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Utilization of Enzymatic Digestion for the Study of the Macromolecular Effect in Complexation Processes. Protonation and Copper Coordination Equilibria of Hyaluronate and Its Fragments

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The effect of enzymatic digestion on the proton and copper(II) binding ability of hyaluronate (HYA), a macromolecular polyelectrolyte, and its fragments were studied via potentiometry. The degree of depolymerization was measured by spectrophotometry. The log K_{app} vs. α functions (where α is the mole fraction of dissociated carboxyl groups in the samples) and the protonation group constants were determined in the native and enzymatically partly and completely decomposed samples. The two different data treatments led to the same chemical consequences. The interaction between copper(II) and HYA was found to be less dependent on the degree of depolymerization than that between H^+ and HYA, the results indicating an almost negligible role of long-range electrostatic forces in the copper(II)–HYA interaction. All the investigations demonstrated that enzymatic digestion can be used advantageously for the characterization of the macromolecular effect in coordination processes.

The secondary structure and high electric charge of macromolecular polyelectrolytes, consisting of repeating monomeric units, may change significantly the basicity and metal ion affinity of electron pair donor groups on the molecule. This so called "macromolecular (polyelectrolyte) effect" can be characterized by the study of analogous protonation or metal coordination equilibria of the macromolecule and its fragments of small molecular weight, respectively. For this purpose either extensive preparative work is needed, i.e. the synthetic preparation of different fragments, or the decomposition of the macromolecule has to be performed under extremely well controlled circumstances. For latter purpose the enzymatic digestion of the macromolecule leading to fragments of different sizes seemed for us the most promising method. Sodium hyaluronate was selected as the first model compound to investigate the practical applicability of enzymatic decomposition of macromolecules for latter purpose.

Hyaluronate (HYA) is the most common glycosaminoglycan constituted of *N*-acetylglucosamine and D-glucuronate dimeric units.[1] Various types of enzyme systems degrade the mucopolysaccharides by an elimination mechanism (see Ref. 2 and the references therein). Hyaluronidase from *Streptococcus disgalactiae* was used to degrade HYA to produce a 4,5-unsaturated sugar at one end, and 2-acetamido-2-deoxy-D-glucose at the other end[3] of the decomposition products (see Fig. 1). The 4,5-unsaturated sugar exhibits an absorption maximum at 232 nm,[2] which permits determination of the concentration of 4,5-unsaturated sugars in the system[4,5] and hence the degree of depolymerization. The digestion can be terminated by heating[6] or by adjusting pH to 2.[2] After exhaustive digestion, the final product is 2-acetamido-2-deoxy-3-*O*-(β-D-hex-4-enopyranosuronyl)-D-glucose.

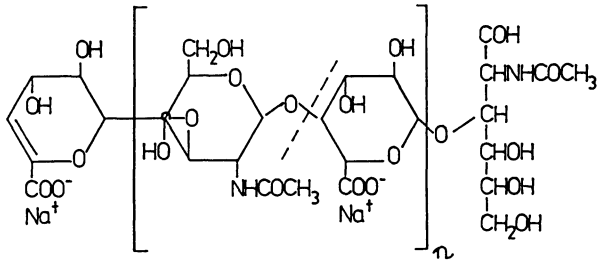

Fig. 1. Products of enzymatic digestion of hyaluronate. The dashed line shows the position of elimination; the products are represented on the right and left hand sides of the formula.

The protonation–deprotonation equilibria of native HYA were described in Refs. 7—10. The conditional protonation constant of HYA was found to be dependent on the initial concentration of HYA, the ionic strength and the degree of dissociation of the carboxyl groups.[8,9]

On the basis of UV-spectrophotometric[11] measurements, the formation of a copper(II) complex with composition CuL_2 was suggested, where L is one disaccharide unit of HYA. This composition was supported by polarographic measurements,[12] but analysis of the ^{13}CNMR spectrum of the complex Cu(II)HYA showed[13] that the formal composition of the complex is CuL. This latter finding was supported by potentiometric, viscosimetric, and electrophoretic data.[14] The conditional stability constant of the complex CuL was proved to be dependent on the degree of formation only at an ionic strength of ca. 0 but became independent of it at rather low ionic strengths ($I \geq 0.020$ mol dm^{-3}).[14]

The aim of the present investigation was to study the changes in the equilibrium properties of the H^+–HYA and Cu^{2+}–HYA systems during enzymatic digestion in

order to demonstrate the difference between the macromolecular and small molecular states and to show the continuous transition from one to the other. The results of this investigation are presented below.

Experimental

Materials. NaHYA of highest purity (protein content <0.1%), and hyaluronidase from *Streptococcus disgalactiae* (nominal activity: 5000 IU/cm^3), obtained from Gedeon Richter Ltd., Budapest, were used for the measurements. For characterization of the NaHYA, the uronic acid content of the sample was determined according to the method of Dische[15] as modified by Bitter and Muir.[16] All other chemicals were of analytical grade.

Methods. The enzymatic decomposition was performed in a 0.005 mol dm^{-3} aqueous solution of NaHYA at pH=6.4 at (37.0±0.1) °C. The ionic strength was adjusted with NaCl (for pH-metry) or $NaClO_4$ (for measurements with a Cu(II) ion-selective electrode) to 0.02 mol dm^{-3}, which has been shown[9,11] to be high enough to keep the mean activity coefficient of the free cations the same in the presence and absence of HYA. Control experiments showed that the presence of the enzyme, in negligible quantity, caused no change in either the electrode calibration curves or the absorption spectrum of the digested sample (see below). Two methods were used to terminate the cleavage.

a) In the pH-metric measurements, a known amount (10.00 cm^3) of the sample was acidified with 2.00 cm^3 of 0.1 mol dm^{-3} standard HCl solution.

b) In the Cu(II) ion-selective electrode measurements, pH of a known amount (10.00 cm^3) of sample was adjusted to pH 4.5 with 0.1 mol dm^{-3} $HClO_4$ from a microburette.

After termination of the digestion, the samples were titrated with standardized NaOH or $Cu(ClO_4)_2$ solution at (25.0 ±0.1) °C, under an inert N_2 atmosphere. Electrode calibration procedures were carried out according to Refs. 18 and 19 with solutions of the same composition as the samples to be measured, but without HYA. For the measurements, a Radiometer G2O2B glass electrode, a Radelkis OP CU7113 Cu(II) ion-selective electrode and a Radelkis OP 08303 double junction Ag/AgCl reference electrode were used (separating solution 0.020 mol dm^{-3} NaCl or $NaClO_4$), which were connected to a computer-controlled automatic titrator described in Ref. 18.

To monitor the degree of depolymerization, the absorption spectrum of the digested sample was recorded on a Varian 634 UV-visible spectrophotometer, at 1 mm path length in the wavelength range 300—180 nm (Fig. 2).

Results and Discussion

pH-Metry. In order to monitor the changes in basicity of the carboxylato groups on the molecule, when the macromolecular structure is destroyed by cleavage, the use was made of the Henderson-Hasselbach (HH) function:[9]

$$\log K_{app} = pH - \log \frac{\alpha}{1-\alpha}, \quad (1)$$

(where α is the mole fraction of dissociated carboxyl groups in the uronic acid moiety of the differently digested samples) and the group constants[20] of the oligomeric sugar acids.

The HH functions of the native and completely digested HYA are presented in Fig. 3. The $\log K_{app}$ vs. α function of the native sample shows a characteristic linear increase. The intercept ($\log K_0$=2.73±0.03) and the slope (0.69) are well compared with the data presented in Ref. 9 ($\log K_0$=2.82±0.01, slope=0.77 at an ionic strength of 0.010 mol dm^{-3}).

After exhaustive digestion, the HH function of the system became independent of α, the result indicating the presence of chemically equivalent small molecular species.

The absorption spectra of HYA samples at different stages of digestion are shown in Fig. 2. After completion of the cleavage, the molar extinction coefficient of

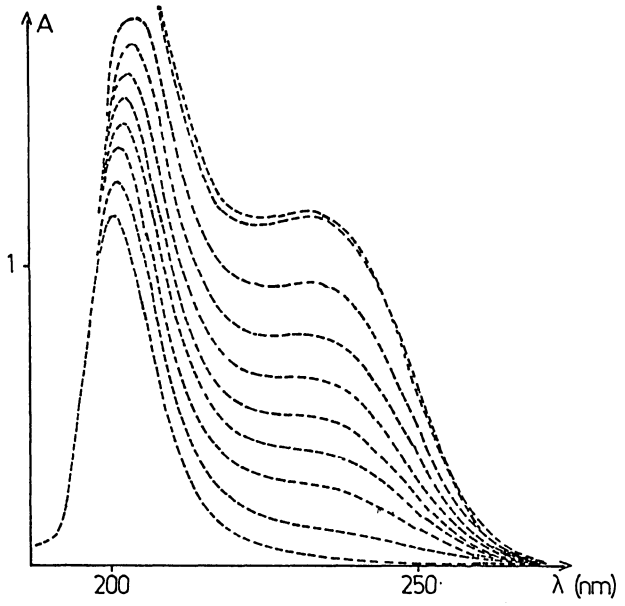

Fig. 2. UV-spectra of hyaluronate during enzymatic digestion (pH=6.4, t=37.0 °C, path length: 1 mm, $C_{hyaluronate}$=1.848×10^{-3} mol dm^{-3}).

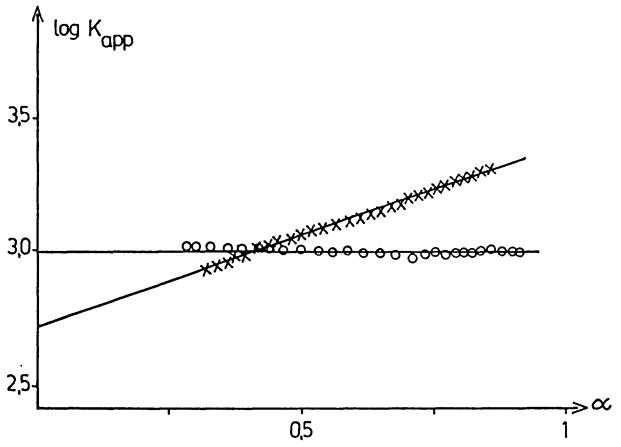

Fig. 3. Conditional protonation constants ($\log K_{app}$) of native (x) and exhaustively digested (o) hyaluronate as a function of the degree of dissociation (α).

the 4,5-unsaturated sugar part was found to be ε=6060 $mol^{-1}\,dm^3\,cm^{-1}$ well compared with that in Ref. 2. The average degree of depolymerization (F) can be calculated from the absorbance measured at different stages of digestion (A) according to the following equation:

$$F = 100\frac{A - A_0}{A_M - A_0}\ (\%), \tag{2}$$

where A_0 and A_M are the absorbances (λ=232 nm) of the samples before digestion and after complete digestion, respectively.

To characterize the samples in the internal stages of the enzymatic processes, the protonation group constants[20] of the two different carboxylate basic centers were calculated. The group constant theory can be used only if the effect of the protonation state of the neighboring group on the site to be studied can be neglected, because the number of σ-bonds between two groups to be studied is more than three.[21] This condition is fulfilled in our system, in which two types of carboxylato groups are present: glucuronate (**I**) and hex-4-enopyranosuronate (**II**), which are separated by 10 σ-bonds.

The appropriate model function is

$$1-\alpha = \frac{C_A}{C_0}\frac{K_A[H^+]}{1+K_A[H^+]} + \frac{C_B}{C_0}\frac{K_B[H^+]}{1+K_B[H^+]}, \tag{3}$$

where K_A and K_B are the group constants, and C_A, C_B and C_0 are the analytical concentrations of compounds **I**, **II**, and HYA, respectively.

The results of these calculations are presented in Fig. 4. The group constants of carboxylates of compounds **I** and **II** decrease with increasing F, and tend to a limiting value. The group constant of the carboxylate for the unsaturated **II** molecule is the same as that of the HH function for the completely digested system in Fig. 3, and K_A tends to approach, the same value as that of the HH function for the native HYA at α=0. This shows that the log K_0 value is characteristic for the basicity of the repeating unit when it is not affected by macromolecular (polyelectrolyte) effects. The data also demonstrate that the HH and group constant treatments of the data can lead to the same chemical consequences.

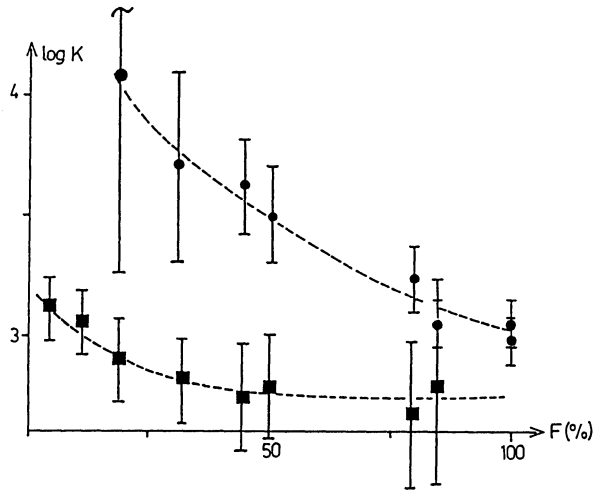

Fig. 4. Protonation group constants (log K) of the glucuronate (■) and hex-4-enopyranosuronate (●) moieties of hyaluronate as a function of the degree of depolymerization (F).

The protonation constants of the unsaturated sugar moiety which is the final product of the digestion is ca. 0.3 log unit higher than log K_0. Since a similar difference is obtained by comparing the log K values of isovaleric acid (4.78) and 3-methyl-2-butenoic acid (5.12),[22] this increase is possibly due to the appearance of the C=C double bond in the near vicinity of the carboxylate.

The tendency of both group constants to increase slightly with increasing average degree of polymerization may be due to the increasing electrostatic field around the macromolecule. This finding indicates experimentally that the enzymatic cleavage is random-like and has no direction (say from one end to the other). In the opposite case, log K_B would be independent of F, and the product of each digestion step would be a small molecular species. This finding supports the data presented in Ref. 6.

Since the experimental errors may cause relatively great ambiguities, an attempt was made to estimate the standard deviations of K_A and K_B at different F's. The functions

$$K_A = f(K_B, x_i) \tag{4}$$

$$K_B = g(K_A, x_i) \tag{5}$$

were used in the explicit form from Eq. 3, where x_i dennotes various chemical quantities measured (x_i= $\alpha[H^+]$, C_A, C_B, C_0). The finite differentials of K_A and K_B

$$\Delta K_A \approx \frac{\partial f}{\partial K_A}\Delta K_B + \sum_{i=1}^{n}\frac{\partial f}{\partial x_i}\Delta x_i \tag{6}$$

$$\Delta K_B \approx \frac{\partial g}{\partial K_A}\Delta K_A + \sum_{i=1}^{n}\frac{\partial g}{\partial x_i}\Delta x_i \tag{7}$$

are approximate values of the standard deviations of K_A and K_B, while ∂x_i's are the estimated standard deviations of the measured quantities x_i's. This equation system was solved for every sample in the pH range 2.6—4.3. The pH-averaged standard deviations for the individual log K_A and log K_B data are also shown in Fig. 4. It is to be seen that, according to expectations, the standard deviations increase markedly with decreasing concentration of species **I** and **II**.

Measurements with a Cu(II) Ion-Selective Electrode. The log K_{app} vs. α function of the Cu(II)–HYA system at an ionic strength of 0.020 $mol\,dm^{-3}$ was found to be independent of α not only for the native[11] but also for the partly and completely digested HYA samples. This allowed characterization of the system at a given stage of cleavage by one log K_{app} value, which can be regarded as the average stability constant of the different com-

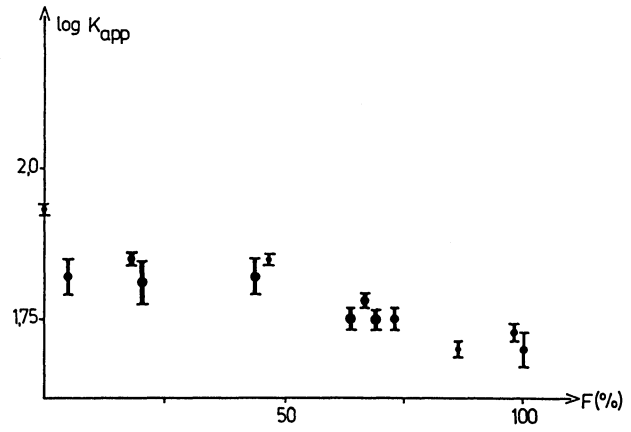

Fig. 5. Conditional stability constants (log K) of copper(II)–hyaluronate complexes as a function of the degree of depolymerization (F).

plexes CuHYA (Cu^{2+} binding to uronic or to hex-4-enopyranosuronate carboxylato groups) present in solution.

This log K_{app} vs. F function is presented in Fig. 5. log K_{app} slightly decreases with increasing degree of depolymerization, but this change is less dramatic than that in the H^+–HYA system. The expected standard deviations of the log K_{app} values (0.02—0.05 log unit) are smaller than the difference between the two extreme (e.g., the native and the completely digested) stages of the system (0.2 log unit). This slightly decreasing tendecy in log K_{app} can be explained by considering two opposite effects: The carboxylato groups in native HYA are less basic than those in the completely digested one, but the ability of the former to bind Cu(II) ions is elevated by the macromolecular character (polyelectrolyte effect) of native HYA.

Conclusion

The use of enzymatic decomposition of sodium hyaluronate and the comparison of protonation and copper complexation of the macromolecule and its fragments, respectively, has demonstrated that latter method can be used advantageously for characterizing the effect of the secondary structure and surface charge of macromolecular polyelectrolytes on the basicity and/or metal ion affinity of donor groups on the molecule. The results of the investigations have also shown that the Henderson–Hasselbach function and the group constant concept are equally suitable for the quantitative treatment of such systems.

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