Monte Carlo simulation of hyaluronidase reaction involving hydrolysis, transglycosylation and condensation

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The action of hyaluronidase on oligosaccharides from hyaluronan is complicated due to branched reaction paths containing hydrolysis, transglycosylation and condensation. The unit component of hyaluronan is a disaccharide, namely GlcA-(β 1 $\rightarrow$ 3)-GlcNAc where GlcA and GlcNAc are D-glucuronic acid and D-N-acetylglucosamine respectively. Hyaluronan is the linear polymer formed by these disaccharide units, linked together with β 1 $\rightarrow$ 4 glycosidic bonds. Bovine testicular hyaluronidase acts only at β 1 $\rightarrow$ 4 glycosidic bonds of hyaluronan. The progress of product distribution from short oligosaccharides was simulated with the Monte Carlo method using the probabilistic model. The model consists only of a single enzyme molecule and a finite number of substrate and water molecules. The simulation is based on a simple reaction scheme and proceeds via an algorithm with minimum adjustable parameters generating random

numbers and probabilities. The experimental data for bovine testicular hyaluronidase using [GlcA-(β 1 $\rightarrow$ 3)-GlcNAc]₄ as the starting substrate were quantitatively simulated with only three adjustable parameters. The simulated data for [GlcA-(β 1 $\rightarrow$ 3)-GlcNAc]₃ and [GlcA-(β 1 $\rightarrow$ 3)-GlcNAc]₅ as the starting substrates agreed semi-quantitatively with experimental data using the same parameters. The mechanism of the hyaluronidase reaction is a combination of branched probabilistic cycles. The condensation reaction is much weaker than the transglycosylation reaction but contributes to product distribution at the final stage of the reaction, preventing complete hydrolysis of the substrates.

Key words: enzyme kinetics, enzyme mechanism, hyaluronan, probabilistic model.

INTRODUCTION

Hyaluronidase [hyaluronoglucosamidase (EC 3.2.1.35) and hyaluronoglucuronidase (EC 3.2.1.36)] catalyses hydrolysis of hyaluronan randomly. Hyaluronan or hyaluronic acid is widely distributed in animal tissues [1–3]. The unit component of hyaluronan is a disaccharide, GlcA-(β 1 $\rightarrow$ 3)-GlcNAc, where GlcA and GlcNAc are D-glucuronic acid and D-N-acetylglucosamine respectively. Hyaluronan is the linear polymer formed by these disaccharide units linked together with β 1 $\rightarrow$ 4 glycosidic bonds. Hyaluronidases also act on chondroitin or chondroitin sulphates [4–7]. Bovine testicular hyaluronidase (EC 3.2.1.35) has been widely studied due to its physiological importance in fertilization. The enzyme acts only at β 1 $\rightarrow$ 4 glycosidic bonds in hyaluronan [1–3,8]; however, due to its complexity, the kinetic mechanism of hyaluronidase has not yet been sufficiently clarified. For oligosaccharides, the enzyme has transglycosylation and condensation activities in addition to hydrolysis. Analysis of the kinetic mechanism by using differential equations from steady-state enzyme kinetics is unsuitable, due to the large number of adjustable parameters involved. In the present study, the Monte Carlo method [9–12] was applied to the kinetic analysis of the hyaluronidase reaction. Since the Monte Carlo algorithm is simple, the progress of product distribution is simulated with minimum adjustable parameters.

METHODS

Active site of hyaluronidase

The active site of bovine testicular hyaluronidase consists of five subsites [3] as shown in Figure 1. GlcA and GlcNAc are defined as A and B respectively and the single unit of the polymers is shown as AB. The residues in the polymer are numbered from the non-reducing end. Each subsite binds with AB. The catalytic site is shown by a $\blacktriangle$ in Figure 1. From the catalytic site, the

subsites are named N1 and N2 towards the non-reducing end, and R1, R2 and R3 towards the reducing end. This subsite map has been determined by Highsmith et al. [3] with detailed analysis of the action patterns against oligosaccharides. At a substrate concentration of approx. 1 mM, AB was not produced by the action of the enzyme at pH 5.2 [8]. Therefore productive binding modes that cover only R1 at the reducing side are negligible. However, productive binding modes that cover only N1 at the non-reducing side were observed in the very low concentration range (approx. 1 nM) of the substrate at pH 7.0 [5,13]. As a result, five possible distinct binding modes exist for the donor as shown in Figure 1. The relative ‘binding frequency’ per molecule for each binding mode was assigned as f_i ($i = 1–5$). The donor covering the catalytic site is divided into two fragments. Then the fraction at the reducing-end side is released and the residual fragment at the non-reducing end remains at the active site. The next product is released by the action of an acceptor. When the acceptor is a water molecule, the reaction is hydrolysis. When the acceptor is an oligosaccharide, the reaction is transglycosylation. The condensation reaction is an exceptional transglycosylation reaction in which the donor binds only at N2N1 without releasing the first fragment. There are two possible acceptor-binding modes for oligosaccharides. The relative binding frequency per molecule of the saccharide is defined as g_1 or g_2 as shown in Figure 1. The relative binding frequency per molecule of water is defined as g_3 .

Probabilistic model for hyaluronidase reaction

The probabilistic model consists of a single enzyme molecule and a finite amount of substrate and water [9–12]. The amount of the starting substrate was usually 10^4 molecules. The number of water molecules was set to be $10^4(55.5/c_0)$, where c_0 is the molar concentration of the starting substrate in the experimental data and 55.5 is the molar concentration of water in dilute aqueous

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Figure 1 Subsite map of bovine testicular hyaluronidase and possible binding modes of substrate

The active site of the enzyme consists of five subsites. Each subsite has the capacity to bind a disaccharide AB. The catalytic site is shown by a $\blacktriangle$. The subsites are numbered from the catalytic site to the non-reducing end as N1 and N2, and to the reducing end as R1, R2 and R3. There are five possible binding modes for donors, shown as f_1 , f_2 , f_3 , f_4 and f_5 . Dotted lines represent possible additional parts of donors outside the active site. There are three possible binding modes for acceptors, shown as g_1 , g_2 and g_3 . The values of f_i and g_j are relative binding frequencies. The position of AB in the polymer is numbered from the non-reducing end, shown as 1, 2, 3, 4, ...

solution. The ratio of the amounts of water and the starting substrate is the same as the molar ratio in the experimental data.

The enzyme reaction in the computer simulation proceeds in a step by step manner with the random number and selection probabilities. The (pseudo) random numbers were produced by a personal computer. Selection probabilities for donors, binding modes and acceptors were calculated with simple rules as described below.

Selection of donor

There are five possible binding or reaction modes for the donor as shown in Figure 1. When the chain length exceeds $(AB)_5$, different binding modes covering all five subsites are possible. If the substrate is $(AB)_i$, the possible binding modes covering all the subsites are $(i-4)$. The 'selection probability' for $(AB)_i$ depends on the 'selection frequency' per molecule and the number of molecules. When the number of $(AB)_i$ is $N(i)$, the selection probability P_i is shown as follows:

$$P_i = \frac{v_i N(i)}{\sum_{i=2}^{\text{Max}} v_i N(i)} \quad 1 < i \leq \text{Max} \quad (1)$$

$$v_i = f_1 \quad i = 2 \quad (2)$$

$$v_i = f_1 + f_4 \quad i = 3 \quad (3)$$

$$v_i = f_1 + f_2 + (i-4)f_3 + f_5 \quad 3 < i \leq \text{Max} \quad (4)$$

where v_i is the selection frequency per molecule of $(AB)_i$. The selection frequency is assumed to be proportional to the sum of possible binding frequencies of $(AB)_i$. Max is the number of AB residues in the longest chain in the system. The value of Max was within 100 in the present simulations. Since AB was not produced at pH 5.2 and at a substrate concentration of approx. 1 mM [5], those binding modes covering only N2N1R1 or N1R1 were neglected. There are four apparently independent parameters for the calculation of P_i , namely f_2/f_1 , f_3/f_1 , f_4/f_1 and f_5/f_1 . However, f_5/f_4 and f_3/f_2 show the same relative contribution for subsite R3. The following relationship is established as a result:

$$\frac{f_5}{f_4} = \frac{f_3}{f_2} \quad (5)$$

The number of independent parameters is therefore only three (f_2/f_1 , f_3/f_1 and f_4/f_1).

Selection of binding mode of donor

The possible binding modes of the donor depend on the chain length. When the donor is $(AB)_2$, there is only one binding mode for the condensation reaction (f_1 mode in Figure 1). For $(AB)_3$, there are two possible binding modes (f_1 and f_4 modes in Figure 1). The probability that binding covers subsites N2N1 for the condensation reaction is as follows.

$$P^* = \frac{f_1}{f_1 + f_4} \quad (6)$$

The probability that binding covers subsites N2N1R1 and releases $(AB)_2$ is $(1 - P^*)$.

Binding probabilities for $(AB)_i$ are listed below ($i > 3$; k is the number of AB residues from the non-reducing end of the donor).

$$P_k^* = \frac{f_1}{f_1 + f_2 + (i-4)f_3 + f_5} \quad k = i \quad (\text{condensation}) \quad (7)$$

$$P_k^* = \frac{f_2}{f_1 + f_2 + (i-4)f_3 + f_5} \quad k = i-2 \quad [\text{release of } (AB)_2] \quad (8)$$

$$P_k^* = \frac{f_5}{f_1 + f_2 + (i-4)f_3 + f_5} \quad k = 1 \quad [\text{release of } (AB)_{i-1}] \quad (9)$$

$$P_k^* = \frac{f_3}{f_1 + f_2 + (i-4)f_3 + f_5} \quad 1 < k < i-2 \quad [\text{release of } (AB)_{i-k}] \quad (10)$$

A water molecule is released in the condensation reaction of eqn (7). The binding modes in eqn (10) are covering all subsites.

Selection of acceptor

There are three selection modes for acceptors as shown in Figure 1. A water molecule is one of the acceptors. The selection probability Q_j is shown as follows:

$$Q_j = \frac{w_j N(j)}{\sum_{j=0}^{\text{Max}} w_j N(j)} \quad 0 \leq j \leq \text{Max} \quad (11)$$

$$w_j = g_3 \quad (j = 0) \quad (12)$$

$$w_j = g_1 \quad (j = 2) \quad (13)$$

$$w_j = g_2 \quad (2 < j \leq \text{Max}) \quad (14)$$

Figure 2 Simulation algorithm

$N(i)$ is the number of $(AB)_i$ and $N(0)$ is the number of water molecules. A random number is produced and the next path is determined by the probability at each of the three selection steps. Simulation procedures are described in detail in the Methods section.

where $N(0)$ is the number of water molecules, w_0 is the relative selection probability for water per molecule, and w_j ($j = 2, \dots, \text{Max}$) is the selection probability for $(AB)_j$ per molecule. There is only one binding mode for any donor as shown in Figure 1. The action of water as an acceptor is prohibited when the intermediate contains only AB, since no AB was released under the present experimental conditions. Although two independent parameters g_2/g_1 and g_3/g_1 are needed for the calculation of Q , only one parameter g_3/g_1 is necessary, since $g_2/g_1 = f_3/f_2$. The total number of independent adjustable parameters for the simulation of the hyaluronidase reaction is therefore four (f_2/f_1 , f_3/f_1 , f_4/f_1 and g_3/g_1).

Degree of the reaction and estimation of best-fit adjustable parameters

Simulated and experimental data were represented as a function of the degree of reaction, in place of time course of the reaction. The degree of reaction was defined as the relative amount of $(AB)_2$, as shown in eqn (15), since it increases monotonically in the experiments from the start to the end of the reaction [8].

$$\text{Degree of Reaction} = \frac{[(AB)_2]}{[(AB)_L]_0} = \frac{N(2)}{N(L)_0} \quad (15)$$

$[(AB)_L]_0$ is the initial molar concentration of the starting substrate in the experiment and $N(L)_0$ is the initial amount of $(AB)_L$ in the simulation. When the reaction is started with $(AB)_4$, the degree of reaction is $[(AB)_2]/[(AB)_4]_0$ or $N(2)/N(4)_0$. Other amounts of

substrates and products were shown as fractions $[(AB)_i]/[(AB)_L]_0$ or $N(i)/N(L)_0$. The best-fit parameters were sought in order to allow ERROR^2 , defined in eqn (16), to be the minimum. ERROR^2 is the sum of the squares of the difference between the observed and calculated values of the fractions.

$$\text{ERROR}^2 = \sum_i \sum_t \left\{ \frac{N(i)}{N(L)_0} - \frac{[(AB)_i]}{[(AB)_L]_0} \right\}^2 \quad (16)$$

Summation is for all observed species. The summation for t covers all experimental points of $(AB)_i$. The best-fit parameters were sought by the trial-and-error method as described previously [12].

Simulation algorithm and procedures

Input data were the chain length of the substrate, initial amount of the substrate molecules (usually 10^4), initial concentration of the substrate and simulation cycles. The simulation is a stacking of the cycles of the algorithm as shown in Figure 2.

The first step is the selection of a donor. A random number between 0 and 1 is produced by the computer, and the chain length i of the donor $(AB)_i$ is selected according to the probability determined by eqn (1). The amount of the donor is decreased by 1 [$N(i) = N(i) - 1$, $i > 1$]. The next step is to select the binding mode of the donor. Another random number is produced and the binding mode is selected according to the probabilities determined by eqns (6–10). The selection procedure finds the k th bond from the non-reducing end of the donor at the catalytic site. If the binding mode covers the catalytic site (hydrolysis or transglycosylation), a fragment at the reducing-end side from the catalytic site is released and the number of fragments is increased by 1 [$N(i-k) = N(i-k) + 1$]. When $k = i$, the binding mode is condensation and no fragment is released from the donor, but the number of water molecules is increased by 1 [$N(0) = N(0) + 1$]. The final step is to select an acceptor. A new random number is produced and the acceptor $(AB)_j$ is selected according to the probabilities determined by eqn (11). The amount of the acceptor is decreased by 1 [$N(j) = N(j) - 1$ ($j > 1$) for condensation or transglycosylation; $N(0) = N(0) - 1$ for hydrolysis]. The final product is released from the enzyme, and the amount of the product is increased by 1 [$N(i+j) = N(i+j) + 1$ for condensation; $N(k) = N(k) + 1$ for hydrolysis; $N(k+j) = N(k+j) + 1$ for transglycosylation]. The cycle was repeated 10^5 – 10^6 times (usually 2×10^5), and the product distributions at 1000 points during all the simulation cycles were obtained.

Programming and experimental data

The simulation program was written in Microsoft Visual Basic (version 6). The simulations were performed with an IBM-compatible personal computer (Intel Pentium 4, 1.8 GHz).

Product distributions from the starting substrates of $(AB)_3$, $(AB)_4$ and $(AB)_5$ by the action of bovine testicular hyaluronidase have been measured with an isocratic reversed-phase ion-pair HPLC at pH 5.2 and 37 °C by Cramer et al. [8]. Initial concentrations were 1.26, 0.8 and 0.8 mM for $(AB)_3$, $(AB)_4$ and $(AB)_5$ respectively. Products longer than $(AB)_5$ were not observed with HPLC due to experimental limitations. In all cases, AB was not produced [8].

RESULTS AND DISCUSSION

Comparison of simulated and experimental data

When $(AB)_4$ was used as a starting substrate, the main products were $(AB)_2$ and $(AB)_3$. Experimental and simulated data are

Figure 3 Comparison of simulated and experimental data from $(AB)_4$

Experimental data are represented as follows: Δ , $(AB)_2$; $\circ$, $(AB)_3$; $\bullet$, $(AB)_4$. In this and subsequent Figures, solid lines represent simulated data. The numbers are the chain lengths of polymers. The x-axis shows degree of reaction and the y-axis shows fractions of polymers. The degree of reaction is $M(2)/M(4)_0$ or $[(AB)_2]/[(AB)_4]_0$. The number of simulation cycles is 2×10^5 .

Figure 4 Simulated product distribution from $(AB)_4$

The amount of the starting substrate is 10^4 molecules and the number of simulation cycles is 2×10^5 . The parameters are the same as those in Figure 3. The x-axis shows simulation cycles and the y-axis shows fractions of polymers; the numbers are the chain lengths of the polymers.

shown in Figure 3. The best-fit parameters were estimated as $f_2/f_1 = 160 \pm 10$, $f_3/f_1 = 2500 \pm 100$, $f_4/f_1 \cong 0$ (< 1), $g_3/g_1 = 0.000095 \pm 0.000005$. Since $f_4/f_1 \cong 0$, the AB transfer reaction is negligible at 37 °C, pH 5.2, and there were thus only three actual independent parameters. That f_2/f_1 is large but not infinite indicates the existence of the condensation reaction. Figure 4 shows progress of the main polymers during the simulation until 2×10^5 cycles. The simulation shows that reaction converges to a steady-state distribution in the final stage. The condensation

Figure 5 Comparison of simulated and experimental data from $(AB)_3$

Experimental data are represented as follows: Δ , $(AB)_2$; $\circ$, $(AB)_3$; $\bullet$, $(AB)_4$. The degree of reaction is $M(2)/M(3)_0$ or $[(AB)_2]/[(AB)_3]_0$. The number of simulation cycles is 2×10^5 .

Figure 6 Comparison of simulated and experimental data from $(AB)_5$

Experimental data are represented as follows: Δ , $(AB)_2$; $\circ$, $(AB)_3$; $\bullet$, $(AB)_4$; $\square$, $(AB)_5$. The degree of reaction is $M(2)/M(5)_0$ or $[(AB)_2]/[(AB)_5]_0$. The number of simulation cycles is 2×10^5 .

reaction contributes to product distribution in the final stage of the reaction, preventing complete hydrolysis of the starting substrate. The simulation also confirms that the number of products longer than $(AB)_4$ is less than the number of shorter products. The final products from glycosidase reactions without condensation reaction are, however, usually complete hydrolysates [12].

Making use of the same best-fit parameters as those used for $(AB)_4$, hyaluronidase reactions using $(AB)_3$ and $(AB)_5$ as the

starting substrates were simulated. The initial stage of $(AB)_3$ reaction is only condensation, but the product, $(AB)_6$, is not accumulated, owing to the following transglycosylation and hydrolysis reactions, as shown in Figure 5. The results and experimental data are shown in Figures 5 and 6. The theoretical curves semi-quantitatively reproduce the characteristics of both reactions. To improve the reliability of the simulation, more detailed experimental data are necessary.

Significance of the probabilistic model

The action of bovine testicular hyaluronidase on bovine nasal cartilage proteoglycan subunits was investigated by light-scattering measurement [14]. The action pattern showed random hydrolysis of these macromolecules. Cramer et al. [8] analysed their own data, which are cited in the present study, with a combined set of simple first- and second-order chemical reactions. Although theoretical curves using six parameters fitted the experimental data quantitatively, the method did not use the principles of enzyme kinetics and the law of conservation of total enzyme concentration was not satisfied [8]. The present probabilistic model, however, defines a single enzyme molecule, and conservation of the total number of enzyme molecules is automatically satisfied throughout the simulation. Since the model simulates enzyme reactions without reference to time course, the algorithm is simpler and independent parameters are minimal. The simulations in the present study clarified the general kinetic mechanism of the hyaluronidase reaction, which involves transglycosylation and condensation in addition to hydrolysis. The estimated parameters are indices of favoured paths in the branched reactions.

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