ELSEVIER

Journal of Chromatography B, 656 (1994) 179–190

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Kinetic investigation of the degradation of hyaluronan by hyaluronidase using gel permeation chromatography

Koen P. Vercruysse*, A.R. Lauwers, J.M. Demeester

University of Ghent, Laboratories of General Biochemistry and Physical Pharmacy, Wolterslaan 16, B-9000 Gent, Belgium

Abstract

A gel permeation chromatography (GPC) system for the analysis of hyaluronan was set up and calibrated by means of the universal calibration method. Using GPC, a kinetic assay of the action of hyaluronidase on hyaluronan has been developed. Applying Michaelis–Menten theory and the direct linear plot we have estimated the kinetic constants of the enzyme. By evaluating the decrease of the various molecular mass averages during the enzymatic reactions, we have given a demonstration of the at random degradation of hyaluronan by hyaluronidase in the initial stage of the reaction.

1. Introduction

Hyaluronan (HA) is a negatively charged high-molecular-mass polysaccharide. It is a linear polymer built from repeated disaccharide units with the . . . [D-glucuronic acid (1-ß-3)N-acetyl-D-glucosamine (1-ß-4)]$_n$ . . . structure [1]. It is ubiquitously distributed in the extracellular space of higher animals, especially in soft connective tissues [1]. Recently some of the physiological and biological functions of HA have been reviewed [1]. Hyaluronidase (HYASE) (hyaluronate-4-glycanohydrolase, EC 3.2.1.35) hydrolyzes the ß-N-acetyl-hexosaminic bonds in HA and also in chondroitin-4- and -6-sulphate and their desulfated derivates, to yield even-numbered oligosaccharides with glucuronic acid residues at the non-reducing end [2]. Hyaluronidases possess important biological functions: they are suspected to be responsible for the pathogenesis of several bacteria [3] and other infectious species [4]; they may contribute to the acceleration of the absorption and diffusion of venoms [5]; HYASE is present in the spermatozoa of many mammalian species and it is assumed to play a major role in fertilization [6,7]. Clinically the enzyme is used in the chemotherapy to enhance the antineoplastic activity of cytostatics [8,9] and in the treatment of acute myocardial infarction [10,11]. The inhibitors of HYASE are investigated as potential anti-allergic compounds [12,13] and as potential non-hormonal contraceptive agents [14]. In view of the importance of the enzyme and its inhibitors, an accurate kinetic assay is required to study the enzyme or the compounds with a potential inhibitory capacity. Quantitative assays of the activity of HYASE are performed by time-dependent total intensity light scattering [15], viscosimetry [16,17], turbidimetry [18], spectrophotometry of complexes between HA and dyes [19] or spectrophotometry of the liberated hex-

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00045-7

osaminic endgroups [20]. Turbidimetry, viscosimetry and spectrophotometry of complexes express the rate of reaction and the activity of the enzyme on an empirical base (decrease of turbidity, viscosity or binding capacity of a HA-solution). The change in physico-chemical properties of HA can hardly be related to the number of bonds broken [17]. Using light scattering or spectrophotometry of the endgroups, the rate of reaction and the activity of the enzyme can be expressed as the number of bonds cleaved per unit of time. This is the only objective way to express the activity of an enzyme degrading a polymeric substrate.

In this work we present a sensitive kinetic assay using gel permeation chromatography (GPC). Statistical descriptions of the at random degradation of a polymer sample yielded, in the initial stage of the reaction, a linear relationship between the reciprocal value of the number average molecular mass (M_n) and the time of reaction [21]. The slope of this relationship provides the rate of reaction, which can be expressed as the number of bonds broken per unit of time. Determining the rate of reaction at different concentrations of HA allowed us to calculate the kinetic parameters of the enzyme. Including the calculation of the other molecular mass averages (MMA), such as mass MMA (M_w), viscosity MMA (M_v), z-MMA (M_z) and $z+1$-MMA (M_{n+1}), we have compared our experimental findings to some theoretical considerations regarding the at random degradation of a polymer.

2. Experimental

2.1. Materials

HA (sodium salt, extracted from rooster comb) was kindly provided by Diosynth (Oss, Netherlands). The testicular hyaluronidase (EC 3.2.1.35) used was the international reference standard provided by the FIP (Ghent, Belgium), containing 330 I.U./mg. Poly(ethylene oxide) TSK standards and pullulan SHODEX P-82 standards with narrow molecular mass distributions ($M_w/M_n < 1.1$) and peak molecular masses in the range of $5.8 \cdot 10^3$–$8.85 \cdot 10^6$ were purchased from Waters/Millipore (Brussels, Belgium). All other chemicals used were of analytical grade. Enzymatic digestions were performed in a sodium phosphate buffer (pH 6.4) containing 140 mM NaCl, 16 mM NaH_2PO_4 and 7 mM Na_2HPO_4 at 37°C.

2.2. Gel permeation chromatography

Ultrahydrogel 1000, 2000 and DNA columns (300 × 7.8 mm I.D.; pore sizes 1000 Å, 2000 Å and 4000 Å, respectively) (Waters/Millipore, Brussels, Belgium) were connected in series to a gel permeation chromatography system (Waters/Millipore, Brussels, Belgium), consisting of a Model 510 pump, a R410 differential refractometer, a U6K injector and was controlled by a System Interface Module. The chromatograms were processed by the Waters Baseline 810 chromatography and data station. The mobile phase was 0.5 M NaCl (pH 6.3) at 0.6 ml/min and the analyses were performed at 25°C. Sample concentrations were 0.3 mg/ml for HA and 0.5 mg polymer/ml for the GPC standards. For each run 300 μl were injected. Ultrahydrogel consists of a cross-linked methacrylate gel packing which is stable in a pH range of 2–12. All samples were dissolved in or diluted with the eluent.

3. Results and discussion

3.1. Calibration

The columns were calibrated using the universal calibration method. This is based on the fact that the retention volume of a polymer is determined by its hydrodynamic volume (HV) rather than its molecular mass [22]. The hydrodynamic volume of a polymer is given by the product of its intrinsic viscosity [η] and its molecular mass M_r. These properties are related to each other by the Mark–Houwink equation:

$$[\eta] = kM_r^{\alpha} \quad (1)$$

where k and α are constants depending on the nature of the polymer, the temperature and the solvent. Thus HV is given by:

$$HV = [\eta]M_r = kM_r^{\alpha+1} \quad (2)$$

As poly(ethylene oxide) (PEO) and pullulan are neutral polymers the values of their Mark–Houwink constants do not change significantly when working with water or in eluents with an elevated ionic strength [23]. The values used for the calibration are those determined in water at 25°C. For PEO, $M_r > 40\,000$, k and α are 0.01192 and 0.76 respectively [24], for PEO, $M_r < 40\,000$, k and α are 0.156 and 0.50 respectively [24] and for pullulan k and α are 0.0191 and 0.67 respectively [25]. Mixtures of standards were analysed several times. The logarithm of the HV of each GPC standard was plotted against its average retention time (T_r) and through these points a third degree polynomial curve was constructed. The equation of this curve was used as the calibration line, relating log(HV) to T_r, and is presented in Fig. 1.

3.2. Calculation of the MMA

Linear relationships between log([η]) and log(M_r) of HA have been reported under several conditions and over a broad range of molecular masses leading to several sets of Mark–Houwink constants of HA [26–28]. The values of the Mark–Houwink constants of HA, in 0.5 M NaCl and at 25°C, are $k = 0.0318$ and $\alpha = 0.777$ [29]. Absolute molecular masses for HA were calculated using this set of Mark–Houwink constants and the calibration line. Three samples (1–3) of HA were analysed three times. The molecular mass distribution (MMD) and the MMA were calculated. The results of the reproducibility of the calculation of the MMA are presented in Table 1.

3.3. Stability of HA solutions

HA dissolved in buffer (0.30 mg/ml) was stored at 4°C for two weeks. Samples were analysed on the day of preparation and during

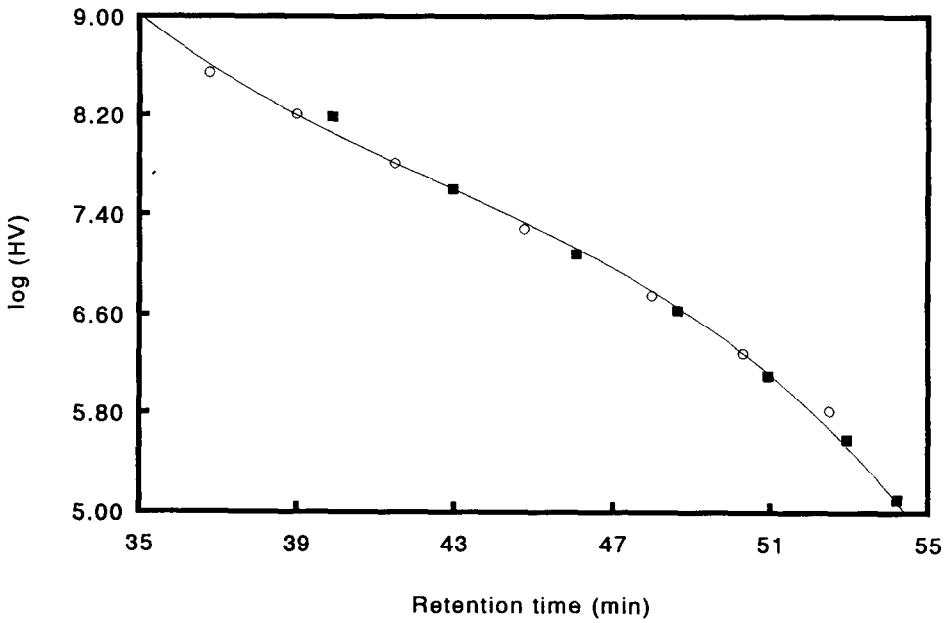

Fig. 1. Universal calibration graph using poly(ethylene oxide) (○) and pullulan (■) standards, describing the relationship between the logarithm of the hydrodynamic volume (HV) of the polymers and the time of retention. HV is the product of the intrinsic viscosity ([η]) and the molecular mass (M_r) of a polymer. A third degree polynomial was constructed through the datapoints. Performed on Ultrahydrogel 1000/2000/DNA columns at 25°C with RI-detection. Eluent 0.5 M NaCl at 0.6 ml/min.

Table 1
Reproducibility of the determination of the molecular mass averages of hyaluronan samples ($n = 3$)

Sample	M_n	M_w	M_v	M_z	M_{z+1}
1	357 000	695 000	663 000	980 000	1 220 500
	±16 500	±23 500	±24 000	±23 500	±40 500
2	379 500	673 000	644 000	945 000	1 208 000
	±31 000	±14 000	±12 500	±21 500	±24 000
3	351 000	707 500	676 500	973 500	1 185 000
	±16 000	±19 500	±20 000	±16 000	±27 000

Analysed on Ultrahydrogel 1000/2000/DNA columns at 25°C with RI-detection; eluent 0.5 *M* NaCl at 0.6 ml/min. A 300-μl sample containing 0.3 mg hyauronan/ml was injected.

storage [$M_{n,4°C} = 500\,500 \pm 11\,500$ ($n = 4$)]. A second solution (0.95 mg/ml) was placed in a water bath at 37°C for 24 h. Samples were taken, diluted with eluent to 0.30 mg/ml and analysed [$M_{n,37°C} = 512\,500 \pm 11\,000$ ($n = 4$)]. The results indicated that in both experiments no degradation had occurred. Solutions of HA can be stored at 4°C for several days and need not to be analysed the day of preparation. At 37°C no spontaneous hydrolysis could be detected. Knowing the variation in calculating the MMA of a sample (Table 1), we concluded that any significant decrease of *e.g.* M_n during an enzymatic reaction would be due to the action of the enzyme.

3.4. Preliminary experiments

A critical point of any kinetic assay is the concentration of enzyme used. To apply Michaelis–Menten theory, the initial rate of the reaction needs to be determined. Moreover the theoretical considerations describing the at random degradation can only be applied in the initial stage of the reaction [21]. The first derivative of the $1/M_n$ function at time $t = 0$ provides the initial rate of the reaction. The concentration of the enzyme should not be too high, otherwise HA would be degraded too fast and one would study the action of HYASE on oligomeric substrates. Evaluating the action of HYASE on a polymeric substrate is a better approach to the *in vivo* situation. Moreover, the lower the concentration of the enzyme, the higher the sensitivity of the assay. Solutions of HA were placed in a water bath at 37°C. HYASE was dissolved in buffer prior to use. Reactions were initiated by adding different amounts of HYASE to the solutions. The concentration of HA was 1.5 mg/ml and the concentrations of HYASE tested were: 15 μg/ml, 7.5 μg/ml, 1 μg/ml and 0.3 μg/ml. A 400-μl volume of eluent was kept cool in an ice-bath. At different times after the addition of the enzyme, samples (100 μl) were taken from the reaction mixtures, diluted to a final concentration of 0.30 mg HA/ml with the 400-μl eluent and analysed immediately. M_n of the initial sample was 469'500. The decrease of M_n in these conditions is presented in Table 2. The results from Table 2 indicate that the use of a concentration of 7.5 or 15 μg HYASE/ml leads to a very rapid decrease of M_n. Using 0.3 μg HYASE/ml the degradation proceeds more slowly, leading to a significant decrease of M_n after 100 min. From these results we decided to continue our investigations using 1 μg HYASE/ml (0.33 I.U./ml).

3.5. Development of a practical assay

We have set up a practical way of working in which we are able to take a sample from the reaction mixture, to inactivate the enzyme and to store the sample until analysis without any further degradation. To a solution of HA, HYASE was added so that the concentrations were 1.5 mg/ml and 1 μg/ml for HA and HYASE respectively. Two hours after starting the reaction, the mixture was divided and poured in five plastic vessels which were kept in an ice-bath (samples

Table 2
Decrease of the number average molecular mass of hyaluronan by hyaluronidase at different concentrations of hyaluronidase

Concentration of hyaluronidase (μg/ml)	Reaction time (min)	M_n
15	110	20 500
7.5	60	42 100
	300	22 700
1	60	261 500
	150	185 500
	280	155 500
0.3	110	406 500
	180	331 500
	320	298 500

Reactions were performed in a sodium phosphate buffer (pH = 6.4) at 37°C; $(M_n)_{t\approx 0} = 469\ 500$. GPC-analyses were performed as described in Table 1.

A–E). From sample A 100 μl were taken, diluted with 400 μl eluent and analysed immediately. All samples were frozen and stored at −20°C. Several days after freezing, a sample was thawn, 100 μl were taken, diluted and analysed. M_n of the sample prior to digestion was 428 000. Table 3 shows M_n of the samples stored at −20°C. After 11 days, two samples (A and D) were thawn and stored further at room temperature. Samples were taken and treated as before. The results are presented in Table 3. With the results from Table 3 and knowing the normal variations of our calculations we concluded that the results of the analyses of the samples performed within days or weeks after sampling, did not differ from the results of the sample which was analysed immediately. After storing at −20°C and consecutive thawing the samples still reflect the MMD of HA as it was at the time of sampling. Freezing the samples irreversibly inactivates the enzyme, as the samples did not show further degradation upon thawing and storing at room temperature. A solution of HYASE stored at 4°C for several days, still showed signs of activity when replaced at room temperature. Similar effects, concerning purified

Table 3
M_n of enzymatic digested hyaluronan solutions stored at −20°C

Sample	Period at −20°C (days)	M_n
A	0	232 500
A	1	227 000
B	4	230 500
C	5	242 000
D	6	247 500
E	7	259 000
A	11	235 000
B	26	242 500

M_n of enzymatic digested samples A and D stored at room temperature, after 11 days at −20°C

	Period at room temperature	M_n
A	18 h	242 500
	15 days	240 500
B	4 days	232 500
	19 days	230 500

Enzymatic digestion of hyaluronan by hyaluronidase was performed. After 120 min, the mixture was divided into 5 samples (A–E) and stored at −20°C. The number average molecular mass (M_n) was calculated immediately and several days after storing at −20°C. Analyses were performed as described in Table 1.

samples of hyaluronidase from snake venoms, were reported before [30]. Activity could be retained by adding bovine serum albumin to the solutions. Other reports owed this deactivation due to adsorption of the enzyme onto glass [31]. Activity was retained by adding detergent or high concentrations of salt. As our samples were stored in plastic vessels, we believe that during the process of freezing the enzyme is irreversibly denaturated and cannot restore its conformation when thawn.

3.6. Linearity of the assay

Mixtures containing 1.5 mg HA/ml and 1.4, 1.2, 0.8 or 0.6 μg HYASE/ml were kept at 37°C. At different times after starting the enzymatic reaction, samples (100 μl) were taken and diluted with 400 μl ice-chilled eluent. The samples were frozen and stored at −20°C. Within two

weeks these samples were thawn and analysed immediately. From the GPC analyses the MMA were obtained and $1/M_n$ was plotted against the time of reaction. Straight lines were obtained (correlation coefficients >0.99) and the slopes of these, the rate of reaction (k_n) expressed as the number of moles of bonds broken per unit of time and per unit of weight HA present, were plotted against the concentration of HYASE (E) present in the mixtures. The rate of reaction was proportional to the concentration of enzyme present in the mixtures, within the range (0.0–1.4 μg HYASE/ml) tested ($k_n = -4.7 \cdot 10^{-14} + 1.1 \cdot 10^{-12} E$; $r^2 = 0.99$).

3.7. Kinetic assay

After establishing and evaluating our practical way of working we have performed some kinetic assays to determine the kinetic constants of the enzyme acting on HA. Samples containing different amounts of HA were placed in a water-bath at 37°C. HYASE was added so that the final concentration was 1 μg/ml. At different times, samples (100 μl) were taken, diluted to 0.3 mg/ml with ice-chilled eluent and stored at −20°C. Within two weeks the samples were analysed and the MMA calculated. The initial rate of the reaction, v_0 (katal/ml), was obtained from:

$$v_0 = [\mathrm{d}(c/M_n)/\mathrm{d}t]_{t=0} \quad (3)$$

where c is the concentration of HA (g/ml) and t is the time of reaction (s). The unit of 'katal' is the catalytic amount which catalyzes as many reaction cycles per second as there are carbon atoms in 0.012 kg of the pure nuclide ^{12}C. Using the direct linear plot [32], the constants of the Michaelis–Menten equation (K_m and V_{max}) were estimated. The Michaelis–Menten equation is given by [33]:

$$v_0 = \frac{V_{max} S}{K_m + S} \quad (4)$$

where S is the concentration of substrate (mg HA/ml) present. V_{max} is the maximum rate (katal/ml) of the reaction. K_m is the Michaelis–Menten constant and is the concentration of substrate (mg/ml) at which the rate of reaction is half the maximum rate. In Fig. 2, c/M_n is plotted against the time of reaction for different concentrations of HA. Fig. 3 shows a direct linear plot used to estimate K_m and V_{max}. The observed results ($n = 3$) were found to be $K_m = 0.44 \pm 0.04$ mg/ml and $V_{max} = 1.8 \cdot 10^{-12} \pm 0.3 \cdot 10^{-12}$ katals/ml or $1.8 \cdot 10^{-9} \pm 0.3 \cdot 10^{-9}$ katals/mg of an enzyme with an activity of 330 I.U./mg. The observed value of K_m can be compared with other values obtained under different conditions [15]. However, since K_m is expressed in mg/ml, it is very probable that the value might depend on the substrate used.

3.8. Other molecular mass averages

From the GPC analyses additional information regarding the MMD of HA during the enzymatic degradation could be obtained. Therefore we have investigated the decrease of the other MMA as a function of the time of reaction. Fig. 4 provides an example of the increase of 1/MMA during an enzymatic reaction. Linear relationships were obtained and the extrapolations to $t = 0$ are compared with the values of the MMA of the original sample in Table 4. Others have reported partial deactivation of the enzyme, due to adsorption onto glass or other mechanisms, within minutes after initiating the reaction [15]. We could not detect any unusual behaviour as our plots of c/M_n against the time of reaction were linear until the last point of sampling (Fig. 2). Moreover the extrapolations to time zero corresponded well with the MMA values of the initial sample (Table 4). As the reciprocal values of the MMA provided linear relationships when plotted against the time of reaction, linear regressions were performed and following equations were set up:

$$\frac{1}{M_n} = \frac{1}{(M_n)_{t=0}} + k_n t \quad (5)$$

$$\frac{1}{M_w} = \frac{1}{(M_w)_{t=0}} + k_w t \quad (6)$$

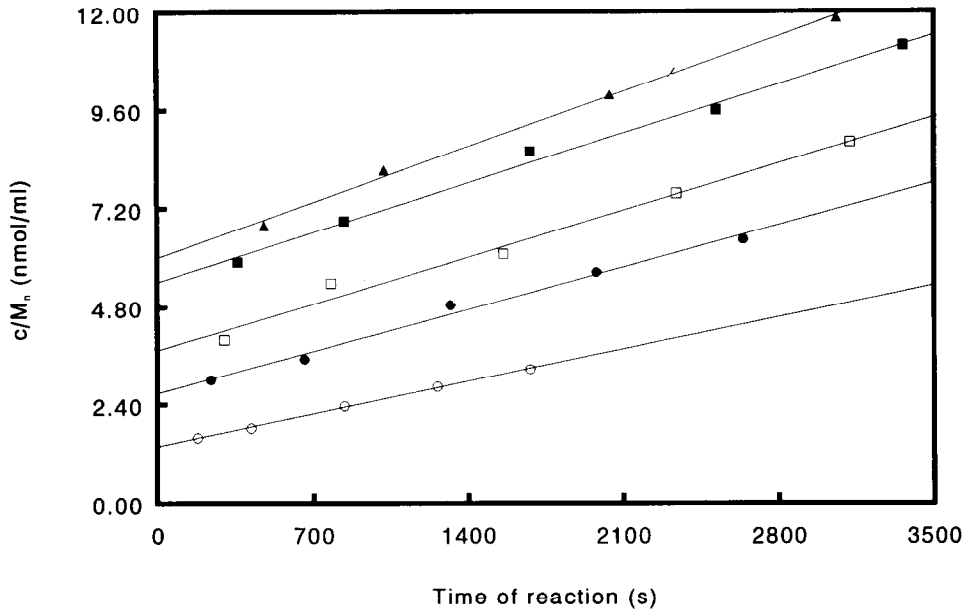

Fig. 2. Decrease of c/M_n during the digestion of hyaluronan by hyaluronidase; c is the concentration of hyaluronan present in the mixture (○) 0.0005 g/ml; (●) 0.001 g/ml; (□) 0.0015 g/ml; (■) 0.002 g/ml; (▲) 0.0025 g/ml). M_n is the number average molecular mass. The concentration of hyaluronidase was 1 μg/ml (0.33 I.U./ml). Reactions were performed in a sodium phosphate buffer (pH 6.4) at 37°C.

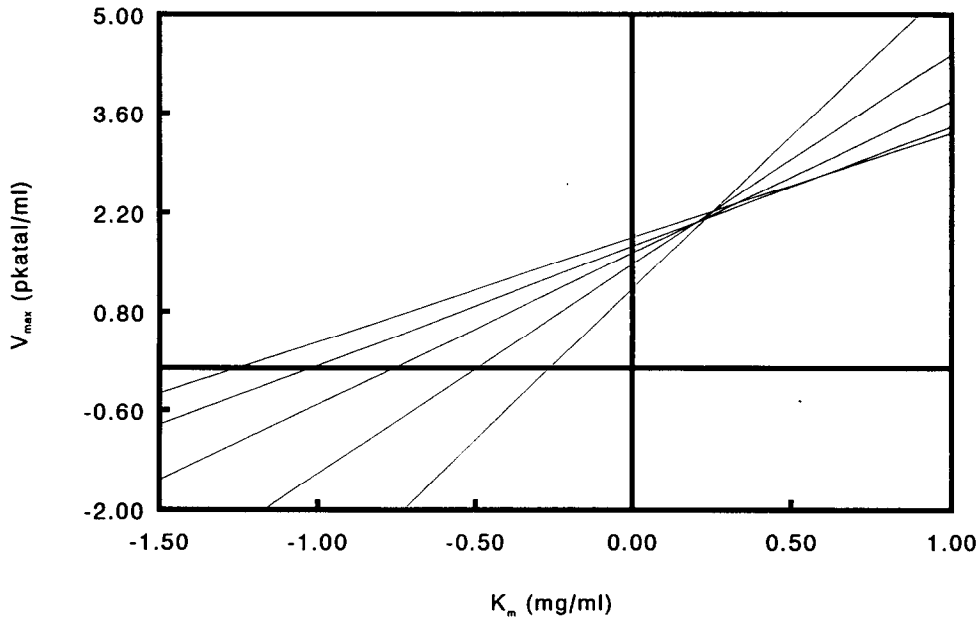

Fig. 3. Direct linear plot [32] used to estimate the kinetic constants of hyaluronidase acting on hyaluronan.

$$\frac{1}{M_v} = \frac{1}{(M_v)_{t=0}} + k_v t \quad (7)$$

$$\frac{1}{M_z} = \frac{1}{(M_z)_{t=0}} + k_z t \quad (8)$$

Table 4
Comparison of the molecular mass averages of the hyaluronan solution prior to digestion and the molecular mass averages obtained after extrapolation to time = 0 following enzymatic digestion

	Extrapolation ($n = 6$)	Initial sample ($n = 3$)
M_n	391 000 ± 14 500	396 000 ± 17 500
M_w	728 000 ± 22 000	728 000 ± 35 000
M_v	698 000 ± 21 500	697 000 ± 34 500
M_z	992 500 ± 35 000	1 000 500 ± 32 500
M_{z+1}	1 217 000 ± 56 500	1 232 500 ± 23 500

Reactions performed in a sodium phosphate buffer (pH = 6.4) at 37°C. The reciprocal value of the molecular mass averages were plotted against the time of reaction and the intercept of the linear regressions were estimated as the molecular mass averages of the initial sample and were compared with the values obtained after analyzing the initial sample. Analyses were performed as described in Table 1.

$$\frac{1}{M_{z+1}} = \frac{1}{(M_{z+1})_{t=0}} + k_{z+1} t \quad (9)$$

where k_n is the rate of reaction and k_w, k_v, k_z and k_{z+1} are all values related to this rate of reaction. From all the enzymatic reactions performed during the kinetic assay, these values were calculated and plotted against the values of k_n (Figs. 5–8). According to statistical descriptions of the at random degradation of a polydisperse sample, k_w should equal $k_n/2$ in the initial stage of the reaction [21]. The correlation we have found experimentally (Fig. 5) is in agreement with theory (compare $0.497 \leftrightarrow 0.5$). Other theoretical descriptions predicted that the at random degradation of a polymer sample will lead to a MMD with following characteristics [34]:

$$M_n = \frac{1}{2} M_w = \frac{1}{3} M_z = \frac{1}{4} M_{z+1} \quad (10)$$

If this holds true then should $k_z = 0.333\ k_n$ and $k_{z+1} = 0.25\ k_n$. Our experimental results seem to confirm these findings (Fig. 7: $0.311 \leftrightarrow 0.333$; Fig. 8: $0.230 \leftrightarrow 0.25$). The differences could be

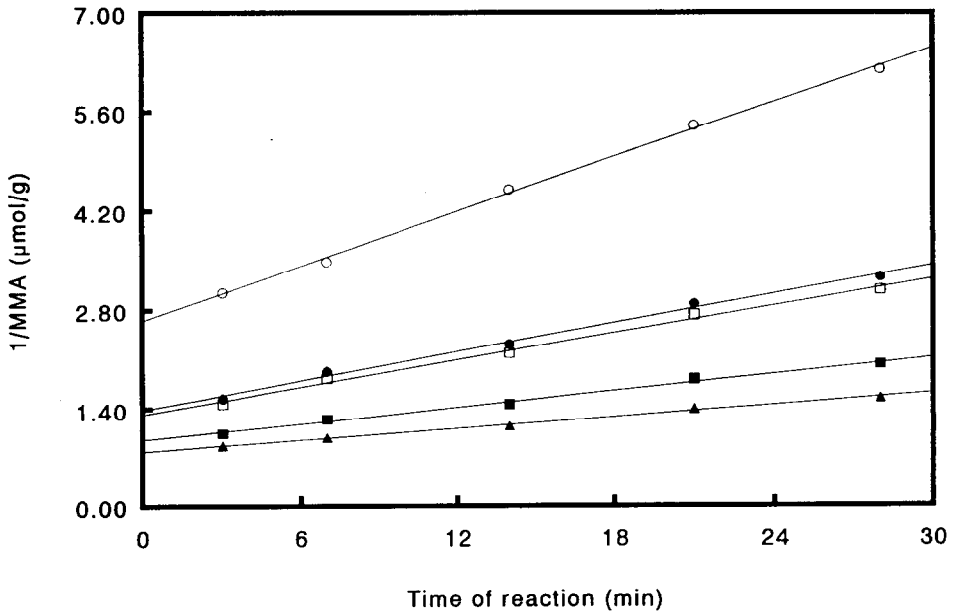

Fig. 4. Linear relationships between the time of reaction and the reciprocal values of molecular mass averages (MMA) during the degradation of hyaluronan by hyaluronidase. (○) M_n (number average M_r); (□) M_v (viscosity average M_r); (●) M_w (weight average M_r); (■) M_z (z-average M_r); (▲) M_{z+1} ($z+1$-average M_r).

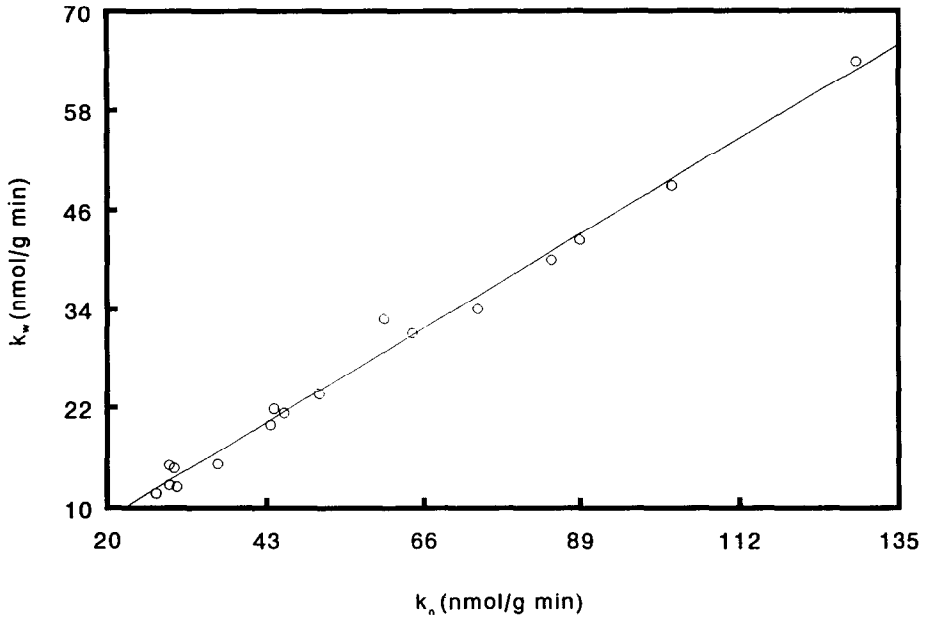

Fig. 5. Correlation between the slopes from eqs. 5 and 6 ($n = 19$). Slope = 0.497 ± 0.013; intercept = 1.115 ± 0.801; standard error = 1.496; correlation coefficient = 0.995.

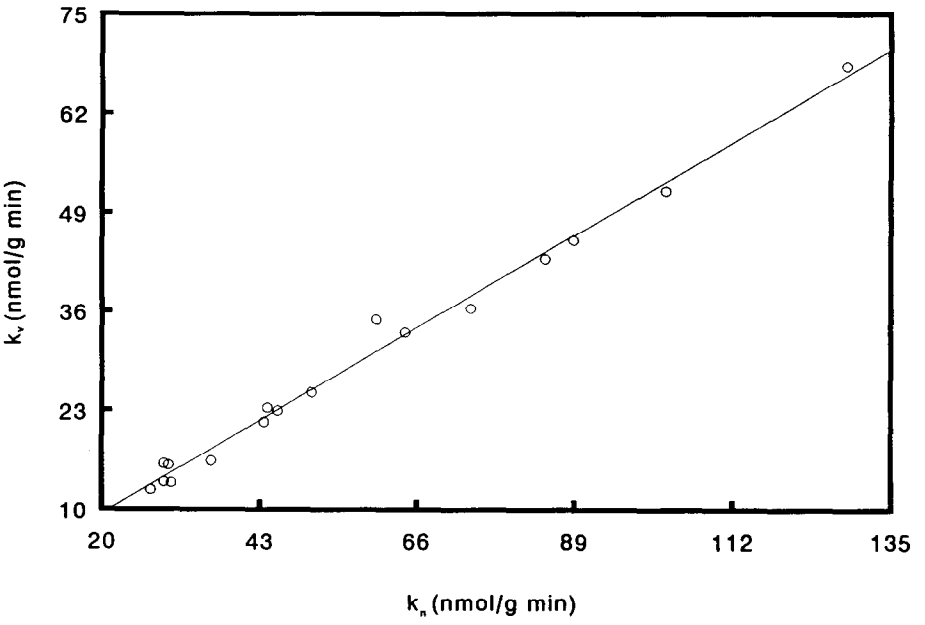

Fig. 6. Correlation between the slopes from equations (5) and (7) ($n = 19$). Slope = 0.529 ± 0.013; intercept = 1.051 ± 0.826; standard error = 1.543; correlation coefficient = 0.995.

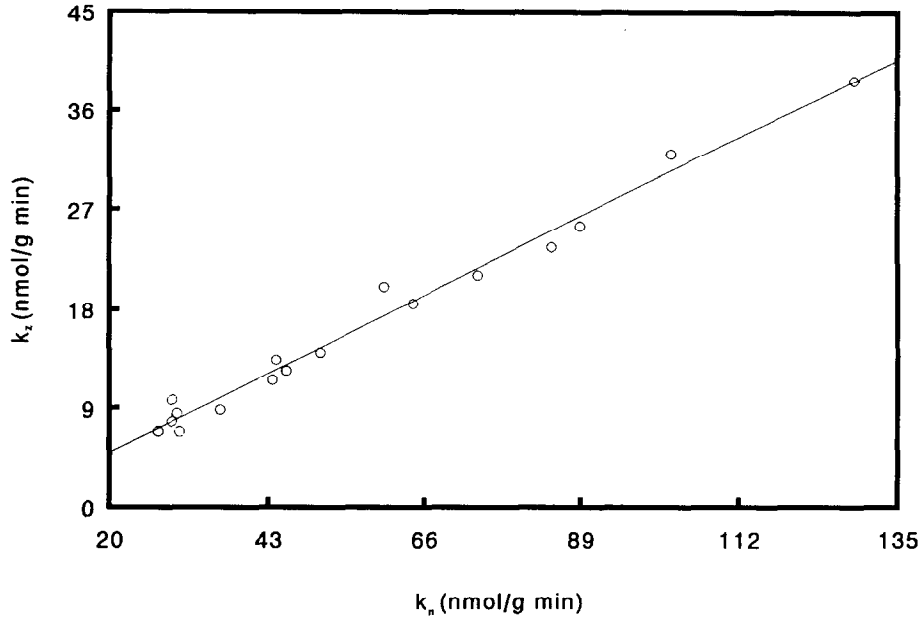

Fig. 7. Correlation between the slopes from eqs. 5 and 8 ($n = 19$). Slope = 0.311 ± 0.010; intercept = 1.329 ± 0.638; standard error = 1.191; correlation coefficient = 0.992.

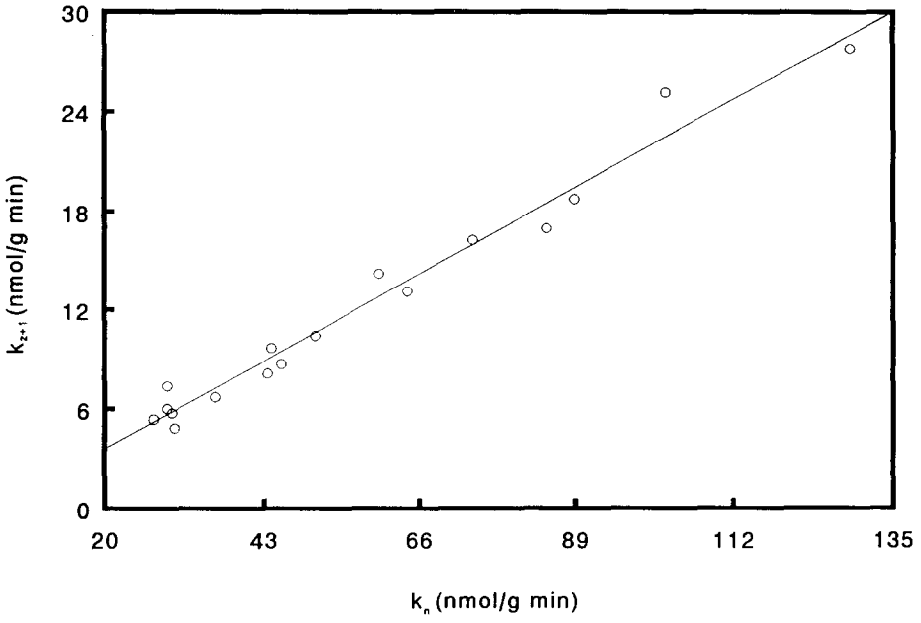

Fig. 8. Correlation between the slopes from eqs. 5 and 9 ($n = 19$). Slope = 0.230 ± 0.009; intercept = 1.004 ± 0.605; standard error = 1.129; correlation coefficient = 0.987.

due to a less accurate calculation of the higher molecular mass averages. Similarly the correlation between M_n and M_v after an at random degradation was predicted to be [35]:

$$M_n = M_v[\Gamma(2+\alpha)]^{-1/\alpha} \quad (11)$$

where α is the exponent of the Mark–Houwink equation and Γ is the gamma function, given by [36]:

$$\Gamma(n) = \int_0^\infty e^{-x} x^{n-1}\, dx \quad (12)$$

Using tabulated values of the gamma function [36] and $\alpha = 0.777$ we calculated k_v to be equal to 0.527 k_n. Experimentally we obtained a correlation of 0.529 (Fig. 6). These findings are a clear proof that the mechanism of the action of hyaluronidase on HA, in the early stage of reaction, is at random. Degradations of polymers which did not involve the at random mechanism were shown not to obey the equations mentioned before [21]. These theoretical considerations of the at random degradation have been employed to describe the kinetics of hyaluronidase, but a clear proof has never been given, although the necessity for doing so was stated before [37]. Other investigations on the mechanism of the action of hyaluronidase often involved the complete digestion of HA and the analysis of the thus formed oligosaccharides [38] or was concerned about the transglycosylation action of hyaluronidase on oligomeric substrates [39].

4. Conclusions

This work has shown that GPC is a powerful tool to investigate the action of a degrading agent on a polymeric sample. The kinetic assay we have developed provides information about the rate and the mechanism of the degradation. We have demonstrated that hyaluronan is degraded by hyaluronidase in an at random way. Our findings provide a firm basis to investigate other kinetic assays of hyaluronidase (*e.g.* viscosimetry) and to study the action of potential inhibitors of hyaluronidase.

5. Acknowledgements

This work was financially supported by the I.W.O.N.L. (Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw).

6. References

[1] T.C. Laurent and J.R.E. Fraser, *FASEB J.*, 6 (1992) 2397.
[2] R. Ruyssen and A. Lauwers (Editors), *Pharmaceutical Enzymes*, E. Story Scientia, Ghent, 1978, Ch. 16, p. 203.
[3] B. Steiner and D. Cruce, *Anal. Biochem.*, 200 (1992) 405.
[4] P.J. Hotez, S. Narasimhan, J. Haggerty, L. Milstone, V. Bhopale, G.A. Shad and F.F. Richards, *Infect. Immun.*, 60 (1992) 1018.
[5] K. Sugahara, S. Yamada, M. Sugiura, K. Takeda, R. Yuen, H.E. Khoo and C.H. Poh, *Biochem. J.*, 283 (1992) 99.
[6] A. Senn, M. Germond and P. De Grandi, *Hum. Reprod.*, 7 (1992) 841.
[7] R.A.P. Harrison, *Biochem. J.*, 252 (1988) 865.
[8] K. Höbarth, U. Maier and M. Marberger, *Eur. Urol.*, 21 (1992) 206.
[9] K. Beckenlehner, S. Bannke, T. Spruß, G. Bernhardt, H. Schönenberger and W. Shiess, *J. Cancer Res. Clin. Oncol.*, 118 (1992) 591.
[10] I. Repa, J.D. Garnic and N.K. Hollenberg, *J. Cardiovasc. Pharmacol.*, 16 (1990) 286.
[11] MILIS Study Group, *Br. Heart J.*, 60 (1988) 290.
[12] Y. Sawabe, K. Nakagomi, S. Iwagami, S. Suzuki and H. Nakazawa, *Biochem. Biophys. Acta*, 1137 (1992) 274.
[13] H. Kakegawa, H. Matsumoto and T. Satoh, *Chem. Pharm. Bull.*, 33 (1985) 642.
[14] C.L. Joyce, S.R. Mack, R.A. Anderson and L.J.D. Zaneveld, *Biol. Reprod.*, 35 (1986) 336.
[15] W.F. Reed, C.E. Reed and L.D. Byers, *Biopolymers*, 30 (1990) 1073.
[16] O.M. Awad, J. Demeester, I. Verhamme and A. Lauwers, *Arch. Int. Physiol. Biochem.*, 96 (1988) B73.
[17] J. Demeester, O.M. Awad, M. Bracke and A. Lauwers, *Fresenius Z. Anal. Chem.*, 330 (1988) 366.
[18] N. Di Ferrante, *J. Biol. Chem.*, 220 (1956) 303.
[19] L.C. Benchetrit, S.L. Pahuja, E.D. Gray and R.D. Edstrom, *Anal. Biochem.*, 79 (1977) 431.

[20] P. Gacesa, M.J. Savitsky, K.S. Dodgson and A.H. Olavesen, *Anal. Biochem.*, 118 (1981) 76.
[21] C. Tanford (Editor), *Physical Chemistry of Macromolecules*, John Wiley and Sons, New York–London, 1961, Ch. 9, p. 611.
[22] J.V. Dawkins, in J. Janča (Editor), *Steric Exclusion Liquid Chromatography of Polymers*, Marcel Dekker, New-York and Basel, 1984, Ch. 2, p. 80.
[23] S. Mori, *Anal. Chem.*, 61 (1989) 530.
[24] C. Rossi and C. Cuniberti, *Polym. Lett.*, 2 (1964) 681.
[25] K. Kawahara, K. Ohta, H. Niyamoto and S. Nakamura, *Carbohydr. Polym.*, 4 (1984) 335.
[26] R.E. Turner, L. Pinyen and M.K. Cowman, *Arch. Biochem. Biophys.*, 265 (1988) 484.
[27] Y. Uneo, Y. Tanaka, K. Horie and K. Tokuyasu, *Chem. Pharm. Bull.*, 36 (1988) 4971.
[28] N.B. Beaty, W.P. Tew and R.J. Mello, *Anal. Biochem.*, 147 (1985) p387.
[29] R.L. Cleland and J.L. Wang, *Biopolymers*, 9 (1970) 799.
[30] C.H. Poh, R. Yeun, M.C.M. Chung and H.E. Khoo, *Comp. Biochem. Physiol.*, 101B (1992) 159.
[31] R.A.P. Harrison, *Biochem. J.*, 252 (1988) 875.
[32] R. Eisenthal and A. Cornish Bowden, *Biochem. J.*, 139 (1974) 715.
[33] R. Ruyssen and A. Lauwers (Editors), *Pharmaceutical Enzymes*, E. Story Scientia, Ghent, 1978, Ch. 1, p. 8.
[34] A. Charlesby, *Proc. Roy. Soc. A*, 224 (1954) 120.
[35] L.H. Peebles, in H.F. Mark and E.H. Immergut (Editors), *Molecular Weight Distibutions In Polymers*, Interscience Publishers, New York–London–Sydney–Toronto, 1971, Ch. 1, p. 8.
[36] R.C. Weast (Editor), *Handbook of Chemistry and Physics*, 52nd ed., The Chemical Rubber Company, Cleveland, OH, 1971–1972, A-191.
[37] Y. Sano, *Bull. Natl. Inst. Agrobiol. Resour.*, 1 (1985) 53.
[38] K. Sugahara, S. Yamada, M. Sugiura, K. Takeda, R. Yuen, H.E. Khoo and C.H. Poh, *Biochem. J.*, 283 (1992) 99.
[39] S. Highsmith, J.H. Garvin and D.M. Chipman, *J. Biol. Chem.*, 250 (1975) 7473.