichloroacetic acid, DCAA) at a weight ratio of components of 10–100:1, depending on the concentration of the chitosan solution.

Pseudo-homogeneous preparation of sulfated chitosan (method N1)
To 30 ml of 2% chitosan solution in an anhydrous mixture of formamide–DCAA (60:1) was added dropwise with stirring and cooling 4.5 ml of $HClSO_3$ for 15 min. The reaction was run at room temperature (22°C) for 4 h and was followed by the formation of gel. At the end of the reaction the gel was diluted with water, neutralized by 20% NaOH and precipitated with methanol. The resultant precipitate was dissolved in water and the solution was submitted to dialysis against demineralized water for 2 days or to filtration on a column (0.9×60 cm) of Dowex-50W×8 (H^+) (Sigma, St Louis). The precipitate was then concentrated and the product either precipitated by ethanol or isolated by lyophilization.

Homogeneous preparation of sulfated chitosan (method N2)
To the sulfating complex obtained above was added with stirring 10 ml of 3% chitosan solution in an anhydrous mixture of formamide–DCAA (40:1) and the reaction was run for 60 min at room temperature (22°C) or 50°C. The reaction solution was treated as described in method N1.

Semi-heterogeneous preparation of sulfated chitosan (method N3)
To the sulfating complex obtained above was added 1 g of solvent including chitosan and the reaction mixture was stirred for 60 min. At an early stage of reaction, when in a swollen state, the polymer was transferred to a solution that was neutralized by 20% NaOH and precipitated by methanol. The product was isolated as described in method N1.

Preparation of sulfated chitosan in aqueous medium (method N4)
To 60 ml of 1.7% aqueous solution of low-molecular weight chitosan ($M = 13\,000$ Da), prepared by partial deamination with sodium nitrite, was added 1.5 g of pyridine–SO_3 (Merck, Schuchardt) complex. The reaction was run with stirring at pH 9 for 60 min. At the end of the reaction the solution was neutralized by 0.1 N H_2SO_4, submitted to desalting on a column (1.5×65 cm) of Sephadex G-10 (Pharmacia, Uppsala) and the filtrate concentrated and precipitated by ethanol. The precipitate was washed with ethanol diethyl ether and dried at 50°C in a vacuum oven.

NMR spectroscopy
^1H and ^{13}C NMR spectra were taken using a Bruker AM-300 spectrometer at 80°C in D_2O with acetone as internal reference (δ_H 2.23 and δ_C 31.45 ppm). The standard Bruker software was used for both the homonuclear and heteronuclear 2D COSY spectra.

RESULTS AND DISCUSSION

Sulfated chitosans (SC) were obtained by different methods and their ^{13}C NMR spectra are shown in Fig. 1.

The simplest spectrum (Fig. 1a) belongs to SC-1 prepared by the first method. This spectrum is very similar to that of SC identified as chitosan-6-sulfate (Naggi *et al.*, 1986). The ^1H NMR spectra of these two SC also proved to be very similar. The unusual low-field signal of H-3 at 4.6 ppm in the spectrum of SC-1 (instead of that characteristic of chitosan-6-sulfate at about 4 ppm) drew our attention and we reinvestigated the assignment in the ^1H NMR spectrum of SC-1 using a 2D COSY experiment.

Analysis of this spectrum showed that we had the ^1H signals were assigned correctly (Naggi *et al.*, 1986). The reason for the low-field position of the

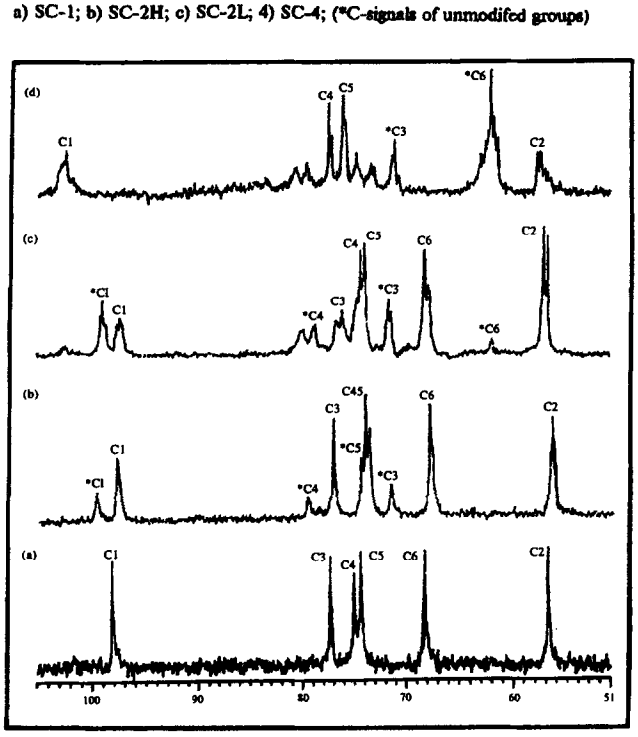

Fig. 1 ^{13}C NMR spectra of chitosan sulfates: (a) SC-1; (b) SC-2H; (c) SC-2L; (d) SC-4. *C = signals of unmodified groups.

H-3 signal became understandable after analysis of the 2D heteronuclear $^{13}C/^{1}H$ COSY spectrum, which showed correlation of H-3 and a carbon with a chemical shift at 77.3 ppm. The low-field position of the carbon signal is undoubtedly connected with the presence of an electronegative substituent at this carbon atom, that is an additional sulfate group. Thus, SC-1 as well as the polymer described in Naggi *et al.* (1986) are chitosan-3,6-disulfates and assignments of their ^{13}C NMR spectra must be as given in Table 1. Additional ^{13}C NMR experiments with variation of the pD of the samples in the interval 1–13 confirmed the assignment of the signal at 77.3 ppm to C-3. This signal (as well as the signal for the anomeric carbon) showed the sharpest dependence on pD, which is characteristic of carbons neighbouring $CHNH_2$ in amino sugars. The change in pD from 1 to 13 provided downfield shift of these signals by 4–5 ppm, whereas the other signals shifted by no more than 1.5 ppm. The comparison of chemical shifts for the corresponding carbon atoms in the ^{13}C NMR spectra of chitosan and SC-1 shows the typical α and β effects of sulfation for pyranoses (Archibald *et al.*, 1981).

The ^{13}C NMR spectrum of SC-2 derived from high-molecular weight chitosan at 50°C using the second method is practically identical to the spectrum of SC-1. Minor signals at 62, 72 and 102 ppm are additionally observed in the spectrum of SC-3 obtained by the third method. The first two signals belong to C-6 and C-3 of the residues without sulfate at these positions, and the third is probably C-1 of the residues with N-acetyl groups. Thus, the SC-3 polymer contains residues that are not sulfated at positions 3 and/or 6.

The spectrum of SC-2H derived from high-molecular-weight chitosan at 22°C by the second method is slightly more complicated (Fig. 1b). It contains minor peaks of 6-substituted residues with diagnostic signals at 67.9 (C-6) and 71.7 (C-3) ppm. (Hirano *et al.*, 1991), together with the set of main signals belonging to 3,6-di-substituted residues. The integral intensity ratio of the major and minor peaks is close to 2:1. The signal with very small intensity at 79.6 ppm may relate to C-3 of 2,3,6-tri- or 2,3-di-substituted residues since N-sulfation as well as N-acetylation shifts the C-3 signal to low field (Hirano *et al.*, 1991).

The spectrum of SC-2L obtained from low-molecular-weight chitosan at 22°C using the second method (Fig. 1c) proved to be the most complicated. Like the spectrum of SC-2H, it contains two sets of signals belonging to 6- and 3,6-O-sulfated sugar residues. In addition, all but a few signals in both sets are split. The splitting is presumably connected with partial N-sulfation and/or with the influence of neighbouring residues with different structure. Additional irregularity may be caused by the presence of residues that are not sulfated at position 6.

Finally, SC-4 (Fig. 1d), produced by the fourth method, did not bear 6-O-sulfates. The absence of signal at about 68 ppm and the presence of an intense signal at 62 ppm justify this conclusion. However, the presence of 3-O-sulfate groups cannot be excluded. This may be responsible for the irregularity of the SC-4 structure reflected in the complexity of its spectrum as well as the presence of a non-stoichiometrical amount of N-acetylated and N-sulfated groups. The absence of C-1 signals at 98–99 ppm, the characteristic signal for chitosan and its O-sulfates (Table 1), suggests total substitution of amino groups.

As was expected, the structural heterogeneity of chitosan sulfates caused by incomplete substitution in each of the three functional groups was clearly revealed for products of homogeneous-phase synthesis. In

Table 1. ^{13}C NMR data for chitosan and its sulfated derivatives

Compound	Chemical shifts (ppm)					
	C1	C2	C3	C4	C5	C6
Chitosan	99.0	57.1	71.6	78.7	76.1	61.7
SC[a] (Naggi *et al.*, 1986)	98.0	56.8	74.3	77.5	74.8	68.2
SC-1[b]	98.1	56.7	77.3	75.1	74.3	68.2
SC-2[b]	97.8	56.7	77.0	75.1	74.4	68.2
SC-3[b]	98.0	56.7	77.2	74.9	74.4	68.4
SC-2H[b,c]	97.8	56.5	77.2	74.6	74.1	67.9
	99.7	—	—	—	—	—
SC-2L[c,d]	99.7	56.5	71.7	77.2	73.7	67.9
	97.8	—	—	—	—	—

[a]Data are given with additive correction by −1 ppm due to different references in Naggi *et al.* (1986) and this work. The assignment of C-3, C-4 and C-5 must be corrected in accordance with data for SC-1 (see also the text).
[b]Data for glucosamine-3,6-disulfate residue.
[c] The first and second values of C-1 chemical shift belong to the residues glycosylating 3,6-di- and 6-mono-sulfated residues, respectively.
[d]Data for glucosamine-6-sulfate residue.

contrast with this, the sulfation reaction proceeding in swollen chitosan (semi-heterogeneous conditions) can promote the formation of zwitterionic structures and H-bonds in products with participating groups at C6 and C2 as well as at C3 and pyranose oxygen. The groups at C2 and C3 are probably submitted to a small amount of sulfation. On the other hand, the same contacts between the groups may also favour a gel formation of sulfated macromolecules in pseudo-homogeneous media (method N1).

As a result of these investigations we have concluded that the chitosan sulfates obtained are as a rule not mono-substituted but are often di-substituted and may also be partially tri-substituted. This means that chitosan sulfates may be considered as copolymers composed of random alternated mono-, di- and tri-substituted units of chitosan. Unmodified (parent) units of chitosan, as well as acetamide groups of chitin, also make a definite contribution to the heterogeneity of structure in chitosan sulfate. The distribution of these and sulfated groups in the products obtained may be estimated by relating the signal intensities of *C1/C1, *C4/C4 and *C6/C6 (where *C = signals of unmodified groups). The investigation of an approach to such an estimation is in progress and may allow the understanding of the fine mechanism of biological action of these substances.

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