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# Hydrolytic degradation of hyaluronic acid

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The hydrolytic degradation of hyaluronic acid (HA) was investigated through kinetic measurements. The first-order rate constants were obtained under different pH conditions on the basis of the decrease in the molecular weight. The mechanism of the hydrolytic degradation of HA was also investigated by theoretical calculations (MNDO-MO) on model compounds of HA.

It was speculated that hydrolysis occurs in acid solution on the glucuronic acid residue and the hemiacetal ring remains, while the destruction of the *N*-acetylglucosamine residue takes place in basic solution.

These speculations are consistent with the structural analysis of the degradation products by $^{13}$C-NMR.

## 1. INTRODUCTION

Hyaluronic acid (HA) is an unbranched high molecular weight polysaccharide. It consists of 2-acetamide-2-deoxy-$\beta$-D-glucose and $\beta$-D-glucuronic acid residues linked 1-3 and 1-4, alternately. It is widely distributed in the connecting tissues[1] of the human body as well as in bacteria.[2] One of the interesting features is that HA has an extremely high molecular weight among glucosaminoglycans.[3] Aqueous solutions of HA show very high viscosity due to its high molecular weight.

On the other hand, taking advantage of some useful features (keeping humidity, high viscosity and the nature of decomposition etc.), there have been developments of new drugs using HA. Therefore, the basic investigation of HA degradation is very important including the kinetics of degradation, degradation product analysis and reaction mechanism. It has been reported that the degradation of HA is caused by hydrolysis[4] and active oxygen in the form of hydroxyl radicals.[5–7] Recently, the energy barrier of glycoside bond cleavage with hyaluronane in acidic condition has been calculated by Pratt *et al*. by means of the MNDO-MO method.[8] In the present study, we have investigated the rate of HA hydrolytic degradation under different pH conditions. Furthermore, on the basis of quantum chemical calculations (MNDO-MO)[10] and $^{13}$C-NMR structure analysis of degradation products, we have predicted the reaction sites and mechanisms of the hydrolysis.

## 2. EXPERIMENTAL

### 2.1. Materials and methods

High molecular weight sodium hyaluronate samples were extracted from the culture broth of *Streptococus equi* and purified by Denki Kagaku Kogyo Co. Ltd. One weight percent aqueous solutions of HA were prepared by stirring for 3 h to dissolve completely. The HA solution was divided into equal parts and sealed in 1 ml ampoules followed by heating in an oil bath at constant temperature. The number-average molecular weight was determined by gel-permeation chromatography (column: Shodex OHpak SB806 ×2, solvent: 0.1M $NaNO_3$ aq, flow rate: 1.2 ml/min, detector: JASCO830RI, column temperature: 40°C, calibrated with standard HA samples of different intrinsic viscosity). The pH conditions were controlled by a wide buffer[9] which was prepared as follows: A-Solution (tris-(hydroxylmethyl)-aminomethane 0.1M, KCl 0.1M, potassium phosphate monobasic 0.1M, citric acid anhydrous 0.1M, and sodium tetraborate 0.1M); B-Solution (HCl aq 0.4M or NaOH aq 0.4M). pH solutions were prepared by adding the

A-Solution diluted 4 times to the appropriate amount of B-Solution. The samples for $^{13}$C-NMR analysis of degradation products were prepared as follows: the acidic sample (pH 1.44) was prepared using aqueous HCl solution and heated at 60°C for 10 days, the basic sample (pH 13.30) was prepared using NaOH solution and heated at 60°C for 7 days.

### 2.2. Rate constant of the hydrolysis reactions of HA

The rate of the hydrolytic degradation of HA in aqueous solution was followed by the measurement of the number-average molecular weight. Assuming the rate of the main chain scission is proportional to the number of glycoside bonds (first-order), we obtain

$$dNb/dt = k_b(M_0 - P) \quad (1)$$

$Nb$: number of main chain scissions, $M_0$: total number of monomer units, $P$: number of polymer molecules, $P = P_0 + Nb$ ($P_0$: number of polymers at $t = 0$), $M_0 - P$: number of glycosidc bonds, $k_b$: first-order rate constant of main chain scission.

The number of main chain scissions at time $t$ is expressed by the number average degree of polymerization $[DP]_n$ at time $t$ and $[DP]_{n0}$ at $t = 0$.

$$Nb = M_0/[DP]_n - M_0/[DP]_{n0} \quad (2)$$

From eqns (1) and (2):

$$d(1/[DP]_n)/(1 - 1/[DP]_n) = k_b \cdot dt \quad (3)$$

Further, $1 \ll /[DP]_n$ for HA with high molecular weight,

$$d(1/[DP]_n)/dt = k_b \quad (4)$$

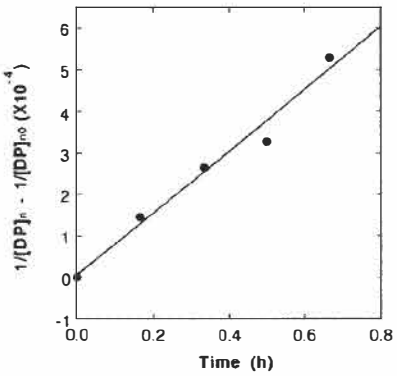


**Fig. 1.** $1/[DP]_n$-$1/[DP]_{n0}$ vs time (h) plot at pH 11, 80°C.

Integrating eqn (4), we obtain

$$1/[DP]_n - 1/[DP]_{n0} = k_b \cdot t \quad (5)$$

According to eqn (5), $1/[DP]_n$-$1/[DP]_{n0}$ plotted against $t$ gives a straight line as shown in Fig. 1. The values of the first-order rate constant $k_b$ were evaluated from the slopes of the straight lines obtained under different conditions. These linear plots show that the degradation reaction of HA is due to random chain scission and accordingly obeys first-order kinetics.

### 2.3. NMR measurement

$^{13}$C normal and DEPT[11,12] spectra were measured on a modified JNM-ALPHA 500 (JEOL).

### 2.4. Calculation

The semi-empirical SCF MO method, MNDO was employed using the MOPAC 5.01 package.[9]

## 3. RESULTS AND DISCUSSION

The rate of the hydrolytic degradation of HA in aqueous solution is strongly dependent on pH. Table 1 shows the pH dependence of $k_b$ at 40°C and 60°C. It is clear that HA is most stable at pH values around neutrality and more labile in acidic conditions than basic conditions. In addition, HA is less stable at higher temperature.

For the theoretical molecular orbital calculation of the HA hydrolysis, two model compounds were chosen as shown in Fig. 2. In order to reduce end-group effects, we focused only upon the central residue, a glucuronic acid residue in the model A and a N-acethylglucosamine residue in model B, and terminal glycoside bonds are substituted by methoxy groups in the model

**Table 1. Rate constant ($k_b$) vs pH at 40°C and 60°C**

| Temperature (degree) | $k_b$ ($s^{-1}$) | | |
|---|---|---|---|
| | pH3 | pH7 | pH11 |
| 40 | $5.0 \times 10^{-10}$ | $5.9 \times 11^{-11}$ | $5.3 \times 10^{-10}$ |
| 60 | $6.1 \times 10^{-8}$ | $2.5 \times 10^{-10}$ | $7.6 \times 10^{-9}$ |

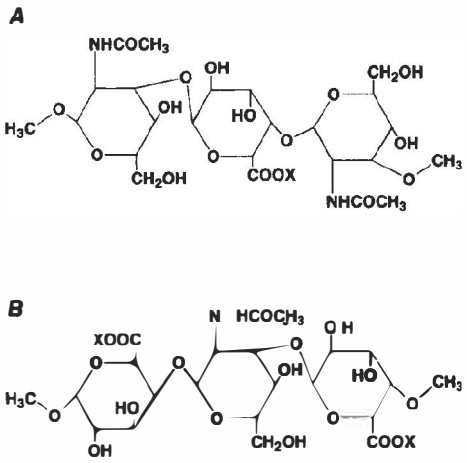


**Fig. 2.** Model compounds for MNDO-MO calculations.

compounds. The molecular orbital calculation shows that the lowest unoccupied molecular orbital (LUMO) is localized on a glucuronic acid residue and the highest occupied molecular orbital (HOMO) on a N-acethylglucosamine residue irrespective of the two model compounds.

This result implies that HA hydrolysis under acid conditions occurs on a glucuronic acid residue because of the nucleophilic nature of acidic hydrolysis.

On the other hand, the basic hydrolysis of HA is anticipated to start on a N-acethylglucosamine residue because of the electrophilicity of basic hydrolysis. The mechanisms of HA hydrolysis under acidic and basic conditions will be discussed in detail.

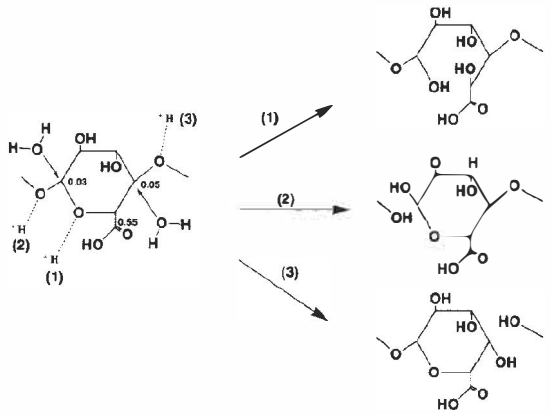


**Fig. 3.** Hydrolysis reaction mechanism of HA in acidic conditions: figures are electron densities of LUMO.

### 3.1. Acidic hydrolysis of hyaluronic acid

The results of the calculation clearly show that the electron density of LUMO on the glucuronic acid (HA units) is localized and that the hydrolytically active sites are on the C1, C4 and carbonyl carbons. Among these, hydrolysis at C1 or C4 contributes to chain scission. In addition, hydrolysis at C1 might involve 2 reaction processes, glycoside bond cleavage 1-3 and ring opening, while hydrolysis at C4 results in a one step reaction for glycoside bond cleavage (1-4) (Fig. 3). On the other hand, from the results of $^{13}$C-NMR (Fig. 5), the hemiacetal ring structure is maintained during the degradation of HA. Thus, the reaction mechanisms predicted from MNDO calculation were supported experimentally.

### 3.2. Basic hydrolysis of sodium hyaluronate

From the results of the calculation, it was clear that the electron density of HOMO on the N-acethylglucosamine (HA units) was localized and the hydrolytically active sites are on the C1, O (ring) and amide nitrogen. Among them, only the C1 position contributes to the lowering of the molecular weight of HA. In this case, the glycoside bond cleavage reaction on the C1 might

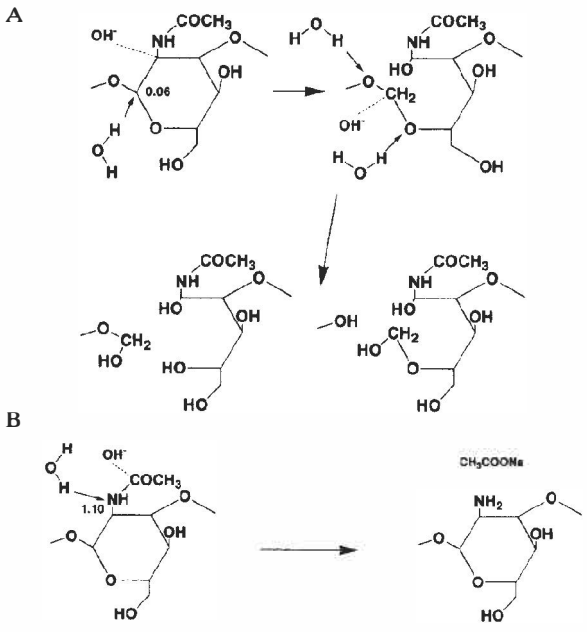


**Fig. 4.** Hydrolysis reaction mechanism of HA in the basic condition: figures are electron densities of HOMO.

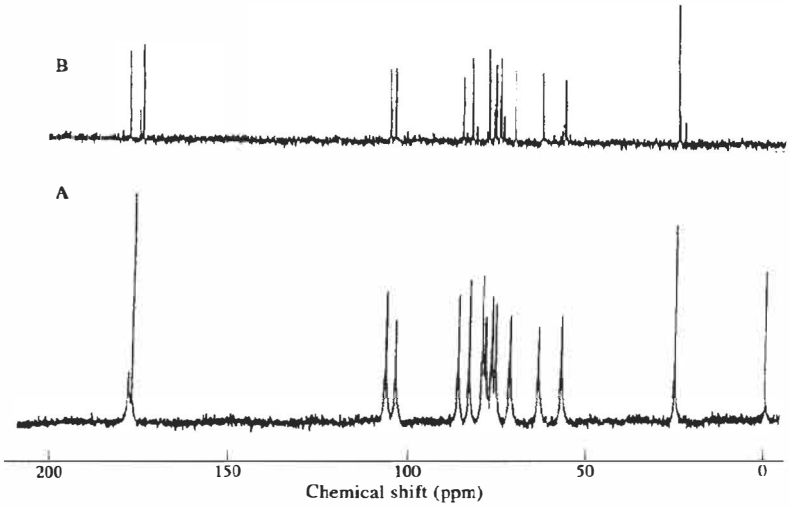


**Fig. 5.** $^{13}$C-NMR spectra (vs TSP): (A) hyaluronic acid, (B) degradation products of hyaluronic acid (pH 1·44, after 10 days).

involve two steps (contrary to acidic hydrolysis): the first step is the cleavage between C1 and C2, and the second step is glycoside bond cleavage (1-4) (Fig. 4). In the first reaction, the production of methylene groups is to be expected.

The $^{13}$C-NMR spectrum of the basic hydrolysis products is shown in Fig. 6. In order to discriminate carbon species, the samples were measured by means of the DEPT method,[10–12] which confirmed the existence of methylene carbon. In addition, the production of sodium acetate was expected from the hydrolysis of pendent functional groups (Fig. 4), and this was also confirmed by $^{13}$C-NMR (Fig. 6).

## 4. CONCLUSIONS

(1) The hydrolytic degradation of HA can be attributed to random chain scission and accordingly obeys first-order kinetics.
(2) From the MNDO calculation on model compounds of HA, it was speculated that acid and base catalysed hydrolyses occur

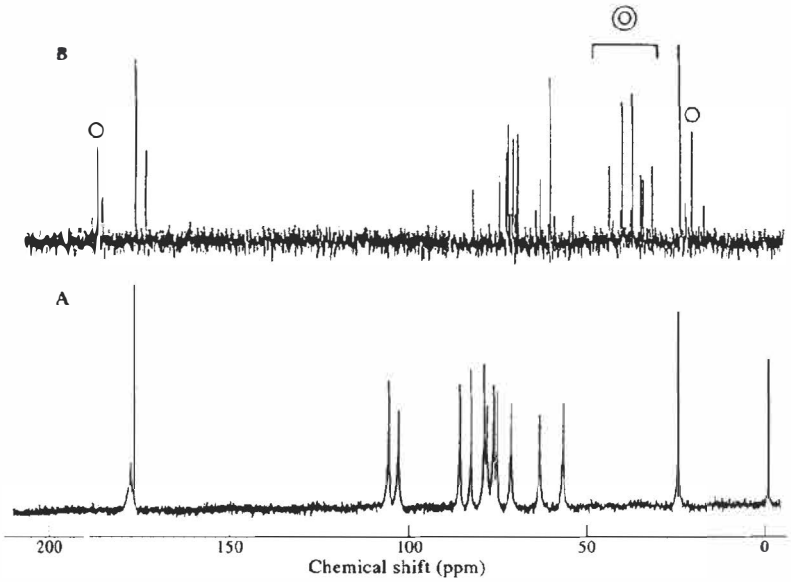


**Fig. 6.** $^{13}$C-NMR spectra (vs TSP): (A) hyaluronic acid, (B) degradation products of hyaluronic acid (pH 13·30, after 7 days), (○) $CH_3COONa$, (◉) methylene carbon.

on glucuronic acid and N-acethyl-glucosamine moieties, respectively. These speculations were supported by the results of $^{13}$C-NMR spectroscopy.

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