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Activity of chondroitin ABC lyase and hyaluronidase on free-radical degraded chondroitin sulfate

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Abstract

High molecular mass-chondroitin sulfate was characterized for M_r, charge density and constituent disaccharides. This glycosaminoglycan was depolymerized by a controlled free-radical process mediated by hydrogen peroxide in the absence or presence of cupric or ferrous ions. Hydrogen peroxide depolymerizes chondroitin sulfate, and the velocity of the reaction increases in the presence of cupric ions and, further, of ferrous ions. Different low molecular mass-chondroitin sulfate fractions were produced and analyzed by high-performance size-exclusion chromatography and polyacrylamide-gel electrophoresis. This last technique strongly supports the hypothesis that the free-radical process proceeds by the destruction of disaccharide units.

The treatment of free-radical chondroitin sulfate samples with chondroitinase ABC and testicular hyaluronidase results in a lower capacity of these enzymes to degrade these glycosaminoglycan derivatives with respect to the natural sample. This was confirmed by polyacrylamide-gel electrophoresis and by the time-courses of enzymatic treatment evaluated by spectrophotometric technique (for treatment with chondroitin ABC lyase).

Keywords: Glycosaminoglycans; Chondroitin sulfate

1. Introduction

Free-radical depolymerization mediated by cupric or ferrous ions has been used to degrade natural polymers such as DNA, hyaluronic acid [1], heparin [2–4], and dermatan

Abbreviations: M_r, relative molecular mass; HPSEC, high-performance size-exclusion chromatography; LMM, low molecular mass.

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sulfate [5,6]. In this paper, high molecular mass-chondroitin sulfate was depolymerized by a controlled free-radical process in the presence or absence of ions. The different low molecular mass (LMM)-chondroitin sulfates obtained were analyzed for peak molecular mass (M_r), using polyacrylamide-gel electrophoresis according to Rice et al. [7], and for the capacity of chondroitin ABC lyase and testicular hyaluronidase to degrade these derivatives. This study could help to understand a radical depolymerization process useful for producing LMM-chondroitin sulfate derivatives with possible application in pharmacological therapy.

2. Materials and methods

Preparation of different free-radical depolymerized chondroitin sulfate.—Chondroitin sulfate from shark donated by IBSA, Institut Biochimique SA, Lugano, Switzerland, was treated with hydrogen peroxide alone or with hydrogen peroxide in the presence of copper acetate, ferrous chloride or ferrous sulfate as follows: 5 g of chondroitin sulfate sodium salt and 0.2 g of copper acetate monohydrate or ferrous chloride or ferrous sulfate (0.02 M) were dissolved in 50 mL of water in a reaction vessel (in the first set of experiments no bivalent cations were added). The temperature was kept at +60°C and the pH adjusted to 7.5 by adding M NaOH solution. A 9% hydrogen peroxide solution was added at a rate of 10 mL/h (1 mL/6 min for the length of the whole reaction). The reaction for chondroitin sulfate was stopped at different times by adding Chelex 100 chelating resin (Bio-Rad, Richmond, CA, USA, cod. 142-2832) to remove contaminating copper or ferrous ions from the product. The pH of the percolate was adjusted to 6.0 by addition of acetic acid, and then two volumes of acetone saturated with sodium acetate were added to precipitate LMM-chondroitin sulfates sodium salt. The precipitate was collected by centrifugation and dried. Different LMM-chondroitin sulfate samples were prepared by stopping the chemical depolymerization process at different times. The sum in weight of all LMM-chondroitin sulfate fractions (sodium salt form) produced about 4.80 g corresponding to a yield of 96%.

Time courses of chondroitin ABC lyase and hyaluronidase degradation of native chondroitin sulfate and free-radical depolymerized samples.—LMM-chondroitin sulfate samples (10 mg/50 μL) were incubated with 125 mU (50 μL) of chondroitinase ABC [E.C. 4.2.2.4] (Seikagaku Kogyo Co.) in 100 μL of 50 mM Tris–HCl buffer, pH 8.0 at 37°C. Aliquots (10 μL) were collected at different times; 5 μL were stored at −20°C until used for polyacrylamide-gel electrophoresis analysis, and 5 μL were tested for spectrophotometric absorbance at 232 nm after adding 500 μL of 30 mM HCl.

LMM-chondroitin sulfate samples (10 mg/50 μL) were incubated with 580 U (2 mg in 100 μL) of testicular hyaluronidase [E.C. 3.2.1.35] (Sigma) in 250 μL of 100 mM phosphate buffer, pH 5.0 containing 0.15 M NaCl at 37°C. Aliquots (10 μL) were collected at different times and stored at −20°C until used for polyacrylamide-gel electrophoresis analysis.

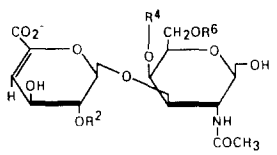
Polyacrylamide-gel electrophoresis.—Electrophoresis was performed on a Bio-Rad Protean IIxi vertical-slab-gel unit connected with an LKB 2219 Multitemp II thermostatic circulator. Power was supplied by an LKB 2197 power supply. The gel was

prepared essentially as reported by Rice et al. [7]. The resolving gel and lower buffer chamber contained 0.1 M boric acid/0.1 M Tris/0.01 M disodium EDTA buffer, pH 8.3. The stacking gel was prepared in the same buffer adjusted to pH 6.3 with HCl. Gels were stained for 30 min in 0.08% (w/v) Azure A in water. Destaining was accomplished with successive rinsing.

Determination of relative molecular mass.—HPLC equipment was from Jasco. Pump mod. 880 PU, system controller mod. 801 SC, ternary gradient unit mod. 880-02, Rheodyne injector equipped with a 100 μL loop, UV detector, mod. 875 UV. The mobile phase was composed of 125 mM Na_2SO_4 and 2 mM NaH_2PO_4 adjusted to pH 6.0 with 0.1 M NaOH. Flow rate was 0.9 mL/min with a back pressure of 25 kg/cm^2. Columns Protein Pak 125 and 300 assembled in series (Waters, 84601 and T72711) were used. Different LMM-chondroitin sulfate samples were solubilized in the mobile phase at a concentration of 10 mg/mL; 10 μL (100 μg) were injected in HPLC. The peak M_r of LMM-chondroitin sulfate samples was determined by a calibration curve plotted with glycosaminoglycan standards, as reported elsewhere [8]. The third grade polynomial regression was calculated by a Macintosh computer program.

Constituent disaccharides quantitation by cleavage with chondroitinase ABC.—100 μg (10 mg/mL in H_2O) of high M_r chondroitin sulfate were incubated with 50 mU of chondroitinase ABC [E.C. 4.2.2.4] in 50 mM Tris-HCl buffer, pH 8.0. The reaction was

Table 1
The percentage of disaccharides derived from the polysaccharide chains of shark chondroitin sulfate (CS) by chondroitin ABC lyase cleavage



Unsaturated disaccharides	Structure (see formula above)			Disaccharide percentage [a]
	R^2	R^4	R^6	CS
ΔDi-0S	H	H	H	5.1
ΔDi-6S	H	H	SO_3^-	39
ΔDi-4S	H	SO_3^-	H	29
ΔDi-2,6diS	SO_3^-	H	SO_3^-	23.3
ΔDi-4,6diS	H	SO_3^-	SO_3^-	3.0
ΔDi-2,4diS	SO_3^-	SO_3^-	H	0.5
ΔDi-2,4,6triS	SO_3^-	SO_3^-	SO_3^-	0.0
M_r				55,720
Anion ratio [b]				1.22

[a] The amount of each identified disaccharide was determined using purified standards and reported as weight percentage. Under experimental conditions, cleavage of native chondroitin sulfate samples with chondroitinase ABC produces about 95% disaccharides.

[b] The sulfate-to-carboxyl ratio was determined by enzymatic degradation after HPLC separation of constituent disaccharides and calculated considering the percentage and the presence of carboxyl and sulfate groups for each disaccharide, and by titrimetric determination as reported elsewhere [5].

stopped after 3 h of incubation at 37°C by 1 min boiling. Constituent disaccharides were determined by strong-anion exchange (SAX)-HPLC on Spherisorb 5 SAX, 250 × 4.6 mm. Isocratic HPLC (Jasco, as above) was performed from 0 to 5 min with 0.10 M NaCl pH 4.00; linear gradient separation from 5 to 60 min with 100% 0.10 M NaCl pH 4.00 to 100% 1.20 M NaCl pH 4.00. Flow was 1.4 mL/min. UV wavelength 232 nm; 20 μg of enzymatically degraded chondroitin sulfate injected. Unsaturated disaccharides were separated using the standards and retention times recommended by Seikagaku Kogyo Co. [9].

3. Results and discussion

Possible contaminant glycosaminoglycans (dermatan sulfate, heparin and heparan sulfate) in the preparation of chondroitin sulfate were determined by agarose-gel electrophoresis, as reported elsewhere [10]. No heparin, heparan sulfate or dermatan sulfate were detected in the native chondroitin sulfate by this technique. A small amount (2–5% w/w) of material resistant to treatment with chondroitinase ABC was detected and classified as keratan sulfate type II on the basis of cellulose acetate electrophoresis [11].

Table 1 reports the physico-chemical properties and disaccharide composition of the chondroitin sulfate purified from shark. This chondroitin sulfate has a high M_r (about 55,720) and sulfate-to-carboxyl ratio (about 1.22) due to a great amount of disulfated disaccharides, in particular of the disaccharide disulfated in position 2 of glucuronic acid and in position 6 of *N*-acetylgalactosamine (about 23%). Chondroitin sulfate from shark has very different physico-chemical properties and structure than that purified from beef trachea [5,12].

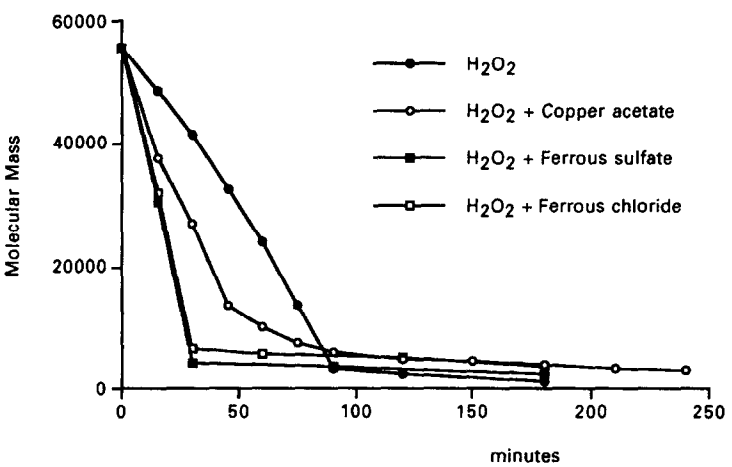


Fig. 1. Decrease of molecular mass depending on time of chondroitin sulfate submitted to treatment with hydrogen peroxide alone and hydrogen peroxide in the presence of copper acetate, ferrous sulfate, and ferrous chloride.

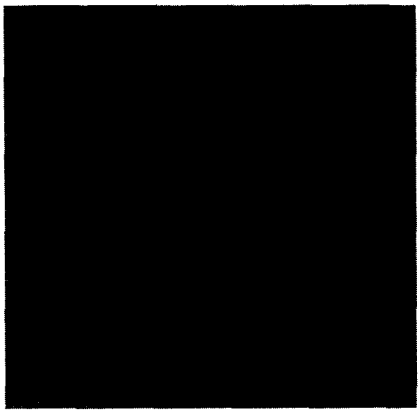


Fig. 2. Polyacrylamide-gel electrophoresis of chondroitin sulfate (**1**, peak M_r = 55,720) and free-radical depolymerized chondroitin sulfate samples (in the presence of hydrogen peroxide and copper acetate) at different times of reaction (**2**: after 15 min, peak M_r = 37,680; **3**: after 30 min, peak M_r = 26,850; **4**: after 45 min, peak M_r = 13,620; **5**: after 60 min, peak M_r = 10,180; **6**: after 75 min, peak M_r = 7,440; **7**: after 90 min, peak M_r = 6,180; **8**: after 120 min, peak M_r = 4,880; **9**: after 150 min, peak M_r = 4,450; **10**: after 180 min, peak M_r = 3,880; **11**: after 210 min, peak M_r = 3,460).

Fig. 1 shows the decrease of M_r depending on time of chondroitin sulfate submitted to treatment with hydrogen peroxide in the absence and presence of copper and ferrous salts. At 60°C and pH 7.0–7.5 hydrogen peroxide is able to degrade chondroitin sulfate. The process of depolymerization is increased in the presence of copper and ferrous salts.

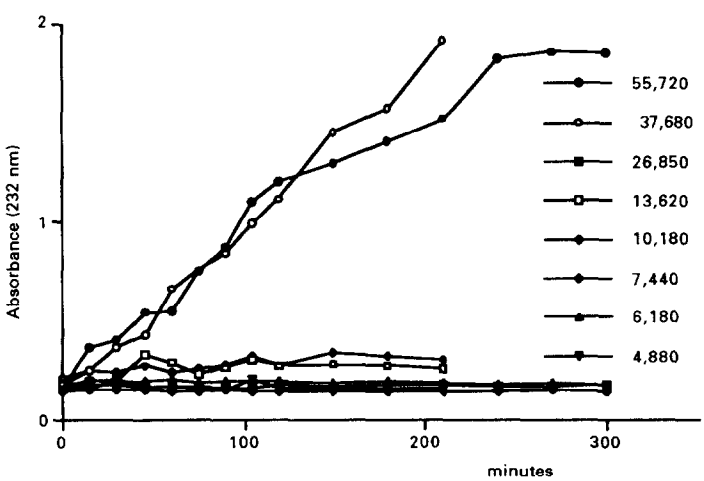


Fig. 3. Time courses of chondroitin ABC lyase degradation of the shark chondroitin sulfate and of free-radical depolymerized chondroitin sulfates samples. The peak M_r of each samples are indicated in the figure.

Moreover, ferrous sulfate and ferrous chloride produce the same effect on the degradation of chondroitin sulfate. No effect was observed in the absence of hydrogen peroxide. The LMM-chondroitin sulfate samples obtained by the radical process do not show significant desulfatation under the experimental conditions used. This was proved by titrimetric evaluation of sulfate-to-carboxyl ratio [5], by their capacity to bind cationic dyes such as toluidine blue and to interact with cationic groups of anion-exchange resins such as Ecteola-cellulose (data not shown).

Fig. 2 shows the polyacrylamide-gel electrophoresis of the native chondroitin sulfate and the free-radical depolymerized chondroitin sulfate samples at different times of reaction. The peak M_r of free radical depolymerized chondroitin sulfate samples evaluated by HPSEC are reported in the figure legend. Analysis by polyacrylamide-gel electrophoresis supports the hypothesis that the free-radical reaction degrades chondroitin sulfate at the disaccharide units. This was also observed for dermatan sulfate depolymerized under the same experimental conditions [13].

Fig. 3 shows the time courses of chondroitin ABC lyase degradation of the native chondroitin sulfate and free-radical depolymerized chondroitin sulfate samples measured by spectrophotometric analysis at 232 nm. As illustrated in the figure, native chondroitin sulfate is extensively degraded by chondroitinase ABC and the enzymatic reactions reach equilibrium after about 4–5 h under the experimental conditions adopted. Free-

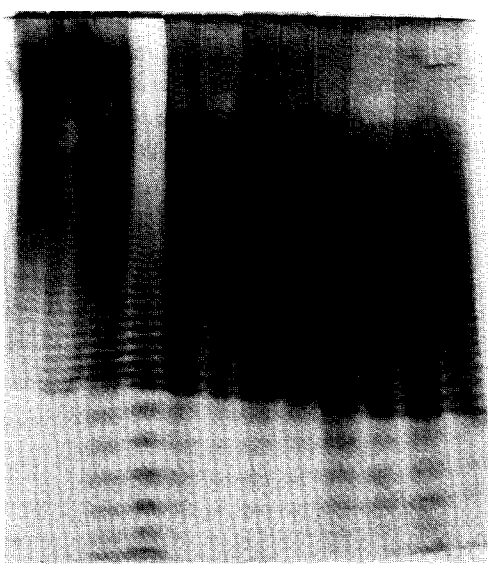


Fig. 4. Polyacrylamide-gel electrophoresis of the time course of chondroitin ABC lyase degradation of shark chondroitin sulfate (**A**: after 2 min, **B**: after 60 min, **C**: after 120 min and **D**: after 180 min of treatment with chondroitinase ABC), of free-radical degraded chondroitin sulfate with M_r = 26,850 (**E**: after 2 min, **F**: after 60 min, **G**: after 120 min and **H**: after 180 min of treatment with chondroitinase ABC) and of free-radical degraded chondroitin sulfate with M_r = 10,180 (**I**: after 2 min, **L**: after 60 min, **M**: after 120 min and **N**: after 180 min of treatment with chondroitinase ABC).

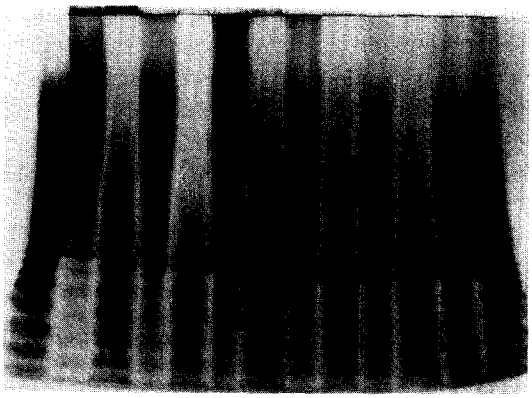


Fig. 5. Polyacrylamide-gel electrophoresis of the time course of testicular hyaluronidase degradation of shark chondroitin sulfate (**A**: after 2 min and **B**: after 4 h of treatment with hyaluronidase), of free-radical degraded chondroitin sulfate with $M_r = 37,680$ (**C**: after 2 min and **D**: after 5 h of treatment with hyaluronidase), of free-radical degraded chondroitin sulfate with $M_r = 26,850$ (**E**: after 2 min and **F**: after 5 h of treatment with hyaluronidase), of free-radical degraded chondroitin sulfate with $M_r = 10,180$ (**G**: after 2 min and **H**: after 5 h of treatment with hyaluronidase), of free-radical degraded chondroitin sulfate with $M_r = 6,180$ (**I**: after 2 min and **L**: after 5 h of treatment with hyaluronidase), and of free-radical degraded chondroitin sulfate with $M_r = 4,880$ (**M**: after 2 min and **N**: after 5 h of treatment with hyaluronidase). Chondroitin sulfate partially treated with chondroitinase ABC was used as standard of oligosaccharide species (**1**).

radical depolymerized chondroitin sulfate sample after 15 min of treatment (corresponding to $M_r = 37,680$) is also extensively degraded by chondroitin ABC lyase. On the contrary, the free-radical treated samples obtained after 30 min of reaction are not depolymerized by lyase even after 4 h of treatment. This is confirmed in Fig. 4. As shown in polyacrylamide-gel electrophoresis separation of saccharide species, native chondroitin sulfate is progressively degraded by chondroitinase ABC (Fig. 4) producing oligosaccharides constituted from 1 to about 25 disaccharide units. On the contrary, free-radical depolymerized chondroitin sulfate after 30 min ($M_r = 26,850$) and after 60 min ($M_r = 10,180$) are not, or only to very limited extent, degraded by lyase even after 180 min of enzymatic reaction. Fig. 4 also demonstrates that free-radical mediated degradation of chondroitin sulfate proceeded at disaccharide units. In fact, as evident after polyacrylamide-gel electrophoretic separation, oligosaccharides with the same degree of polymerization are produced for native chondroitin sulfate treated with lyase and for chondroitin sulfate samples obtained after 30 min and, even more, after 60 min of chemical degradation mediated by hydrogen peroxide and copper salt.

Hyaluronidase, an hydrolase involved in the catabolic processes of hyaluronic acid and proteoglycans in human, was also tested for its capacity to degrade the free-radical depolymerized chondroitin sulfate. As this enzyme acts through hydrolysis, rather than through an eliminase mechanism producing unsaturated oligosaccharide products, its activity was measured by polyacrylamide-gel electrophoretic separation of product of reaction (Fig. 5). As illustrated in the figure, hyaluronidase is able to degrade native chondroitin sulfate (after 4 h of enzymatic treatment) and free-radical samples produced

after 15 (M_r = 37,680), 30 (M_r = 26,850), 60 (M_r = 10,180) and 90 (M_r = 6,180) min of chemical process (after 5 h of enzymatic treatment). On the other hand, hyaluronidase (also after 5 h of treatment) is unable to act on free-radical chondroitin sulfate obtained after 120 (M_r = 4,880) min of chemical process. In every case, testicular hyaluronidase was more able to degrade free-radical depolymerized chondroitin sulfate fractions than chondroitin ABC lyase, in particular those with M_r higher than about 5,000.

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