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Depolymerization of hyaluronan by sonication

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High molecular weight hyaluronan (M_r 400 000) obtained from human umbilical cord was depolymerized by sonication for 10 h into small molecules and finally into molecules of constant size (M_r 11 000). The molecular size of the depolymerized hyaluronan was unaltered even under different conditions of sonication. After sonication, the main sugar residues at the reducing and non-reducing termini of depolymerized hyaluronan were *N*-acetylglucosamine (86%) and glucuronic acid (98%), respectively. Hyaluronans derived from rooster comb (M_r 1×10^6) and *Streptococcus zooepidemicus* (M_r 1.2×10^6) were depolymerized into molecules of different but characteristic sizes by sonication. On the other hand, neither chondroitin sulfate nor glycogen was depolymerized by sonication. These results suggest that high molecular weight hyaluronan may have some weak linkages related to *N*-acetylglucosamine in the chain, which are extremely sensitive to sonication. At present, however, the nature of these linkages is still unclear.

Keywords: hyaluronan (hyaluronic acid); sonication; depolymerization

Abbreviations: HA, hyaluronan; PA, 2-aminopyridine.

Hyaluronan (hyaluronic acid, HA) is a major constituent of extracellular matrix and plays some important biological roles [1, 2]. It has also been isolated from certain strains of bacteria, such as Streptococcus [3, 4]. The current concept of the fundamental structure of HA is a long linear structure without side chains, composed of repeating disaccharide units: *N*-acetyl-D-glucosamine and D-glucuronic acid [4–7]. High molecular weight HA has been shown to undergo spontaneous decrease in viscosity in solution and depolymerization upon heating, phenomena which do not occur with other glycosaminoglycans such as chondroitin sulfate.

Moreover, it is also known that HA molecules are depolymerized by UV irradiation [8], oxidative reaction with substances such as L-ascorbic acid, cysteine and ferrous salts [9, 10], and sonication [11, 12]. In the present study, we examined the depolymerization of HA by sonication.

Materials and methods

Materials

HA was isolated from human umbilical cord by the method of Danishefsky and Bella [13], and then further purified by anion-exchange chromatography on Dowex 1-X2 (Dow Chemical Co. Midland, MI, USA) and gel filtration on Sephacryl S-200 (Pharmacia, Uppsala, Sweden) in 4 M guanidine-HCl [14]. The purified HA (M_r 400 000) was completely digestible with Streptomyces hyaluronidase (Seikagaku Kogyo, Tokyo, Japan). Other HAs (M_r 1×10^6 from rooster comb and M_r 1.2×10^6 from *Streptococcus zooepidemicus*) and glycogen (M_r 1×10^6) were purchased from Sigma (St. Louis, MO, USA).

Chondroitin 6-sulfate (M_r 43 000, from shark cartilage), chondroitin 4-sulfate (M_r 31 000, from whale cartilage) and heparan sulfate (M_r 15 000, from bovine kidney) were purchased from Seikagaku Kogyo. Chondroitin (M_r 8000), dodeca- (M_r 2294), deca- (M_r 1915), hexa- (M_r 1156) and tetrasaccharide (M_r 777) derived from HA were the same as described previously [14, 15].

Sonication methods

Five milliliteres of a 5 mg ml^{-1} solution of HA in distilled water were transferred to a plastic tube in an ice-bath. The water used had been distilled twice, and degassed with a sonicator. The probe of a sonicator (Bronson, Model 250) was immersed in the HA solution, and sonication was performed at 20 kHz and 7.5 W. The sonication time was

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prolonged successively. After sonication, the HA sample was monitored by HPLC.

High performance liquid chromatography

A liquid chromatograph (Hitachi, L-6000) equipped with a refraction index monitor (Hitachi, L-3300) and a fluorescence spectrometer (Hitachi, F-1050) was used. For detection of fluorescence of pyridylaminated (2-aminopyridine, PA) sugar, an excitation wavelength of 320 nm and an emission wavelength of 400 nm was used [16].

HPLC for size fractionation analysis was carried out with a Shodex OHpak KB-803 (8 mm × 300 mm) and a KB-805 (8 mm × 300 mm) column (Showa Denko, Tokyo, Japan) using 0.2 M NaCl at 30 °C.

Determination of reducing sugar

One milligram of HA (before and after sonication) was reduced with NaB^3H_4 (100 μCi, specific activity 40 Ci mmol^{-1}, ICN Radiochemicals, Irvine, CA, USA) in 50 mM sodium borate buffer, pH 8.0, at room temperature for 2 h according to the method of Majima *et al.* [17]. After recovering the radioactive sugar alcohol at the reducing termini with trifluoroacetic acid and nitrous acid, the sugar alcohol was separated into acidic and basic fractions using Dowex 1-X2 and Dowex 50W-X2.

Finally [^{3}H]gulonolactone and [^{3}H]glucosaminitol, derived from glucuronic acid and *N*-acetylglucosamine by reduction at the reducing termini with NaB^3H_4, were identified by paper chromatography (solvent, 1-butanol: pyridine:water, 6:4:3 by vol) and assayed using a liquid scintillation counter (Aloka, LSC 3500).

Determination of non-reducing terminal sugar

One milligram of HA was incubated with rabbit liver exo-β-glucuronidase (exo-β-*N*-acetylglucosaminidase-free, Sigma) [14]. In another experiment, 1 mg HA was incubated with bovine epididymal exo-β-*N*-acetylglucosaminidase (Sigma), together with 0.1 mg ml^{-1} saccharo-1,4-lactone as an inhibitor of exo-β-glucuronidase. The monosaccharides released from the non-reducing terminus of HA in each experiment were coupled separately with PA, and the amounts of PA-monosaccharide (PA-glucuronic acid and PA-*N*-acetylglucosamine) were determined using HPLC according to the method of Takagaki *et al.* [16].

Results

HA depolymerization by sonication

High molecular weight HA depolymerized into small molecules by sonication at 20 kHz and 7.5 W. The relationship between the sonication period and degree of depolymerization of HA was monitored by HPLC using a Shodex OHpak KB-805 (Fig. 1). Within 1 min after the start of sonication, depolymerization of HA was observed. Prolonged

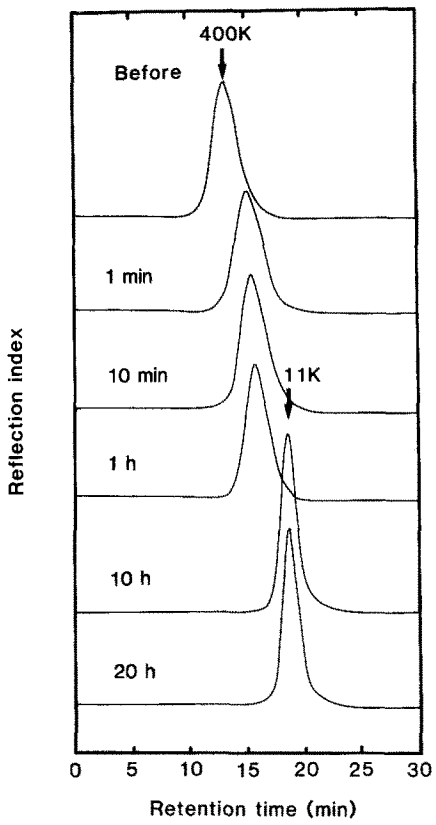

Figure 1. HPLC of sonicated HA. Depolymerization of HA by sonication was monitored by size fractionation HPLC (column, KB-805). As molecular weight markers, HA (M_r 1 × 10^6), chondroitin 6-sulfate (M_r 43 000), chondroitin 4-sulfate (M_r 31 000), heparan sulfate (M_r 15 000), chondroitin (M_r 8000), and dodeca- (M_r 2294), deca- (M_r 1915), hexa- (M_r 1156) and tetrasaccharide (M_r 777) derived from HA, were used.

sonication resulted in gradual depolymerization of HA, but after 10 h no further depolymerization was evident. Under different conditions of sonication at 28 kHz and 20 W, the molecular size of the depolymerized HA was the same as that obtained by sonication at 20 kHz and 7.5 W (data not shown). These results indicated that HA obtained from human umbilical cord was depolymerized in an orderly manner into units of a final constant size. As proteoglycans are degraded by superoxide radicals [18], glutathione (10 mmol ml^{-1}) was added to the HA solution as an active oxygen scavenger during sonication. However, the addition of glutathione did not influence HA depolymerization. The depolymerized HA did not repolymerize after it was left to stand in water at 4 °C for 30 days.

The molecular weight of the depolymerized HA was estimated to be about 11 000 by HPLC using a Shodex OHpak KB-803. No oligosaccharides or monosaccharides were found other than the M_r 11 000 depolymerized HA. As no increase in absorbance at 232 nm was observed after sonication, the depolymerization could not have occurred through an elimination reaction. Furthermore, the

Table 1. Depolymerization of HA under various conditions. After sonication and chemical treatments of HA, depolymerization was checked by size fractionation HPLC.

Treatment	*Depolymerization*[a]
Sonication (20 kHz, 7.5 W, 10 min, 0 °C)[b]	+
Sonication (28 kHz, 20 W, 10 min, 0 °C)[c]	+
7 M Urea (20 °C, 12 h)[c]	−
4 M Guanidine-HCl (20 °C, 12 h)[c]	−
2% EDTA (20 °C, 12 h)[c]	−
Delipidation with chloroform-methanol (2:1)[b]	−
2% β-Mercaptoethanol (50 °C, 24 h) and alkylation with 0.75 M iodoacetate[b]	−
0.05 N NaOH − 1 M $NaBH_4$ (50 °C, 48 h)[b]	−
0.01 N HCl (80 °C, 1 h)[b]	−
Pronase digestion in 25 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$[b]	−

[a] +, Depolymerized; −, not depolymerized.
[b] The solvent used for HPLC was 0.2 M NaCl.
[c] The solvent used for HPLC was the same as the solution used in each treatment.

depolymerized HA was completely digestible with Streptomyces hyaluronidase. These results suggested that the fundamental structure of HA was still preserved after sonication.

Chemical treatment of HA

In order to examine the nature of the chemical bond in high molecular weight HA related to depolymerization by sonication, HA was treated with various chemicals (Table 1), and the HPLC elution profiles before and after each treatment were compared. The elution profiles of HA did not change after treatment with 7 M urea, 4 M guanidine-HCl or 2% EDTA. Furthermore, depolymerization was observed after other chemical treatments, such as delipidation [19], reduction with β-mercaptoethanol [20], hydrolysis with alkali (0.05 N NaOH) [21] or acid (0.01 N HCl) [22] and Pronase digestion. No weak bonds susceptible to chemical treatments were found in HA.

Reducing and non-reducing terminal sugar of depolymerized HA

After sonication (20 kHz and 7.5 W for 16 h), depolymerized HA was recovered, and then the reducing and non-reducing termini of the HA were examined. As shown in Table 2, *N*-acetylglucosamine residues accounted for the majority of the reducing termini of non-sonicated HA. After sonication, the quantity of these residues at the reducing termini was increased 26-fold. On the other hand, all of the non-reducing terminal sugars of non-sonicated HA were glucuronic acid, and only glucuronic acid residues were found to increase after sonication. These results indicated that the depolymerization of HA by sonication occurred at the *N*-acetylglucosaminide linkages in the HA chain.

Table 2. Reducing and non-reducing terminal sugars of HA before and after sonication.[a]

Terminal sugar	*Sonication*	
	Before	*After*
Reducing terminal sugar	100(*1)	2623
Glucuronic acid	7	379
N-Acetylglucosamine	93	2244
Non-reducing terminal sugar	100(*2)	4800
Glucuronic acid	100	4700
N-Acetylglucosamine	0	100

[a] The total glucuronic acid and *N*-acetylglucosamine residues at the reducing (*1) and non-reducing termini (*2) before sonication were estimated to be 100%, respectively. Reducing terminal sugars were determined as follows: 1 mg HA (before and after sonication) was reduced with NaB^3H_4. After hydrolysis with 4 N HCl at 100 °C for 4 h, the hydrolysate was separated into acidic and basic fractions using Dowex 1-X2 and Dowex 50W-X2. Finally, [^{3}H]gulonolactone and [^{3}H]glucosaminitol were identified by paper chromatography (solvent, 1-butanol: pyridine:water, 6:4:3 by vol) and assayed by liquid scintillation counting. Non-reducing terminal sugars were determined as follows: Each 1 mg of HA was incubated with exo-β-glucuronidase or exo-β-*N*-acetylglucosaminidase. The monosaccharides released from the non-reducing terminus of HA were coupled with 2-aminopyridine, and the amounts of pyridylaminated glucuronic acid and *N*-acetylglucosamine were determined using HPLC.

Depolymerization of HAs from different sources

HAs obtained from different sources, human umbilical cord, rooster comb and *Streptococcus zooepidemicus*, were sonicated under the same conditions at 20 kHz and 7.5 W at 10 °C for 10 h. Each was depolymerized into units of different sizes (Table 3). Depolymerization of some polysaccharides was also examined; chondroitin 4-sulfate, chondroitin 6-sulfate and glycogen were not depolymerized at all under the same

Table 3. Effect of sonication on HAs derived from various sources.[a]

Source of HA	*Molecular mass*	
	Before	*After*
Human umbilical cord	400	11
Rooster comb	1000	3
Streptococcus zooepidemicus	1200	60

[a] Sonication was performed at 20 kHz, 7.5 W at 0 °C and continued until no further degradation was observed by HPLC.

conditions as those used for HA depolymerization (data not shown).

Discussion

High molecular weight HA (M_r 400 000) was depolymerized by sonication, finally breaking down into molecules of constant size (M_r 11 000). The molecular size of the depolymerized HA did not change under different conditions of sonication. However, HAs obtained from different sources were depolymerized into unit of different and characteristic sizes. Moreover, chondroitin 4-sulfate and 6-sulfate and glycogen were not depolymerized at all by the sonication. Therefore, it seems that the depolymerization of HA by sonication is essentially specific for the HA molecule of high molecular weight. Furthermore, it is likely that the HA molecule includes a special chemical structure, which is sensitive to sonication.

In this experiment, no monosaccharides or oligosaccharide fragments of HA were found in the sonication products, and no increase in absorbance at 232 nm was observed. Therefore, the depolymerization did not occur through an elimination reaction of glycosidic linkages in the HA molecule. The depolymerized HA was completely digested by Streptomyces hyaluronidase, which is absolutely specific for HA. Therefore, the depolymerized HA still retained the fundamental structure of HA polymers. The results of treatment with various chemicals indicated that the HA molecule did not contain disulfide bonds, hydrogen bonds, hydrophobic bonds, ion linkages, or linkages sensitive to weak alkali or acid.

The reducing and non-reducing terminal residues of the depolymerized HA were *N*-acetylglucosamine and glucuronic acid, respectively, indicating that the depolymerization of the HA chain by sonication occurred at the *N*-acetylglucosaminide linkage. It was unclear whether an unknown special glycosidic linkage sensitive to sonication, other than the already established linkages, is present.

High molecular weight HA undergoes catabolic depolymerization in tissues and organs, and the maximum molecular weight of HA excreted in urine seems to be about 12 000 kDa [23]. Recently, we reported that HA in culture medium of human skin fibroblasts was depolymerized into small molecules of constant size by an enzyme different from already known hyaluronidases. This constant size of the depolymerized HA may indicate that HA is composed of equal subunits.

In cell or tissue culture systems, high molecular weight HA is synthesized very rapidly without any intermediate product. Despite numerous investigation of HA biosynthesis, the mechanisms responsible for producing a molecule as large as HA in such a short time is still unclear. Recent studies using cell-free systems, however, have shown that the molecular weight of the synthesized HA is less than 5000 [10, 24].

These results and ours suggest that high molecular weight HA may be composed of low molecular weight subunits. As shown in this study, each HA from different sources is depolymerized into molecules of characteristic sizes by sonication. Therefore, it is highly likely that each HA is composed of different subunits, but of constant size. Therefore, the products of HA digestion with testicular hyaluronidase have been examined by mass spectrometry [15]. However, no abnormal oligosaccharides have been found in the digestion products other than the usual ones obtained as testicular hyaluronidase digestion products. Accordingly the linkage sensitive to sonication is still unclear.

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