

Ultrasonic depolymerization of hyaluronic acid

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Abstract

Hyaluronic acid (HA) was depolymerized by ultrasonication. Changes in molecular weight and molecular weight distribution were observed by size exclusion chromatography with a low-angle laser light scattering photometer. We investigated the influence of sonication intensity, temperature, HA concentration, coexisting cations and ionic strength. Results demonstrated that, with an increase of intensity, initial depolymerization rate (k) increased and ultimate depolymerized molecular weight (M_{lim}) converged to smaller size. The factors that change high-order structure of HA molecules had great influence upon the k , but not so much upon the M_{lim} . For example, continuous sonication with 55 W depolymerized the HA to almost the same M_{lim} (approximately 0.1×10^6), with a few exceptions. Where exceptions occurred, they were in concentrated monovalent cation solutions; the M_{lim} increased up to about 0.3×10^6 . Consequently, by regulating the sonication conditions, HA with the desired lower molecular weight and a narrow distribution could be prepared from high molecular weight samples. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Hyaluronic acid; Ultrasonic depolymerization; Molecular weight; Molecular weight distribution; Cation

1. Introduction

Hyaluronic acid (HA) is a linear anionic polysaccharide, composed of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid. Molecular weight of HA obtained by extraction from bacterial culture broth or animal tissues is in the range 10^5 – 10^7 . High molecular weight HA forms an entanglement network in dilute solution of the order of $10^{-2}\%$, indicating unique rheological properties [1–3]. The characteristic viscoelasticity plays an important role in physical protection and integrity in living tissues, for example, articular cartilage, synovial fluid, skin and vitreous humor. Industrial HA samples are now obtained mostly from rooster combs and bacterial culture broth of *Streptococci*. Present extraction technologies supply HA at a relatively low price and in large quantities, facilitating the further study of HA.

Ambitious medical applications of HA have been tried because of its biological safety and rheological characteristics. Features rendering it are difficult to substitute by other artificial polymers or polymers, whose lubricating, shock-absorbing and humidity-controlling ability

are inferior to HA. Joint injections for osteoarthritis [4,5], space-makers for ophthalmologic operation [4,6] and eye lotion for xerophthalmus [7] are the present practical uses of sodium HA preparations. In recent reports, it is also suggested that damage induced by ultrasonication during phacoemulsification and aspiration for cataract surgery, could be avoided by pre-injection of HA preparation into the anterior chamber [6,8].

In the field of polymer chemistry, ultrasonication has been used as a conventional method to simply and efficiently obtain polymers of lower molecular weight by degradation of the original high molecular weight polymer. A number of mechanisms have been suggested, however, it is now generally accepted that ultrasonic depolymerization proceeds by mechanical force [9]. Propagation of acoustic energy causes rapid pressure variation to form small bubbles in the liquid. Formation of the bubbles (cavitation) and subsequent bubble collapse is responsible for the breakage of polymers by the generation of large velocity gradients.

Previously, we reported mechanical depolymerization of HA during measurements by a rotational viscometer [10]. The molecular weight was decreased by the load of shearing stress. In that report, HA in a dilute solution, having characteristic viscoelasticity, showed unique depolymerization behaviour. For instance, the ruptured

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point in a polymer chain differed depending on the shearing rate. Also with increased NaCl concentration up to 1 M, the depolymerization proceeded more immediately. Nevertheless, under such strong ionic strength conditions, the HA molecules were supposed to contract further to reduce stress loading. As ultrasonication is also a mechanical method, some unique observations were expected. There are rather few reports available on the ultrasonic depolymerization of HA [11–16], and in those that exist experimental conditions were limited and systematic detailed study was not fully done. Therefore, we investigated the influence of temperature, HA concentration, coexisting cations and ionic strength; these factors considerably effect the high-order structure of the HA molecules. While obtaining initial depolymerization rates, changes of molecular weight distribution were also observed. Finally, we tried preparing low molecular weight HA samples with a narrow molecular weight distribution.

2. Experimental

2.1. Materials

HA, extracted from the culture medium of *Streptococcus equi*, was mainly used except where otherwise stated. It had a weight average molecular weight (M_w) of 2.6×10^6 and a number average molecular weight (M_n) of 2.3×10^6 . Two other HA samples were also used; one was obtained from *Streptococcus zooepidemicus* (M_w 1.8×10^6 , M_n 1.7×10^6), and the other was obtained from rooster combs (M_w 1.0×10^6 , M_n 0.8×10^6). Other reagents were of analytical grade. All the water used was distilled once and then Millipore filtered.

2.2. Ultrasonication

Ultrasonication was performed using an ASTRASON Model XL2020 ultrasonic liquid processor (20 kHz, MISONIX Inc.). For the ultrasonic probe, a cupped model surrounding water jacket (431A) was used to keep the temperature constant at 37.0°C during the sonication, and also to avoid direct contact between the probe and the sample solutions. Samples were sonicated in a glass vessel hanging in the center of the water jacket. After a defined sonication period, a portion of sample solution was removed, appropriately diluted and subjected to molecular weight measurement.

2.3. Molecular weight measurement

The molecular weights of HA were measured using size exclusion chromatography with a low-angle laser light scattering photometer (SEC-LALLS, Tosoh Co. Ltd.). SEC-LALLS was performed on three columns, a

TSK-guard column PW_{XL} (6.0×40 mm), a TSK-G6000PW_{XL} (7.8×300 mm) and a TSK-G3000PW_{XL} (7.8×300 mm), with 0.2 M NaCl solution. The flow rate was 0.3 or 0.5 ml/min; it was slowed down to obtain more reliable LALLS response for the measurements of severely depolymerized HA. A 500 μl portion of the diluted sample containing about 0.02–0.1% HA was injected, and the peak elution was monitored with a LALLS photometer (Tosoh LS-8000) and a differential refractometer (Tosoh RI-8012). The estimation of molecular weight was performed through the Tosoh GPC-LALLS data processing program on the LALLS mode. Each determination was carried out at least thrice and the mean value was given as the result.

2.4. Viscosity measurement

A cone-plate rotational viscometer of E type (Tokimec Co. Ltd.) was used to measure the HA solution viscosity. The diameter and angle of the cone were 24 mm and 1° 34'. Measurements were performed at 37.0°C, maintained by circulating water.

Intrinsic viscosities were measured in three Ubbelohde capillary viscometers, with a flow time of 199–215 s for distilled water at 25°C.

3. Results and discussion

3.1. Influence of the cavitation on ultrasonic depolymerization

It is now generally accepted that ultrasonic depolymerization is caused by the mechanical energy that arises on formation and collapse of cavitation bubbles [9]. Strong velocity gradients, generated close to the collapsing cavitation bubbles, cause breakage of the macromolecules in the solutions. On a long molecular chain, which cannot follow the flow of the solvent, sufficient tension to break the chain will concentrate. At one time, thermal effect was proposed to be another factor responsible for depolymerization, but it is now known not to be directly responsible. Without thermal control, the temperature surely rises due to the absorption of sound energy. However, our experimental investigations also demonstrated that depolymerization was not a thermal effect (Fig. 1). An ordinate of Fig. 1 is plotted as percentages of the initial M_n . The depolymerization was less at 60°C than at 10 or 37°C. If the reaction was thermal, the depolymerization should proceed faster at a higher temperature. Heating would affect the depolymerization by changing the viscosity of the HA solution, the magnitude of vapor pressure inside the cavitation bubbles and the amount of dissolved gases in the solution. With an increase of temperature, the degree of freedom of HA molecular motion increased

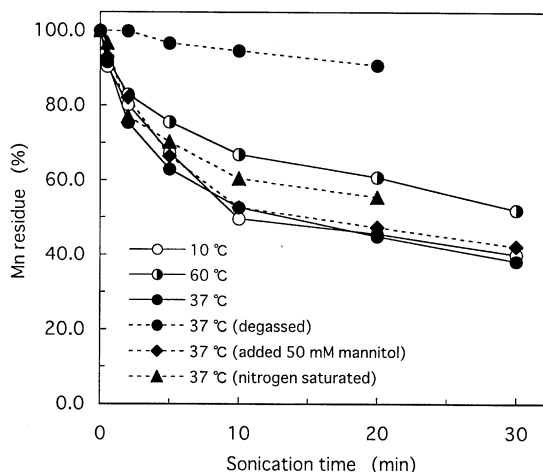


Fig. 1. Ultrasonic depolymerization of HA under several thermal conditions. Sample solutions were 0.05% HA in 0.2 M NaCl.

and the loaded mechanical stress was more easily relieved as motive energy. Simultaneously, the vapor pressure of the solvent being raised by heating, solvent molecules evaporated into the cavitation bubbles during their growth, and that resulted in diminution of the collapsing shock because of a cushioning effect. Moreover, to begin with, the gas solubility into the aqueous solution decreases with an increase of temperature. Samples degassed by aspirator for 3 min immediately before the sonication showed a small change in molecular weight (---●---). It substantiated the role of cavitation. Decrease in dissolved gas as nuclei for the cavitation bubbles would suppress the depolymerization.

Chemical effects also played a minor role. As HA is known to be depolymerized by active oxygen [17–19], especially hydroxyl radical, mannitol was added to the solution as a scavenger (---▲---) or the solution was deaerated by nitrogen saturation immediately before sonication (---◆---). However, the depolymerization occurred within the first 0.5 min. Under such suppressed conditions, chemical reaction should not be instantly pronounced. In the case of nitrogen saturated solution, slight repression of the depolymerization was recognized 5 min after sonication. For that reason, an external pressure above the solution was assumed. The experiment was conducted in sealed glass ampoules, therefore, after a certain period, the increased external vapor pressure in the hermetically sealed vessels would prevent cavitation.

As mentioned above, the depolymerization of HA was affected sensitively by the dissolved gas, therefore, the initial gas amount should be controlled. For ideal conditions, it should be defined either being degassed or air-saturated. However, there were some problems in actuality; one was an inhibition of depolymerization in the degassed solutions, and the other was the difficulty to make the initial HA molecular weight uniform. Bubble generation through the solution depolymerized HA mechanically. In obtaining the data in the nitrogen

saturated solutions, the molecular weight of the sample after the gas saturation, but before sonication, was also measured. The molecular weight decreased to about 90% of the original value, namely, in the relative comparison with other results in Fig. 1, the denominator for the percentage calculation was numerically different from others. This minor difference of initial molecular weight does not matter in the practical investigation of depolymerization characteristic, but it could be thought to be a cause of errors and preferably avoided. For these reasons, the following experiments were performed without both deaeration and air-saturation.

To counterbalance this, the samples were kept under steady temperature and atmospheric pressure before the operations to ensure the same conditions. Also, it had been confirmed beforehand that with such a deliberation, the measurements did not vary widely with repetition. The standard deviation in 3–5 repeated experiments was less than 5.0.

Among the series of factors influencing the cavitation, the ultrasonic intensity is one of the most important [9]. The intensity defines the acoustic pressure amplitude, and the cavitation can not be formed under a certain pressure variation. With increase of intensity, both the number of bubbles and their maximum size increase, resulting in a strengthening of cavitation activity. In consequence, the depolymerization proceeded faster and the degree of depolymerization was enhanced. Since the plot of percentages of M_n on a log scale versus sonication time indicated a linear relationship at the very beginning of the reaction, a first-order reaction was considered, and then the rate constants were estimated from the slopes of the straight lines (Fig. 2). The lines in the figure are drawn from the equation obtained by the least squares method. A relation between the ultrasonic intensity and the initial depolymerization rate (k) is demonstrated in Fig. 3. In this experiment, various

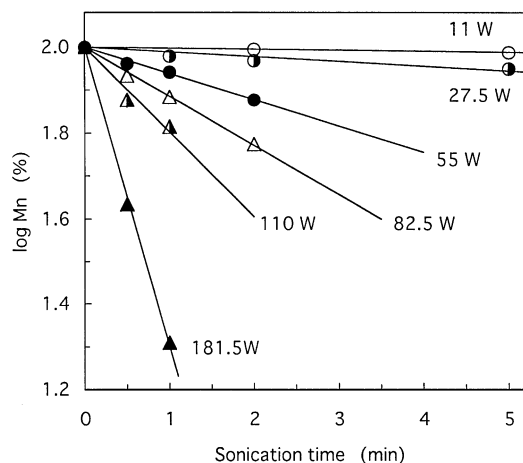


Fig. 2. Effect of ultrasonic intensity on initial depolymerization rate. Sample solutions were 0.05% HA in 0.2 M NaCl, and sonication was done at 37°C.

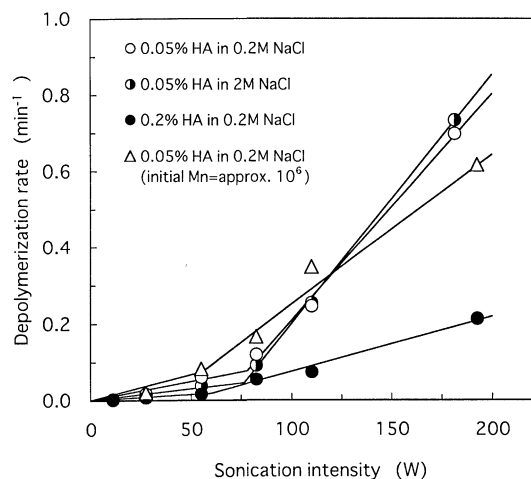


Fig. 3. Relation between ultrasonic intensity and initial polymerization rate in several HA solutions.

solutions of different HA concentration, ionic strength, or initial molecular weight were subjected to an ultrasonic treatment at increasing intensity levels. In every solution, k increased markedly above 55 W. The minimum intensity of 11 W, however, seemed to be higher than threshold value for the cavitation formation, because the depolymerization proceeded faster than without sonication.

3.2. Characterization of ultrasonic depolymerization

Contrasts to chemical or thermal depolymerization, non-random kinetics are the characteristics of mechanical depolymerization, and a limiting molecular weight, where the ultimately depolymerized molecules converge, exists [9]. In our HA ultrasonic depolymerization, these two points were confirmed with observation of changes in SEC chromatograms related to the sonication. Fig. 4 illustrates the (a) RI chromatograms and (b) LALLS chromatograms of 0.05% HA in water sonicated with 192.5 W up to 1920 min. Numbers in the figure indicate duration in minutes of the sonication period. Peak elution by the LALLS detector became smaller as the sonication period increased. The depolymerization proceeded rapidly in the early stage of the reaction, but it slowed down with prolonged sonication. It is found from the RI chromatograms that starting from a HA sample with a relatively narrow molecular weight distribution, the ultrasonication broadened the distribution reaching the maximum after 2–10 min, then it became narrower again over further passage of time. Values of the molecular weight distribution were 1.22 at 0 min, 1.87 after 10 min and 1.07 after 480 min. On further sonication, the distribution broadened once more; it was 1.33 at 1080 min. In repeating the increase and decrease in the distribution, the peak elution converged to a certain retention time. This indicates a non-random depolymerization process. Assuming a random depolymerization and no limiting molecular weight, the

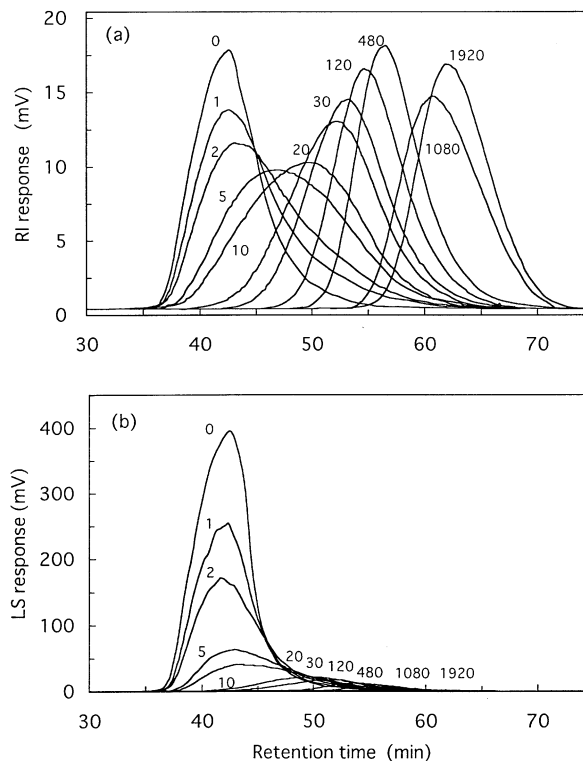


Fig. 4. Changes in RI and LALLS chromatograms; 0.05% HA in water was sonicated with 192.5 W at 37°C.

peak height of longer retention time would have risen from the beginning of the sonication. Actually, however, a new peak was found to focus in the low-molecular-weight region, then another successive peak of lower molecular weight appeared; that is to say that the transition of peak elution was in steps. Finally, no particular change in the chromatograms was recognized. It was considered that the depolymerization ceased on reaching the M_{lim} . A detailed discussion of the M_{lim} will be given later.

Further, as the ultrasonic depolymerization is mechanical in relation to the cavitation, it is understandable that polymer chains having higher molecular weight are more easily degraded because of the difficulty in following the perturbation [9]. Larger polymer molecules show more resistance to flow and accumulate greater stresses than the critical tensile strength of the chemical bonds, which leads to more frequent rupture than in the case with shorter polymer chains. It is clearly shown that the changes in molecular abundance against sonication period were different for different initial molecular weight (Fig. 5). In the range of high molecular weights, exemplified by HA with M_n of 2.8×10^6 , only depolymerization took place and the concentration of this molecular weight decreased consistently with time. At low molecular weights, exemplified by HA with M_n of 0.1×10^6 , no molecule was initially present. After a certain period of sonication, the presence increased steadily due to the depolymerization of larger ones. In

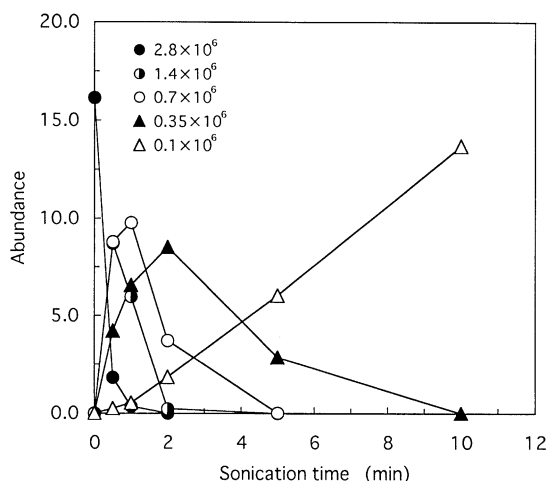


Fig. 5. Variation in abundance of HA molecules having definite molecular weight; 0.05% HA in water was sonicated with 192.5 W at 37°C.

the intermediate region of molecular weights, exemplified by the amount of HA with M_n of 1.4×10^6 , 0.7×10^6 and 0.35×10^6 , the changes are determined by a balance between the molecules that are depolymerized, and those formed by depolymerization of molecules having higher molecular weights. The larger the molecular weight, in the earlier stage the maximum of the abundance appeared, and the amount fell one-sidedly thereafter with time. This diagram illustrates that the ultrasonic depolymerizing effect is more pronounced for larger chains.

3.3. Effect of HA concentration on initial depolymerization rate

Since ultrasonic depolymerization occurs according to the load of shearing stress in the turbulent field during cavitation collapse, the configuration of molecules in the solution was expected to have an influence on the depolymerization. HA is known to form an entanglement network exhibiting viscoelastic properties [1–3]. By rheological measurements, it has been proved that the number of entanglements is highly dependent on the polymer length and concentration. When the molecular weights are equal, the entangled points increase with increased in the concentration, and the elastic properties more strongly appear. In our previous report about mechanical depolymerization of HA by shearing stress, an optimal concentration was observed; the depolymerization rate slowed down in both above and below the concentration [10]. Also in the ultrasonic depolymerization, the influence of HA concentration on the initial depolymerization rate constants was investigated.

The k at the same intensity of 55 W but at different HA concentrations are given in Fig. 6. There appeared to be a maximum at a concentration of 0.05% in NaCl solution, and of 0.10% in LiCl solution. In KCl solution, the rate constants decreased consistently with increase of polymer concentration in the range of 0.02–

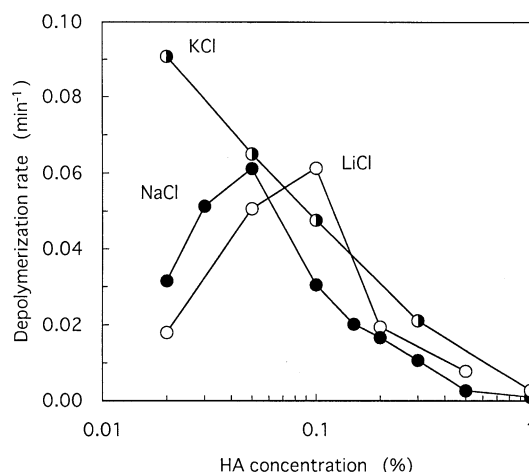


Fig. 6. Effect of HA concentration on initial depolymerization rate with 0.2 M monovalent cations. Sonication was done with 55 W at 37°C.

1.0%, and the maximum was predicted in lower concentrations. The effect of the concentration is very complicated, since the high order structure of HA in the perturbed flow due to cavitation cannot be described by simple mathematical relations. At the optimal concentration, however, it was considered that the polymer chains began to overlap while forming entanglements strong enough to store stresses greater than the critical tensile strength required to rupture the covalent bonds. As the concentration was further increased, the network became very dense and the elasticity of the HA solution was noticeably pronounced. This elastic solution behaved generally as if it were a gel, consequently, there was a reduction in the stress on each molecule, and also a restriction on the flow of the solvent. In the extremely dilute solution, on the other hand, HA molecules could move considerably freely without restriction by entanglements. It was predictable that the depolymerization would be reduced. The forces, which oppose each other, would induce the optimal concentration for depolymerization.

3.4. Effect of co-existing cations and ionic strength

The conformation of HA molecules would be predicted to vary in the presence of different cations. In Fig. 6, comparing the three monovalent cations in the same molar of 0.2 M, the maximum k is shown at higher HA concentration in order of $\text{Li}^+ > \text{Na}^+ > \text{K}^+$. As the polymer chains became hard to entangle and the individual molecule contracted more, higher concentrations would be needed to form a sufficient network construction. Therefore, it was supposed that the expansion of HA molecules would be small in line with the order Li^+ , Na^+ , and K^+ . Next, the influences of the co-existing cations and their ionic strength were investigated.

As shown in Fig. 7, the influence of ionic strength on k was not remarkable in high HA concentration, and the values of k were generally small. If the HA solution

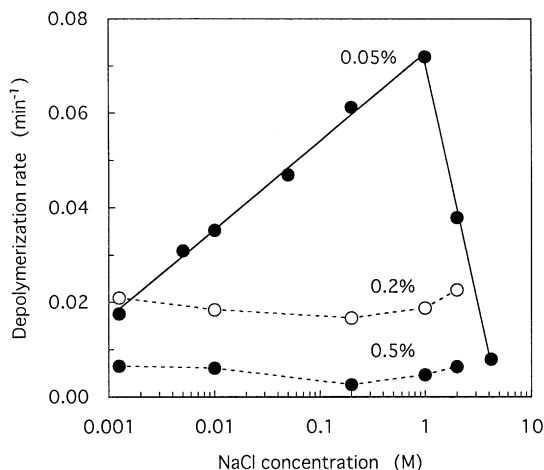


Fig. 7. HA depolymerization susceptibility to NaCl concentration.

was concentrated to a high level, the molecules formed a highly constrained network even under unusually high ionic strengths like 2 M. In excessively entangled structures, the flow of the solvent was greatly reduced, resulting in little depolymerization. Contrary to this, the rate constants drastically changed in 0.05% HA solutions with different NaCl concentrations. Therefore, the moderate 0.05% HA solution was selected for investigations with various cations.

Generally, polymers tend to be easily applied with mechanical stress with more expansion [20,21]. As HA is a polyelectrolyte, it dissociates in water forming polyions. In the lower ionic strength solutions, the electrostatic repulsion of the charged side groups will be pronounced and the chains will expand more. Hypothesising from the general knowledge of polymers, in such situations, polymer molecules could be expected to be depolymerized. From the results illustrated in Fig. 8, however, the opposite tendencies concerning salt concentration were shown in the low ionic strength region, in every case. The k increased with increase of the ionic

strength. A similar behaviour was observed in our previous study of HA depolymerization under shearing stress [10]. The explanation of these opposite tendencies is that the low ionic strength intensified the electrostatic repulsion causing an enhancement of elasticity. The solution began to behave as a whole gel, so that the stress on individual molecules was reduced.

It was interesting to note that the rate constants started to fall rapidly above the square root of an ionic strength of around 0.4 for KCl, MgCl₂, BaCl₂, 0.8 for SrCl₂, and 1.0 for LiCl, NaCl. For this reason, three factors were taken into consideration. First, the reduction of molecular expansion, as in the same manner as common polymers. By suppressing the electrostatic repulsion, the molecular chains would be assumed to shrink to compact coils. However, it was somewhat doubtful that the electrostatic effect had a greater influence on the configuration of dissolved polyelectrolytes over an order of 0.1 ionic strength. Let us assume that differences in intrinsic viscosity ($[\eta]$) of polyelectrolyte solution become smaller in such a sufficient ionic strength region. We obtained the results in NaCl solution of HA from *Streptococcus zooepidemicus* as listed below: $[\eta]$ = 79.9 in 0.001 M, 45.3 in 0.01 M, 31.5 in 0.05 M, 26.9 in 0.1 M, 23.7 in 0.2 M, 19.8 in 0.5 M and 16.3 in 1.0 M. This means that the expansion of molecular chains is not altered very much under high salt concentration. Second, a hydration of co-existing cations can be given. HA viscosities, measured by a cone-plate rotational viscometer, are illustrated in Fig. 9. The measurements were done in the presence of various kinds of monovalent, divalent or trivalent cations. The viscosity decreased with increase of ionic strength up to a certain level. However, above that it somewhat increased except in KCl solutions. Approximately 4 M KCl is a saturated solution, so that experiments with higher concentration were impossible. With other polyelectrolytes, similar profiles were observed, accordingly this was not a HA characteristic.

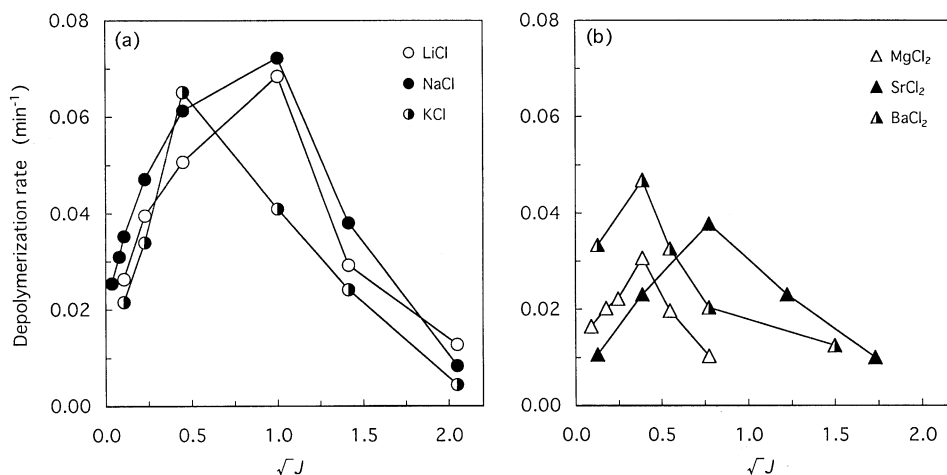


Fig. 8. Effect of ionic strength on initial depolymerization rate; 0.05% HA was sonicated with 55 W at 37°C.

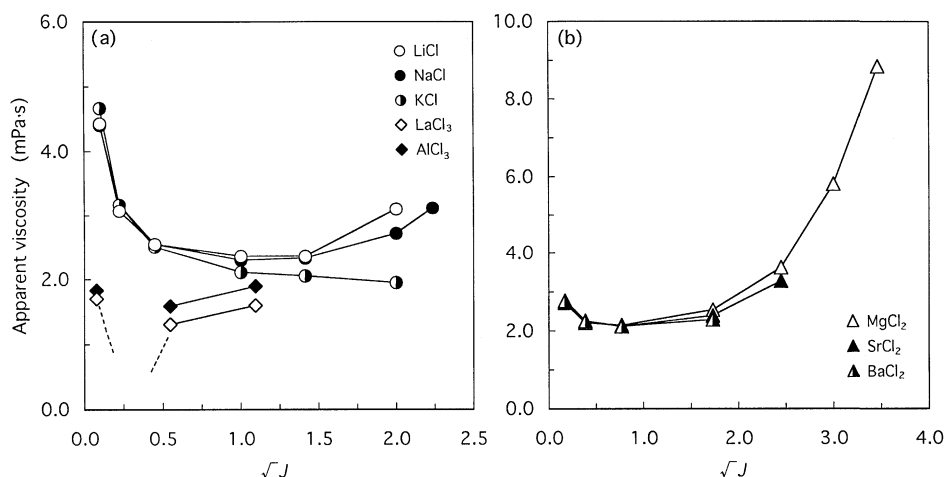


Fig. 9. Viscosity of HA solution with mono-, di- or trivalent cations. Apparent viscosity was measured under 100 rpm at 37°C with 0.05% HA solution.

Some polyelectrolytes can be precipitated by the salting-out effect. Also with HA solutions in trivalent ion (AlCl₃ and LaCl₃) solutions of 0.005–0.02 M, the viscosity could not be measured by precipitation. No HA precipitation occurred with mono- or divalent cations; however, it is conceivable that some variation in solubility took place. It was not for charged polymers, though, the effect of solvation on mechanical depolymerization has been studied [9,20,21]; the depolymerization rate decreased in poor solvents. Above all, suspensions of polymers in nonsolvents were not degraded. The HA molecules were in a fully dissolved state even in high salt concentrations, but there might be the possibility that they were not easily depolymerized. This last consideration is related to dissolved gases. As stated in Section 3.1, the elimination of dissolved gases inhibits ultrasonic depolymerization. High salt concentration increases the surface tension, and decreases gas solubility [22,23]. Surely, these physical properties are closely related to the changes in water structure, and that results in a reduction of cavitation. We end this section by noting that, at present, we have no reliable understanding of the curious relationship between k and the high ionic strength. The effect of co-existing cations on the configuration of HA molecules in dissolved states is under detailed investigation now. The results will be presented in the future.

3.5. The limiting molecular weight

M_{lim} is another characteristic of mechanical depolymerization. It is considered to correspond to the critical molecular length that can diffuse the loaded mechanical stress without a breakage of covalent bonds. In mechanical depolymerization, the entangling molecules are preferentially ruptured at regions close to their midpoints of two entanglements. It was expected that such factors as HA concentration, ionic strength, or the sort of co-

existing ions, influencing the high-order structure of HA, changed the M_{lim} . Strictly speaking, over a long period, depolymerization by chemical reaction occurs a little in warmed solutions. Practically, the convergence to the M_{lim} was assumed when the SEC chromatograms after t min nearly overlapped with those after $1/2t$ min. The results are summarized in Table 1, and some of them are illustrated in Fig. 10.

The M_{lim} obtained with 0.05% HA in 0.2 M NaCl decreased with increasing ultrasonic intensity ranging from 27.5 to 192.5 W. The M_{lim} obtained with 192.5 W sonication was too small to estimate accurately, hence it

Table 1
The values of M_{lim} and molecular weight distribution of ultrasonicated HA

	Cation (M)	HA concn (%)	Intensity (W)	M_{lim} and M_w/M_n		
				M_{wlim} ($\times 10^{-4}$)	M_{nlim}	M_w/M_n
LiCl	0.2	0.05	55	15.2	14.3	1.06
	7.0	0.05	55	32.6	30.8	1.06
NaCl	0.001	0.05	55	7.0	6.6	1.07
		0.05	55	10.5	9.3	1.13 ^a
	0.2	0.02	55	10.2	9.4	1.09
		0.05	27.5	36.0	34.6	1.04
		0.05	55	15.8	14.1	1.12
		0.05	82.5	1.4	1.1	1.26
		0.20	55	12.0	11.3	1.06
1.0	0.05	55	19.7	18.7	1.05	
3.6	0.05	55	32.5	27.6	1.18	
4.0	0.05	55	31.6	28.9	1.09	
KCl	0.2	0.05	55	13.6	12.3	1.11
	2.0	0.05	55	22.8	21.1	1.08
MgCl ₂	0.02	0.05	55	11.4	10.9	1.05
	0.3	0.05	55	12.2	11.5	1.06
	1.0	0.05	55	12.0	10.6	1.13
	4.0	0.05	55	9.5	9.2	1.03
SrCl ₂	1.0	0.05	55	13.5	12.1	1.12
BaCl ₂	0.2	0.05	55	9.4	8.6	1.09

^a HA, obtained from *Streptococcus zooepidemicus*.

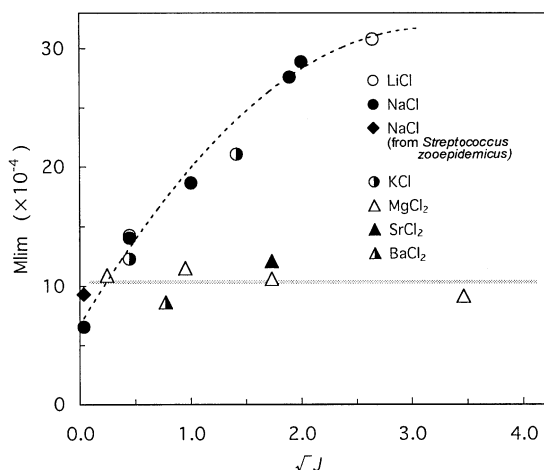


Fig. 10. Relation between limiting molecular weight and ionic strength; 0.05% HA was sonicated with 55 W at 37°C.

is omitted from the table. HA was depolymerized to a smaller size by higher mechanical stress under enhanced cavitation activity. However, it was an experimental result inconsistent with that of Kubo et al. [13]; they obtained the same molecular size under different conditions of sonication with 20 kHz, 7.5 W and 28 kHz, 20 W. These disparate results would probably be caused by the ultrasonic conditions. We unified the frequency at 20 kHz and changed only the acoustic intensity, while they changed both the frequency and the intensity. It was reported that the maximum radius of the cavitation bubble was inversely proportional to the frequency of the ultrasound within a large range [9]. As the frequency is increased, the radius of cavitation bubbles decreases, resulting in less cavitation intensity. In the case of Kubo et al., it was supposed that the effects of increasing frequency and intensity compensate for each other.

Contrary to expectations, the values of M_{lim} were almost equal with regard to the origin of HA, polymer concentration and nature of co-existing cations under the same ultrasonic intensity. The time needed for the convergence depended on the solutions; however, the M_n converged to approximately 0.1×10^6 in every case. The HA chains depolymerized to a certain degree could no longer form entanglement networks, so that they had not been influenced very much by the factors effecting the high-order structure of HA. But the fact is that there were some exceptions in concentrated monovalent solutions. When increasing the salt concentration, the M_{lim} became longer, reaching a peak around 0.3×10^6 as shown by the dotted line in Fig. 10. The molecular weights were kept unchanged over 200 h of sonication. It would be reasonably understandable that a similarly increasing tendency of M_n was also observed in the divalent cation solutions. We need to further investigate this issue.

As listed in the right column of Table 1, the molecular weight distribution (M_w/M_n) was rather narrow. After freeze-drying, the samples had the same molecular

weight as they had before treatment. Enzymatic, thermal and ultrasonic depolymerization methods are used widely as effective procedures for producing samples with lower molecular weight. In enzymatic procedures, the enzyme has to be separated from the sample afterwards. In thermal depolymerization, the molecular weight distribution is broadened by random scission, and the fractionation will be required for narrow samples. In ultrasonication, the obtained samples can be used without further purification and fractionation. Further, it was proved that a desired low molecular weight sample could be prepared by regulating the sonication intensity and/or the constitution of the solution.

4. Conclusion

HA was depolymerized by ultrasonication. The factors effecting the high-order structure of HA chains, such as HA concentration, nature of co-existing cations and ionic strength had great influence upon the k , but not so much upon the M_{lim} , with a few exceptions in concentrated monovalent cation solutions. By regulating the conditions, it was suggested that HA with the desired lower molecular weight could be prepared from an original high molecular weight sample.

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References

- [1] Yanaki T, Yamaguchi T. *Biopolymers* 1990;30:415–25.
- [2] De Smedt SC, Dekeyser P, Ribitsch V, Lauwers A, Demeester J. *Biorheology* 1993;30:31–41.
- [3] Kobayashi Y, Okamoto A, Nishinari K. *Biorheology* 1994; 31:235–44.
- [4] Balazs EA, Denlinger JL. *Ciba Foundation Symposium* 1989; 143:265–80.
- [5] Huskisson EC, Donnelly S. *Rheumatology* 1999;38:602–7.
- [6] Arshinoff SA. *Ophthalmic Practice* 1995;13:98–104.
- [7] Mengher LS, Pandher KS, Bron AJ, Davey CC. *Br J Ophthalmol* 1986;70:442–7.
- [8] Miyauchi S, Horie K, Morita M, Nagahara M, Shimizu K. *J Ocul Pharmacol* 1996;12:27–34.
- [9] Basedow AM, Ebert K. *Adv Polym Sci* 1977;22:83–148.
- [10] Miyazaki T, Yomota C, Okada S. *J Appl Polym Sci* 1998; 67:2199–206.
- [11] Chabreck P, Soltes L, Kallay Z, Novak I. *Chromatographia* 1990;30:201–4.
- [12] Chabreck P, Soltes L, Orvisky E. *J Appl Polym Sci Appl Polym Symp* 1991;48:233–41.
- [13] Kubo K, Nakamura T, Takagaki K, Yoshida Y, Endo M. *Glycoconj J* 1993;10:435–439.

- [14] Orvisky E, Soltes L, Chabreck P, Novak I, Stancikova M. *Chromatographia* 1993;37:20–2.
- [15] Rehakova M, Bakos D, Soldan M, Vizarova K. *Biol Macromol* 1994;16:121–4.
- [16] Soltes L, Mislovicova D, Sebille B. *Biomed Chromatogr* 1996; 10:53–9.
- [17] Wong SF, Halliwell B, Richmond R, Skowronek WR. *J Inorg Biochem* 1981;14:127–34.
- [18] Presti D, Scott JE. *Cell Biochem Funct* 1994;12:281–8.
- [19] Miyazaki T, Yomota C, Okada S. *Colloid Polym Sci* 1998; 276:388–94.
- [20] Price GJ, Smith PF. *Eur Polym J* 1993;29:419–24.
- [21] Nakano A, Minoura Y. *J Appl Polym Sci* 1978;22:2207–15.
- [22] Craig VSJ, Ninham BW, Pashley RM. *J Phys Chem* 1993; 97:10192–7.
- [23] Pugh RJ, Weisseborn P, Paulson O. *Int J Miner Process* 1997; 51:125–38.