Hyaluronic acid of tailored molecular weight by enzymatic and acid depolymerization.

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ABSTRACT

Hyaluronic acid (HA) is a glycosaminoglycan crucial for homeostasis of tissues, and its role on cell signalling and regulation of tissue injury and repair largely depends on HA molecular weight. Therefore, HA application in a variety of fields requires HA of defined size. While a number of enzymatic, chemical and physical methods exist for HA depolymerization, limited information is currently available for accurate planning of experiments. In the present work, we propose a pseudo-mechanistic model to describe depolymerization kinetics of HA with hyaluronidase, chondroitinase ABC and phosphoric acid. Data to feed the model was provided by monitoring molecular weight reduction by gel permeation chromatography with light scattering detection over 24 h. Five enzyme to substrate ratios and three temperatures were used for enzymatic and chemical reactions respectively, allowing for selection of operational parameters in a range of conditions. The model adequately reproduces the resulting data providing flexibility in the planning of the reactions to obtain HA of the desired molecular weight.

Keywords: Hyaluronic acid; enzymatic depolymerization; acid hydrolysis.

1. Introduction

Glycosaminoglycans are linear polysaccharides consisting of *O*-linked disaccharide units endowed with a range of biological activities [1-3]. Within this group, hyaluronic acid (HA) stands as the only non-sulfated representative, forming long chains of D-glucuronic acid (GlcA) linked to *N*-acetyl-D-glucosamine (GlcNAc) through alternating $\beta(1,3)$ and $\beta(1,4)$ glycosidic bonds. Despite its apparent simplicity, HA appears as a critical component of the extracellular and pericellular matrices, regulating essential cellular events such as adhesion, mobility, proliferation and differentiation [4, 5].

More strikingly, HA displays antagonistic effects on certain biological functions, such as inflammation, cell migration and proliferation [4]. Among other factors, these contradictory effects can be explained by molecular weight size and distribution. High molecular weight HA (>1 MDa) has been associated to wound healing, inflammation suppression, protection of the epithelial tissue, tissue homeostasis, and antiproliferative and antiangiogenic effects, while HA between 250 kDa and 1MDa appears as a pro-inflammatory agent [6]. HA fragments of 117 kDa have shown to inhibit migration and invasion of cancer cells, whereas 35 kDa produced the opposite effects [7]. Disruption of the extracellular matrix homeostasis driven by cancer and oxidative stress seems to occur along with increased concentrations of HA of less than 250 kDa and ultimately oligomers. The latter are able to produce contrasting effects on inflammation, invasion, angiogenesis and cell proliferation [6]. This serves to illustrate the importance of HA chain length and the need for reliable methods to produce HA of defined molecular weight.

HA cannot be currently synthesized and must be obtained from animal tissue or by bacterial fermentation of certain *Streptococci* species [8, 9]. Either way, the molecular

weight of purified HA typically exceeds 1MDa, thus obtention of lower molecular weight material requires depolymerization. In animals, HA fragmentation occurs through enzymatic catalysis by hyaluronidases and free radicals [6], although *in vitro* depolymerization is also possible with bacterial enzymes and by physicochemical means [10]. Vertebrate hyaluronidases are endo- β -*N*-acetyl-hexosaminidases which randomly hydrolyse $\beta(1\rightarrow4)$ glycosidic bonds [11]. In HA, they cleave GlcNAc-GlcA linkages without modification of the polysaccharide structure. Some forms are also active on chondroitin sulfate [12]. A number of works have described hydrolysis kinetics of hyaluronidase on HA, defining reaction parameters generally based on analysis of the oligosaccharides produced, i.e. by HPLC [13] or capillary electrophoresis [14], or reducing end formation [15, 16]. More rarely, the reaction has been followed by taking advantage of absolute molecular weight estimates provided by gel permeation chromatography with light scattering detection (GPC-LS) [17]. These studies have provided good insight into reaction mechanisms and kinetics, however planning of depolymerization experiments is not straightforward based on the information provided.

HA depolymerization with bacterial enzymes has not been so extensively described as with vertebrate hyaluronidases, because the former degrade preferentially chondroitin sulfate and dermatan sulfate [18]. Bacterial enzymes act as *N*-acetylhexosaminidases, producing β -elimination across the $1\rightarrow4$ link which results in the formation of an unsaturated bond between C-4 and C-5 of the acid sugar. Within this group, chondroitinase ABC (CASE) from *Proteus vulgaris* is the most widely used for glycosaminoglycan degradation [12]. CASE actually consists of two enzymes, an endolyase (CASE-I) and an exolyse (CASE-II). CASE-I produces tetrasaccharides and disaccharides as end products, while CASE-II acts processively from the non-reducing end releasing disaccharides [19]. Enzymatic activity on HA has been described by increase of absorption at 232 nm due to the formation of a double bond on the sugar

acid [18, 19], but to the best of our knowledge the reaction has never been followed by GPC-LS.

Physicochemical methods include acid and alkaline hydrolysis, ultrasonic and thermal degradation, attack by oxidants and exposure to radiation of different types [10].

Among these, acid hydrolysis appears as a cost effective method to depolymerise HA [20]. The process produces random scission of glycosidic bonds preferentially at $\beta(1\rightarrow4)$ links, although cleavage of $\beta(1\rightarrow3)$ bonds also occurs to a lesser extent [21, 22]. Temperature and pH have shown to influence kinetics of the reaction, generally carried out with strong acids such as HCl [22, 23]. Rate constants and other kinetics parameters have been calculated from these experiments, as well as from numerical simulations applied to model the hydrolytic process [20]. However, as is the case for enzymatic methods, design of depolymerization processes is not straightforward based on the information provided.

In the present work we propose to fill this gap by proposing a pseudo-mechanistic model capable of predicting the conditions of reaction for the depolymerization of HA with bovine testicular hyaluronidase (HASE), CASE, and phosphoric acid. To this end, each reaction was followed by GPC-LS over 24 h, registering absolute molecular weight at specific times. Enzymatic reactions were studied at four enzyme to substrate ratios and acid hydrolysis at three different temperatures. This allows for selection of operational parameters in a range of conditions, providing flexibility in the planning of the reaction and ultimately enabling to obtain HA of the desired molecular weight.

2. Materials and methods

2.1. Depolymerization of hyaluronic acid

Hyaluronic acid (HA) was produced by microbial fermentation from *Streptococcus equi subsp. zooepidemicus* ATCC 35246 according to the processes previously described [24, 25]. Then, HA was isolated and purified from bacterial post-incubates by a combination of chemical and ultrafiltration treatments [9] to obtain HA of purity greater

than 98% and 1.2 MDa of molecular weight. This final HA was depolymerized with chondroitinase ABC (CASE) from *Proteus vulgaris* (EC 4.2.2.4., 0.86 U mg⁻¹, Prod. No. C2905, Sigma-Aldrich), hyaluronidase (HASE) from bovine testes type I-S (EC 3.2.1.35., 587 U mg⁻¹, Prod. No. H3506, Sigma-Aldrich), and phosphoric acid (85%, Prod. No. 20624.295, VWR Chemicals). HA was dissolved at 1 g/L, in 50 mM TRIS-HCl / 150 mM sodium acetate at pH 8 for reaction with CASE; in 5 mM NaH₂PO₄ / 150 mM NaCl at pH 4 for HASE depolymerization; and in ultrapure water for hydrolysis with H₃PO₄. Enzymatic reactions were carried out in triplicate at 37°C under magnetic stirring by adding the appropriate amounts of either enzyme to tubes containing 10 mL of HA solutions to reach enzyme to substrate ratios of 0.01, 0.025, 0.05, 0.075 and 0.01 units of CASE and 0.5, 1.0, 2.0, 7.0 and 12.0 units of HASE per mg of HA. At various times, 0.6 mL aliquots were taken and reactions stopped by heating at 70°C for 25 min. After centrifugation at 15000g for 30 min, supernatants were filtered through 0.2 µm polyethersulfone (PES) membranes and stored at -20°C until analysis. Acid hydrolysis was performed in duplicate after addition of 5 mg H₃PO₄ per mg HA (pH=1.71) to 10 mL of 1 g/L HA solutions at 40 °C, 60 °C and 75 °C. At various times, 0.6 mL aliquots were taken, reactions stopped by neutralization with NaOH, filtered through 0.2 µm PES membranes and stored at -20°C until analysis.

2.2. Molecular weight determination

Filtered samples were injected onto a GPC system (Agilent1260) consisting of quaternary pump (G1311B), injector (G1329B), column oven (G1316A), refractive index (G1362A) and dual angle static light scattering (G7800A) detectors.

Chromatographic separation was carried out with a set of four Suprema columns (PSS, Mainz, Germany): precolumn 5 µm, 8x50 mm; 30Å 5 µm, 8x300 mm; 100Å 5 µm, 8x300 mm; and ultrahigh 10 µm, 8x300 mm. A sample volume of 100 µl was injected onto the system and eluted at a flow rate of 1 mL/min with 0.1 M NaN₃ and 0.01 M NaH₂PO₄ at pH 6.6. Column oven and light scattering detector were kept at 30°C, while

refractive index detector was maintained at 40°C. Both detectors were calibrated with a polyethylene oxide standard (PSS, Mainz, Germany) of 106 kDa (Mw) and polydispersity index (PDI) 1.05, dissolved in the mobile phase at 1g/L. Absolute molecular weights were determined combining refractive index and dual angle light scattering signals using refractive index increments (dn/dc) of 0.150.

2.3. Depolymerization modelling and estimation

Enzymatic depolymerization kinetics follows the next expression:

$$\frac{dM_n}{dt} = -k(M_n - M_n^*)^n \quad (1)$$

where M_n is the number average molecular weight of HA (in Da), n is the kinetic order (dimensionless), k the rate of decay (in h⁻¹) and M_n^* the molecular weight after depolymerization for 24 h (in Da). The enzyme to substrate ratio (E) affects both the kinetic rate and the molecular weight after depolymerization for 24h, and was modeled with the following expressions:

$$k(E) = \frac{V_m E}{K_m + E} \quad (2)$$

$$M_n^*(E) = M_n^e e^{-\mu E} \quad (3)$$

where V_m is the maximum reaction rate (in h⁻¹), K_m the Michaelis-Menten constant (in U mg⁻¹), μ a coefficient of fitting (in mg U⁻¹) and M_n^e denotes the estimated initial molecular weight (in Da). There are four parameters (V_m , K_m , M_n^e and μ) that are unknown a priori, but can be estimated from depolymerization data with, at least, two levels of enzyme.

For acid hydrolysis, eq. (2) assumes the form of Arrhenius equation, whose substitution in (1) at $n=2$ results in:

$$\frac{dM_n}{dt} = -Ae^{-\left(\frac{E_a}{RT}\right)} (M_n - M_n^d)^2 \quad (4)$$

where M_n^d is the molecular weight of a saturated disaccharide (397.3 Da), A the pre-exponential factor (in $\text{Da}^{-1} \text{h}^{-1}$), R the Universal gas constant ($0.0083145 \text{ kJ mol}^{-1} \text{K}^{-1}$), T the absolute temperature (in K) and E_a the activation energy (in kJ mol^{-1}).

For the estimation of the parameters we minimized the residual sum of squares with the AMIGO2 toolbox [26]. For the implementation, we used an estimation protocol previously described [27] with the derivative form of expression (1).

3. Results and discussion

3.1. Molecular weight distribution profiles

Fig. 1 illustrates the decrease in molecular weight during enzymatic and chemical depolymerization of HA monitored by GPC-LS. In all cases, the distributions shift toward lower retention volumes as reaction time increases, consistent with a reduction in molecular weight. In the process, peak heights of the depolymerized fragments decrease compared to original HA, especially in enzymatic hydrolysis, but areas of the distributions remain virtually unchanged in acid and HASE hydrolyses, consistent with random cleavage. In the case of CASE, the reaction not only widens the distributions but also diminishes their area. This agrees with the –exo action of CASE, as disaccharides stripped off the chain do not remain in the main HA distribution and typically appear as a single peak at higher retention volumes [18, 19]. Increases in the disaccharide peak may give an indication on the extent of the –exo action. In our case, Fig. 1 does not show the expected disaccharide peak, most likely because of overlap with the negative solvent signal starting at 27.3 min. However, -exo action of CASE

Figure 1. Illustrative GPC eluograms (refractive index detector signals) of hyaluronic acid (HA) from *S. zooepidemicus* depolymerized with (A) hyaluronidase (12 U/mg HA), (B) chondroitinase ABC (0.1 U/mg HA), and (C) phosphoric acid (1:5 w/w; 75°C) at different reaction times.

can still be evaluated by the variation in the area of the main polymeric peak as molecular weight decreases (Fig. 2). We have fitted the data represented by the following expression:

$$A = M_n^0 (1 - e^{-0.0188(M_w - 27.6792)})$$

Where A is the refractive index area of the HA peak measured by GPC and M_n^0 the initial molecular weight. The fitting is reasonably good, with R_{adj} of 0.7010. The model displays a near-zero slope from original HA down to around 300 kDa, indicating predominant –endo action. From this value downwards the slope dramatically increases as random cleavage is replaced by the processive mechanism, possibly because of diminished aggregation of HA [11]. In practical terms, this implicates minimal loss of HA mass down to around 300 kDa. As peak area is directly related to HA mass, we can estimate the mass of HA released as disaccharides using the above model, resulting for example in losses of 0.3% at 400 kDa, 1.2% at 300 kDa, 5.3% at 100 kDa and 23.1% at 100 kDa.

Figure 2. Area of the main HA peak (GPC – refractive index detector) versus estimated molecular weight (M_n) for CASE depolymerization (scatter). Solid line represents the fitting model.

3.2. Depolymerization kinetics

In all experiments reduction of molecular weight was more pronounced at the start of the reaction (Fig. 3). In enzymatic digestions, the decrease is very slight after 5-6 hours, reaching an almost static state. Hence, we considered depolymerization after 24 h as the final point of the enzymatic reactions, even though they could theoretically progress to lower molecular weight oligosaccharides and disaccharides [18, 28]. In acid

Figure 3. Evolution of number average molecular weight (M_n) of HA during depolymerization reactions with (a) chondroitinase and (b) hyaluronidase at five enzyme to substrate ratios and (c) phosphoric acid (5:1 w/w) at three temperatures. Model solution represented by solid lines; scatter data: average M_n (kDa) at each sampling time; error bars: standard deviation ($n=3$ for HASE and CASE; $n=2$ for phosphoric acid).

hydrolysis, this phenomenon does not appear. Because at the reaction pH (1.7) cleavage occurs preferentially at the $\beta(1\rightarrow4)$ glycosidic bond between GlcNAc and

GlcA [22], the lowest molecular weight for the enzymatic reaction was set to 397.3 Da (saturated disaccharide)

The model proves capable of reproducing the experimental data within the enzyme to substrate ratios, temperatures and timeframes defined, as shown in Fig. 3. In enzymatic digestions, reaction rate was left free in the model, resulting in reaction rates of 1.83 for CASE and 2.70 for HASE (Table 1), while in acid hydrolysis, reaction rate was set to 2 [22]. Among the model parameters shown in Tables 1 and 2, adjusted correlation coefficients of 0.986 for CASE, 0.991 for HASE and 0.978 for acid hydrolysis demonstrate the goodness of fit of the model.

	V_m	K_m	μ	n	M_n^e	R_{adj}^2
CASE	7.36×10^{-5}	0.019036	39.5139	1.8305	645878	0.9862
HASE	6.62×10^{-4}	5×10^5	0.82979	2.6913	565542	0.9907

Table 1. Fitting parameters (M_n) of enzymatic depolymerization of HA.

A	E_a	M_n^d	R_{adj}^2
1.381×10^8	91.693	365.8	0.9780

Table 2. Fitting parameters (M_n) of acid depolymerization of HA. E_a in kJ mol^{-1} ; A in $\text{Da}^{-1} \text{h}^{-1}$; M_n^e in Da.

In enzymatic reactions we assumed that the enzyme to substrate ratio (E) affected the kinetic rate (k) and the molecular weight after depolymerization for 24h (M_n^*). A

Michaelis-Menten adjustment for k proved adequate (2), and dependence of M_n^* with E was modeled with a negative exponential equation (3), as linear or second order polynomials produced worse fits.

In acid hydrolysis, k was estimated using the Arrhenius equation, with values for pre-exponential factor (A) and activation energy (E_a) of $1.38 \times 10^{11} \text{ kDa}^{-1} \text{h}^{-1}$ and 91.7 kJ mol^{-1}

¹ respectively (Table 2). Although to the authors' knowledge, HA hydrolysis with phosphoric acid has not been previously reported, E_a of 137 kJ mol⁻¹ was estimated for HA depolymerization with 0.1 M HCl [22]. To determine the source of the discrepancy, we can use the same calculation method applied in [22] for our data. This method consists in a linear regression applied to the log-transformed Arrhenius equation, with kinetic rate constants previously determined by linearization of a second order kinetic equation. As a result, we obtain a much closer value of 133 kJ mol⁻¹. The difference seems then to arise from the double linearization applied. In fact, fitting of the molecular weight data with 133 kJ mol⁻¹ achieves R_{adj}^2 of 0.893, compared to 0.978 obtained when parameters are jointly estimated, which indicates the suitability of the method presented here.

3.2. Design of depolymerization experiments

The proposed model allows to define the conditions of reaction required to obtain HA of the molecular weight desired. Fig. 4 depicts the possible combinations of E and reaction time for enzymatic depolymerization, and temperature and reaction time for acid hydrolysis, as a function of M_n . As can be seen, a given M_n can be reached by increased t at low E (or T) or vice versa. Reaction times at chosen E and T can be calculated integrating differential equation (1):

$$\frac{1}{(M_n - M_n^*)^{n-1}} = \frac{1}{(M_n^0 - M_n^*)^{n-1}} + (n-1)kt \quad (5)$$

Where

$$t = \left(\frac{1}{(n-1)k} \right) \left(\frac{1}{(M_n - M_n^*)^{n-1}} - \frac{1}{(M_n^0 - M_n^*)^{n-1}} \right)$$

For enzymatic depolymerization, substituting relationships (2) and (3) in (5):

Figure 4. Variation in Mn of HA with reaction time and (a,b) enzyme to substrate ratio (E) and (c) temperature (T) .

$$t = \left(\frac{K_m + E}{V_m E(n-1)} \right) \left(\frac{1}{(M_n - M_w^e e^{-\mu E})^{n-1}} - \frac{1}{(M_n^0 - M_n^e e^{-\mu E})^{n-1}} \right) \quad (6)$$

For chemical hydrolysis, substituting relationship (4) in (5):

$$t = A e^{-\left(\frac{E_a}{RT}\right)} \left(\frac{1}{M_n - M_n^d} - \frac{1}{M_n^0 - M_n^d} \right) \quad (7)$$

Where M_n^0 is the initial molecular weight. When selecting particular reaction conditions it must be born in mind that shortening reaction times by increasing E or T results in a more abrupt decrease of M_n . Stopping the reaction under these conditions may compromise M_n accuracy, as small variations in time can result in large changes in M_n . This is particularly relevant for enzymatic reactions since inactivation of the enzymes by heat is not instantaneous and may pose operational challenges at large scale. Besides accuracy, other aspects may influence the selection of a particular reaction. Phosphoric acid may represent an interesting option because of its low cost compared to enzymes, but only down to medium-sized HA, as either high temperature or long reaction times are required to achieve low molecular weight fragments. For the latter, enzymatic depolymerization may represent a better option. CASE is considerably more expensive than HASE, has less affinity for HA and as already discussed, the yield of depolymerized CS decreases dramatically below 300 kDa because of disaccharide stripping by the α -exo form. Therefore, HASE may appear as a more attractive option for enzymatic depolymerization. On the other hand, the chemical structure of depolymerized HA products may be of great importance. HASE preserves the chemical structure of undepolymerized HA, whereas CASE yields reaction products carrying unsaturation at the non-reducing end. For detection purposes, this is an advantage as unsaturation allows HA to absorb UV radiation. In other applications, the possibly altered bioactivity introduced in HA by such structural changes may pose a problem, as it might trigger immune reactions, or represent an advantage for materials more resilient to biogenic degradation.

4. Conclusions

We have described the depolymerization of hyaluronic acid with hyaluronidase, chondroitinase and phosphoric acid through a pseudo-mechanistic model. Good fits

are obtained with adjusted correlation coefficients greater than 0.978 in all cases. The proposed equations allow to define combinations of enzyme amount and reaction time, or temperature and reaction time for acid hydrolysis, to obtain HA of the desired molecular weight.

Conflict of interest

The authors declare no conflict of interest.

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