Kinetic investigation of the action of hyaluronidase on hyaluronan using the Morgan–Elson and neocuproine assays

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In this paper we describe kinetic investigations of the action of testicular hyaluronidase on hyaluronan. We have compared the use of two spectrophotometric assays, the first based on the Morgan–Elson reaction and the second on the neocuproine reaction. With the neocuproine reaction K_m was found to be 0.46 mg/ml and V_{max} to be 126 nmol l⁻¹ s⁻¹. Because of a low

sensitivity and the production of interfering precipitates, the Morgan–Elson assay cannot be used for kinetic investigation of the enzyme. Furthermore this assay is prone to interference from compounds such as disodium cromoglycate, (+)-catechine, penicillamine, CaCl₂ and acetate buffer.

INTRODUCTION

Hyaluronan (HA) (Figure 1) is a negatively charged high-molecular-mass polysaccharide built of β -D-glucuronic acid-(1 $\rightarrow$ 3)- β -N-acetyl-D-glucosamine disaccharide units linked 1 $\rightarrow$ 4 and has various important biological functions [1]. Hyaluronidases

(HYASEs) are a class of enzymes that degrade glycosaminoglycans such as HA. These enzymes are commonly divided according to their hydrolytic mechanism (Figure 1) into three main classes: hyaluronate 4-glycanohydrolase (EC 3.2.1.35; testicular, lysosomal and venom HYASE), hyaluronate 3-glycanohydrolase (EC 3.2.1.36; leech HYASE) and hyaluronate lyase (EC 4.2.2.1; bacterial HYASE) [2]. As the first group of HYASEs liberate hexosaminic end groups from the substrate, a spectrophotometric assay, based on the Morgan–Elson reaction for the quantification of hexosaminic carbohydrates, has often been used to investigate their properties [3,4]. This chemical approach has been claimed to be less sensitive than physicochemical methods such as turbidimetry or viscosimetry [5]. However, turbidimetry and viscosimetry provide an empirical estimation of enzymic activity based on the changes in the polymeric properties of the substrate. These changes can hardly be related to the actual number of bonds hydrolysed. We have developed a sensitive assay for HYASE activity based on gel-permeation chromatography (GPC) [6]. With this approach we can determine the kinetic parameters of the enzyme and express enzymic activity in katal units. Furthermore we have investigated the degradation mechanism and provided clear evidence that the enzyme acts on HA in a random fashion. On the basis of these findings we have developed a viscosimetric approach for determining the kinetic parameters of the enzyme and expressing enzymic activity in katal units [7]. In this paper we describe our kinetic investigations of the action of testicular HYASE on HA using two spectrophotometric assays: one based on the Morgan–Elson reaction and the second based on the neocuproine method for the quantification of reducing sugars [8]. The latter method is unspecific and prone to interference from any reducing substance, but it should allow investigation of the action of any type of HYASE on HA. We have also studied the possible interference of several compounds with the Morgan–Elson reaction.

EXPERIMENTAL

Materials

HA (sodium salt extracted from rooster comb) was kindly provided by Diosynth B. V. (Oss, The Netherlands). The testicular HYASE (EC 3.2.1.35) used was the international reference

Figure 1 Chemical structures of HA and hydrolytic products

(a) HA; (b) hydrolytic product of testicular, lysosomal or venom HYASE; (c) hydrolytic product of leech HYASE; (d) hydrolytic product of bacterial HYASE.

Figure 2 Chemical structure of neocuproine

standard provided by the Fédération Internationale Pharmaceutique (FIP Ghent, Belgium) and contained 328 units/mg. GlcNAc, 4-(dimethylamino)benzaldehyde and neocuproine were obtained from Janssen Chimica (Geel, Belgium). $K_2B_4O_7 \cdot 4H_2O$, HA disaccharide and (+)-catechin were obtained from Sigma (Bornem, Belgium). Na_2CO_3 and $CuSO_4 \cdot 5H_2O$ were obtained from Vel/UCB (Leuven, Belgium). Glycine was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Penicillamine was a gift from Merck, Sharp & Dohme B. V. (Haarlem, The Netherlands). Disodium cromoglycate was a gift from Fisons Pharmaceuticals N. V. (Leuven, Belgium). Chondroitin sulphate A (molecular mass 25 kDa) was a gift from Luitpold Pharma (München, Germany). All other chemicals were of analytical grade.

GPC analysis

All GPC analyses were performed as described previously [6].

Spectrophotometric measurements

Spectrophotometric analyses were carried out on a Biochrom 4060 UV-visible spectrophotometer from Pharmacia LKB (Cambridge, U.K.). Water was used as the blank for all measurements.

Morgan–Elson assay [8]

$K_2B_4O_7 \cdot 4H_2O$ (6.1 g) is dissolved in 100 ml of water (reagent A). 4-Dimethylaminobenzaldehyde (10 g) is dissolved in a mixture of 11 ml of conc. HCl, 1.5 ml of water and 87.5 ml of acetic acid; 10 ml is diluted to 100 ml with acetic acid before use (reagent B). Then 50 μ l of reagent A is added to 250 μ l of sample, standard or control and the mixtures are incubated at 100 °C for 3 min. After being cooled rapidly to room temperature, 1.5 ml of reagent B is added and the mixtures are incubated at 37 °C for 20 min. Absorbance is determined at 585 nm. It is assumed that treatment with alkali at 100 °C results in the formation of anhydro intermediates [9], which condense with 4-(dimethylamino)benzaldehyde in an acid medium at 37 °C.

Neocuproine assay [8]

Na_2CO_3 (40 g), glycine (16 g) and $CuSO_4 \cdot 5H_2O$ (450 mg) are dissolved in 1 litre of water (reagent C). Neocuproine (150 mg) is suspended in about 80 ml of water to which a few drops of 1 M HCl are added until all is dissolved; the solution is made up to 100 ml with water and stored in a dark bottle (reagent D). Then 400 μ l of reagent C and 400 μ l of reagent D are added to 200 μ l of sample, standard or control. The mixtures are incubated at 100 °C for exactly 12 min. After the solution has been cooled rapidly to room temperature, 1 ml of water is added and A_{450} is measured. In this assay Cu^{2+} is reduced to Cu^+ , which binds to neocuproine (Figure 2) resulting in the shift of the absorbance spectrum of this compound.

Enzymic reactions

Enzymic 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. To 2 ml of a HA solution, dissolved in buffer and equilibrated at 37 °C, was added 100 μ l of HYASE solution, dissolved in buffer. Reactions were terminated after a fixed time by incubating the mixtures at 100 °C for 15 min. The samples were stored frozen at –20 °C until analysis. This approach allowed us to obtain samples on which both spectrophotometric assays could be performed in triplicate.

RESULTS AND DISCUSSION

Reproducibility of the assays on GlcNAc and calibration

Both assays were performed in water and buffer, and for the Morgan–Elson reaction the absorbances (mean $\pm$ S.D.; $n = 9$) were -0.012 ± 0.021 (water) and -0.017 ± 0.028 (buffer). For the neocuproine assay these values were 0.265 ± 0.018 (water) and 0.255 ± 0.017 (buffer). GlcNAc was dissolved in both solvents in various concentrations and assayed by the Morgan–Elson and neocuproine reactions. The mean absorbances obtained were independent of the solvent used. These findings suggest that, for calibration of the assays, GlcNAc standard solutions could be prepared in either solvent, but in our experience GlcNAc standard solutions are more stable when prepared in water. When dissolved in buffer a precipitate formed within a few days and the absorbance decreased as a function of time. For all further experiments the calibration graph was constructed using GlcNAc standard solutions prepared in water, but for the kinetic experiments the control reactions were performed with phosphate buffer. Both assays were performed on several GlcNAc standard solutions and good linearity ($r^2 = 0.998$) was observed in the concentration range 0–700 μ M. Furthermore the two assays were found to have comparable sensitivity towards GlcNAc.

Evaluation of the assays on HA

Both reactions were performed on HA solutions (1.5–2.5 mg/ml) prepared in buffer. With the Morgan–Elson reaction a string of precipitated material was obtained on addition of reagent B and the supernatant had the same absorbance as the control reaction. The addition of reagent B renders the mixture very acidic and under such conditions the polyanionic polymer becomes uncharged, which probably accounts for the observed precipitation with samples of undegraded HA. With the Morgan–Elson reaction we could not estimate the number of mol of end groups present in the substrate solution before digestion. For the neocuproine reaction, only at concentrations above 2 mg/ml, were absorbances significantly higher than the control reaction obtained. At such concentrations HA solutions are rather viscous and not easily handled in a quantitative way. By performing several assays on an HA solution (2.3 mg/ml) we could estimate (mean $\pm$ S.D.; $n = 10$) that 1 mg of HA contained about 15.8 ± 8.7 nmol of reducing end groups determined as GlcNAc. From GPC analyses we could ascertain that the experimental conditions of the neocuproine assay did not degrade the substrate and thus would not artificially produce additional reducing end groups.

Preliminary investigations

The action of HYASE (final concentration 0.33, 1.65 or 6.6 units/ml) was investigated on HA (final concentration 0.5 mg/ml). Depending on the conditions, the enzymic reactions were stopped at different time intervals (between 0 and 100 min)

Figure 3 GPC profiles of HA after 10 min digestion with testicular HYASE at different concentrations

GPC analyses of HA (0.5 mg/ml) after 10 min degradation by HYASE at a concentration of 0.33, 1.65 or 6.6 units/ml. GPC analyses were performed as described elsewhere [6].

and both spectrophotometric assays were performed on all samples. With the Morgan–Elson assay, many samples contained precipitated material. It appears therefore that the HA chains must be degraded to a minimal molecular mass before assay in order to prevent precipitation. A sample of enzymically degraded HA can still contain some molecules of high molecular mass. These could produce precipitation, not necessarily visible, and thus increase the absorbance. Any kinetic investigation based on this assay requires high amounts of enzyme or long reaction times, e.g. 1 h incubation with an enzyme concentration of 6.6 units/ml. The absorbance (mean $\pm$ S.D.; $n = 3$) then obtained, corrected for the control reaction, was 0.546 ± 0.006 . When the neocuproine assay was used, the result was 0.810 ± 0.027 . These results suggest that the neocuproine method is much more sensitive towards degraded HA than the Morgan–Elson reaction, despite their equivalent sensitivities towards GlcNAc. This difference in sensitivity between the two assays was also observed with solutions of chondroitin sulphate A and HA disaccharide.

The Morgan–Elson assay yielded results that were 87% (chondroitin sulphate A) and 55% (HA disaccharide) lower than those yielded by the neocuproine assay. As both calibration methods are comparable, the estimation of the reaction rate, expressed as the number of mol of end groups released per unit of time and per unit of reaction volume, will provide different results when using the Morgan–Elson and the neocuproine assays. With a HYASE concentration of 0.33 unit/ml the amount of reducing sugars increased from 8 to about $50 \mu\text{M}$ within 100 min. These reaction conditions were the same as in our previous kinetic investigations [6,7], but obviously the spectrophotometric assay based on the neocuproine method would require long reaction times to produce a significant increase in the amount of reducing sugars. Even at a concentration of 1.64 units/ml the amount of reducing sugars increased very slowly (from 8 to about $70 \mu\text{M}$ within 100 min), but at a concentration of 6.6 units/ml a linear increase was observed within 60 min. These preliminary kinetic investigations suggested that the spectrophotometric assays could not monitor the action of HYASE on HA as sensitively as GPC or viscosimetric assays [6,7].

Figure 3 shows GPC profiles of the HA sample after 10 min of

Figure 4 Relationship between the reaction rate and HA concentration as obtained with the neocuproine assay

Linear relationship between the concentration of HYASE and the reaction rate for the action of the enzyme on HA (1 mg/ml) as determined by the neocuproine assay.

enzymic degradation by HYASE at a concentration of 0.33, 1.65 or 6.6 units/ml. These chromatograms indicate the large differences in the polymeric properties of the substrate when the kinetic experiments are performed at different enzyme concentrations. The addition of high concentrations of enzyme leads to a more rapid decrease in the mean molecular mass of the substrate and the action of the enzyme on oligomeric substrates is investigated. By performing GPC analyses before, during and at the end of the enzymic reactions, we had a clear idea of the polymeric properties of the substrate in our previous kinetic assays [6]. We could ascertain that, under the experimental conditions we employed, the action of the enzyme on high-molecular-mass HA was being investigated. Such experimental conditions are a better reflection of the situation *in vivo* as HA extracted from biological sources always has a high molecular mass [1].

Kinetic investigations using the neocuproine assay

Our preliminary investigations suggested that the spectrophotometric assay based on the neocuproine method would be more sensitive than any assay based on the Morgan–Elson reaction. Therefore our first kinetic investigations were performed with the neocuproine assay. We investigated the reducing properties of our enzyme preparation (0–500 $\mu\text{g/ml}$) and found that 1 μg of HYASE corresponds (mean $\pm$ S.D.; $n = 7$) to 0.85 ± 0.37 nmol of reducing sugars calculated as GlcNAc. For all kinetic investigations the enzyme should be added to the control reaction. The action of HYASE (final concentrations ranging between 1.65 and 8.25 units/ml) was investigated on an HA solution (final concentration 1 mg/ml). The reactions were stopped at fixed times as described in the Experimental section and the amount of reducing sugars liberated was plotted as a function of the reaction time. Straight lines were obtained and the reaction rate (v in $\mu\text{mol l}^{-1}\text{s}^{-1}$) was estimated from the slopes of these lines. Figure 4 presents the relationship observed between the enzyme concentration and v . From these results and our preliminary investigations we decided to perform kinetic investigations using an enzyme concentration of 6.6 units/ml. This concentration allows us to observe significant increases in the amount of reducing sugars within reasonable reaction times, and the contribution of the enzyme to the reducing capacities of the reaction mixtures is limited.

The action of HYASE (6.6 units/ml) was investigated on solutions containing 0.3–2 mg/ml HA. The enzyme reactions

Figure 5 Kinetic investigation of the action of HYASE (6.6 units/ml) on HA using the neocuproine assay

Amount of reducing sugars, calculated as GlcNAc and determined by the neocuproine assay, released from HA (■, 0.2 mg/ml; ●, 0.5 mg/ml; □, 1.2 mg/ml; ○, 1.7 mg/ml) by the action of HYASE (6.6 units/ml) as a function of reaction time.

were terminated at different time intervals and the neocuproine reaction was performed in triplicate on each sample. The amount of reducing sugars, calculated as GlcNAc, was plotted as a function of the reaction time. Figure 5 presents some typical plots obtained for the action of HYASE on HA at different concentrations. The initial rate of reaction, expressed as the number of mol of reducing end groups liberated per unit of time and reaction volume, was determined from the slopes of the regression lines through the experimental data. The results were fitted to the Michaelis–Menten equation and the kinetic parameters of the enzyme were estimated using the direct linear plot [10]. The results obtained were $K_m = 0.46 \text{ mg/ml}$ and $V_{max} = 126 \text{ nmol l}^{-1} \text{ s}^{-1}$ or $6.3 \text{ nmol mg}^{-1} \text{ s}^{-1}$ for an enzyme preparation containing 328 units/mg.

The value of K_m corresponds well to the values obtained with our GPC (0.44 mg/ml) and viscosimetric (0.45 mg/ml) assays [6,7], but the value of V_{max} obtained is much larger than previous results (GPC: $1.8 \text{ nmol l}^{-1} \text{ s}^{-1}$; viscosimetry: $1.34 \text{ nmol l}^{-1} \text{ s}^{-1}$). We can propose at least two suggestions to explain these results. First, with spectrophotometry the rate of reaction depends on the standard used for calibrating the assay and on the assay used to determine the end groups released by the enzyme. Employing a different kind of standard or using another kind of assay will probably give different estimations of the reaction rate. As discussed above, the use of the Morgan–Elson assay, if it can be used, will probably lead to different estimations of enzyme activity. Secondly, the molecular-mass properties of the substrate during the kinetic assays should be considered. From Figure 3 the different polymeric properties of the substrate, under different experimental conditions, can be judged. Under the conditions employed in our spectrophotometric assay the action of the enzyme is being investigated on a substrate of much lower molecular mass than in the GPC or viscosimetric assay. We cannot rule out the possibility that the enzyme acts on the two types of substrate with different kinetics.

Kinetic investigations using an enzyme concentration of 82.5 units/ml

By using much higher enzyme concentrations than in our previous investigations, we intended to find reaction conditions that would allow us to perform a kinetic investigation based on the Morgan–Elson reaction. The action of HYASE at a concentration of 82.5 units/ml was investigated on HA solutions with

final concentrations ranging between 0.3 and 2 mg/ml as described in the Experimental section. Reactions were stopped at fixed times and a first sample was obtained after 60 s. This allowed us to observe how the reaction was initially proceeding. The Morgan–Elson and neocuproine reactions were performed on all samples in triplicate. The enzyme preparation did not interfere with the Morgan–Elson assay and was therefore excluded from the control reaction. As mentioned above the enzyme preparation must be added to the control reaction when the neocuproine assay is performed. The amount of end groups released by the enzyme, as determined by both methods, was plotted as a function of the reaction time. Many samples still contained precipitated material when the Morgan–Elson reaction was used. Furthermore, under the conditions employed, the substrate was rapidly degraded and the initial rate of the reaction could not be determined. These observations suggest that kinetic parameters cannot be properly determined by means of a spectrophotometric assay based on the Morgan–Elson reaction. A spectrophotometric assay based on the neocuproine method is more suitable, but it is very sensitive towards interfering substances; any compound that possesses reducing properties will interfere. Therefore it is possible that this assay could not be applied to biological sources of HYASE (e.g. venoms or extracts).

The Morgan–Elson assay and some HYASE inhibitors

The spectrophotometric assay based on the Morgan–Elson reaction has often been employed to investigate the capacity of a compound to inhibit or activate the action of HYASE on HA [11–17]. Owing to the lack of specificity of the neocuproine assay, we did not use it for any investigations of HYASE inhibition. Using the Morgan–Elson assay, we investigated the possible interference of three compounds, disodium cromoglycate, (+)-catechin and penicillamine, all of which have been claimed to inhibit the action of HYASE [13,15,18]. The reaction was performed on solutions containing the inhibitor, GlcNAc or enzymically degraded HA and on mixtures of the compounds with GlcNAc or degraded HA, all dissolved in buffer. All assays were performed in triplicate. The reaction performed on a disodium cromoglycate solution (13 mM) produced a suspension of white flocks of precipitated material, resulting in very high absorbance. (+)-Catechin produced an interfering coloured substance and its presence reduced the absorbance obtained with GlcNAc or HA (50% reduction in a concentration of 1.7 mM). Penicillamine, CaCl_2 and 0.1 M acetate buffer (pH 3.5) reduced the colour formation of GlcNAc in the Morgan–Elson assay (25% reduction at 6.7 mM penicillamine; 25% reduction at 150 mM CaCl_2). These results indicate that the Morgan–Elson reaction, although specific towards hexosaminic carbohydrates, is prone to interference. This has been observed previously [19]. It therefore appears that any investigation of the kinetics, inhibition or activation of HYASE or any other type of enzyme [19] using the Morgan–Elson reaction should be performed with great care.

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