Absolute and empirical determination of the enzymic activity and kinetic investigation of the action of hyaluronidase on hyaluronan using viscosimetry

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We describe an investigation of the action of hyaluronidase on hyaluronan using viscosimetry. A new viscosimetric approach was developed for determining the activity of the enzyme in katal units. This approach requires knowledge of several parameters (e.g. Mark–Houwink constants) which were determined by combining viscosimetric measurement and gel-permeation chromatography analysis. Using all the necessary parameters we determined the kinetic parameters of the enzyme and found that 250 i.u. correspond to 1 nkat. An empirical viscometric was used to estimate the activity of the enzyme, and the K_m was determined using the kinetic dilution method. The estimates produced by the absolute and empirical approaches were in good agreement. We demonstrate that the empirical estimation of the reaction rate is related to the rate of reaction expressed in absolute units and thus provides a good estimate of enzyme activity. Furthermore, we have found an empirical relationship which enables investigation of the kinetics of the enzyme in a simple and sensitive way by viscosimetry.

INTRODUCTION

Hyaluronidases (HYASES) are endoglycosidases that can degrade glycosaminoglycans such as hyaluronan (HA), chondroitin, chondroitin 4- and 6-sulphate. These enzymes can be divided into three main classes according to the mechanism of hydrolytic reaction [1]. Testicular HYASE (hyaluronate 4-glycanohydrolase, EC 3.2.1.35), like lysosomal or venom HYASE, degrades glycosaminoglycans by hydrolysing β-*N*-acetylhexosaminic bonds. HA is a negatively charged high-molecular-mass polysaccharide made up of β-D-GlcA-(1 → 3)-β-D-GlcNAc disaccharide units linked 1 → 4 [2]. The biological and physiological properties of HA [2] and HYASE [3] have been extensively reviewed.

Quantitative assay of HYASE activity is usually performed by turbidimetry [4] or spectrophotometric determination of the liberated hexosaminic end groups [5]. These assays often lack specificity and sensitivity. Viscosimetric assay provides an empirical measurement of enzyme activity that is simple and sensitive [6]; however, the reaction rates observed are not easily related to the actual number of bonds broken per unit of time. Attempts have been made to relate the decrease in viscosity to the number of bonds broken per unit of time [7], but assumptions were made that are questionable or not proven. In a previous paper we described an assay for HYASE based on gel-permeation chromatography (g.p.c.) [8]. This method enabled determination of the rate of reaction expressed as mol of bonds broken per unit of time and per unit of reaction volume. Furthermore, we provided a clear proof that HA is randomly degraded by HYASE. In the present paper we describe, on the basis of our g.p.c. results, a viscosimetric approach to determine the number of bonds broken per unit of time, expressing HYASE activity in katal units. Using the empirical viscosimetric method, enzyme activity is estimated from the slope of the plot of the reciprocal value of the natural logarithm of the relative viscosity ($\eta_{rel.}$) as a function of reaction time [6]; this method has been accepted by the European Pharmacopoeia for assay of pharmaceutical HYASE preparations [9]. A kinetic dilution method has been developed for estimating the rate of reaction at different substrate concentrations by this approach [10]. However, this method is rather complicated. In the present paper we describe the determination of the kinetic parameters of the enzyme by the absolute and empirical viscosimetric approach. Furthermore, we demonstrate that the empirical reaction rate is related to the actual number of bonds broken and thus provides a good estimate of enzyme activity. We also describe a simple empirical method for estimating the rate of reaction at different substrate concentrations by viscosimetry.

EXPERIMENTAL

Materials

HA (sodium salt extracted from rooster comb) was kindly donated by Diosynth B.V. (Oss, The Netherlands). Testicular HYASE was the international reference standard provided by the Fédération Internationale Pharmaceutique (FIP Ghent, Belgium) and contained 328 i.u./mg. Poly(ethylene oxide) (PEO) standards with narrow molecular-mass distributions [weight-average molecular mass (M_w)/number-average molecular mass (M_n) < 1.1] and peak molecular masses between 5.8 and 885 kDa were purchased from Waters/Millipore (Brussels, Belgium) and from Filter Service N.V. (Eupen, Belgium). All other chemicals were of analytical grade. Enzyme reactions were investigated in sodium phosphate buffer (pH 6.4) containing 140 mM NaCl, 16 mM NaH_2PO_4 and 7 mM Na_2HPO_4 at 37 °C, and all HA solutions were prepared in this buffer. Sonication was performed with a low-power ultrasonic cleaning apparatus (Bransonic 32 sonifier from Voor't labo, Eeklo, Belgium).

Abbreviations used: HYASE, hyaluronidase; HA, hyaluronan; g.p.c., gel-permeation chromatography; PEO, poly(ethylene oxide); HV, hydrodynamic volume.

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G.p.c.

The g.p.c. system, columns and operating conditions were as described previously [8], except that the analyses were performed at 37 °C with buffer as the eluent.

Viscosimetric measurements

Viscosimetric measurements were made with automated apparatus equipped with a calibrated micro-Ubbelhode viscosimeter from Schott Geräte G.m.b.H. (Hofheim a. Ts., Germany) placed in a thermostatically controlled water bath (37±0.05 °C). The outflow times (t in s) were automatically recorded by an electronic timer. The kinematic viscosity (ν in mm²/s) of a solution is related to t by:

$$\nu = k\left(t - \frac{B}{t^2}\right) \tag{1}$$

where k is the viscosimetric constant of the viscosimeter and B is a correction factor for kinetic energy effects during the outflow. B has a value of 412 s³ for type-I viscosimeters ($k = 0.01$ mm²/s²) and 84 s³ for type-Ic viscosimeters ($k = 0.03$ mm²/s²). As the densities of HA solutions and buffer are almost the same, the relative viscosity ($\eta_{rel.}$) of a solution was approximated by:

$$\eta_{rel.} \approx \frac{\nu}{\nu_o} \tag{2}$$

where ν_o is the kinematic viscosity of the buffer.

Determination of the Mark–Houwink constants of PEO

These constants (K and α) are the parameters of the Mark–Houwink equation, relating the intrinsic viscosity ([η]) of a polymer to its viscosity-average molecular mass (M_v):

$$[\eta] = KM_v^{\alpha} \tag{3}$$

Of two PEO standards (molecular mass 340 and 570 kDa) a serial dilution (0.2–1.0 mg/ml) was prepared in buffer and analysed viscosimetrically at 37 °C. All other standards were dissolved in buffer and their relative viscosites determined at 37 °C. Concentrations (C in g/ml) were fixed such that the intrinsic viscosity (in ml/g) could be determined by means of the empirical equation [7]:

$$[\eta] = \frac{8}{C}\left[\sqrt[8]{(\eta_{rel.})} - 1\right] \tag{4}$$

The Mark–Houwink constants are obtained by plotting log [η] as a function of log M_v.

Determination of the Mark–Houwink constants of HA

These constants were determined in buffer at 37 °C as described by Price et al. [11]. At least two samples of HA with different molecular-mass properties and a g.p.c. system, calibrated by the universal calibration method [12], are needed. The g.p.c. system was calibrated as described previously [8] using the PEO standards and their Mark–Houwink constants as determined by us. By considering a broad-distribution polymer as a series of monodisperse fractions, eqn. (3) can be rewritten as:

$$[\eta]_i = KM_i^{\alpha} \tag{5}$$

where $[\eta]_i$ is the intrinsic viscosity of the ith fraction of molecular mass M_i. Two polymer fractions (A and B) eluted at the same g.p.c. volume exhibit the same hydrodynamic volume (HV) [12]. HV is the product of the intrinsic viscosity of a polymer and its molecular mass (M):

$$HV = [\eta]M \tag{6}$$

It can be shown for fractions A and B that:

$$\ln M_{i,B} = \frac{\ln(K_A/K_B) + (1+\alpha_A)\ln M_{i,A}}{1+\alpha_B} \tag{7}$$

where (K_A, α_A) and (K_B, α_B) are the Mark–Houwink constants of fractions A and B respectively. A is the calibration polymer (PEO in our case) for which these constants and the molecular mass are known. B is the polymer for which the Mark–Houwink constants need to be determined (HA in our case).

A hydrodynamic volume parameter (J_i) is defined as:

$$J_i = M_i[\eta]_i = M_i KM_i^{\alpha} = KM_i^{1+\alpha} \tag{8}$$

The intrinsic viscosity of a polymer can be written in terms of the weight fractions (w_i) of the individual fractions and their intrinsic viscosities:

$$[\eta] = \sum_i w_i[\eta]_i \tag{9}$$

For two samples of HA, eqns. (5), (7), (8) and (9) can be rearranged to:

$$\frac{[\eta]_1}{[\eta]_2} = \frac{\sum_i^{(1)} w_i J_i^{\alpha/(1+\alpha)}}{\sum_i^{(2)} w_i J_i^{\alpha/(1+\alpha)}} \tag{10}$$

Both J_i and w_i are obtained by chromatography of the samples and the intrinsic viscosities are determined independently. α is varied continuously until both sides of eqn. (10) are equal and K of HA is then obtained from:

$$K = \left(\frac{[\eta]_1}{\sum_i^{(1)} w_i J_i^{\alpha/(1+\alpha)}}\right)^{1+\alpha} \tag{11}$$

By preparing several HA fractions with different polymeric properties, multiple estimates of the Mark–Houwink constants can be obtained.

Degradation of HA

An HA solution (2.37 mg/ml) was prepared in buffer and divided into four fractions. These were sonicated for 0, 15, 45 or 60 min. A serial dilution (0.12–0.95 mg/ml) was prepared of all fractions and analysed viscosimetrically at 37 °C. One of each series was analysed by g.p.c. A second HA solution (1.93 mg/ml) was prepared in buffer. HYASE (final concentration 0.5 μg/ml) was added to 20 ml of this solution. Mixtures were incubated at 37 °C for 0.5, 2 or 3 h and placed in a boiling-water bath for 1 h to inactivate the enzyme. All samples were stored frozen until analysis. For each sample a serial dilution (0.15–1.54 mg/ml) was prepared and analysed viscosimetrically at 37 °C. Again one of each series was analysed by g.p.c. Undegraded HA was analysed viscosimetrically at 37 °C at a concentration range of 0.08–2.13 mg/ml. Table 1 presents a summary of all the HA fractions prepared and the abbreviations used to describe them throughout the paper. To determine [η] of the HA samples from the viscosimetric data, the suitability of eqn. (4), the Huggins equation [eqn. (12)] [13], the Kramer equation [eqn. (13)] [13], the

Table 1 Preparation of HA fractions

Summary of the preparation of the different HA fractions and their abbreviations. Enzymic digestion and sonication were performed as described in the text.

HA preparation	Abbreviation
Undegraded	HA
Sonicated	
15 min	SON-1
45 min	SON-2
60 min	SON-3
Digested	
0.5 h	ENZ-1
2 h	ENZ-2
3 h	ENZ-3

Martin equation [eqn. (14)] [6] and the Fuoss equation [eqn. (15)] [14] was evaluated.

$$\frac{\eta_{rel.}-1}{C} = [\eta]+k_H[\eta]^2C \quad (12)$$

$$\frac{\ln\eta_{rel.}}{C} = [\eta]-k_K[\eta]^2C \quad (13)$$

$$\log\left(\frac{\eta_{rel.}-1}{C}\right) = \log[\eta]+k_M[\eta]C \quad (14)$$

$$\frac{C}{\eta_{rel.}-1} = \frac{1}{[\eta]}-\frac{k_F}{[\eta]}\sqrt{(C)} \quad (15)$$

k_H, k_K, k_M and k_F are the viscosimetric constants of these equations.

Viscosimetric estimation of HYASE activity expressed in katal units

An HA solution prepared in buffer was equilibrated at 37 °C. At reaction time $T = 0$ s, HYASE (dissolved in buffer) was added (final concentration 1 μg/ml) to the substrate. After mixing, 4 ml was placed in the thermostatically controlled viscosimeter and t measured continuously throughout the reaction. Reactions were monitored between 60 and 750 s reaction time and the outflow times typically ranged between 100 and 45 s, depending on the type of viscosimeter used. As the reaction proceeds during the outflow, the actual reaction time at which t is measured, is $T+(t/2)$. Using the appropriate equation, $[\eta]$ was calculated from the knowledge of $\eta_{rel.}$ during the reaction. From eqn. (3) and the Mark–Houwink constants of HA in buffer at 37 °C, M_v was obtained. Theoretical considerations of the random degradation of a polymer produced, in the initial stages of the reaction, a linear relationship between the reciprocal value of the number-average molecular mass (M_n) and the reaction time [15]. The slope of this line (k_n) gives the initial rate of reaction expressed as mol of bonds broken per unit of time and per unit of weight of polymer present in the mixture. We have shown that these theories can be applied to the action of HYASE on HA [8] and demonstrated that the reciprocal values of the other molecular-mass averages give a straight line as a function of reaction time. We demonstrated that the slopes of these lines are related to k_n as predicted for random degradation of a polymer [15,16]. The slope (k_v) of the plot of $1/M_v$ as a function of reaction time is related to k_n by [16]:

$$k_n = k_v[\Gamma(2+\alpha)]^{1/\alpha} \quad (16)$$

where α is the exponent of eqn. (3) and Γ is the gamma-function given by [17]:

$$\Gamma(n) = \int_0^\infty e^{-x}x^{n-1}dx \quad (17)$$

The rate of reaction at different substrate concentrations (C) is obtained by multiplying the slopes of the plots of C/M_v as a function of reaction time with the factor of eqn. (16). In this way the action of HYASE on HA was investigated in the concentration range 0.10–1.80 mg of HA/ml. All reactions were performed in triplicate. The results were fitted to the Michaelis–Menten equation [18]:

$$v = \frac{V_{max.}C}{K_m+C} \quad (18)$$

where v is the initial reaction rate, $V_{max.}$ is the maximum reaction rate and K_m is the Michaelis constant. These parameters were estimated using a direct linear plot [18].

Viscosimetric estimation of enzyme activity in empirical units

From the experimental data obtained for the enzyme reactions described above, the empirical rate of reaction was estimated from the slope of the plot of $(\ln\eta_{rel.})^{-1}$ as a function of $T+(t/2)$ [5]. Then the relationship between enzyme concentration and reaction rate was investigated. To an HA solution (final concentration 0.2 mg/ml) were added different amounts of HYASE such that its final concentration ranged between 0.25 and 1 μg/ml, and the rate of reaction was determined empirically as described above.

Determination of K_m by the kinetic dilution method

The kinetic dilution method [10] was developed to investigate the action of HYASE on HA at different concentrations. In this method all kinetic experiments are performed at a common minimum HA concentration (C_o). The rate of reaction at C_o is determined empirically as described above. Enzyme is then added to higher concentrations (C), and the mixtures are incubated at 37 °C. At well-defined reaction times ($T = T_{dil.}$) the mixtures are diluted with buffer to the lowest concentration and mixed. Then 4 ml is placed in the viscosimeter and the outflow times are recorded for a further 15 min. By plotting $(\ln\eta_{rel.})^{-1}$ as a function of $T+(t/2)$ for each dilution, straight lines are obtained and extrapolated to the time of dilution. The extrapolated values of $(\ln\eta)^{-1}$ are plotted against T_{dil} and the slope of this line, multiplied by the dilution factor (C/C_o), gives the rate of reaction (v) at higher concentrations of HA. The experimental data were fitted to the Michaelis–Menten equation [eqn. (18)] and the kinetic parameters estimated by means of a direct linear plot.

RESULTS AND DISCUSSION

Determination of the Mark–Houwink constants at PEO

Eqn. (4) predicts a linear relationship between the eighth root of $\eta_{rel.}$ and C. This is shown for two PEO standards in Figure 1. A straight line was obtained for values of $\eta_{rel.}$ between 1 and 1.2. Therefore we used this equation to calculate $[\eta]$ of the other PEO standards at a single concentration. Concentrations were fixed such that the values of $\eta_{rel.}$ did not exceed 1.2. As the PEO standards have narrow molecular-mass distributions, the indi-

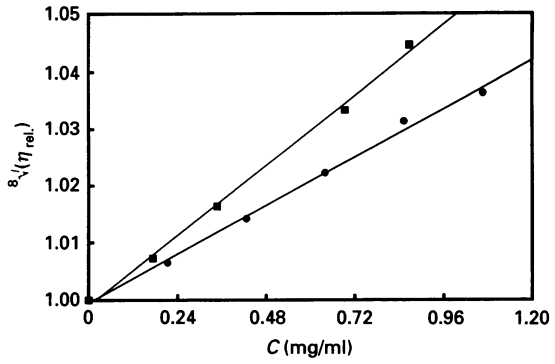

Figure 1 Regression analysis according to eqn. (4)

The empirical relationship between the eighth root of $\eta_{rel.}$ and C for two [570 kDa (■) and 340 kDa (●)] PEO standards is shown.

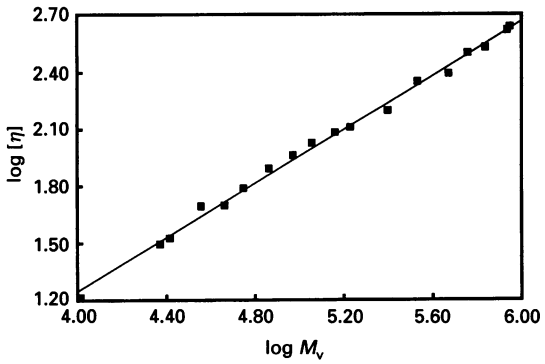

Figure 2 Mark–Houwink relationship of PEO

The relationship between log [η], as calculated by using eqn. (4), and log M_v of the PEO standards is presented.

cated value of the molecular mass was taken as an approximate value of M_v. Figure 2 presents a plot of log [η] as a function of log M_v. Regression analysis ($r^2 = 0.996$; $n = 18$) yielded values ± standard error of estimate of $\alpha = 0.71 \pm 0.01$ and $K = 0.026 \pm 0.002$ for PEO.

Viscosimetric analysis of the HA fractions

Plots and regression analyses according to eqns. (12)–(15) were performed for the dilution series of all HA fractions prepared (Table 1). Table 2 presents the results obtained. In all cases a linear relationship was observed within the indicated concentration range. The estimated values of [η] obtained with the Huggins, Kramer and Martin equations are in good agreement with each other, but the values obtained with the Fuoss equation are systematically lower. The latter is an empirical formula used to describe the observed relationship between the viscosity and

Table 2 Regression analyses according to eqns. (12)–(15)

A survey of the results of regression analyses of the dilution series of all HA fractions (Table 1) is presented. Mean estimate ± S.E.M. [η] and the constant for each equation is shown; the correlation coefficient and the number of observations (r^2; n) and the concentration range (C) where linearity was observed are also shown.

Fraction	[η] (ml/g)	k_H, k_K, k_M or k_F	r^2 (n)	C (mg/ml)
Huggins equation [eqn. (12)]				
HA	2288 ± 89	0.413 ± 0.032	0.984 (20)	0.08–1.24
SON-1	2287 ± 75	0.309 ± 0.020	0.980 (5)	0.12–0.95
SON-2	642 ± 17	0.410 ± 0.021	0.959 (5)	0.12–0.95
SON-3	740 ± 9	0.416 ± 0.011	0.989 (5)	0.12–0.95
ENZ-1	1318 ± 41	0.522 ± 0.033	0.994 (6)	0.15–1.54
ENZ-2	788 ± 3	0.396 ± 0.003	0.999 (6)	0.15–1.54
ENZ-3	606 ± 1	0.375 ± 0.001	0.999 (6)	0.15–1.54
Kramer equation [eqn. (13)]				
HA	2295 ± 47	0.116 ± 0.005	0.945 (20)	0.08–1.24
SON-1	2255 ± 42	0.147 ± 0.005	0.971 (5)	0.12–0.95
SON-2	642 ± 11	0.114 ± 0.004	0.901 (5)	0.12–0.95
SON-3	744 ± 5	0.121 ± 0.002	0.961 (5)	0.12–0.95
ENZ-1	1366 ± 18	0.105 ± 0.003	0.975 (6)	0.15–1.54
ENZ-2	791 ± 2	0.129 ± 0.001	0.999 (6)	0.15–1.54
ENZ-3	607 ± 1	0.138 ± 0.001	0.999 (6)	0.15–1.54
Martin equation [eqn. (14)]				
HA	2343 ± 108	0.130 ± 0.003	0.986 (22)	0.08–2.13
SON-1	2300 ± 61	0.114 ± 0.007	0.987 (5)	0.12–0.95
SON-2	644 ± 15	0.157 ± 0.024	0.956 (5)	0.12–0.95
SON-3	746 ± 6	0.154 ± 0.007	0.994 (5)	0.12–0.95
ENZ-1	1409 ± 17	0.135 ± 0.003	0.998 (6)	0.15–1.54
ENZ-2	798 ± 2	0.139 ± 0.002	0.999 (6)	0.15–1.54
ENZ-3	612 ± 2	0.137 ± 0.003	0.999 (6)	0.15–1.54
Fuoss equation [eqn. (15)]				
HA	2106 ± 39	0.518 ± 0.018	0.978 (22)	0.08–2.13
SON-1	2081 ± 36	0.463 ± 0.046	0.981 (5)	0.12–0.95
SON-2	598 ± 8	0.259 ± 0.026	0.980 (5)	0.12–0.95
SON-3	690 ± 8	0.279 ± 0.024	0.979 (5)	0.12–0.95
ENZ-1	1225 ± 19	0.442 ± 0.022	0.990 (6)	0.15–1.54
ENZ-2	724 ± 7	0.300 ± 0.018	0.993 (6)	0.15–1.54
ENZ-3	555 ± 2	0.254 ± 0.007	0.998 (6)	0.15–1.54

Table 3 Statistics of the values of the constants of eqns. (12), (13) and (14)

Mean, standard deviation and 95% confidence interval limits of the values of the constants of the Huggins (k_H), Kramer (k_K) and Martin (k_M) equations as obtained from regression analyses of the HA fractions (Table 2; $n = 7$) are shown.

Statistic	k_H	k_K	k_M
Mean	0.406	0.124	0.138
Standard deviation	0.063	0.014	0.015
95% confidence interval limits	0.347–0.464	0.111–0.137	0.125–0.152

the concentration of a polyelectrolytic polymer [14]. We believe that eqn. (15) can describe some aspects of this complex relationship, but the intercept is probably not a good estimate of $[\eta]$. We have investigated the possible relationships between the constants of eqns. (12)–(15) and $[\eta]$. With k_H, k_K and k_M no correlation could be observed. The value of k_F decreased with increasing intrinsic viscosity. This fact and the observed differences in the estimate of $[\eta]$ make the Fuoss equation not suitable for calculating $[\eta]$ of an HA sample during enzymic digestion. In Table 3 we present some statistics on the values of the constants of eqns. (12), (13) and (14) obtained from regression analyses (Table 2). The values agree well with previous results [6,19].

Calculation of intrinsic viscosity

From the viscosimetric measurements of the HA fractions (Table 1) at the concentrations tested, $[\eta]$ was calculated according to eqns. (12), (13) and (14) using the mean and 95% confidence interval limits of the constants given in Table 3. These three estimates of $[\eta]$ were averaged and the coefficient of variation [20] was taken as a measure of the variation in the calculation of $[\eta]$ due to the experimental error in the estimate of the constant of the equation applied. Values for coefficient of variation did not exceed 5% over the whole viscosity range investigated for all three constants. As the regression analyses according to eqns. (12)–(14) yielded approximately the same value of $[\eta]$ (Table 2), the mean of these three values was taken as an estimate of the real value of $[\eta]$. Using the mean values of the constants of the above mentioned equations (Table 3), $[\eta]$ of each fraction, at each concentration, was calculated. $[\eta]$ at each concentration was also calculated according to eqn. (4). All these values were compared with the real value of $[\eta]$. With eqn. (4) large deviations (> 10%) were observed for $\eta_{rel.}$ values above 2. This equation can only be applied to polymer solutions with low viscosities, as observed here for HA and PEO. Eqns. (12) and (13) yielded large deviations (> 20%) for $\eta_{rel.}$ values above 10. Only the Martin equation provided good approximations (maximum deviation 5%) over the whole range tested. This is also reflected in the regression analyses (Table 2), as for fraction HA only the Martin equation gave a linear relationship over the complete concentration range analysed. Therefore to calculate $[\eta]$ at a single concentration at any time during an enzymic reaction we used eqn. (14) with $k_M = 0.138$.

Determination of the Mark–Houwink constants of HA

Figure 3 presents g.p.c. profiles of HA degraded by sonication. These can be compared with the chromatograms of HA degraded by HYASE (Figure 4). Degradation of HA by HYASE proceeds in a random fashion [8] and the profiles are bell-shaped.

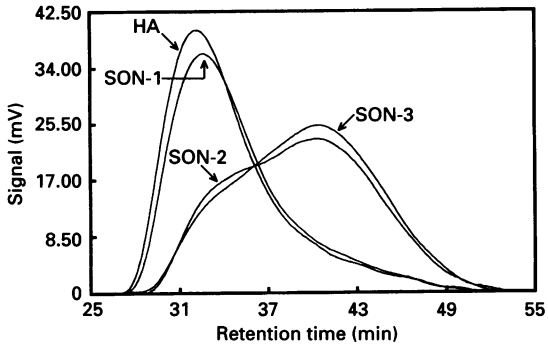

Figure 3 G.p.c. of HA degraded by sonication

Chromatograms of HA sonicated for 0 (HA), 15 (SON-1), 45 (SON-2) or 60 (SON-3) min using a low-power ultrasonic cleaning apparatus are presented.

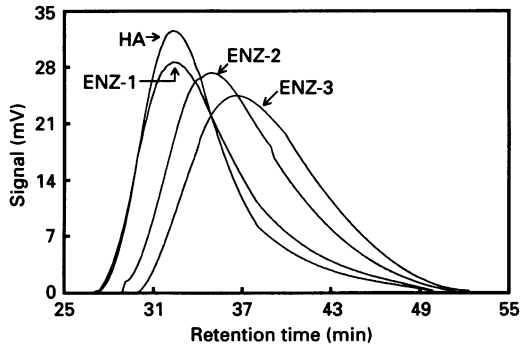

Figure 4 G.p.c. of HA degraded by HYASE

Chromatograms of HA degraded by HYASE (0.5 μg/ml) for 0 (HA), 0.5 (ENZ-1), 2 (ENZ-2) or 3 h (ENZ-3) are presented.

Sonication appears to degrade HA in a non-random fashion. This can be seen in Figure 3, as the chromatograms, after 45 and 60 min sonication, tend to take on a bimodal shape, suggesting that high-molecular-mass fractions are degraded more slowly than low-molecular-mass fractions. Furthermore, degradation seems to stop at a certain molecular mass, as the second maximum after 45 or 60 min sonication does not shift to higher retention times. This fact is also reflected in the estimates of intrinsic viscosity (Table 2). The intrinsic viscosity of the sample after 60 min of sonication is slightly higher than that after 45 min of sonication. As the molecular-mass distributions of sonicated HA appear to differ radically from the HA distribution after enzymic degradation, Mark–Houwink constants of HA were estimated from the analyses of fractions HA and ENZ-1 to 3 (Table 1). The values of the constants obtained (mean ± S.D.) were $\alpha = 0.72 \pm 0.07$ ($n = 6$) and $K = 0.036 \pm 0.001$ ($n = 4$).

Viscosimetric estimation of HYASE activity expressed in katal units

$[\eta]$ and M_v were calculated at any time during an enzyme reaction by use of eqn. (14) ($k_M = 0.138$) and the Mark–Houwink constants as obtained by us. For all enzyme reactions, C/M_v was plotted as a function of reaction time, and straight lines were obtained. The factor of eqn. (16) was calculated as 1.872. This

Table 4 Estimation of the rate of reaction of HYASE on HA by viscosimetry (expressed in absolute or empirical units)

Mean absolute ($v_{abs.}$) and empirical ($v_{app.}$) reaction rates (±S.D.; $n = 3$) of the action of HYASE on HA at different concentrations were determined by viscosimetry. $v_{abs.}$ was determined from the slopes of the plots of C/M_v as a function of reaction time, and $v_{app.}$ is the slope of the plot of $\ln(\eta_{rel.})^{-1}$ as a function of reaction time.

HA (mg/ml)	$v_{abs.}$ (pmol/ml per s)	$10^3 \times v_{app.}$ (s^{-1})
0.09	0.23±0.01	14.81±0.183
0.23	0.44±0.02	4.36±0.063
0.36	0.60±0.01	2.85±0.049
0.54	0.69±0.02	1.50±0.006
0.72	0.86±0.01	1.06±0.005
0.90	0.90±0.01	0.72±0.001
1.18	0.91±0.03	0.49±0.003
1.45	1.02±0.03	0.31±0.009
1.81	1.08±0.05	0.25±0.012

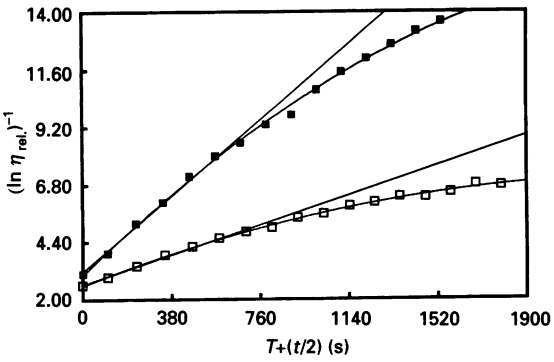

Figure 5 Action of HYASE on HA monitored by viscosimetry

The action of HYASE (1 μg/ml) on HA [0.17 mg/ml (■) and 0.18 mg/ml (□)] in sodium phosphate buffer (pH 6.4) at 37 °C was determined. Plots of the reciprocal value of $\ln\eta_{rel.}$ as a function of reaction time (time T from the start of the reaction plus half the outflow times t) are presented.

enabled us to determine the rate of reaction of HYASE on HA expressed as mol of bonds broken per unit of time and per unit of reaction volume ($v_{abs.}$). Table 4 presents the mean reaction rates obtained for HA in the concentration range 0.09–1.81 mg/ml. The kinetic parameters obtained were K_m = 0.45±0.04 ($n = 3$) mg/ml and $V_{max.}$ = 1.34±0.07 ($n = 3$) pmol/s per ml or 1.34±0.07 ($n = 3$) nmol/s per mg of enzyme with an activity of 328 i.u./mg. These results agree with our previous work using g.p.c. and allow expression of enzyme activity in katal units [21]. Under optimal reaction conditions, about 0.75 mg of enzyme preparation corresponds to 1 nkat. Similarly one can calculate that about 250 i.u. corresponds to 1 nkat.

Using a similar approach, Sano [7] determined the activity of HYASE acting on chondroitin sulphate. The intrinsic viscosity at any time during the enzyme reaction was calculated by using the Huggins equation, without investigating the possible relationship between the constant of this equation and the molecular mass or intrinsic viscosity of the polymer. Furthermore, M_v in eqn. (3) was replaced by M_w, the weight-average molecular mass. This substitution is only valid when the exponent of the Mark–Houwink equation (α) approaches 1. It has been observed that replacing M_v in eqn. (3) with M_w leads to systematic errors in the estimation of the number of bonds broken [22]. Furthermore, Sano assumed, without any proof, that the degradation occurred randomly and that at any stage during the reaction M_w/M_n equalled 2. This ratio depends strongly on the polydispersity of the initial substrate, especially in the initial stage of the reaction, as observed for the action of cellulase on hydroxyethylcellulose [23]. Using g.p.c. we have investigated the polydispersity of the substrate during the reaction [8] and determined accurately the relationships between M_n and the other average molecular masses.

Light-scattering analysis was used by Reed et al. [24] to calculate the scission rate of HA by HYASE. The authors made several assumptions (e.g. mass independence of second virial coefficient) and adapted their method to be useful even with simple light-scattering instruments (measurements at high scattering angles, cheap monochromatic light source) and measured the scattered intensity as a function of the reaction time. Initial reaction velocities are calculated as μmol of bonds broken/h, considering all the approximations. In this method the decrease in molecular mass is not determined. The initial rate of degradation can be obtained by measuring, by light scattering, the decrease in the weight-average molecular mass [23]. During the reaction, Reed et al. [24] observed deviations from linearity and they assumed, without any experimental proof, that enzyme deactivation was the cause. In the present work we have shown that the estimation of the degradation rate of a polymer by viscosimetry is not obvious. Only after careful investigation of the degradation mechanism and the viscosimetric properties of HA, before and after degradation, could we determine HYASE activity, expressed in katal units.

Sensitivity and linearity of the empirical viscosimetric assay

Table 5 presents the reproducibility of the determination of $\eta_{rel.}$ based on the viscosimetric analyses of fraction HA (Table 1). From these results we decided to use 0.2 mg/ml as the minimal HA concentration (C_o) for the kinetic dilution method. At this concentration the relative viscosity of the HA solution is still much higher than that of buffer ($\eta_{rel.} = 1$). The small experimental errors made in the viscosimetric measurements allow us to observe the smallest decreases in viscosity caused by the action of the enzyme. Furthermore, the enzyme concentrations for the kinetic assay can be kept low, thus increasing the sensitivity of the assay. A linear relationship was observed between enzyme concentration, ranging from 0 to 1 μg/ml, and observed reaction rate ($r^2 = 0.98$; $n = 4$). Therefore we decided to perform all further kinetic investigations with an enzyme concentration of 1 μg/ml (0.328 i.u./ml).

Viscosimetric estimation of enzyme activity expressed in empirical units

As described in the Experimental section, the activity of the enzyme is empirically estimated from the slope of the plot of $(\ln\eta_{rel.})^{-1}$ as a function of $T+(t/2)$. Figure 5 presents typical plots obtained for the action of HYASE (1 μg/ml) on HA (0.17 and 0.18 mg/ml) for prolonged reaction times. The curves tend to bend downward, but a good linearity is obtained during the initial stage (up to 750 s) of the reaction. Similar plots were obtained for the action of HYASE on HA at several concentrations. The slopes of these lines provide an apparent reaction rate ($v_{app.}$) and means ±S.D. are included in Table 4. It can be observed that $v_{app.}$ decreases with increasing substrate concentration. The enzyme reactions are investigated under different 'viscosimetric' conditions and the slopes of the plots do not intersect at the same point. Thus rates of reaction obtained at different substrate concentrations cannot be compared.

Table 5 Reproducibility of the determination of the relative viscosity of HA solutions

Mean (±S.D.; $n = 5$) values of the relative viscosity ($\eta_{rel.}$) of HA dissolved at different concentrations (C) in sodium phosphate buffer (pH 6.4) at 37 °C are presented.

c (mg/ml)	$\eta_{rel.}$
0.08	1.1866 ± 0.0001
0.09	1.2401 ± 0.0022
0.10	1.2609 ± 0.0025
0.12	1.3032 ± 0.0009
0.18	1.4903 ± 0.0005
0.19	1.5144 ± 0.0001
0.20	1.5145 ± 0.0028
0.21	1.5895 ± 0.0010
0.24	1.6633 ± 0.0007
0.31	1.9367 ± 0.0008
0.38	2.1241 ± 0.0023
0.39	2.1846 ± 0.0003
0.41	2.3039 ± 0.0015
0.47	2.5659 ± 0.0003
0.52	2.6978 ± 0.0047
0.56	2.9555 ± 0.0007
0.62	3.2032 ± 0.0111
0.71	3.7904 ± 0.0009
1.03	5.5381 ± 0.0034
1.24	7.3575 ± 0.0024
1.68	13.5081 ± 0.0034
2.13	26.2271 ± 0.0257

As HA is known to have exceptional macroscopic properties and a very expanded structure, it would be of interest to know whether special structures have an impact on the behaviour of HYASE. The network structure of the HA solutions used in this work has been investigated by static and dynamic rheological measurement. From a critical concentration of about 1 mg/ml the HA molecules begin to entangle [25]. Using fluorescence recovery after photobleaching, we determined the diffusion coefficients in dilute, semi-dilute and concentrated HA solutions of fluorescein isothiocyanate dextrans with molecular masses ranging from 70 to 500 kDa and diameters ranging from 11 to 30 nm [26]. A very interesting phenomenon was observed: the probe molecules moved much faster through the network than predicted on the basis of macroscopic zero-shear-rate viscosities of HA. In the concentration range used in this study (between 0 and 2 mg of HA/ml, about $4 \times K_m$), only a small steric hindrance is observed for diffusion of the fluorescein isothiocynate dextran probe molecules through HA solution. Moreover the mean ± S.D. mesh size of HA in a concentration of 0.5 and 3 mg/ml was measured as 96 ± 7 and 35 ± 5 nm respectively. HYASES have molecular masses ranging between 60 and 90 kDa [27,28]. Radii of gyration for serum albumin (molecular mass 66 kDa) [29] and catalase (molecular mass 225 kDa) [30] are 3 and 4 nm respectively. HYASES probably have similar dimensions. Hence the network structure of HA has important effects on its mechanical properties, but the mesh size is large enough to permit free movement of HYASE molecules. This is in agreement with our kinetic findings: random degradation with equal accessibility of the different glycosidic bonds of HA and no diffusion control of the decrease in reaction rate with increasing HA concentration. To investigate the relationship between the rate of reaction (expressed in empirical viscosimetric units) and the substrate concentration, the kinetic dilution method [9] needs to be employed as described in the Experimental section.

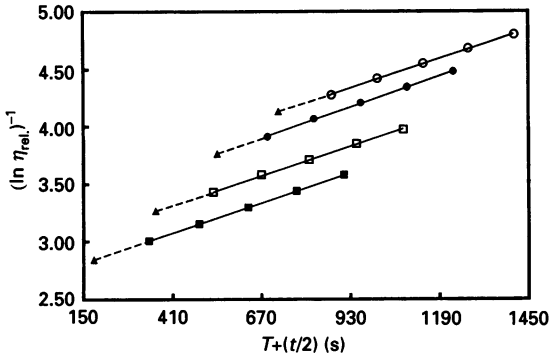

Figure 6 Example of the kinetic dilution method

The action of HYASE (1 μg/ml) on HA (0.8 mg/ml) in sodium phosphate buffer (pH 6.4) at 37 °C was determined. The reaction mixtures were diluted after 180 (■), 360 (□), 540 (●) or 720 (○) min. Plots of the reciprocal value of $\ln(\eta_{rel.})$ and the reaction time (time T from the start of the reaction plus half the outflow times t) are presented. $(\ln\eta_{rel.})^{-1}$ values were extrapolated to the time of dilution (▲).

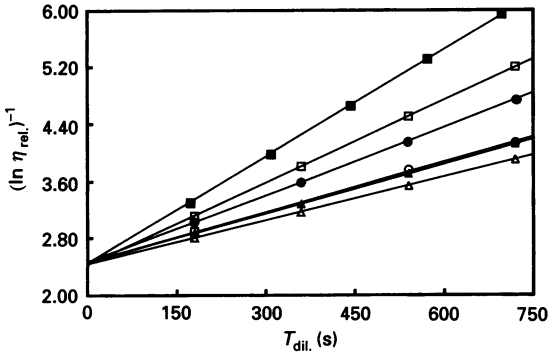

Figure 7 Estimation of the rate of reaction using the kinetic dilution method

Plots of the extrapolated values of the reciprocal values of $\ln\eta_{rel.}$ as a function of the time of dilution ($T_{dil.}$) for several HA concentrations [0.4 (□), 0.6 (●), 0.8 (○), 1.0 (▲) and 1.2 (△) mg/ml] are shown. A plot of the reciprocal value of $\ln\eta_{rel.}$ and the reaction time for HA at a concentration of 0.2 mg/ml (■) is also shown.

Determination of K_m constant by the kinetic dilution method

Figure 6 presents an example of the kinetic dilution method. To four HA solutions ($C = 0.8$ mg/ml) was added HYASE (final concentration 1 μg/ml), and the mixtures were incubated at 37 °C. These solutions were diluted to the minimal HA concentration ($C_o = 0.2$ mg/ml) at $T_{dil.} = 180$, 360, 540 and 720 s. Figure 6 presents the plots of $(\ln\eta_{rel.})^{-1}$ as a function of $T+(t/2)$ for each dilution and the extrapolation to the time of dilution. The same procedure was performed for HA concentrations ranging from 0.4 to 1.2 mg/ml. At each concentration, extrapolated values of $(\ln\eta_{rel.})^{-1}$ were plotted as a function of $T_{dil.}$ (Figure 7). The slopes of these lines multiplied by the dilution factor (C/C_o) give the real viscosimetric reaction rate ($v_{vis.}$). The rate of reaction of HYASE with HA at the minimum concentration was determined directly from the plot of $(\ln\eta_{rel.})^{-1}$ as a function of reaction time; this plot is also included in Figure 7. It can be observed that all lines intersect at approximately the same point. The enzyme reactions were performed at different substrate concentrations, but, by diluting to a common concentration, were investigated under common viscosimetric con-

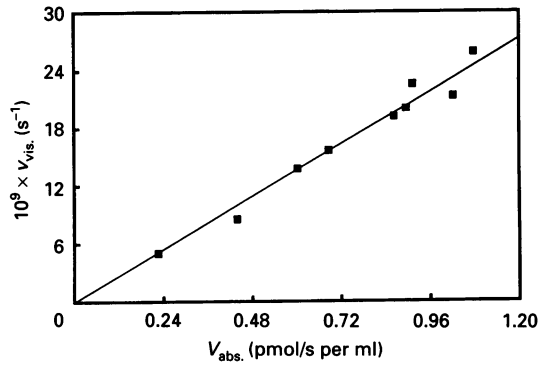

Figure 8 Relationship between the viscosimetric estimation of HYASE activity and the activity expressed in katal units

Plot of the real viscosimetric reaction rate ($v_{vis.}$) as a function of rate of reaction expressed as mol of bonds broken per unit of time and per unit of reaction volume ($v_{abs.}$).

ditions. Using the kinetic dilution method we investigated the action of HYASE on HA in the concentration range 0.2–1.2 mg/ml and estimated the K_m using a direct linear plot. The investigations were performed in triplicate and the mean value ± S.D. of K_m was 0.51 ± 0.05 mg/ml. This value is in excellent agreement with our previous investigations. As the rate of reaction is determined by viscosimetry and expressed in empirical units (s^{-1}), the value of $V_{max.}$ obtained cannot be compared with our previous results. The observed value of $V_{max.}$ was 0.0175 ± 0.005 s^{-1}.

Empirical correlations

The action of HYASE on HA can be readily and sensitively measured by viscosimetry using the plot of $(\ln\eta_{rel.})^{-1}$ as a function of reaction time. However, the apparent reaction rates ($v_{app.}$) obtained at different concentrations cannot be compared. To do this, the kinetic dilution method needs to be used, but this is rather complicated. We determined an empirical relationship between the apparent reaction rate ($v_{app.}$) (Table 4) and the real viscosimetric reaction rate ($v_{vis.}$) using the kinetic parameters of the enzyme determined by the kinetic dilution method. This relationship is described by the following equation:

$$\log\left(\frac{v_{vis.}}{v_{app.}}\right) = \log a + b \log C \qquad (19)$$

where a and b are empirical constants obtained by the regression analysis. Regression analysis ($r^2 = 0.999$; $n = 9$) yielded values ± standard error of estimate of log $a = 1.28 \pm 0.03$ and $b = 1.92 \pm 0.03$. The values of these constants depend only on the viscosimetric properties of HA and can be used to investigate any HYASE preparation. Knowing these values, the rate of reaction of HYASE with HA at any concentrations can be calculated from the apparent reaction rate, without the need to use the kinetic dilution method. Furthermore, one can derive the viscosimetric rate of reaction by plotting $C^b/\ln\eta_{rel.}$ as a function of reaction time and multiplying the slope by a. This approach allows us to estimate enzyme activity from plots of $(\ln\eta_{rel.})^{-1}$ as a function of reaction time at any concentration of HA. We have demonstrated that, in the case of random degradation, the reciprocal value of M_v gives a straight line when plotted as a function of reaction time. The slope of this line is related to the actual number of bonds broken. In this work we investigated an empirical approach to estimating enzyme activity from the slope of the plot of $(\ln\eta_{rel.})^{-1}$ as a function of reaction time. At all concentrations investigated, straight lines were obtained. In Figure 8 we plotted the viscosimetric reaction rate ($v_{vis.}$), calculated using the empirical relationship obtained above, as a function of absolute reaction rate ($v_{abs.}$) obtained previously and expressed as mol of bonds broken per unit of time and per unit of reaction volume. A straight line was obtained ($r^2 = 0.970$; $n = 9$) and the slope ± standard error of estimate of this line was 22.8 ± 0.5 μl/mol. This provides clear proof that the empirical estimate of the reaction rate obtained by viscosimetry is related to the actual number of bonds broken and provides a good estimate of enzyme activity.

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