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Enzymatic Precision Polymerization for Synthesis of Glycosaminoglycans and Their Derivatives

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Summary: Hyaluronidase (HAase)-catalyzed polymerization was performed to provide synthetic hyaluronan (HA), chondroitin (Ch) and their derivatives. The 2-methyl oxazoline derivatives derived from their repeating disaccharides of *N*-acetylhyalobiuronate (**1a**) and *N*-acetylchondrosine (**3a**) were effectively polymerized by the enzyme, giving rise to synthetic HA and synthetic Ch in good yields through regio-selective and stereo-controlled ring-opening polyaddition. The oxazoline derivatives of 2-ethyl (**1b**, **3b**), 2-*n*-propyl (**1c**, **3c**), 2-isopropyl (**1d**, **3d**), 2-phenyl (**1e**, **3e**), 2-vinyl (**1f**, **3f**) and 2-isopropenyl (**1g**) were synthesized and subjected to the enzymatic reaction. Monomers **1b**, **1c**, **1f**, **3b** and **3f** were polymerized to corresponding polysaccharides **2b**, **2c**, **2f**, **4b** and **4f**, all of which are unnatural glycosaminoglycans. Compounds **1d**, **3c** and **3d** were also catalyzed by the enzyme, affording oligomers of **2d**, **4c** and **4d** were produced in trace amounts. Monomers **1e**, **1g** and **3e** were not catalyzed by HAase.

Keywords: chondroitin; enzymatic polymerization; glycosaminoglycan; hyaluronan; hyaluronidase

Introduction

Glycosaminoglycans (GAGs) are naturally occurring linear hetero-polysaccharides, which are found as carbohydrate components of proteoglycans (PGs) in extracellular matrices (ECMs) and on the cell surface. GAGs include six biomacromolecules of hyaluronan (hyaluronic acid, HA), heparin/heparan sulfate, chondroitin (Ch), Ch sulfate (ChS), dermatan sulfate and keratan sulfate. GAG has a repeated disaccharide structure, which consists of a hexosamine and an uronic acid. GAGs containing *N*-acetyl-D-glucosamine (GlcNAc) as a hexosamine constituent are called as glucosaminoglycans, and those containing *N*-acetyl-D-galactosamine (GalNAc) as galactosaminoglycans. HA and Ch are the most well-known glucosaminoglycan and galactosaminoglycan, composed of $\beta(1\rightarrow4)$-linked β-D-glucuronyl-$(1\rightarrow3)$-*N*-acetyl-D-glucosamine (GlcA$\beta(1\rightarrow3)$GlcNAc; *N*-acetylhyalobiuronate) and β-D-glucuronyl-$(1\rightarrow3)$-*N*-acetyl-D-galactosamine (GlcA$\beta(1\rightarrow3)$GalNAc; *N*-acetylchondrosine) as repeating units, respectively. HA, Ch and

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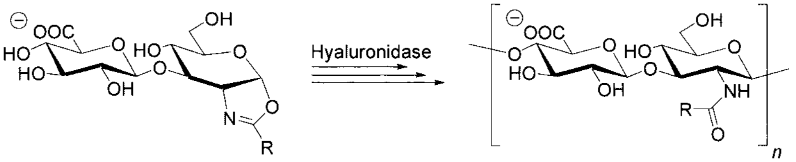
ChS are main constituents of ECMs in dermis and cartilage, in which large molecular complexes are formed by association of these molecules[1]. GAGs play critical roles in a wide variety of biological events such as regulation of cell differentiation[1–3], proliferation and migration leading to tissue morphogenesis and remodeling[4–9], matrices formation around cumulus cells during ovulation and fertilization[10–12]. Investigation of their more detailed functions is actively ongoing; however, it requires GAG samples with well-defined structure and synthesis of such GAGs is still challenging in synthetic chemistry[13–15]. Therefore, a facile and efficient method to prepare these biomacromolecules is demanded.

During a series of studies on enzymatic polymerization utilizing glycoside hydrolases as catalysts[16–21], synthesis of structurally well-defined natural and unnatural oligo- and polysaccharides has been achieved: Cellulose and xylan were prepared by cellulase[22–24], chitin by chitinase[25,26], and a cellulose-xylan hybrid polysaccharide by xylanase[27]. The polymerization employs a sugar monomer activated at the anomeric carbon, which reduces activation energy for the reaction, resulting in a polymer via repeated regio-selective and stereo-controlled glycosylation. Such structurally well-defined samples are essential to elucidate their molecular functions for vital activities in living system.

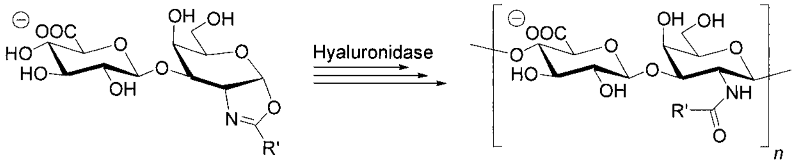
The present paper focuses on precision synthesis of HA and its derivatives (Scheme 1)[28,29] and natural-type Ch and its derivatives (Scheme 2)[30] via hyaluronidase (HAase)-catalyzed ring-opening polyadditions of sugar oxazoline derivative monomers. These reactions provide a facile and efficient approach to synthesis of GAGs with perfectly controlled structures.



1a; R= -CH_3
1b; R= -CH_2CH_3
1c; R= -$CH_2CH_2CH_3$
1d; R= -$CH(CH_3)_2$
1e; R= -Ph
1f; R= -$CH=CH_2$
1g; R= -$C(CH_3)=CH_2$

2a; R= -CH_3 (*Synthetic Hyaluronan*)
2b; R= -CH_2CH_3
2c; R= -$CH_2CH_2CH_3$
2d; R= -$CH(CH_3)_2$
2e; R= -Ph
2f; R= -$CH=CH_2$
2g; R= -$C(CH_3)=CH_2$

Scheme 1. Enzymatic polymerization of monomers (**1**) to synthetic HA and its derivatives (**2**).



3a; R'= $-CH_3$	**4a**; R'= $-CH_3$ (*Synthetic Chondroitin*)
3b; R'= $-CH_2CH_3$	**4b**; R'= $-CH_2CH_3$
3c; R'= $-CH_2CH_2CH_3$	**4c**; R'= $-CH_2CH_2CH_3$
3d; R'= $-CH(CH_3)_2$	**4d**; R'= $-CH(CH_3)_2$
3e; R'= -Ph	**4e**; R'= -Ph
3f; R'= $-CH=CH_2$	**4f**; R'= $-CH=CH_2$

Scheme 2. Enzymatic polymerization of monomers (**3**) to synthetic Ch and its derivatives (**4**).

Results and Discussion

Monomer design and synthesis. Oxazoline monomers (**1a**–**1g** and **3a**–**3f**) were designed based on the concept of "transition state analogue substrate monomer"[16–21,28–30]. Structures of HA and Ch are illustrated in Figure 1. There are two kinds of glycoside hydrolases responsible for their catabolism[31–33]. One is endo-*β*-*N*-acetylhexosaminidase (HAase), which can hydrolyze *β*(1→4)-*N*-acetylhexosaminide linkages[31,34]. The other is endo-*β*-glucuronidase catalyzing hydrolysis of *β*(1→3)-glucuronide linkages[31,35–38]. These two distinct enzymes are classified to the "retaining" enzymes, by which the stereochemistry of the anomeric carbon retains the *β*-form after hydrolysis. According to their catalysis mechanisms, monomers for the synthesis of HA and Ch can be considered via two modes of molecular designs, that is, (A) oxazoline-type monomers for HAases, and (B) fluoride-type monomers for *β*-glucuronidases. The former is feasible due to availability of the commercial enzymes, on the other hand the latter is not practical because the enzymes are difficult to obtain. Thus, we selected the route using HAase and oxazoline-type monomers **1** and **3** for the synthesis of HA and Ch. Monomers **1** and **3** were prepared according to the multi-step chemical procedures[28–30].

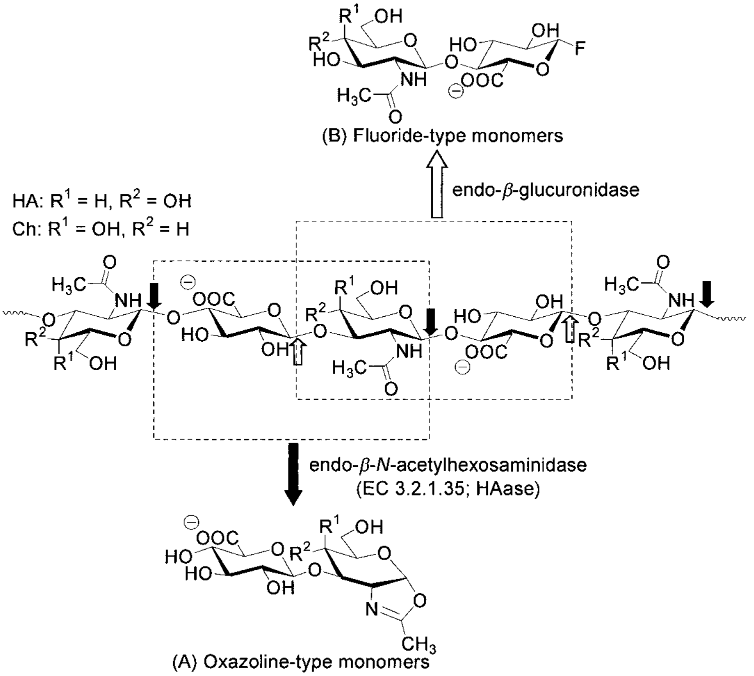
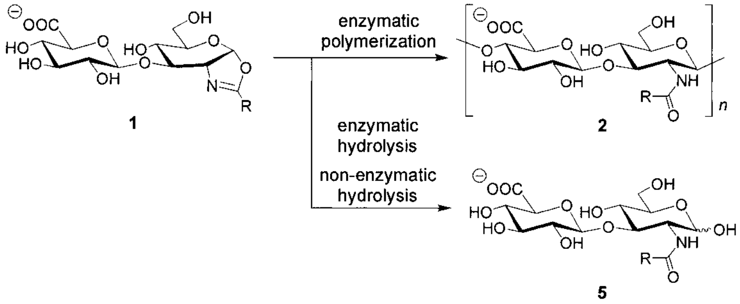


Figure 1. Possible monomer designs for the synthesis of HA and Ch. Allows show possible bonds for enzymatic cleavage by HAase (black arrows) and endo-*β*-glucuronidase (white arrows).

Synthesis of natural and unnatural HAs. Monomers **1a**–**1g** for the synthesis of HA and its derivatives were subjected to the enzymatic reaction catalyzed by ovine testicular HAase (OTH) at pH 7.5 and 30°C. In the enzymatic polymerization three kinds of reaction are possible to occur (Scheme 1); polymerization of **1** to product polymers (**2**), enzymatic and non-enzymatic hydrolyses of **1** resulting in formation of *N*-acylhyalobiuronates (**5**). All of the reactions performed in the absence of enzyme produced the disaccharides **5** through hydrolysis of the corresponding monomers.



Scheme 3. Three kinds of reaction occur during the polymerization of **1**.

Table 1 illustrates the results of the polymerization reactions of monomers **1a**–**1g**. OTH catalyzed the perfect regio-selective and stereo-controlled ring-opening polyaddition of **1a**, giving rise to **2a** (synthetic HA; M_n 13300, approx. 66 saccharides; entry 1) in a 53% yield. Unnatural HA derivative bearing *N*-propionyl groups in respective every glucosamine unit (**2b**, M_n 9700, approx. 46 saccharides; entry 2) was synthesized from 2-ethyl oxazoline monomer **1b** in a 52% yield. Monomer **1c** was also recognized and catalyzed by the enzyme effectively, affording the corresponding polymer **2c** (M_n 8000, approx. 38 saccharides; entry 3) in a 40% yield. However, 2-isopropyl oxazoline monomer **1d** was difficult to polymerize by the enzyme (entry 4). Oligomers of **2d** (4–14 saccharides) were formed in a trace amount. Monomer **1e** was not catalyzed by the enzyme (Entry 5), affording the hydrolyzed compound **5e** (69%) and unchanged **1e** (31%) after 168 h. Polymerization of **1f** smoothly proceeded by OTH, resulting in the formation of *N*-acryloyl derivative **2f** having M_n 9100 (approx. 44 saccharides) in a 41% yield (entry 6). Neither the polymer **2g** nor its oligomers were formed from **1g** (entry 7). The hydrolyzed compound **5g** (84%) and unchanged **1g** (16%) were confirmed after 168 h. Thus, not only natural-type HA **2a** but also HA derivatives **2b**, **2c** and **2f** were prepared in good yields via HAase-catalyzed polymerization. All ^1H and ^{13}C NMR data of product polymers confirmed their structures, indicating that the stereochemistry and regioselectivity were perfectly controlled during the polymerization.

All these behaviors of the monomers from Table 1 suggest the reactivity order of HAase-catalyzed reaction; **1a** (2-methyl) > **1b** (2-ethyl) ~ **1f** (2-vinyl) > **1c** (2-*n*-propyl) >> **1d** (2-isopropyl) > **1g** (2-isopropenyl) ~ **1e** (2-phenyl). This can be understood primarily in terms of a steric factor of the substituents.

Table 1. Enzymatic polymerization of monomer **1** to polymer **2**.[a]

Entry	Monomer	Time[b] (h)	Polymer **2**		
			Yield[c] (%)	M_n[d]	M_w[d]
1	**1a**	48	53	13300	22000
2	**1b**	48	52	9700	18500
3	**1c**	60	40	8000	16900
4	**1d**	96	trace		
5	**1e**	168[e]	0		
6	**1f**	48	41	9100	19500
7	**1g**	168[e]	0		

[a] In a carbonate buffer at pH 7.5, 50 mM; initial monomer concentration, 0.10 M; enzyme amount, 10 wt% (OTH, 560 units/mg) for monomer; reaction at 30 °C. [b] Reaction time for the complete consumption of the monomer unless otherwise indicated. [c] Isolated yields after purification. [d] Determined by SEC measurements calibrated with hyaluronan standards. [e] Reaction was terminated at the indicated time.

Synthesis of natural and unnatural Chs. Polymerization reaction of monomers **3a**–**3f** with H–OTH, which has higher hydrolysis activity units than OTH, was performed under the conditions of pH 7.5 and 30 °C. Table 2 indicates polymerization results of the monomers. Synthetic Ch (**4a**) was obtained via HAase-catalyzed polymerization of **3a** in a 50% yield (entry 1). It is notable that the molecular weight value (M_n) of **4a** at 2 h reached 4600 (entry 2), which is similar to that of naturally occurring Ch[39]. Monomers of 2-ethyl (**3b**; entry 3) and 2-vinyl (**3f**; entry 7) oxazolines were significantly catalyzed by the enzyme; unnatural *N*-propionyl (**4b**) and *N*-acryloyl (**4f**) derivatives of Ch were obtained in a 46% yield with M_n 2700 and in a 19% yield with M_n 3400, respectively. Monomers of 2-*n*-propyl (**3c**; entry 4) and 2-isopropyl (**3d**; entry 5) oxazoline derivatives were catalyzed to produce oligomers of **4c** and **4d** in low yields. The 2-phenyl oxazoline derivative (**3e**; entry 6) was not catalyzed by the enzyme, only affording a non-enzymatically hydrolyzed compound. Ring-opening polyaddition of the monomers **3a**–**3d** and **3f** proceeded in a regio-selective and stereo-controlled manner. Reactivity order of HAase-catalyzed reaction indicated from Table 2 is 2-methyl (**3a**) > 2-ethyl (**3b**) > 2-vinyl (**3f**) >> 2-*n*-propyl (**3c**) > 2-isopropyl (**3d**) > 2-phenyl (**3e**). These results show that structure of the oxazoline part is critical for the enzyme catalysis.

Table 2. Enzymatic polymerization of monomer **3** to polymer **4**.[a]

Entry	Monomer	Time[b] (h)	Polymer **4**		
			Yield[c] (%)	M_n[d]	M_w[d]
1	**3a**	23	50	2100	2500
2	**3a**	2	19	4600	6800
3	**3b**	35	46	2700	3600
4	**3c**	122	trace		
5	**3d**	168	trace		
6	**3e**	239	0		
7	**3f**	24	19	3400	4600

[a] In a phosphate buffer at pH 7.5, 50 mM; initial monomer concentration, 0.10 M; enzyme amount, 10 wt% (H–OTH, 2502 units/mg), for monomer; reaction at 30 °C. [b] Reaction time for the complete consumption of the monomer unless otherwise indicated. [c] Determined by HPLC containing products with molecular weight higher than tetrasaccharides. [d] Determined by SEC measurements calibrated with hyaluronan standards.

Polymerization Mechanism. The present ring-opening polyaddition of the oxazoline monomers **1** and **3** utilizes hydrolysis enzyme HAase as catalyst. Figure 2(A) shows the possible transition states (or intermediates) in the hydrolysis of natural Ch with HAase, which is generally accepted. In the hydrolase enzyme, there are donor and acceptor sites and two carboxylic acid groups are involved in the catalysis. Protonation onto the oxygen atom in the $\beta(1\rightarrow4)$ linkage occurs after the recognition of the substrate Ch by the enzyme as seen in stage (a). Then, the carbonyl oxygen atom of GalNAc attacks from the α direction onto the anomeric C1 carbon and at the same time cleaves the C-O bond to form an oxazolinium ion via substrate-assisted stabilization as given in stage (a) to (b). Water molecule attacks nucleophilically the C1 of the oxazolinium to open the ring, giving rise to the hydrolysis product from stage (b) to (c).

On the other hand, as shown in Figure 2(B) polymerization employs the oxazoline monomer **3** which is easily recognized at the donor site. There is no need to protonate the less basic oxygen atom but only need to protonate the more basic oxazoline nitrogen atom. Therefore, the polymerization lacks stage (a) and starts from stage (b). The structures of a transition state (intermediate) of (b) in both (A) and (B) are very close to each other. Thus, oxazoline monomer **3** can be regarded as a transition-state analogue substrate monomer; the structure of **3** facilitates recognition and further activation by the enzyme, with lowering the activation energy for the subsequent reactions. The oxazolinium ion readily formed from the monomer is attacked from the *β* direction by 4-OH group of GlcA of the other monomer or of the growing chain located at the acceptor site, inducing the ring-opening of the oxazolinium ion to give a *β*(1→4) glycosidic bond as well as the *N*-acylgalactosamine unit from stage (b) to (c). Repetition of stages (b) and (c) eventually produce Ch or Ch derivatives with perfectly regulating these complicated structures.

It is also noteworthy that HAase catalysis allowed the production of unnatural GAGs having various amido groups as well as natural-type GAGs. This suggests that the enzyme possesses a space at the active site enough for sterically larger *N*-substituents compared with *N*-acetyl group, or HAase is dynamic during polymerization catalysis as a host for the larger group guests. It is also suggested that recognition of 4-OH position in the hexosamine units is not very strict from the fact that the enzyme catalyzed the polymerization of both monomers **1** and **3**. Thus, precise design of monomer allows the production of structurally well-defined GAG derivatives having various amido functional groups under mild reaction conditions.

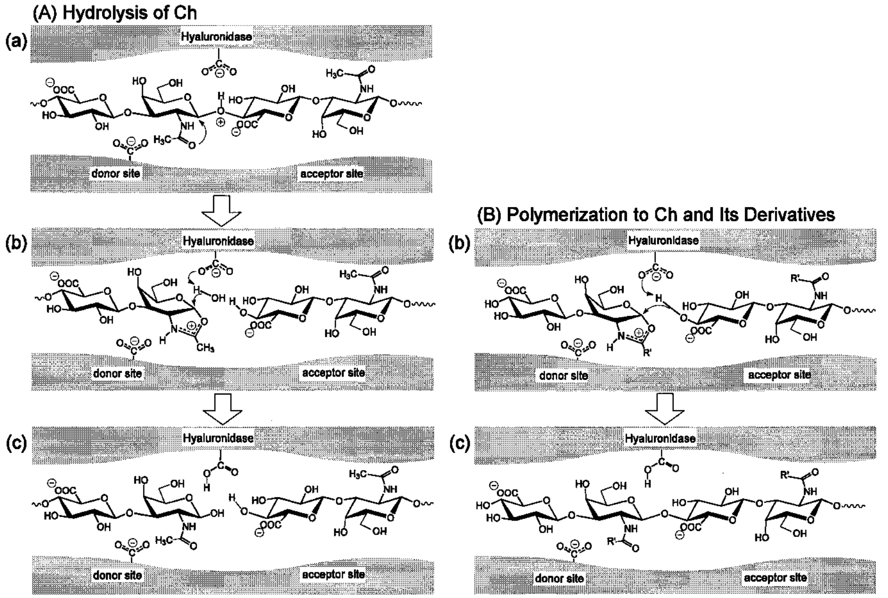


Figure 2. Comparison of mechanism in the HAase catalysis. (A) Hydrolysis of natural Ch and (B) Polymerization of the oxazoline monomers to Ch and its derivatives.

Conclusion

Enzymatic polymerizations to synthetic HA, synthetic Ch and their derivatives were achieved by the catalysis of HAases using transition state analogue substrate monomers, *N*-acylhyalobiuronate oxazolines and *N*-acylchondrosine oxazolines. The polymerization proceeded under neutral conditions in a perfect regio-selective and stereo-controlled manner. The synthetic HA having M_n 10000~20000 is laborious to be obtained by chemical and enzymatic degradation of naturally occurring HA as well as the other synthetic methodologies. HA in mitotic cells has a similar molecular weight to that of the synthetic HA[40,41], therefore, synthetic HA is expected to be a potent tool for investigation of bioactivities of intracellular HA. M_n value of the synthetic Ch was close to that of naturally occurring Ch produced in nematodes, which plays essential roles in the morphogenesis[42,43]. In addition, unnatural GAG derivatives **2b**, **2c**, **2f**, **4b** and **4f** were obtained via HAase-catalyzed polymerization. These polymers possess perfectly controlled structure, and it is difficult to produce such unnatural GAGs by biosynthetic pathways. Furthermore, *N*-acryloyl derivatives of HA (**2f**) and Ch (**4f**) are useful for the production of functionalized HA and Ch derivatives with a well-defined structure such as new macromonomers, telechelics and gels utilizing the reactive vinyl group. Thus, enzymatic polymerization is an effective method to construct not only natural-type GAGs but also unnatural derivatives that are applicable in various fields of science such as medicinal chemistry, pharmacology, physiology, biochemistry, polymer chemistry and enzymology.

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