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Note

Stability studies of chondroitin sulfate

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Abstract

The stability of chondroitin sulfate (CS) was studied under acidic, neutral and basic conditions at 30 and 60 °C. CS is remarkably stable under neutral conditions at low temperature, while it degrades at 60 °C producing low-molecular-mass fragments and desulfated products. This decomposition process begins at ca. 500–600 h and is consistent with an acid-catalyzed hydrolysis of glycosidic linkages caused by a drop in pH resulting from acidic products. Under basic conditions, a breakdown of glycosidic linkages causes a decrease in molecular mass due to the β-elimination reaction, confirmed by a strong increase of absorbance at 232 nm and ^{1}H NMR. Virtually no loss of *O*-sulfate groups can be detected in the base-treated CS. Under acidic conditions, the molecular mass decreases probably through hydrolysis of polysaccharidic linkages resulting in an increased number of reducing end groups. Little or no β-elimination occurs. A loss of *O*-sulfate groups was detected, producing desulfated derivatives.

Keywords: Chondroitin; Glycosaminoglycans; Stability studies; Chemical depolymerization

Chondroitin sulfate (CS), a sulfated, linear polysaccharide formed of alternating β-GalNAc-(1→4)-β-HexA-(1→3)-, is utilized as a chondroprotective drug in the treatment of osteoarthritis [1–3]. Like heparin and other glycosaminoglycans used as drugs, CS is usually stored at neutral pH. For this reason, a study was undertaken to evaluate CS stability in a pH 7.0 solution at elevated temperatures. A second important goal is to identify the chemical processes responsible for CS's degradation. In addition to this, research also investigated possible new chemical approaches to depolymerize CS, as several CS derivatives have been studied with purportedly better biological and pharmacological (pharmacokinetic and availaibility) properties [4–6].

Bovine trachea CS used in this study has a molecular mass of ca. 26,000 (from 60,000 to 10,000) and a sulfate-to-carboxyl ratio of ca. 0.98. This preparation is a mixture of CS A (chondroitin 4-sulfate, 56%) and CS C (chondroitin 6-sulfate, 38%) (Table 1).

Fig. 1(A) shows a strong absorbance measured at 232 nm for unsaturated HexA for CS submitted to basic conditions at 30 and 60 °C, while samples examined under acidic conditions present a slight increase in optical density. The same results have been obtained by Jandik and co-workers [7] on treating heparin

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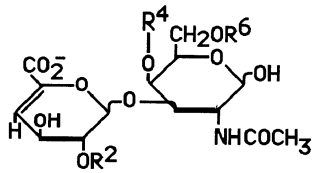
under comparable experimental conditions, suggesting that a strong base can fragment glycosaminoglycans through a β-elimination reaction forming an unsaturated HexA at the non-reducing end of each newly formed chain.

After 960 h in acidic conditions at 30 °C, no appreciable depolymerization of polysaccharide was observed, while total degradation occurred at 60 °C after 96 h, evaluated by HPSEC and PAGE. Basic treatment at 60 °C resulted in a decrease of CS molecular mass and the formation of low-molecular-mass products after about 190 h. It is noteworthy that a complete CS depolymerization occurred after 96 h of acidic treatment, while strongly basic conditions were unable to degrade polysaccharide totally, even after 960 h. Acidic treatment at 30 °C also decreased the staining intensity of electrophoretic bands as a function of time, suggesting a possible *O*-desulfation of the polysaccharide (see below).

Fig. 1(B) demonstrates a very strong rise in reducing ends in the sample treated at 60 °C, while no such increase takes place in samples treated with base. These observations can be reconciled if the aldehyde groups (detected by reducing sugar assay) are destroyed under basic conditions, as reported for polysaccharides [8] and heparin [7] treated with base.

Table 1
Disaccharide composition, peak molecular mass (M_r) and charge density values of CS used in this study



[a]	R^2	R^4	R^6	% Unsaturated disaccharides
ΔDi-0S	H	H	H	4
ΔDi-6S	H	H	SO_3^-	38
ΔDi-4S	H	SO_3^-	H	56
ΔDi-2,6diS	SO_3^-	H	SO_3^-	1.5
ΔDi-4,6diS	H	SO_3^-	SO_3^-	0.5
ΔDi-2,4diS	SO_3^-	SO_3^-	H	0.0
ΔDi-2,4,6triS	SO_3^-	SO_3^-	SO_3^-	0.0
M_r				26,000
SO_3^-/COO^-				0.98

[a] ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-galactose 6-sulfate; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-galactose 4-sulfate; ΔDi-2,6diS, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid 2-sulfate)-D-galactose 6-sulfate; ΔDi-4,6diS, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-galactose-4,6-disulfate; ΔDi-2,4diS, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid 2-sulfate)-D-galactose-4-sulfate; ΔDi-2,4,6triS: 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid 2-sulfate)-D-galactose-4,6-disulfate.

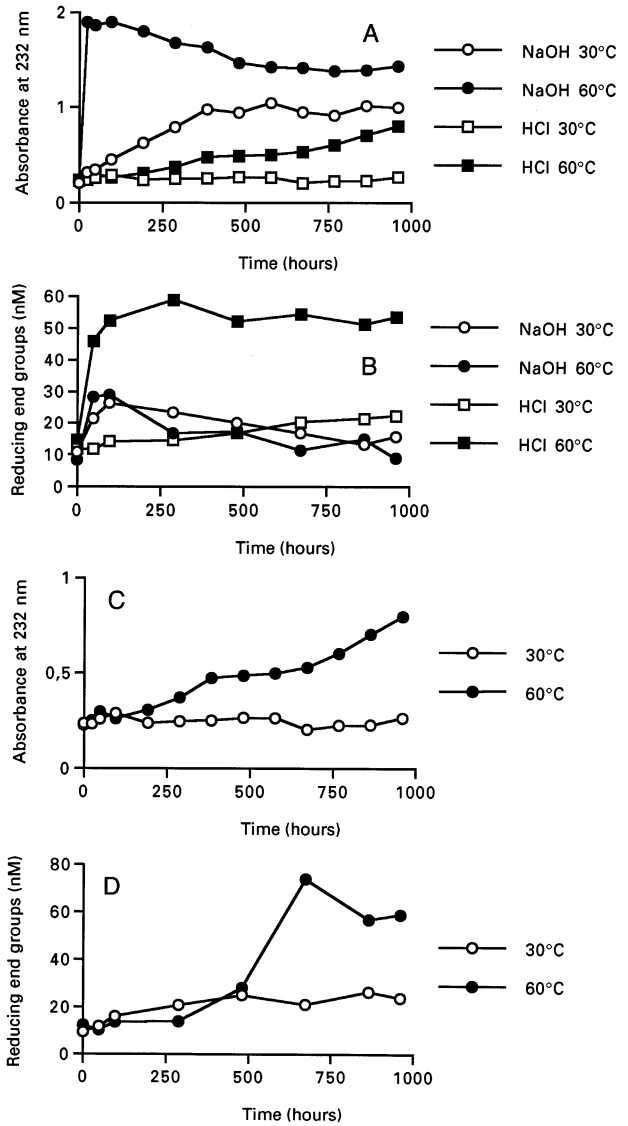


Fig. 1. (A) Absorbance at 232 nm and (B) reducing end groups as a function of time performed as reported [7] for CS treated under basic and acidic conditions. (C) Absorbance at 232 nm and (D) reducing end groups as a function of time of CS under neutral conditions. The average of three independent experiments is reported.

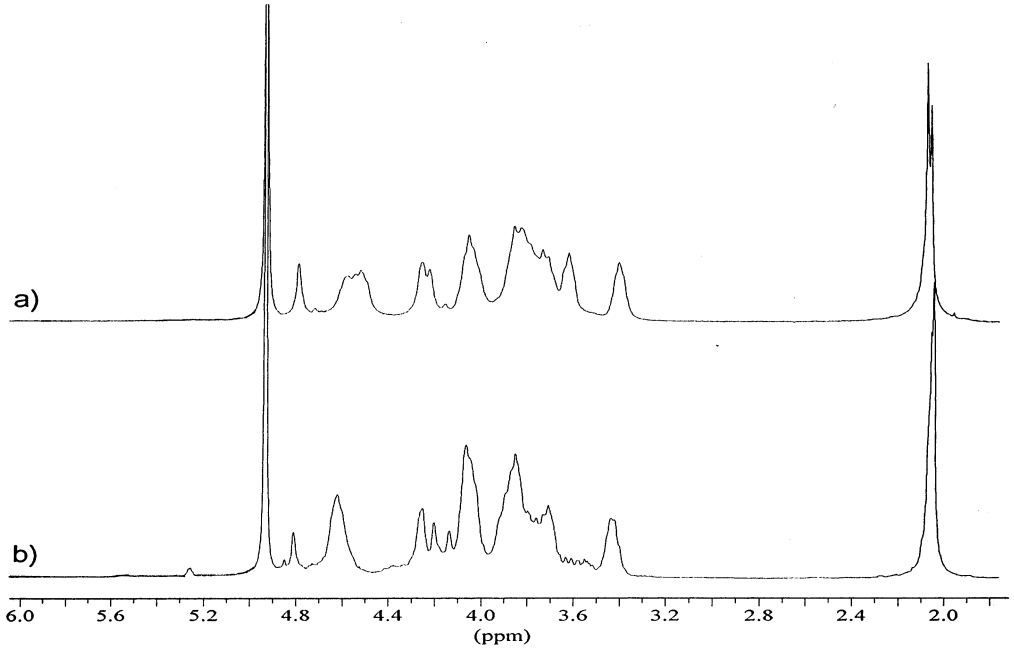


Fig. 2. ^{1}H NMR spectra of (a) CS and (b) acid-treated CS at 30 °C showing the presence of signals from terminal UH-3 and UH-4 at ca. 3.6 ppm indicating a shortening of the polysaccharide chains. The increase of NH-4 signal at 4.14 ppm is due to partial desulfation.

No appreciable desulfation of polysaccharide was observed even after 960 h under basic conditions at 30 °C and, after 480 h at 60 °C, while a reasonable level of desulfation occured in HCl at 30 °C after 960 h (ca. 14% non-sulfated disaccharide; sulfate-to-carboxyl ratio of 0.86). Under acidic conditions at 60 °C, desulfation was observed for samples after 24 h (sulfate-to-carboxyl ratio of 0.84) and 48 h (sulfate-to-carboxyl ratio of 0.79) treatment (not shown).

The ^{1}H NMR spectrum of base-treated CS at 30 °C essentially confirms the above, showing no desulfation and a slight depolymerization of polysaccharide with the appearance of new and sharp signals in two regions, from 1.7 to 2.8 and 3.4 to 4.4 ppm (not shown). Furthermore, a doublet at 5.9–6.0 ppm corresponding to the UH-4 (U = HexA) of the unsaturated HexA residue [9] supports the contention that β-elimination has taken place. In contrast, the ^{1}H NMR spectrum, 2D DQS and TOCSY spectra (not shown) of acid-treated CS at 30 °C reveal terminal saturated GlcA unit (terminal UH-3 and UH-4) signals in the 3.5–3.6 ppm region, and a significant desulfation of the polymer with an increase of non-sulfated regions (NH-4 at 4.14 ppm [N = GalNAc]) (Fig. 2).

At the beginning of the experiment, the pH of the samples was carefully adjusted to 7.0 and oxygen was excluded to eliminate oxidative degradation, according to the conditions used to study heparin stability [7]. Fig. 3 illustrates a scan of absorbance between 200 and 600 nm and Fig. 1(C) reports the absorbance at 232 nm for samples treated at 30 and 60 °C, showing a CS degradation with the formation of decomposition product(s). This effect is confirmed by PAGE (Fig. 4) and HPSEC (not shown), and by an increase of

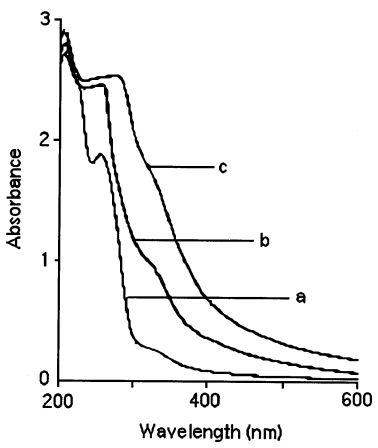


Fig. 3. Absorbance spectrum at different wavelengths (nm) of CS (a) under neutral conditions at 30 °C (b) and 60 °C (c) after 960 h.

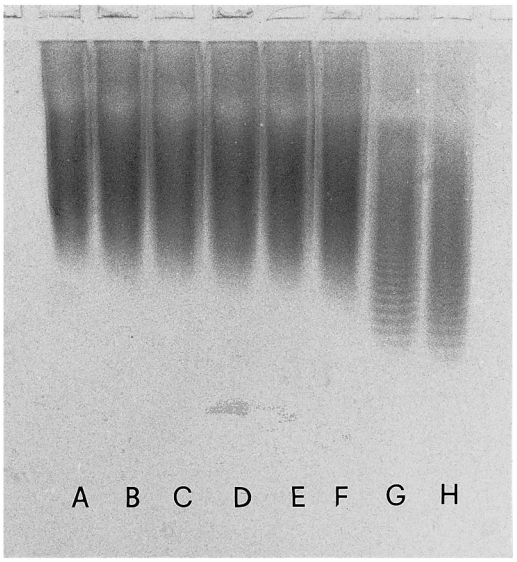


Fig. 4. PAGE according to Rice and co-workers [12] of CS under neutral conditions heated at 60 °C as a function of time. (A) control; (B) after 24 h; (C) after 48 h; (D) after 96 h; (E) after 288 h; (F) after 480 h; (G) after 768 h; (H) after 960 h.

reducing end groups (Fig. 1(D)). Analysis of unsaturated disaccharides after treatment with chondroitinase shows desulfation of polysaccharides after 960 h in neutral conditions at 30 °C (a sulfate-to-carboxyl ratio of 0.96 versus 0.98 of the control) and 60 °C (a sulfate-to-carboxyl ratio of 0.93). The ^{1}H NMR spectrum of CS treated at 60 °C confirmed a decrease of the molecular mass and a loss of sulfate groups by the appearance of new signals at 2.15, 4.67, 5.3 and 6.0 ppm (not shown).

Low-molecular-mass glycosaminoglycan (heparin, dermatan sulfate, CS and mixtures) derivatives are of great importance in therapy due to their better pharmacokinetics and availability. Apart from fractionation using gel permeation chromatography, various controlled enzymatic or chemical processes have been adopted to produce depolymerized derivatives [9,10]. One of the goals of these processes is to produce low-molecular-mass products with limited or no loss of sulfate groups, due to their paramount importance in the interaction with proteins and cellular components conferring pharmacological properties to these polyanions. In this study we observe that basic treatment of CS produces low-molecular-mass derivatives by cleaving glycosidic linkages by a β-elimination mechanism resulting in the formation of unsaturated HexA, with no or a small loss of sulfate groups, depending on temperature conditions, and a controlled base-catalyzed process could be used to produce CS derivatives with the desired molecular mass and a comparable charge density of the native polysaccharide.

1. Experimental

Materials.—Bovine trachea CS was from IBSA (Institut Biochimique SA, Lugano, Switzerland). Hydrochloric acid (0.1 M solution) was from Mallinckrodt, Paris, KY. Sodium hydroxide (0.1 M solution), anhydrous sodium phosphate dibasic, anhydrous sodium carbonate, copper sulfate, disodium 2,2′-bicinchoninate (4,4′-dicarboxy-2,2′-biquinoline, disodium salt), GlcNAc, and L-aspartic acid were from Sigma, St Louis, MO. The reagents for electrophoresis were acrylamide, bis-*N*′,*N*′-methylenebisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylenebisacrylamide (TEMED), ammonium persulfate and Alcian Blue from Sigma. All other organics and inorganics were reagent grade.

All spectrophotometric measurements were made with a Jasco V-550 UV–Vis spectrophotometer.

Treatment of CS under acidic, basic and neutral conditions.—CS (10 mg/mL) was degraded under both acidic and basic conditions, performed in 0.1 M HCl (pH 1.50) and 0.1 M NaOH (pH 12.10) solutions, respectively, essentially as reported by Jandik and co-workers for heparin [7]. These solutions were incubated in water baths at 30 and 60 °C. Aliquots were removed at various times (from 0 to 960 h) and neutralized with an equal volume of either 0.1 M NaOH or 0.1 M HCl, followed by an equal volume of 50 mM sodium phosphate buffer (pH 7.0). A 10.0 mg/mL CS solution was prepared in 10 mM sodium phosphate pH 7.0, and aliquots (1 mL) were put in 5 mL clear glass ampules under nitrogen and incubated in water baths at 30 and 60 °C. At various time intervals two ampules were removed, cooled to room temperature (rt), opened, combined, and stored

frozen at −20 °C for further analysis. The absorbance of each sample was determined at 232 nm and the samples were then stored frozen at −20 °C.

Measurement of sample color.—A sample (10 μL) was diluted with distilled water (990 μL) in a 1.0 mL quartz cuvette. The absorbance (from 200 to 800 nm) of the diluted sample was determined against a water blank.

Analysis of reducing capacity.—Samples were analyzed to determine the moles of reducing sugar present at each time point. The assay was essentially performed as reported [7].

Analysis of molecular mass.—The peak molecular mass of CS samples was determined by high-performance size-exclusion chromatography (HPSEC) as previously reported [11], against a calibration curve plotted with glycosaminoglycans standards. Heat-treated CS samples were also analyzed using gradient polyacrylamide gel electrophoresis (PAGE), essentially as reported by Rice and co-workers [12].

Analysis of constituent disaccharides and sulfate-to-carboxyl ratio.—Quantitative evaluation of CS constituent disaccharides was obtained by degradation of polysaccharide samples with chondroitinase ABC and separation of unsaturated disaccharides by HPLC at 232 nm [13]. The sulfate-to-carboxyl ratio was determined by enzymatic degradation and HPLC separation of constituent disaccharides.

^{1}H NMR analysis.—The ^{1}H NMR spectra of CS samples were recorded with a Bruker AMX 400 Wb spectrometer operating at 400.13 MHz. The samples were previously lyophilized three times with D_2O. The ^{1}H chemical shifts (δ) were quoted with respect to external sodium 4,4-dimethyl-4-silapentene-1-sulfonate (0.0 ppm). DQS [14] parameters: spectral width (f_2) = 5 ppm, 2048 complex point; spectral width (f_1) = 10 ppm, 512 t_1 increments with 32 scans per t_1 value; relaxation delay = 0.2 s, 18–70 ms evolution time, 135° read pulse. NOESY–TPPI [15] parameters: spectral width (f_2) = 6.5 ppm, 2048 complex point; spectral width (f_1) = 6.5 ppm, 256–512 t_1 increments with 64 scans per t_1 value; relaxation delay = 0.2 s; mixing time = 50 ms. TOCSY–TPPI [16] parameters: spectral width (f_2) = 6.5 ppm, 2048 complex point; spectral width (f_1) = 6.5 ppm, 256 t_1 increments with 32 scans per t_1 value; relaxation delay = 0.2 s; spin-lock field of 4 kHz applied for 70 ms.

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