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SULFATED *N*-(CARBOXYMETHYL)CHITOSANS: NOVEL BLOOD ANTICOAGULANTS

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ABSTRACT

N-(Carboxymethyl)chitosan was subjected to sulfation in a mixture of concentrated sulfuric acid (oleum) and *N,N*-dimethylformamide, under anhydrous conditions. The resulting product contained 11% of sulfur and degree of substitution: *N*-acetyl, 42%; *N*-carboxymethyl, 58%; and sulfate, 100%. Sonication of the sulfated *N*-(carboxymethyl)chitosan gave two main fractions whose molecular weights were 39,000 and 80,000. In human blood, complexes of sulfated *N*-(carboxymethyl)chitosan and antithrombin inhibited both thrombin and factor Xa, and produced neither hemolysis nor alterations in erythrocytes and lymphocytes. Sulfated *N*-(carboxymethyl)chitosan is therefore proposed as a blood anticoagulant.

INTRODUCTION

The similarity of chitosan and heparin has been pointed out previously[1] and a number of modifications, such as sulfation[2], sulfation combined with carboxylation[3], or depolymerization[4,5], and the introduction of sulfoamino[6] or *N*-formyl[7] groups, to afford chitosan derivatives having anticoagulant activity, have been described. Other reports relate to the anticoagulant activity of such products as chitosan–dextran sulfate[8] and chitosan–(carboxymethyl)dextran complexes[9] and to dextrans containing carboxymethyl, sulfate, benzyl sulfate, and α-amino acid groups[10].

Although studies have been performed with a number of other polysaccharides[11,15] and synthetic polymers[16], chitosan appears to be more suitable than most of them for preparing heparin-like substances because of its (1→4) linkages, its linearity, and the presence of amino and acetamido groups, which, otherwise, are difficult to introduce.

Based on previous work, it may be reasoned that introduction of *O*-sulfate and carboxymethyl–preferably as α-amino acid–groups, together with a decrease of molecular weight may afford derivatives having high anticoagulant activity[17]. To

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avoid side reactions and other complications, the reaction should be as mild as possible and result in an even distribution of the substituents, and the chitosan should be reasonably pure and well defined[18].

The present article describes the preparation of such compounds by sulfating *N*-(carboxymethyl)chitosan[19–24] followed by molecular-weight decrease through sonication[25].

EXPERIMENTAL

Preparation of sulfated N-*(carboxymethyl)chitosan.* — *N*-(Carboxymethyl)chitosan (8 g) prepared from *Euphausia superba* chitosan having degree of acetylation 42 ±4% was suspended in *N,N*-dimethylformamide (200 mL) and stirred overnight in a sealed flask. The flask was then transferred to a refrigerated bath and *N,N*-dimethylformamide–sulfur trioxide complex (450 mL) was added dropwise with stirring and under anhydrous conditions. The complex was prepared by slowly adding oleum (150 mL, 30% sulfur trioxide) to *N,N*-dimethylformamide with stirring, cooling, and under exclusion of moisture. The times of reaction were 3 h for the first preparation and 15 h for the second. The temperature of the mixture was not allowed to rise >25°. At the end of the reaction time, a solution of sodium hydroxide in 1:3 water–methanol mixture was slowly added to neutralize the sulfuric acid; after decanting, the solid phase was collected and submitted to dialysis against demineralized water for 5 days, to remove sodium sulfate and products of low molecular weight. Sulfur was determined after combustion with a Leco model 621-500 analyzer, equipped with a radiofrequency oven, by iodometric titration of the resulting sulfur dioxide.

Sonication. — Solutions of sulfated *N*-(carboxymethyl)chitosan, refrigerated at ~2°, were submitted to sonication for a total of 30 min, during fifteen 2-min periods, alternated with 2-min pauses. The instrument used was a Branson model B-12.

Gel chromatography. — Sonicated, sulfated *N*-(carboxymethyl)chitosan in acetate buffer (50 mg/mL), filtered through 0.45-μm Millipore membranes, was submitted to gel chromatography at 20° in a column (76 × 1.6 cm) of Bio-Gel P-100 (exclusion limit 100,000) with an LKB chromatographic system model 2137 at a flow-rate of 0.5 mL/min in 0.35M acetate buffer, pH 4.5. LKB equipment, including a fraction collector (model 7000 Ultrorac), detector, and control units (model 2089 Uvicord III), and a chart recorder was used. Molecular-weight standards of 40,000 and 80,000 were used.

Optical equipment. — Optical instruments used were a Perkin–Elmer model 544 u.v.–visible spectrophotometer, a Leitz Orthoplan microscope, and a Jasco model A-500 spectropolarimeter; the circular-dichroism spectra showed a negative Cotton band at 203 nm; molar ellipticity at pH 8.0 was ~200,000 (140,000–234,000) depending on the dialysis time.

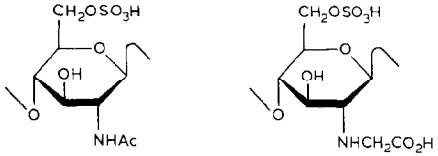
Alkalimetry. — The samples of sulfated *N*-(carboxymethyl)chitosan sodium

salt (0.5 g) were dissolved in 0.1M hydrochloric acid (50 mL) also 0.1M in sodium chloride, and were titrated with a mixture of sodium hydroxide and sodium chloride (both 0.1M) under nitrogen.

Human blood-coagulation tests. — An excess of sulfated *N*-(carboxymethyl)chitosan was used as an accelerator of the reaction between plasma antithrombin and a preparation of bovine thrombin: the remaining thrombin activity was then measured by using a chromogenic substrate. The materials were supplied by Kabi Diagnostica, Stockholm, Sweden. Sulfated *N*-(carboxymethyl)chitosan was also determined as a complex with antithrombin: the sample was incubated at 37° with an excess of coagulation factor Xa. The remaining amount of free factor Xa catalyzed the splitting of *p*-nitroaniline from a substrate, and *p*-nitroaniline was determined spectrophotometrically at 405 nm. Experimental details may also be found in a previous publication[13].

RESULTS AND DISCUSSION

Sulfated *N*-(carboxymethyl)chitosan was found to contain 11.0% of sulfur, indicating reaction with all of the primary alcohol groups. Accordingly, the i.r. spectra of sulfated *N*-(carboxymethyl)chitosans insolubilized with acetone at pH 1 (hydrochloric acid) and the spectra of sulfated *N*-(carboxymethyl)chitosans obtained by lyophilizing a solution of pH 8.3 showed absorption bands at 1230 and 800 cm^{-1}, assigned to the sulfate group: such bands do not occur in the spectra of *N*-(carboxymethyl)chitosan (Fig. 2 of ref. 22). In the spectrum of the polymer isolated at acidic pH, the 1730-cm^{-1} band provides evidence for the undissociated carboxyl group.



Percent. 42 ± 4 58 ± 4

Sulfur content Calculated 11.0%, found 11.0%

Average mol wt 292.5

The titration curve obtained with sulfated *N*-(carboxymethyl)chitosan sodium salt is shown in Fig. 1, where the neutralization points and the pK value are indicated. The inflection points are 4.0, 7.2, and 10.3; the first one corresponds to the final point of the carboxyl-group titration; the second is the pK_a for $-CH_2OSO_3^-$, $-N^+H_2CH_2CO_2^-$/$-CH_2OSO_3^-$, $-NHCH_2CO_2^-$, and the third one corresponds to complete titration. The isoelectric point is near pH 2.0 because of the

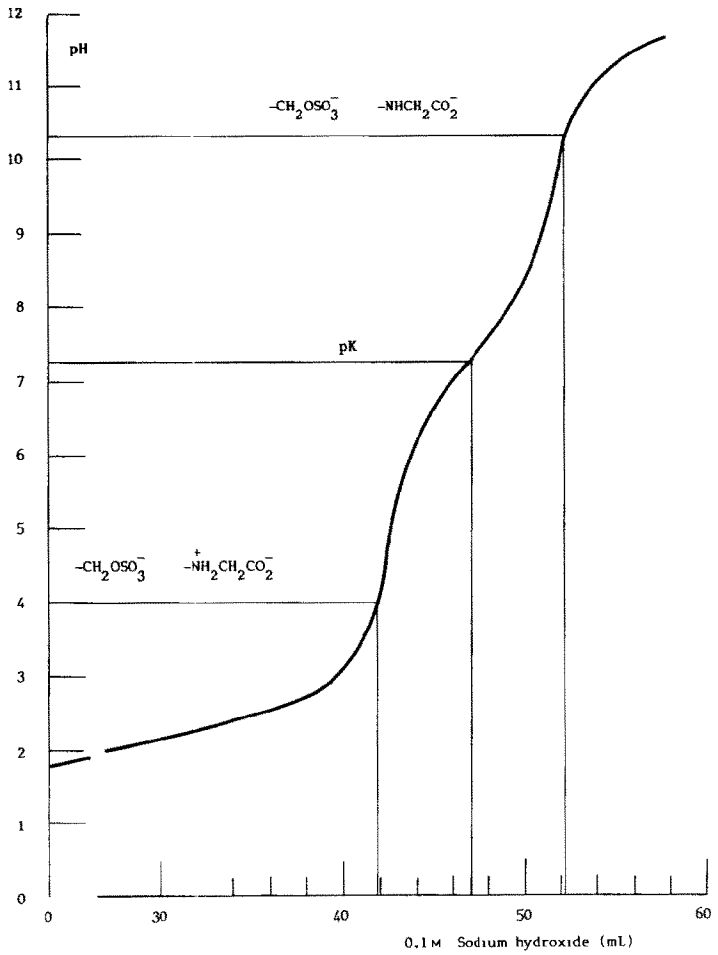


Fig. 1. Alkalimetric curve recorded for sulfated *N*-(carboxymethyl)chitosan sodium salt (0.5 g) in 0.1M hydrochloric acid (50 mL in the presence of 0.1M sodium chloride) at 25°, under nitrogen.

large proportion of acidic groups: in fact, the material is insoluble at pH below 3.5. Titration of the amino groups required 10.2 mL, in agreement with the calculated value. In comparison with data for *N*-(carboxymethyl)chitosan (Fig. 6, ref. 22) the titration interval for amino groups is narrower (10.2 mL instead of 12.5), in agreement with the increased, average molecular weight (292.5) of the repeating units. Also, the end point is closer to the theoretical value of 50.0 mL for the sulfated product, because the latter was produced and weighed as the sodium salt, whereas *N*-(carboxymethyl)chitosan was produced in acidic medium and weighed as the protonated polymer. Sulfated *N*-(carboxymethyl)chitosan contained 11% of sulfur after 3 and 15 h of sulfation, indicating that a 3-h sulfation period is sufficient.

The detection of eluates at 206 and 280 nm indicated that sonication gener-

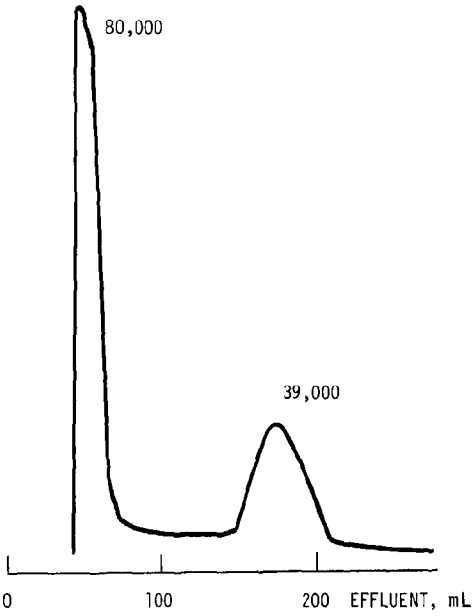


Fig. 2. Chromatogram of sulfated *N*-(carboxymethyl)chitosan (50 mg/mL, acetate buffer) on a column (76 × 1.6 cm) of Bio-Gel P-100 at 20°, flow-rate 0.5 mL/min. Automatic recordings at 206 and 280 nm were identical. Peak values determined against mol.-wt. standards of 40.000 and 80.000, under the same conditions.

ated two major fractions, as shown by Fig. 2. The elution peaks were centered at mol. wt. 39,000 and 80,000, and corresponded to ~6 and ~33% of the amount of sulfated *N*-(carboxymethyl)chitosan used; the remainder was constituted of lower oligomers.

The results of blood-anticoagulant activity tests indicated that sulfated *N*-(carboxymethyl)chitosan bound to antithrombin inhibits the thrombin present according to the following reactions, where SNCMC denotes sulfated *N*-(carboxymethyl)chitosan:

$$\text{AT} + \text{SNCMC}_{\text{excess}} = |\text{AT-SNCMC}| + \text{SNCMC}_{\text{remaining}}$$

$$|\text{AT-SNCMC}| + \text{Thrombin}_{\text{excess}} = |\text{AT-SNCMC-Thrombin}| + \text{Thrombin}_{\text{remaining}}$$

$$\text{Chromogenic substrate} \xrightarrow{\text{Thrombin}_{\text{remaining}}} \text{Peptide} + p\text{-nitroaniline}$$

Similarly, sulfated *N*-(carboxymethyl)chitosan forms a complex with antithrombin (AT) and inhibits factor Xa, thus proportionally decreasing the splitting of *p*-nitroaniline from the substrate:

$$\text{SNCMC} + \text{AT}_{\text{excess}} = |\text{SNCMC-AT}|$$

$$|\text{SNCMC-AT}| + \text{FXa}_{\text{excess}} + |\text{SNCMC-ATFXa}| + \text{FXa}_{\text{remaining}}$$

$$\text{Chromogenic substrate} \xrightarrow{\text{FXa}_{\text{remaining}}} \text{Peptide} + p\text{-nitroaniline}$$

Sulfated *N*-(carboxymethyl)chitosan was therefore effective as a blood anticoagulant, exerting its action for prolonged periods of time. Differences were observed between the sonicated and filtered samples, and the untreated samples. Untreated, sulfated *N*-(carboxymethyl)chitosan, still containing fractions of high molecular weight, when added to human blood caused a certain degree of hemolysis, platelet aggregation, and adverse phenomena in the cellular structures. In contrast, the sonicated and filtered, sulfated *N*-(carboxymethyl)chitosan (a mixture of mol. wt. 39,000 and 80,000) caused no appreciable hemolysis; the lymphocytes observed at a magnification of 400×, 24 h after treatment, did not show any undesired alteration, and resembled those in heparinized human blood. Erythrocytes were not altered in shape and volume, and no evidence of osmotic shock was observed. Their behavior on spontaneous sedimentation was quite analogous to that observed for samples treated with established products. The dose of the low-molecular-weight, sulfated *N*-(carboxymethyl)chitosan necessary for complete and definite anticoagulant action was found to be 50 I.U./mL of human blood, that is, only slightly higher than that currently used with heparin.

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