Enzymatic Polymerization to Artificial Hyaluronic Acid Using a Transition State Analogue Monomer

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Summary: Artificial hyaluronic acid has been synthesized *in vitro* via enzymatic polymerization catalyzed by testicular hyaluronidases, which is the first successful example of the hyaluronic acid synthesis via non-biosynthetic pathways. The novel GlcAβ(1→3)GlcNAc oxazoline derivative was designed and synthesized as a transition state analogue monomer for the hyaluronidase catalysis. The oxazoline monomer was efficiently recognized by the enzymes at pH 7.1 to 9.0 and the polymerization reaction proceeded in a regio- and stereo-selective manner to give rise to artificial hyaluronic acid with molecular weight higher than 15000. These results strongly suggest that the transition state of these testicular hyaluronidases catalysis corresponds to a sugar oxazolinium ion.

Introduction

Hyaluronic acid (HA) is well-known as a natural polysaccharide classified into glycosaminoglycans (GAGs), widely existing not only in extracellular matrix (ECM) but also living cells such as vitreous of the human eye[1], synovial joint fluid[1], a rooster comb, smooth muscle cells[2] and fibroblasts.[2] HA exibits important functions as a member of ECM *in vivo*, for instance, tissue proliferation, regeneration, wound healing etc.[3] A typical heteropolysaccharide of HA consists of two kinds of sugar units, glucuronic acid (GlcA) and 2-acetamido-2-deoxy-D-glucose (GlcNAc), and has a structure of GlcAβ(1→3)GlcNAc disaccharide repeating unit connecting through β(1→4) glycosidic linkage. This biologically important HA is produced *in vivo* with catalysis of HA synthase, via alternating addition of GlcA and GlcNAc to the growing chain toward outside of cells using their activated nucleotide sugars as substrates, UDP-GlcA and UDP-GlcNAc, respectively.[4] Generally HA used for medical or cosmetic purposes is prepared by extraction from a rooster comb or bacterial culture fluid of *Streptococcus*. Studies on syntheses of HA reported are *in vitro* method using HA synthase via biosynthetic pathways[5] and a transglycosylation catalyzed by

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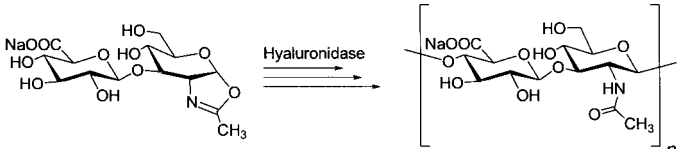
hyaluronidase.[6]

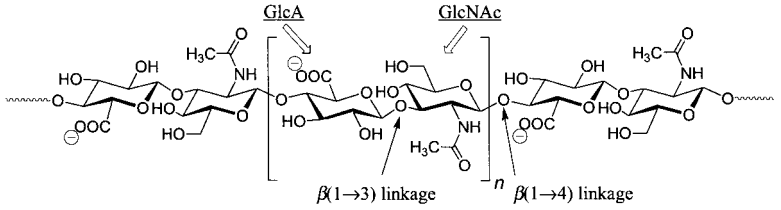
Enzymatic polymerization using a glycosyl hydrolase as catalyst has been shown very effective in synthesizing several natural and unnatural polysaccharides such as cellulose[7], xylan[8], chitin[9], and a cellulose-xylan hybrid polysaccharide[10], all of which have a $\beta(1\rightarrow4)$ glycosidic structure.[11] As extension of our enzymatic polymerization, we reports the successful synthesis of HA via nonbiosynthetic pathways (Scheme 1).[12]



Scheme 1.

Results and Discussion

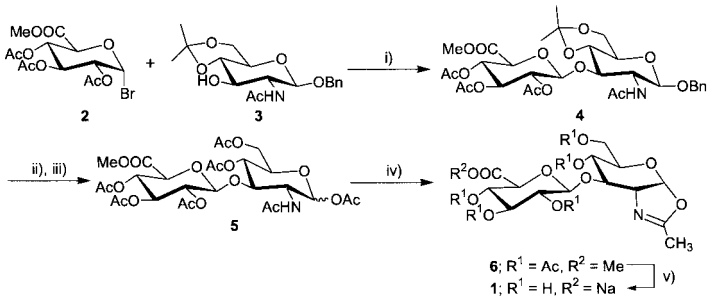
Design and Synthesis of Substrate Monomer 1. HA has a structure of GlcA unit and GlcNAc unit alternatingly arrayed (Scheme 2).



Scheme 2.

Hyaluronidases[13], endo-type hydrolysis enzymes of HA, are classified into three kinds of enzymes; hyaluronoglucosaminidase (HAGNase, EC 3.2.1.35), hyaluronoglucronidase (HAGUase, EC 3.2.1.36), and hyaluronate lyase (EC 4.2.2.1). Among them, HAGNase and HAGUase hydrolyze HA at $\beta(1\rightarrow4)$-*N*-acetylglucosaminide linkage and $\beta(1\rightarrow3)$-glucuronide linkage, respectively, resulting in the production of GlcA$\beta(1\rightarrow3)$GlcNAc disaccharide by HAGNase and GlcNAc$\beta(1\rightarrow4)$GlcA disaccharide by HAGUase as a minimum unit. These observations allowed us to conceive two possibilities for monomer design, an oxazoline derivative of an activated GlcNAc form as a donor side of HAGNase or a glycosyl fluoride of an activated GlcA form as a donor side of HAGUase. Thus, we designed and synthesized a

novel GlcAβ(1→3)GlcNAc disaccharide oxazoline derivative (**1**) on the hypothesis that the oxazoline derivative serves as a transition state analogue substrate monomer for HAGNase catalysis, giving rise to artificial HA.



Scheme 3. i) AgOTf, $Me_2NC(O)NMe_2$ / CH_2Cl_2, -40 °C ~ rt, 22h, 29%, ii) Pd-C, H_2 / MeOH, rt, 72h, 98%, iii) Ac_2O / Pyridine, rt, 6h, 98%, iv) TMSOTf / $ClCH_2CH_2Cl$, 50 °C, 7h, quant., v) MeONa / MeOH, then carbonate buffer (50 mM, pH 10.6)

The novel GlcAβ(1→3)GlcNAc oxazoline monomer (**1**) was chemically synthesized (Scheme 3): Glycosidation of methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate (**2**) with benzyl 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-β-D-glucopyranoside (**3**) using silver triflate and *N,N'*-tetramethylurea catalyst produced the corresponding disaccharide (**4**) in 29% yield. Hydrogenation of compound **4** was carried out by using 10% palladium on activated carbon in methanol followed by acetylation to give the acetylated disaccharide derivative (**5**) in 96% yield (2 steps). Then compound **5** was treated with trimethylsilyl triflate in 1,2-dichloroethane to afford the oxazoline derivative (**6**) in a quantitative yield. All *O*-acetyl protecting groups of compound **6** was removed by using sodium methoxide in methanol followed by removing methyl ester by *in situ* hydrolysis in carbonate buffer (50mM, pH 10.6) to give the desired oxazoline monomer (**1**).

Ring-Opening Polyaddition of 1. Catalysis behavior for **1** was firstly investigated by employing ovine testicular hyaluronidase (OTH) or bovine testicular hyaluronidase (BTH), both of which belong to HAGNase (Figure 1). The reaction (0.1M of **1** in D_2O, carbonate

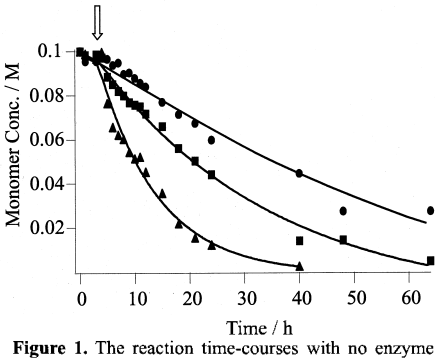


Figure 1. The reaction time-courses with no enzyme (●), OTH (▲), and BTH (■). The arrow shows the addition of the enzymes.

buffer, pH 7.1, OTH or BTH 10 wt% for **1**) was carried out in a NMR probe tube at 30°C. The reaction proceeded homogenously. The consumption of **1** was followed by means of ^{1}H NMR spectroscopy, from which the integration ratio of anomeric proton to methyl proton of acetamido group was calculated. It became significantly faster by addition of the enzymes at 3.5 h compared with the reaction without enzyme (control). These results show that the designed monomer **1** was successfully recognized by the enzymes causing the ring-opening reaction of oxazoline ring by the enzyme catalysis, resulting in the polymerization products. Without enzyme, the only product confirmed was a hydrolysis product of **1**, 2-acetamido-2-deoxy-3-*O*-(sodium β-D-glucopyranosyluronate)-D-glucopyranose. The polymerization of **1** under higher pH (8.0 and 9.0) also gave the polymerization products but in smaller yields. It should be noted that the product polysaccharide was not further subjected to hydrolysis under the reaction conditions of pH 7.1 or higher (Figure 2). This irreversible behavior of hyaluronidases catalysis is probably because of that the optimal pH of the enzyme hydrolysis is reported as pH 4.0~6.0.[13]

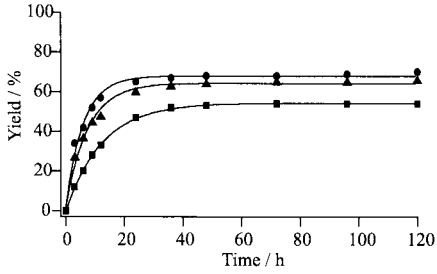


Figure 2. The time-course yield of the artificial HA with OTH under the pH 7.1 (●), 8.0 (▲), and 9.0 (■). Yields were determined by HPLC.

Characterization of the products. After the reaction, the enzymes were denatured by heating over 90°C for 3 min followed by addition of tetrahydrofuran. The precipitates were separated by centrifugation followed by purification through Sephadex G-10, to give a polysaccharide isolated in 52% yield with OTH after 36 h, and in 39% yield with BTH after 64 h, respectively. In the ^{1}H NMR spectrum (not shown), specific signals from two kinds of anomeric protons were observed at 4.55 (GlcNAc) and 4.47 ppm (GlcA) with the coupling constants of 7.48 and 6.96 Hz, respectively, showing the formation of β-glycosidic linkage. In the ^{13}C NMR spectrum (not shown), characteristic signals due to two anomeric carbons were observed at 103.89 (GlcA) and 101.34 ppm (GlcNAc). The signals at 83.43 and 80.79 ppm are ascribed to internal glycoside C3 of GlcNAc and C4 of GlcA, respectively. ^{1}H and ^{13}C NMR spectra of the polysaccharide

obtained were almost identical with those of natural HA[15], strongly supporting that the product is "artificial HA." The molecular weight was measured by size-exclusion chromatography (SEC) using HA samples having different molecular weight for calibration standards; molecular weight values (*Mn*) of two artificial HAs were determined as 1.74×10^4 and 1.35×10^4, respectively.[14] Circular dichroism (CD) behaviors further supported the structure of artificial HA.[15]

Catalysis mechanism for the enzyme are explained as follow: In the hydrolysis, a bond cleavage at the C-O via nucleophilic attack of water onto the anomeric carbon of GlcNAc is postulated to involve an oxazolinium ion intermediate stabilized by the carboxylate group of the hyaluronidases active site (Figure 3A and B). The oxazolinium intermediate in part B is structurally corresponding to the protonated species of monomer **1** in the active site as shown in part C. Thus, **1** can be considered as a transition state analogue monomer that is recognized very readily at the donor site of the enzyme and activated via protonation, lowering the activation energy for the reaction. 4-Hydroxyl group in GlcA of another **1** or of the growing chain end located at the acceptor site nucleophilically attacks onto the oxazolinium of **1** from β-side to form $\beta(1\rightarrow4)$ glycosidic linkage between GlcNAc and GlcA as given in part C. Repetition of this regio- and stereoselective glycosylation is a ring-opening polyaddition of **1** catalyzed by the enzyme, giving rise to artificial HA of relatively high molecular weight with perfectly controlled structure. This enzymatic polymerization is due to an extremely specific catalysis of hyaluronidase; the enzyme inherently catalyzes the $\beta(1\rightarrow4)$ bond cleavage of HA *in vivo*,

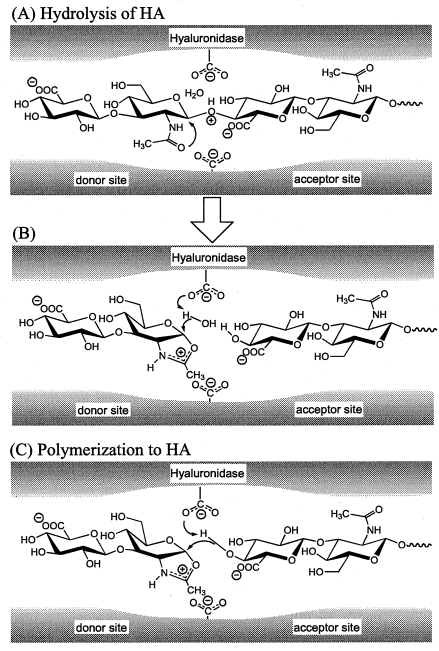


Figure 3. Postulated transition states of the hyaluronidases for the hydrolysis of natural HA (B), and for the polymerization of the monomer **1** to HA (C).

whereas it catalyzed the $\beta(1\rightarrow4)$ bond formation to lead to HA *in vitro*.[11d]

In conclusion, this study provides a novel method of synthesizing a natural heteropolysaccharide of HA containing both $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ glycosidic linkages. It is well-known that preparation of HA of the present molecular weight range by enzymatic digestion of HA or other preparative techniques is extremely difficult, and hence, this method is very convenient for preparation of such HA samples, which are expected to be a useful class of tools for exploring *in vivo* functions of HA.[16] There are many other polysaccharides containing an *N*-acetylhexosamine (HexNAc) residue among naturally occurring carbohydrates. GAGs are one of the most complicated polysaccharides containing HexNAc in their repeating units, which are associated with a core protein and highly sulfated. The present principle allows the approach to various natural and unnatural GAGs, which are very difficult to prepare via conventional methods. In addition, this reaction strongly suggests the mechanism of *in vivo* HA synthesis where the hydroxyl group at the growing chain end of non-reduced terminal attacks the anomeric carbon of UDP-GlcA or UDP-GlcNAc. The present study may play an important role for understanding the HA synthesis *in vivo*.

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