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ORIGINAL CONTRIBUTION

T. Miyazaki
C. Yomota
S. Okada

Degradation of hyaluronic acid at the metal surface

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T. Miyazaki (✉) · C. Yomota · S. Okada
National Institute of Health Sciences, Osaka
1-1-43 Hoenzaka
Chuo-ku, Osaka 540
Japan

Abstract When the viscosity of hyaluronic acid (HA) solution was measured with a rotational viscometer, depolymerization occurred only by leaving the sample in the cup of the viscometer, while the solution in a glass test tube at the same temperature, 37 °C, was practically stable during the entire period of investigation. As the parts where the sample solution had contact with the viscometer are stainless steel, the participation of metal ions in the depolymerization was suspected. Therefore, experiments were carried out with stainless-steel beads in the HA solution. The results show that the molecular weight of HA decreased on exposure to the beads under aerobic conditions. The addition of 10 beads to 2 ml of a 0.1% HA solution prepared with 0.2 M NaCl reduced the molecular weight to about 75% of the initial value after 4 h. The degradation rate was proportional to the surface area of the beads. However, no dissolution of metal ion was detected, and therefore, this reaction was thought to proceed at the surface of the metal.

Key words Hyaluronic acid – degradation – molecular weight – metal surface – hydroxyl radical

Introduction

We previously studied the phenomenon and reason for a decrease in the viscosity of a dilute hyaluronic (HA) aqueous solution during measurements using a cone-plate rotational viscometer [1]. Since HA exhibits a characteristic entanglement network, it was considered to store applied shearing energy in the secondary intermolecular bond causing scission of the main chain. High molecular weight HA was shown to be easily degraded by mechanical stress even under such conditions as encountered with routine viscometric measurement and owing to the reduction in molecular weight, the viscosity decreased.

Moreover, it was noted that without shearing stress, a slight decrease in molecular weight occurred when the HA sample was left in the cup of the viscometer. The temperature of the viscometer was controlled with circulating water at 37.0 °C, so thermal stability was suspected. However, HA solutions in test tubes, made of glass, immersed in a water bath at the same temperature showed little decrease of molecular weight. Thus, the degradation that occurred in the stationary viscometer was not caused by mechanical stress or heating. The main difference between the two situations, in the viscometer and in the test tubes, was the material with which the sample solutions had contact. With regard to HA degradation, there have been many reports on oxygen derived free radicals catalyzed by metal ions [2–7]. Since the cup and cone of the viscometer used were made of stainless steel, the influence of metal was suspected. There seems to be no other report about HA degradation induced by contact with a solid-state metal. Thus, we performed several experiments with some stainless-steel beads added to a HA solution.

Experimental

The HA sample (sodium salt), extracted from the culture medium of *Streptococcus equi*, had a weight average molecular weight (M_w) of about 2.5×10^6. In most of the experiments, a solution prepared at a concentration of 0.1% HA in 0.2 M NaCl was used. Various metal chlorides were of the best grade commercially available and were used with no further purification. Other reagents were of analytical grade. All the water used was deioniezed and then Millipore filtered.

The molecular weights of HA were measured using gel permeation chromatography equipped with a low-angle laser light scattering (GPC-LALLS, Tosoh Co., Ltd.). GPC-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 a 0.2 M NaCl solution. The flow rate was 0.5 ml/min. A 500 μl portion of the diluted sample containing about 0.02% 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 with the Tosoh GPC-LALLS data processing program in the LALLS mode. Each determination was carried out at least in triplicate and the mean value was given as the result.

To study the influence of solid state metal on the HA molecular weight, several kinds of metallic beads with a diameter of about 5 mm were used. They were added to test tubes containing 2 ml of the sample solutions. For the experiments under nitrogen atmosphere, glass ampules were used instead of test tubes and the necks sealed with a burner. After the desired exposure times, sample solutions were removed and subjected to GPC-LALLS after the appropriate dilution. The stainless-steel beads were made of SUS304 and had the same contents as the cup and cone of the viscometer, which consisted of less than 0.08% carbon, 19% chromium and 9% nickel. They were commercially available ball bearings. The copper beads prepared by Fukuda Metal Foil & Powder Co., Ltd. (Kyoto, Japan) were of pure grade. The tungsten–carbide beads were composed of 93.5–94.5% tungsten–carbide and 5.5–6.5% cobalt. They were commercial products of MORITEX Co., Ltd. (Tokyo, Japan) as grade 25 Tungsten Carbide Precision Balls.

The effects of metal ions on the degradation of HA were also studied using various kinds of metal chlorides at several concentrations. After the desired period, sample solutions were removed and diluted with 0.2 M NaCl containing 20 mM mannitol to inhibit further degradation before being subjected to GPC-LALLS as soon as possible.

To measure the amounts of iron and chromium dissolving into the HA solution from stainless–steel beads and copper dissolving from copper beads during the exposure period, an atomic absorption spectrophotometer (AA-845, Nippon Jarrell Ash Co., Ltd.) was used. The combustible gas was acetylene and the supporting gas was air. For the light source, hollow cathode lamps were used by adjusting the wavelength to 248.3 nm for iron, 357.9 nm for chromium and 324. 8 nm for copper.

Results and discussion

HA degradation by stainless steel

HA degradation proceeded gradually in the cup of the viscometer (E type, Tokimec Co., Ltd.) without shearing stress at 37 °C (Table 1). The M_w was reduced to about 86% of the initial value after 2 h, and 74% after 4 h by only leaving the sample solution in the stationary viscometer. On the other hand, the M_w of the sample solution in a glass test tube decreased little during the entire period of the experiment at the same temperature. The reduction was only 6% after 4 h. The main difference between the two situations was the material with which the sample solutions had contact. The cup and cone of the viscometer were made of stainless steel, SUS304, possessing excellent properties for resisting corrosion and abrasion. However, the stainless steel was suspected as the cause of the degradation due to dissolving trace amounts of metal ions into the sample solutions. For model purposes, various experiments were performed using several stainless-steel beads made of the same composition as the components of the viscometer.

The stainless-steel beads reduced the M_w of 0.1% HA by as much as that of the viscometer (Fig. 1a). With an increasing number of bead added to the solutions, the degradation proceeded further. To control for the loss in surface area which would have occurred by piling the solid metals, we used spherical beads, because contact with each other would be theoretically a point. However, despite this care, a comparison of the degradation behavior between solutions with 10 and 20 beads showed little difference.

Table 1 Changes in weight average molecular weights of HA

Time [h]	$M_w \times 10^{-6}$				
	0	0.5	1	2	4
In the viscometer	2.52	2.47	2.44	2.17	1.87
In the test tube	2.42	2.35	2.37	2.29	2.27

The values are means from at least three independent measurements.

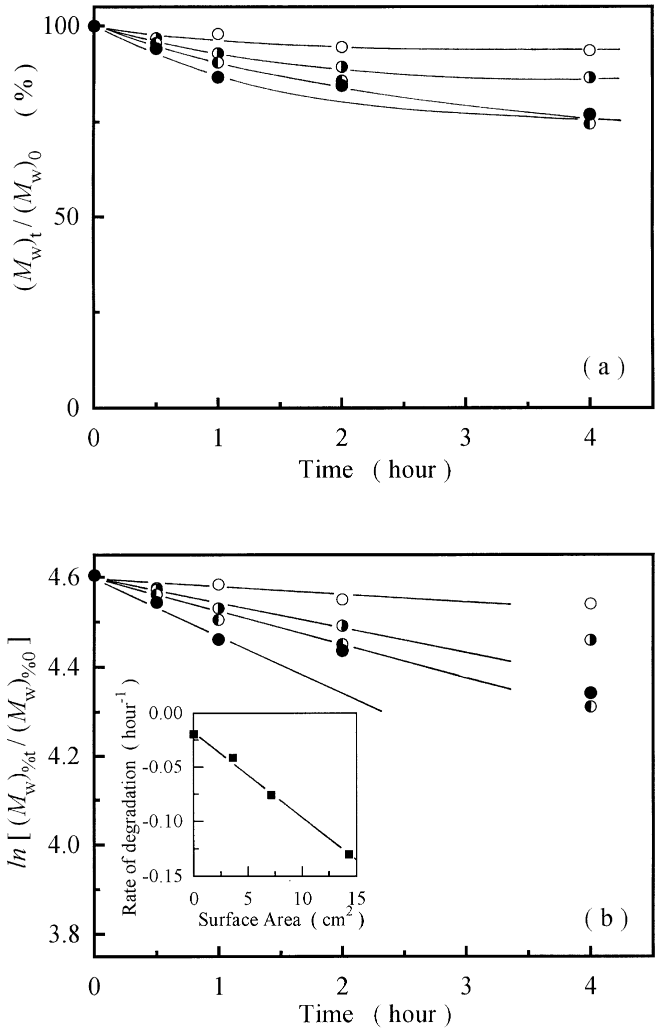

Fig. 1 HA degradation induced by addition of stainless-steel beads to 2 ml sample solutions at 37 °C. Number of beads: (○) 0; (◑) 5; (◐) 10; (●) 20

Table 2 HA degradation by several kinds of metal ions

Concentration [mM]		Reduction of M_w [%]			
Time [h]		0.5	1	2	4
None		96.9	98.0	94.5	93.6
Ni^{2+}	0.1	96.4	94.1	96.3	94.7
Zn^{2+}	0.1	94.9	95.4	95.2	94.5
Mn^{2+}	0.1	97.7	96.1	94.6	93.9
Co^{2+}	0.1	96.6	94.0	88.4	87.8
Cr^{3+}	0.002	99.9	98.6	93.5	–
	0.1	96.1	92.5	88.0	83.5
Cu^{+}	0.002	98.6	99.0	96.0	94.8
	0.02	91.2	86.9	83.6	66.8
	0.1	35.6	–	31.7	26.3
Cu^{2+}	0.1	94.7	90.9	87.5	82.1
Fe^{2+}	0.005	96.5	96.7	95.1	94.8
	0.01	96.5	94.0	95.2	92.0
	0.1	94.3	89.4	83.6	77.5
	1	92.3	83.9	71.8	54.6

However, by semi-logarithmic plotting (Fig. 1b), the relationship between the surface area of the beads and the rate of HA degradation was clear. The time dependence of the logarithmic percentage reduction of M_w from the initial value indicated a linear relationship at the initial stage of the degradation. Thus, the decay lines of molecular weights may be described by the relation,

$$(M_w)_{\%t} = 100 \times e^{-kt} , \tag{1}$$

where $(M_w)_{\%t}$ is the percent weight average molecular weight at time t, and k is the rate constant of the degradation. The rate constants estimated from the slope of the straight lines were proportional to the surface area of the beads (as indicated in Fig. 1b). In that case, two inferences could be made. One was that the HA degradation by the catalyzation of metal ions dissolved from the beads depended on the surface area, and the other was that the reaction proceeded on the surface of the beads; that is, the more beads provided a larger effective area for the reaction. Although stainless-steel resists corrosion, trace metal ions may have dissolved in a high electrolytic solution such as 0.2 M NaCl. The effect of metal ions on the molecular weight of HA was then investigated.

HA degradation by metal ions

There have been many reports by others that HA degradation is mediated by oxygen derived free radicals produced by contaminating trace amounts of metal ions, especially iron and copper ions. Hydroxyl radicals appear to be the most effective reactive oxygen species to depolymerize HA [4, 8]. They are formed by the metal-catalyzed Fenton reaction [9] as follows (2)–(4):

$$M + O_2 \rightarrow M^+ + O_2^{\cdot -} , \tag{2}$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 , \tag{3}$$

$$M + H_2O_2 \rightarrow M^+ + {}^{\cdot}OH + OH^- \tag{4}$$

(M; metal ions at their reduced valence state, M^+; metal ions at their oxidized valence state).

The degradation behavior was investigated by adding 0.1 mM of various kinds of metal chlorides, including iron, nickel and chromium: which are constituents of the stainless-steel beads. As listed in Table 2, the strongest degradation behavior was shown by copper(I), and 0.1 mM CuCl degraded HA to about one-third of the original molecular weight after 30 min. Iron(II), copper(II), chromium(III) and cobalt(II) also enhanced the degradation to some degree, but nickel(II), zinc(II) and manganese(II) showed little or no effect at this concentration. The system of the Fenton reaction to form hydroxyl radicals requires the metal ions at their reduced valence state, therefore the degradation that occurred by adding copper(II) or chromium(III) could be due to a very small amount of some other contaminating ion at its low valence state. In the case of chromium, chromium(II) has reducing potency, however the experiment using it was impossible under aerobic condition, because the oxidation rate chromium(II) to chromium(III) was too fast to prepare the sample solution.

In addition, we studied the quantitative effects of iron(II), chromium(III) and copper(I). We chose iron and chromium, because they are major components of SUS304 and also have comparatively stronger potencies to degrade HA than nickel. Copper(I) was chosen because of its marked effect. With decreasing amounts of metal chloride, the degradation was slowed down by various degrees (Table 2). In the case of iron(II) at the concentration of 0.01 mM, it had virtually no effect on the M_w over the period studied. Even copper(I) scarcely depolymerized HA at a concentration of 0.002 mM. The small amounts of metal ion without reducing agents, that reduce the ions in their oxidized form to the lower valence state, could not generate enough hydroxyl radicals to observe a decrease in molecular weight. Next, the amounts of iron and chromium dissolving from the stainless-steel beads into the sample solutions during the exposure were measured using an atomic absorption spectrophotometer. Up until 4 h, no iron or chromium was detected in the sample solution containing ten beads. The detection limits of the detector used were 50 ppb for iron and 20 ppb for chromium. Namely, the concentrations of 0.005 mM iron(II) and 0.002 mM chromium(III), as shown in Table 2, correspond to about 5 times the concentration of the detection limits. Although HA was negligible be degraded by adding sufficient amounts of iron or chromium ions to be detected by the atomic absorption spectrophotometer, ten stainless-steel beads depolymerized HA with no dissolving metal detected. It was thus speculated that the HA degradation occurred predominantly at the surface of the beads.

Differences in degradation behavior by various kinds of metal beads

We found that HA degradation did not occur at the same rates with the various kinds of metal ions. To investigate whether the differences could also be seen in the case of solid state metals, we replaced the stainless-steel beads with other metal beads, such as copper or tungsten-carbide, with approximately of the same size. Table 3 shows the differences in the rate of degradation according to the material of the beads. Beads made of tungsten-carbide had no influence on the HA molecular weight. After 16 h, the M_w was reduced about 10%, which was almost equal to the decrease obtained in a control experiment, without beads. From this result, tungsten, which made up 94% of the beads, was considered to have little or no reducing effect. Although tungsten has been reported to have some oxidation states, the interconversion of the two valence states would not be easy. Another metallic component of the beads is cobalt, but, its proportion was small, in the order of 6%, as compared with the percentage of chromium in a stainless-steel bead which was 19%. Further, 0.1 mM cobalt chloride degraded HA, but the effect was less than iron chloride and chromium chloride of the same concentration (Table 2). Therefore, the change in M_w of HA was negligible. In contrast, copper beads markedly depolymerized HA. The addition of only two beads decreased the M_w to about 80% after 15 min, and after 1 h it had decreased to one-half of the initial value. However, copper beads are less resistant to corrosion and abrasion than stainless-steel and tungsten-carbide beads, that is, they are chemically active such that the surface rusts by oxidation and some copper dissolves into the sample solution with an increasing exposure period in 0.2 M NaCl. In the solution with two beads, 0.0016 mM copper was detected after 30 min when the reduction of M_w was about 30%, and 0.018 mM copper was detected after 4 h when the M_w was reduced by about 82%. However, with the addition of 0.002 and 0.02 mM CuCl, the decrease in M_w after 4 h were only 5% and a little more than 30%, respectively (Table 2). Thus, most of the degradation proceeded at the surface of copper beads as was found for the stainless-steel beads.

Table 3 Differences in the degradation behavior induced by various kinds of metal beads

Time [h]	Reduction of M_w [%]					
	0.25	0.5	1	2	4	16
Without bead	–	96.9	98.0	94.5	93.6	89.8
10 stainless-steel beads	–	95.6	90.5	85.7	74.6	–
2 copper beads	82.2	71.0	49.9	29.4	18.1	–
10 copper beads	65.0	40.9	18.2	13.3	12.0	–
10 tungsten–carbide beads	–	–	–	100.4	96.2	89.2

Beads were cast into a 2 ml sample solution at 37 °C.

Radical species formed at the surface of the beads

It is well known that with microscopic observation at the surface of a solid state metal in an electrolyte solution, the

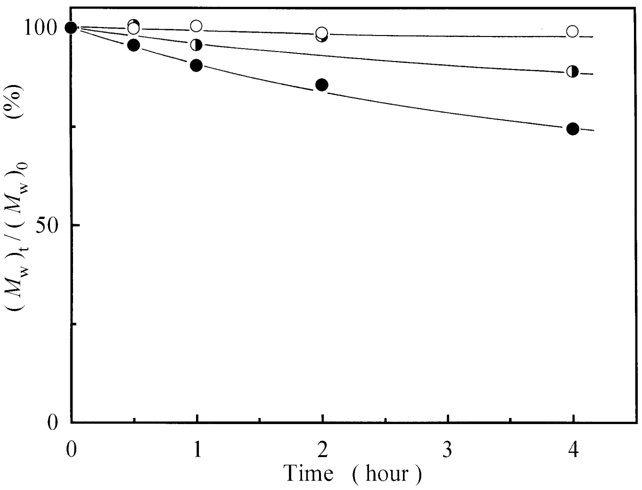

Fig. 2 Inhibition of HA depolymerization by addition of mannitol or deoxygenation. (●) with 10 stainless-steel beads; (◑) with 10 stainless-steel beads + 5 mM mannitol; (○) with 10 stainless-steel beads under a nitrogen atmosphere

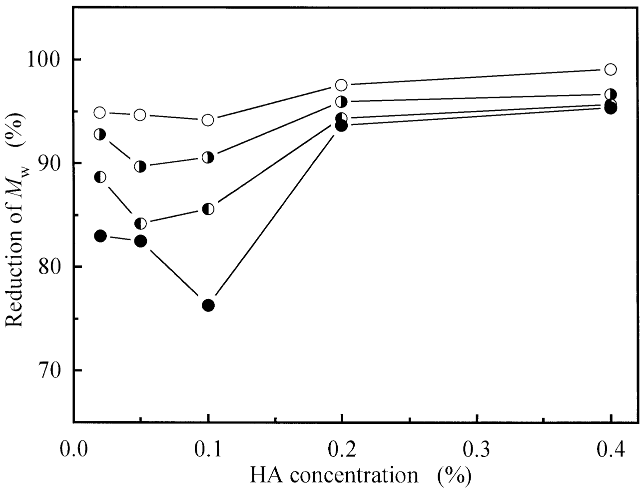

Fig. 3 Effect of HA concentration on the degradation induced by 10 stainless-steel beads at 37 °C. Exposure period: (○) 30 min; (◑) 1 h; (◐) 2 h; (●) 4 h

metal atoms dissolve into the solution by cationic charging with hydration and form an electrical double layer with the remaining electrons. Namely, at the interface between the solid-state metal and the solution-charged metal ions exist. With regard to HA degradation by iron and copper ions, hydroxyl radicals, which would be formed through the Fenton reaction, have been reported to be the main active species. Assuming that the effective species was the hydroxyl radical, then the degradation could be inhibited by mannitol which is a scavenger of hydroxyl radicals [10, 11]. In our experiments, 5 mM mannitol inhibited the degradation induced by 0.1 mM $FeCl_2$. Also in the case of metallic beads, it was expected that oxygen radicals would be generated by the cations formed at the surface. To confirm the participation of hydroxyl radicals, the influence of mannitol was investigated. In the presence of ten stainless-steel beads, HA depolymerization was inhibited by adding 5 mM mannitol under aerobic conditions (Fig. 2). These results suggested that hydroxyl radicals were the cause of the degradation that proceeded on the surface of the beads. In addition, when beads were added to a nitrogen saturated HA solution in the ampules, no reduction of M_w was observed. Oxygen would be necessary for the generation of hydroxyl radicals probably through reactions (2)–(4).

Effects of polymer and salt concentration

Concerning the degradation of polyelectrolytes induced by radicals, the dissolution state of the molecular chains has great influence on the reactivity [12, 13]. With increasing salt concentration, the electrostatic repulsion of the charged side groups along the chains will be suppressed and the extension of molecules will be small. Also with increasing polymer concentration, the molecules will shrink due to the influence of such factors as intermolecular interactions and the increase of the ions from the polyelectrolyte itself. Generally, polyelectrolytes have the tendency that the more contracts that form, the less the molecules are attacked by the radicals, that is, the chances of scission decrease with increasing polymer and salt concentrations. Therefore, the effects of HA and NaCl concentrations were studied using stainless-steel beads.

Figure 3 shows the results obtained by the experiments performed with ten stainless-steel beads and HA concentrations ranging from 0.02 to 0.4% in 0.2 M NaCl solution. Above 0.1%, the degradation was slowed down with increasing concentration. However, the tendency shown below 0.1% was the opposite, in that the degradation was slightly reduced with the decreasing concentration. At every observation time, either the 0.05% or 0.1% HA solutions showed the maximum reduction of M_w. This indicates a decrease in the chance of HA molecules to collide with active points on the surface of the beads. To complete the reaction, sufficient approaches would be needed, but in so dilute solution as 0.02%, the chance of collision itself would decrease. This would be the rate-determining step; thus, the degradation was considered to be suppressed.

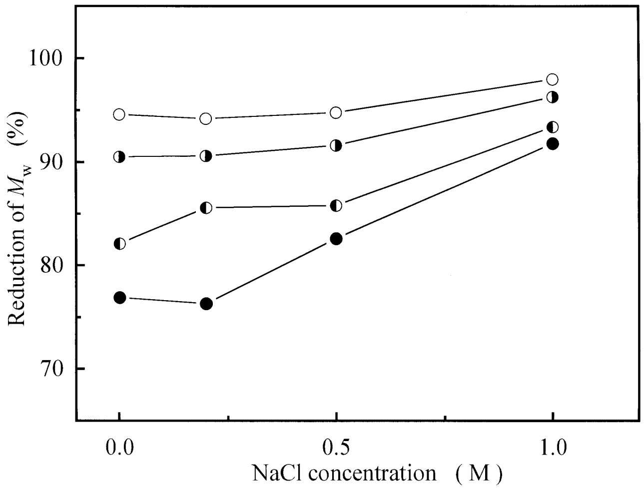

Fig. 4 Effect of NaCl concentration on the degradation induced by 10 stainless-steel beads at 37 °C. Exposure period: (○) 30 min; (◑) 1 h; (◐) 2 h; (●) 4 h

By varying the salt concentration in the range from 0.001 to 1 M, the M_w decreased more slowly with the increasing NaCl concentration as shown in Fig. 4. Shrinking of the molecules by suppressing the electrostatic repulsion might make it hard for the HA to be attacked by radicals.

Kinetic study of HA degradation by stainless-steel beads

With regard to HA chain scission behavior, the changes in polydispersity during the course of exposure to metal beads or ions were small. For example, by adding two copper beads to the solution, the polydispersity increased from 1.03 to 1.40, while the M_w decreased to about one-fifth of the original M_w value after 4 h. In polymer degradation, there appear to be three types of chain scission behavior: central, random and terminal scission. In the case of terminal degradation, a marked change of polydispersity would be expected [1, 14]. The present small change of polydispersity shown in the degradation by metal beads suggests random or central chain scission. Alternatively, random degradation could prevail. If the degradation occurs mechanically by loading stress, the energy will concentrate at some particular region around the center on the polymer molecules, and central chain scission will likely occur. However, in the chemical degradation induced by the formation of hydroxyl radicals in the entire sample solution, random chain scission probably occurs.

As the degradation proceeded randomly, semi-logarithmic plots such as illustrated in Fig. 1b indicated linear lines and the first-order rate constants could be evaluated from the slopes at their initial stages. Therefore, the thermal effect on the rate constant was investigated under the aerobic condition with ten stainless-steel beads in the range from 10 to 60 °C. At higher temperatures the degradation proceeded faster. The scission of HA molecules in this experiment resulted from a complex series of reactions such as ionization of the metal surface, the chain reactions described by Eqs. (2)–(4) to form hydroxyl radicals catalyzed by the ions at their reduced valence state, the approach between the polymer and the active point on the bead, and an abstraction of a hydrogen atom from the polymer chain by the radical. Still the logarithmic values of the rate constant, k, indicated a linear relationship versus the reciprocal of the absolute temperature (Fig. 5). The kinetics of HA degradation could be described using the Arrhenius equation,

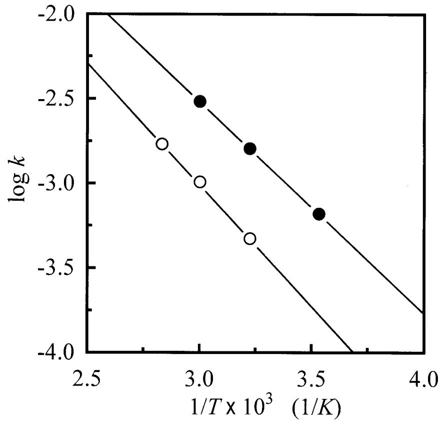

Fig. 5 Arrhenius plots for the degradation of 0.1% HA in 0.2 M NaCl. (○) without beads; (●) with 10 stainless-steel beads in 2 ml sample solutions

$$k = A \exp(-E/RT), \qquad (5)$$

Although a similar thermal dependence was shown in the case without beads, HA was considerably stable even at 80 °C.

The present study indicated a reduction in the M_w during the routine measurement using a rotational viscometer. Therefore, attention should be paid to the handling of the samples. The possibility cannot be excluded that a HA solution can come in contact with metals during preparation; for example, a spatula made of stainless steel could be left in the solution. We found that only slight degradation occurred during brief contact with metal at room temperature. However, in view of quality control, it should be taken care during the treatment of samples. We found that the M_w of HA decreased during the measurement of viscosity and flow. A rotational viscometer without metal parts is commercially available. Unless such an apparatus is used, attentions should be paid to reducing the time for the measurements.

Currently, ultrasonication [15–17], enzymatic treatment [16, 18, 19] and exposure to high temperatures in an autoclave [20] are commonly used for preparation of low molecular weight HA by degradation of the original high molecular weight sample. Among them, ultrasonic degradation has been reported to be highly effective for preparing HA of various molecular weights, because the chemical structure is unchanged and the polydispersity is low. However, a special apparatus is required for sonication. In the case of enzymatic degradation, the enzyme has to be separated from the samples after the treatment. On the other hand in the degradation induced by stainless-steel beads, the obtained samples can be used for any purpose without further purification, because there is no possibility of contamination. In addition, no special apparatus is needed. Moreover, samples with a desired molecular weight and comparatively low polydispersity can be easily prepared, because the possible reaction estimated from the rate equation proceeds under relatively mild thermal conditions. More detailed investigations such as the chemical characterization after the radical attack should be explored to obtain a useful approach used for this HA depolymerization.

Conclusions

HA was degraded by contact with solid-state metals. Beads made of stainless-steel or copper reduced the molecular weight of HA under comparatively mild thermal conditions. This degradation was inhibited by mannitol, suggesting the involvement of hydroxyl radicals catalyzed by metal ions. However, since insufficient amounts of dissolving metal ions for inducing the depolymerization were detected in the sample solutions after exposure to the beads, it was concluded that the degradation occurred at the surface of the solid-state metal. This reaction can be applied to the preparation of lower molecular weight HA from the original high molecular weight sample. However, from the view point of quality control, attention should be paid to the stability of the sample.

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