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Chitosan *N*-sulfate. A water-soluble polyelectrolyte

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Abstract

Chitosans having degrees of acetylation (da) of 0.04, 0.10, and 0.22, respectively, were *N*-sulfated under a variety of reaction conditions. The derivatives obtained ranged in degree of sulfation (ds) from 0.4 to 0.86 (± 0.05). All were soluble in water, and the rheological properties of their solutions varied markedly with da and ds values. Both ionic strength and pH had an effect on their solubility properties, and also on interactions that they exhibited with *O*-(carboxymethyl)cellulose, xanthan gum, and heparin. Being compatible with other polyelectrolytes such as these, the chitosan derivatives may be useful in some aqueous formulations. © 1997 Elsevier Science Ltd.

Keywords: Chitosan; Polyelectrolyte

1. Introduction

Chitosan, and its parent biopolymer chitin, are objects of substantial research into their utilization by industry and biotechnology [1,2]. This interest has given rise to the preparation of chitosan-based materials in a variety of *N*-, *O*-, and *N*,*O*-substituted forms, having substituents that include alkyl, acyl, and imino groups [3]. In general, hydrophilic adducts such as carbohydrate branches [4,5], or carboxyalkyl substituents [6–8], render the chitosan soluble. Alternatively, some derivatives are of interest less for their solubility than their ability to form membranes [9] or films [8,10], or act as metal-chelating agents [7,11]. Furthermore, there has been a growth in research activity into the biological and biomedical properties of chitosan and chitosan-derived materials [1]. In particular, derivatives [12–16] having *N*- and/or *O*-sulfate groups either alone or in conjunction with other substituents have been examined as potential heparinoids. Another approach for the preparation of heparinoid polymers involves the attachment of heparin oligosaccharides to chitosan [4,5,17,18].

The solubility properties of chitosan and its derivatives, and their use in biomedical applications, entail some problems in common, particularly for use as a polymer support or carrier material. Our objective was to prepare soluble materials that might have interesting rheological properties. Another concern was the solution compatibility of these derivatives with anionic polymers, because chitosan and some of its derivatives form insoluble complexes with such important polymers as *O*-(carboxymethyl)cellulose, xanthan gum, alginate, and heparin [21,22]. Both purposes were achieved by converting amino groups of chitosan preparations of low *N*-acetyl content into anionic centers, through selective *N*-sulfation. The products, designed to vary in their levels of derivatization, were evaluated as to their rheological charac-

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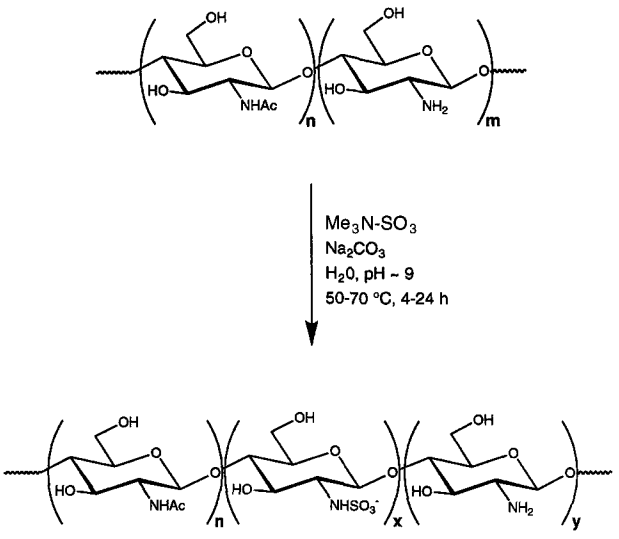
teristics, and their solution compatibility with other anionic polymers.

2. Results and discussion

Trimethylamine–sulfur trioxide ($Me_3N–SO_3$), which is known [12] to effect selective *N*-sulfation of amino-alcohols, was used in this study, which supplements the use of the pyridine–SO_3 complex and related reagents with chitosan reported earlier [19,20]. Three chitosan samples were selected, having residual degrees of *N*-acetylation of 0.04, 0.10, and 0.22, respectively. Previously lyophilized, each was dispersed in dilute sodium carbonate solution and treated with $Me_3N–SO_3$ under a variety of conditions (see Scheme 1 and Table 1). The products obtained are also described in this table, in terms of their degrees of *N*-sulfation (ds) and *N*-acetylation (da), and free amine content (dn). All three sets of values were obtained by a combination of ^1H and ^{13}C NMR spectroscopy, the latter method being particularly useful for derivatives that gave highly viscous solutions. Separation of the ^1H signals for H-2s (sulfoamino) and H-2n (amino) was enhanced by recording the spectra at pD ~ 9, rather than at neutrality, the observed chemical shifts then being δ 3.15 and 2.74, respectively (see Fig. 1). Also well resolved in this region of the spectrum is the acetamido methyl signal, at δ 2.10, its intensity differing notably in Fig. 1A in reflecting the three different levels of *N*-acetylation among samples. Fig. 1B illustrates variations in the level of *N*-sulfation effected at a given *N*-acetyl content, depending on the experimental conditions. In a representative ^{13}C NMR spectrum (Fig. 1C), the C-2n, -2a, and -2s signals at δ 60.1, 59.1, and 63.5, respectively, provide a ready measure of the relative proportions of 2-amino, -acetamido, and -sulfoamino groups in this particular derivative. Positions C-1, C-3, and C-4 also exhibit some splitting as a result of structural heterogeneity. Tentatively assigned on the basis of relative peak heights are resonances for C-3a, C-4a, C-3, and C-4.



Scheme 1. Synthesis of *N*-sulfated chitosan.

It is worth noting that the initial *N*-acetyl content of the chitosan had a bearing on the *N*-sulfation reaction. That is, the sample having fewer *N*-acetyl groups required longer reaction periods to become fully solubilized, which occurred when the ds level reached about one-half its final value. With the most

Table 1
Reaction conditions and characterization data for derivatives 2a–f

Product	Molar ratio		Time [a] (h)	Temp. (°C)	ds [b]		da [b]	dn [b]	O.R. (°)
	$Me_3N–SO_3$	Na_2CO_3			NMR	E.A. [c]			
2a	2.8	2.3		65	0.45	0.40	0.10	0.45	−11.8
2b	2.3	1.7	12	55	0.59	0.58	0.10	0.32	−9.5
2c	2.8	3.0	3	65	0.63	0.50	0.10	0.27	−4.5
2d	3.4	1.9	24	55	0.72	0.73	0.10	0.18	−10
2e	3.3	2.0	6	65	0.77	0.75	0.10	0.14	−6.5
2f	5.0	3.3	12	65	0.86	0.81	0.10	0.05	n.d.
3a	2.9	3.0	4	65	0.52		0.22	0.25	n.d.
3b	2.9	3.0		65	0.46		0.22	0.32	−10
4a	2.9	3.0	12	65	0.63		0.04	0.33	n.d.
4b	2.9	3.0			0.54		0.04	0.42	n.d.

[a] Time corresponds to duration of heating after obtaining a clear solution.
[b] ($\pm$0.05).
[c] From elemental analysis data.

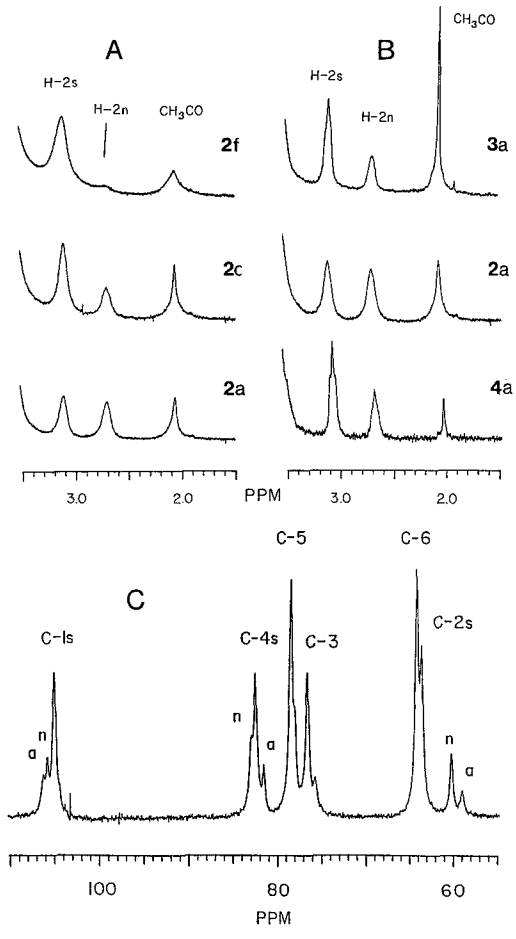


Fig. 1. (A) 300-MHz ^{1}H NMR spectra of derivatives 2f, 2c, and 2a, showing the changes in H-2n and H-2s, as ds increases. (B) 300-MHz ^{1}H NMR spectra of *N*-sulfated chitosan derivatives 3a, 2a, and 4a, showing variations in degree of acetylation (da). (C) 75-MHz ^{13}C NMR spectrum of derivative 2c, having a ds of 0.63.

highly acetylated material, by contrast, dissolution of the solids occurred at relatively lower ds levels. As well, qualitative observations suggest that the solubility of the isolated products was dependent upon da for materials having similar ds values. These observations suggest that not only the introduction of sulfoamino substituents, but also the co-occurrence of a substantial proportion of acetamido groups, is important for efficient disruption of interchain associations within a chitosan suspension, and that solubility is imparted more readily by a specific combination of the two substituents, e.g. 4:1 *N*-sulfate–*N*-acetyl. Presumably, an initially broad distribution of acetamido groups also contributes to a more favorable overall substitution pattern. Optical rotations for some of the analogs were determined and all yielded similar rotations, independent of the degree of *N*-sulfation or degree of *N*-acetylation.

Solution properties.—Isolated as sodium salts, all of the derivatives were soluble in distilled water; 0.5% solutions had pH ~ 8. They were less soluble in aqueous acidic media, giving either higher viscosity solutions, gels, or precipitates, depending on the ds. To illustrate these effects, rheological characterizations of 0.5% solutions of samples 2a–f in distilled water, and of 2b,c,e, and f in acidic media, were carried out (see Table 2). All of the solutions tested were pseudoplastic and were adequately modelled using the Power-law equation

$$\eta = m\gamma^{(1-n)} .$$

The solution viscosity was measured at 16 different shear rate values, over the range 0.5–500 s^{-1}. Linear regression of the logarithm of viscosity against the logarithm of shear rate provided the parameters *n* and *m* (Table 2) according to the Power-law model. The parameters $\eta_{1.0}$ and η_{100} give a measure of the pseudoplasticity (shear thinning) of the fluid, where *m* is numerically equal to the solution viscosity at a shear rate of 1.0 s^{-1} ($\eta_{1.0}$), and facilitate a comparison of the rheological properties of the various samples and reference solutions.

In general, solution viscosities varied widely, depending on the ds, for samples at pH 8. Derivative 4b was the most viscous, having a higher $\eta_{1.0}$ value than the well-known industrial polysaccharide, xanthan, at an equal concentration. Product 2b was the only other notably high viscosity sample ($\eta_{1.0}$ 3900), whereas 2a,c,e, and f had intermediate viscosities. Compounds 2d, 3a,b, and 4a gave visibly non-viscous 0.5% solutions, and were not evaluated further. The ds values of both 4b and 2b are ~ 0.55, whereas their da values are 0.04 and 0.10, respectively, with the higher da material corresponding to the less viscous solution. Another feature worth noting is that both 3a and 3b, which have high da values, gave non-viscous solutions. This may indicate that a higher da, in conjunction with a similar level of *N*-sulfation (ds ~ 0.50) relates inversely to the viscosity of the solution.

The high ds derivative, 2f, showed no significant change in solution viscosity upon acidification, whereas the solutions of derivatives 2b and 2c had reduced viscosities at pH 6. By contrast, derivative 2c showed a marked increase in viscosity upon acidification. It is noteworthy that 2b and 2c are so different in their solution properties, when both appear to have close to the level of sulfation optimal for achieving

Table 2
Viscosities (η) of 0.5% w/v solutions [a] of *N*-sulfated chitosan derivatives, and their mixtures with xanthan gum, at shear rates of 1.0 and 100 s^{-1}, at pH 6 and 8

Sample	ds	Viscosities			
		pH 8.0		pH 6.0	
		$\eta_{1.0}$	η_{100}	$\eta_{1.0}$	η_{100}
2a	0.45	150	32	[b]	[b]
2b	0.59	3900	175	1750	70
2c	0.63	800	78	3100	100
2d	0.72	24	12	[c]	[c]
2e	0.77	760	88	500	67
2f	0.86	1050	85	1060	80
3a	0.52	11	6	[c]	[c]
3b	0.46	60	22	[b]	[b]
4a	0.63				
4b	0.54	6200	220	[b]	[b]
XAN + 2a		1100	60	[b]	[b]
XAN + 2c		3600	115	4900	140
XAN + 2f		3100 –	100	2200	85
0.5% XAN				3000	65
0.25% XAN				825	29

[a] All solutions had total polysaccharide concentrations of 0.5% (w/v). Mixtures were composed of a 1:1 ratio of the derivative to xanthan gum (XAN).
[b] These materials precipitated at pH 6.
[c] Solutions were visually observed to be non-viscous and were not further analyzed.

high viscosity. Possibly, the optimum levels for the protonated and unprotonated forms do not occur at the same ds values.

Polyelectrolyte-based interactions of chitosan and some of its derivatives have been reported. The variations in ionic functionality of the present family of derivatives made them suitable candidates for an analogous investigation. Mixtures of 2a, 2c, and 2f with xanthan gum (XAN) were prepared having a 1:1 weight ratio, and a total polysaccharide content of 0.5% w/w. Mixtures XAN2c and XAN2f gave $\eta_{1.0}$ values similar to that of XAN05 (a 0.5% w/w solution of xanthan gum), whereas XAN2a was closer in viscosity to XAN025. These results indicate that there is sufficient interaction between derivative 2c and 2f and xanthan, to impart viscosities similar to that of xanthan alone, at equivalent total polysaccharide concentrations. All of the mixtures gave higher $\eta_{1.0}$ values than the solution containing only an equivalent xanthan concentration as the mixtures (XAN 025), illustrating further that these derivatives do interact with xanthan. As the resultant viscosities ($\eta_{1.0}$) were greater than would be expected from the viscosities of the individual components, a synergistic polyelectrolyte interaction probably occurred. Although the mixtures were not as pseudoplastic as the xanthan solution, they were more so than most of the 0.5% derivative solutions. This observation was not unexpected, as the interactions that give xanthan its highly pseudoplastic character were being augmented or replaced by different, probably ionic interactions, which could affect the shear rate dependence of the solutions. It is noteworthy that acidification of XAN2c caused a marked increase in $\eta_{1.0}$, whereas that of XAN2f was lowered. These results are analogous to the effect of acidification upon the 2c and 2f solutions themselves.

The foregoing results indicate that ionic interactions play an important role in determining the solution properties of chitosan *N*-sulfate derivatives. Although this is not surprising in the light of descriptions of ionic and polyelectrolyte associations of chitosan, the type of derivative described here offers a method for varying the degree of interaction. In the sodium form, all of the derivatives were found to be compatible in solution with ionic polymers, heparin, *O*-(carboxymethyl)cellulose (CMC, ds 0.9), and xanthan gum. By contrast, the first two form polyelectrolyte complexes with chitosan that precipitate from aqueous media. It is well known that associations between ionic polymers can be exploited in terms of generating a variety of stable rheological systems in the form of gels, emulsions, and foams. Based on the results reported here, it seems likely that *N*-sulfated

chitosan derivatives could find applications in such systems.

3. Experimental

Materials.—Chitosan samples 1A and 1B were purchased from Sigma Chemical Co. (Lot #46F-0268 and 109C-04511, respectively). Chitosan 1C was a generous gift from Protan laboratories, Inc. Dialysis tubing was purchased from Fisher Chemical Co. and had a molecular weight cut-off of ~ 3500 (Spectropor R 1). The Sephadex G-15 and G-100 gel filtration media were obtained from Pharmacia. All other reagents and chemicals were purchased from commercial sources and used as supplied.

Methods.—Workup and purification of products entailed exhaustive dialysis against distilled water with Spectropor cellulose membrane tubing (3500 MW cut-off). Sephadex G-15, 100 or 200, was used for liquid chromatography, and product MW profiles were analyzed by FPLC with a Pharmacia system equipped with a Sepharose-12 column and a variable UV detector (Spectra Physics SP8440) at *l* 214 nm. Elemental microanalyses (C, H, N, S) were performed by Guelph Chemical Laboratories. The degree of sulfation (ds) was calculated from the S/C ratio, which, with the degree of acetylation (da) allowed the determination of the molecular formula. After introducing a water correction factor, all molecular formulae were within acceptable limits of the analytical data (i.e. $\pm 0.35\%$).

The NMR spectra were recorded with a Varian XL300 spectrometer, operating at 300 MHz for ^{1}H and 75.4 MHz for ^{13}C. For ^{1}H NMR, the samples were dissolved in deuterium oxide (D_2O) that contained Na_2CO_3 (pD ~ 9), and were repeatedly (3–4 times) evaporated and redissolved in D_2O, to give a final concn of ~ 2% w/v. For the native chitosan samples the procedure was the same except that the solvent was 1% w/v CH_3COOH-d_4 in deuterium oxide. All ^{1}H NMR spectra were recorded at 70 °C, and were referenced to internal sodium 4,4-dimethyl-4-silapentanoate-2,2,3,3-d_4 (TSP). Samples for ^{13}C NMR were typically 5–10% w/v in D_2O containing Na_2CO_3 (pD ~ 9); their spectra were recorded at 70 °C and referenced with respect to external Me_4Si. Optical rotations were recorded using a Perkin–Elmer Model 241 polarimeter, with a sodium source. Measurements were performed using solns at 5 mg/mL concn in 100 mM, pH 8, sodium phosphate buffer, in a 1.0-cm cell at 22 °C.

General sulfation procedure.—Chitosan (1.0 g, 6.2 mmol glucosamine equivs) which had previously been lyophilized, was dispersed in 50–200 mL of distilled water, and treated with Na_2CO_3 (1.3–2.2 g, 12.3–20.8 mmol, 2.0–3.3 equiv) and Me_3N–SO_3 (2.5–4.5 g, 18.0–32.3 mmol, 3–5 equiv). The mixture was heated at temperatures between 50–70 °C, until a clear viscous soln or gel formed (4–12 h). Isolation at that point provided low ds products (~ 0.50), whereas continued heating resulted in the formation of the additional range of compounds described in Table 1. The cooled mixture was then dialyzed exhaustively against (in succession) distilled water, distilled water containing a suspension of Amberlite IR 120, (H^+) exchange resin, 0.025 M aq NaOH (4 L), and finally against distilled water. The dialyzed soln was then lyophilized to yield a white fluffy solid (1.1–1.5 g, 80–95%). More specific reaction parameters are provided for each derivative in Table 1. Attempts to prepare similar products by pre-dissolving the chitosan in aq CH_3COOH, then neutralizing, and introducing the Me_3N–SO_3 without isolation of the dispersed material, were unsuccessful. (The comparable use of HCl as a solvent has been described [12].)

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