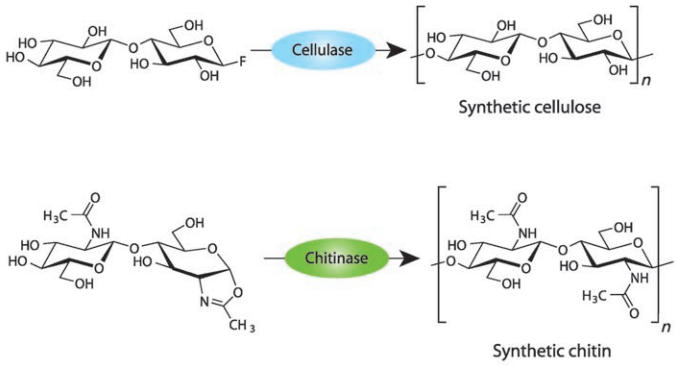
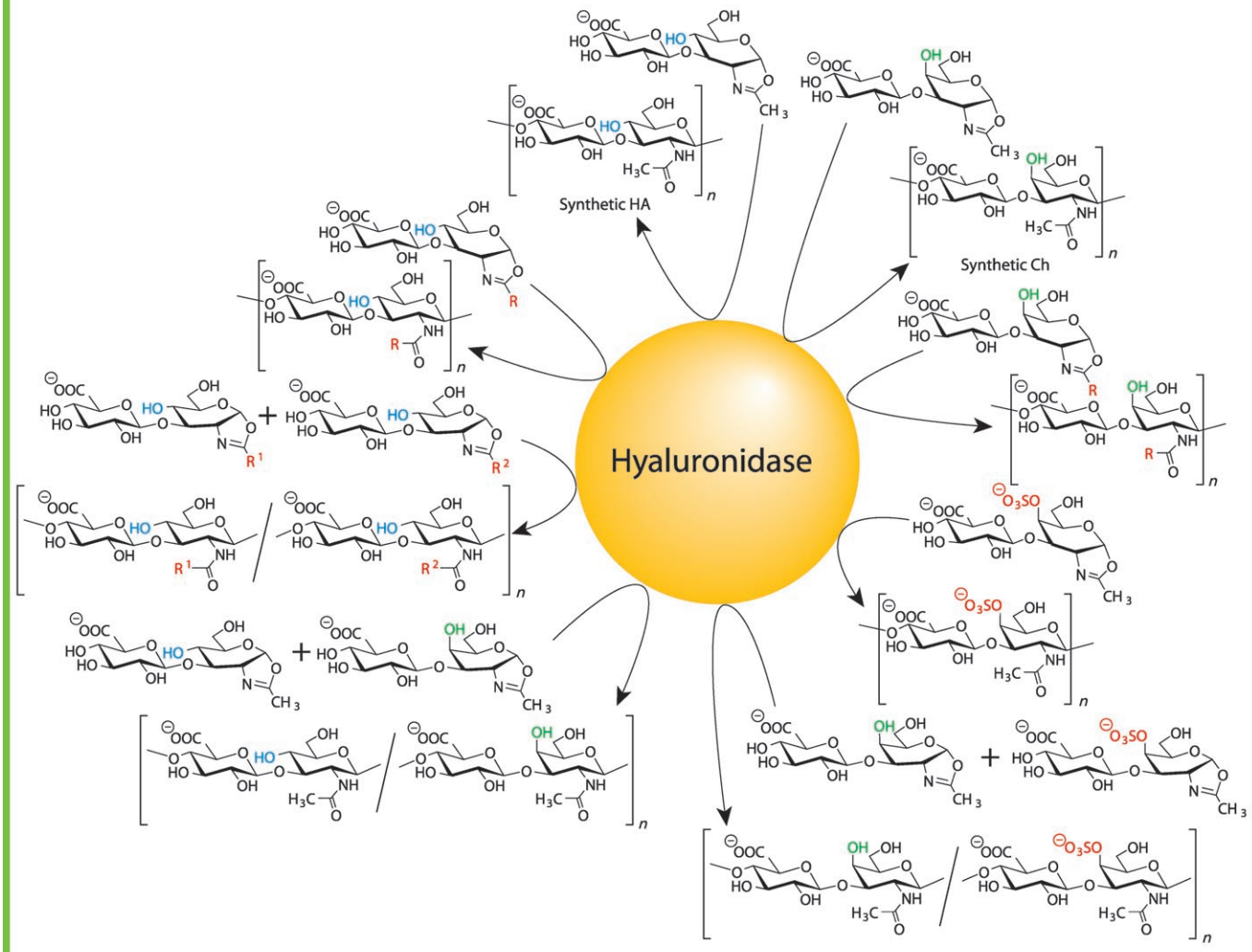
## Normal Enzyme Catalysts




## A Hyaluronidase Supercatalyst



DOI: 10.1002/chem.200600191

# A Hyaluronidase Supercatalyst for the Enzymatic Polymerization to Synthesize Glycosaminoglycans

**Shiro Kobayashi,*[a, b] Masashi Ohmae,[a] Hirofumi Ochiai,[a] and Shun-ichi Fujikawa[a]**

**Abstract:** Hyaluronidase (HAase) catalyzes multiple enzymatic polymerizations with controlling regio- and stereoselectivity perfectly. This behavior, that is, the single enzyme being effective for multireactions and retaining the enzyme catalytic specificity, is not usual, and hence, HAase is a *supercatalyst*. Various sugar oxazoline monomers prepared based on the concept "transition-state analogue substrate" were successfully polymerized and copolymerized with HAase catalysis, yielding natural and unnatural glycosaminoglycans.

**Keywords:** enzyme catalysis · glycosaminoglycans · hyaluronidase · polymerization

## Introduction

Polysaccharides are one of the naturally occurring three major biomacromolecules, together with proteins and nucleic acids, that exist in many cases as glycoconjugates, such as glycoproteins and glycolipids, in living cells. Over the last decades it has been disclosed that carbohydrates play critical roles in a number of biological events;[1] for example, regulation of signaling, differentiation and proliferation of cells, and immune responses.[2–4] In particular, glycosaminoglycans (GAGs), one of the classes among natural polysaccharides, participate in all of these events. These important carbohydrate polymers are synthesized in vivo by a variety of glycosyltransferases,[5,6] followed in some cases by modification with accessory enzymes such as sulfotransferases and epimerases.[6,7] Thus, polysaccharide chains with complicated structures are not encoded directly in the genome in contrast to proteins. Therefore, synthesis of polysaccharides is an important and challenging problem in the postgenome era. There are a number of reports describing synthetic methodologies utilizing chemical[8] and biochemical approaches;[9] however, in many cases such methods require difficult manipulations.

Enzymatic polymerization that utilizes hydrolases as the catalyst is an effective method for synthesis of various natural and unnatural polymers, in particular polysaccharides, through a nonbiosynthetic pathway.[10–15] This method enables a single-step production of a polysaccharide through polymerization of a simple sugar monomer activated at the anomeric carbon. For example, cellulose was synthesized by means of polymerization of β-cellobiosyl fluoride catalyzed by cellulase,[16–19] xylan by xylanase with β-xylobiosyl fluoride,[20] and chitin by chitinase with *N*,*N*′-diacetylchitobiose oxazoline.[21–23] Thus, each polysaccharide was prepared by a combination of a single enzyme and a single monomer. This situation was explained by the "key and lock" theory proposed by Emil Fischer in 1894, that is, only the correctly shaped key (a substrate monomer) fits into the key hole (active site) of the lock (an enzyme), leading to a catalysis of a single reaction.[10–14] Recently, we have achieved the production of hyaluronan,[24] chondroitin,[25] chondroitin sulfate,[26] and their derivatives[25,27] by utilizing the catalysis of a single enzyme, that is, hyaluronidase (EC 3.2.1.35).[28] The single-enzyme catalysis enabled the production of various polysaccharides for the first time. These polysaccharides belong to the family of GAGs exhibiting important functions in living cells. In the present paper we discuss the synthesis of such GAGs and their derivatives by means of enzymatic polymerization. A hyaluronidase shows a wide spectrum of catalysis for enzymatic polymerization of a variety of substrate monomers with perfect control of the stereochemistry and regioselectivity; this control is not usual in view of enzymatic catalysis specificity. Thus, hyaluronidase is a *supercatalyst* for enzymatic polymerization.

[a] Prof. Dr. S. Kobayashi, Dr. M. Ohmae, Dr. H. Ochiai, Dr. S.-i. Fujikawa
Department of Materials Chemistry
Graduate School of Engineering, Kyoto University
Kyoto 615-8510 (Japan)
E-mail: kobayash@kit.ac.jp

[b] Prof. Dr. S. Kobayashi
Present Address: R & D Center for Bio-based Materials
Kyoto Institute of Technology, Kyoto 606-8585 (Japan)
Fax: (+81) 75-724-7688

## Discussion

**Glycosaminoglycans**: GAGs are one of the naturally occurring linear heteropolysaccharides, consisting of an alternating structure of a hexosamine (D-glucosamine and D-galactosamine) and an uronic acid (D-glucuronic acid and L-iduronic acid). The "glycosaminoglycans" is a general term for the polysaccharides that include important biomacromolecules such as hyaluronan (hyaluronic acid, HA), heparin/heparan sulfate, chondroitin (Ch), chondroitin sulfate (ChS), dermatan sulfate and keratan sulfate.[29] (Figure 1)

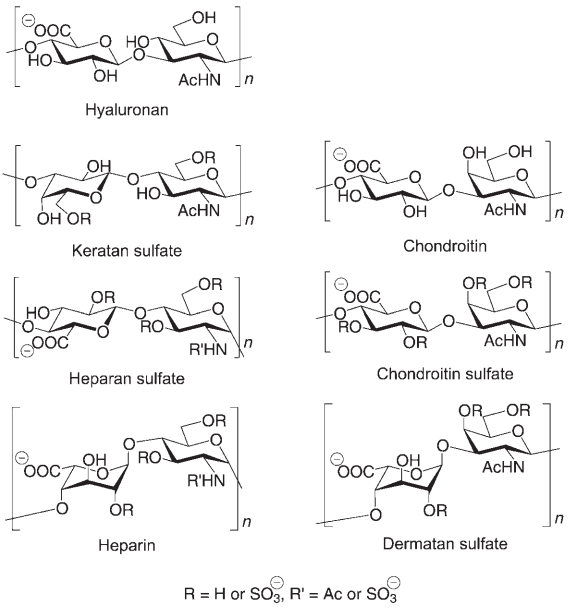



Figure 1. Chemical structures of naturally occurring GAGs.

These polysaccharides exist widely in the living body as components of the extracellular matrices (ECMs)[3] and on the cell surface.[30] In particular, HA, Ch, and ChS are present in higher concentration in the dermis and cartilage, in which large molecular complexes are formed by interaction of these molecules. GAGs directly associate with cellular differentiation and proliferation through signaling interactions with growth factors and morphogens, leading to tissue morphogenesis and wound healing.[3,4,31–34]

GAGs show a variety of structural diversity causing discrete structural forms within a molecule generated by complex patterns of deacetylation, sulfation, and epimerization. These are normally found in many tissues and influenced by some diseases and aging.[35,36] HA and Ch containing a glucosamine and a galactosamine, respectively, as a hexosamine constituent are the most well-known glucos- and galactosaminoglycan, respectively.[29]

The multifunctional HA[37] is biologically synthesized in the plasma membrane through alternating addition of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) catalyzed by hyaluronan synthase (EC 2.4.1.–) with two types of sugar nucleotides as substrates, uridine 5′-diphospho (UDP)-GlcNAc and UDP-GlcA.[38]

Ch exists as a carbohydrate part of proteoglycans in *C. elegans*[39] or in mammalians as a precursor of ChS, mainly existing in cartilage, cornea, and brain matrices.[40] A number of reports have been published that describe the biological functions of Ch and ChS;[41–43] in particular it is essential for the development of the central nervous system, which is closely associated with the sulfation patterns.[44] Biosynthesis of Ch is performed in the Golgi apparatus by the catalysis of chondroitin synthase (EC 2.4.1.–) and other glycosyltransferases with UDP-*N*-acetyl-D-galactosamine (UDP-GalNAc) and UDP-GlcA as substrates,[34,45–48] followed by selective sulfation by several kinds of specific sulfotransferases.[7,49]

Thus HA, Ch, and ChS play crucial roles in living systems; structurally well-defined samples are essential to elucidate their molecular functions for vital activities in living system. Their chemical or biochemical synthesis is challenging,[50] and a facile and efficient method to prepare these biomacromolecules with complicated structures has been an important unsolved problem.

**Hyaluronidase**: Hyaluronidase (HAase) is a hydrolase belonging to the glycoside hydrolase (GH) family 56,[51] which cleaves (1→4)-β-*N*-acetylhexosaminide linkages in HA, Ch, and ChS. GH56 hyaluronidases in Homo sapiens are well investigated in a gene level, which are classified into six members.[52] Three genes designated as HYAL1, HYAL2, and HYAL3 are clustered on chromosome 3p21.3, and another two genes, HYAL4 and PH20, and a pseudogene, HYALP1, are on chromosome 7p31.3.[53] HAases of Hyal-1, Hyal-2, Hyal-3, Hyal-4, and PH-20 are the gene products from HYAL1, HYAL2, HYAL3, HYAL4, and PH20, respectively; Hyal-1 and PH-20 have the ability to hydrolyze ChS as well as HA, and Hyal-4 is a chondroitinase first identified in vertebrate tissues without activity for HA hydrolysis. Each enzyme has a high degree of amino acid identity to that from other vertebrate, for example, mouse Hyal-3 has approximately 80% identity to that of human beings. Now, commercially available HAases are from mammalian testes and from bee venom that contain PH-20. Therefore, these enzymes are the candidate catalysts for the synthesis of GAGs by enzymatic polymerization.

**Monomer design**: PH-20 HAase hydrolyzes HA, Ch, and ChS through substrate-assisted mechanism.[54] Based on the concept of a "transition-state analogue substrate" (TSAS) monomer[10–14] proposed first in the synthesis of chitin,[21] oxazoline monomers were designed for the synthesis of HA and Ch by HAase-catalyzed polymerization (Figure 2A).

Other enzymes that hydrolyze HA and Ch are the *endo*-β-glucuronidases, which cleave the (1→3)-β-glucuronide linkages. The glycosyl fluorides of GlcNAcβ(1→4)GlcA and of GalNAcβ(1→4)GlcA are potential monomers for HA and Ch synthesis, respectively, catalyzed by the glucuronidases (Figure 2B). However, these enzymes are found only

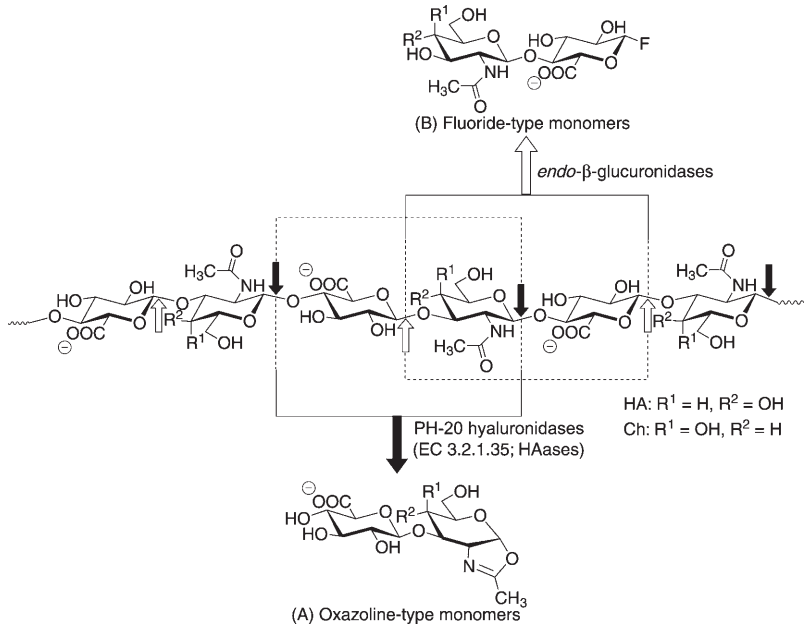
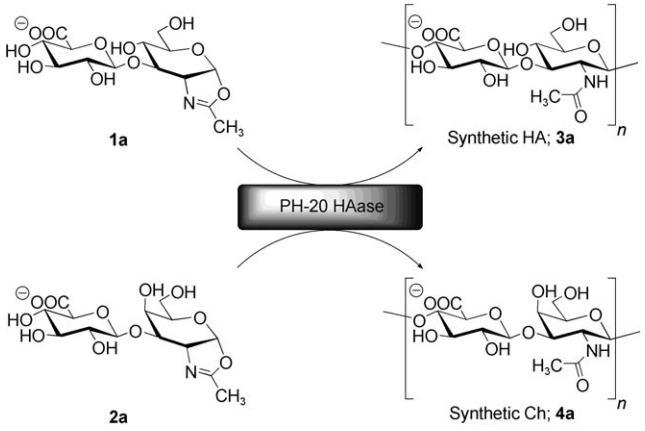



Figure 2. Monomer design for the synthesis of HA and Ch via enzymatic polymerization. Allows show possible bonds for enzymatic cleavage by HAases (black arrows) and *endo*-β-glucuronidases (white arrows).

in leeches and small marine crustaceans[55] for HA and in rabbit liver[56] for Ch. Therefore, combination of the oxazoline monomers (**1a** and **2a**) and HAase is feasible for the production of HA (**3a**) and Ch (**4a**) by enzymatic polymerization (Scheme 1).




Scheme 1. Enzymatic polymerization to synthetic HA and Ch catalyzed by PH-20 HAase.

**Mechanistic aspects**: As a typical example Figure 3 illustrates the postulated reaction mechanism catalyzed by HAase. Hydrolysis of HA occurs by the following steps as shown in Figure 3A–C. Protonation of the oxygen atom in the β(1→4) glycosidic linkage occurs after the recognition of the substrate HA by the enzyme as depicted in Figure 3A. Subsequently, the carbonyl oxygen atom of GlcNAc at the donor site attacks its own anomeric carbon atom from the α-side to assist in cleavage of the glycosidic bond, which results in a high-energy oxazolinium ion species (Figure 3B). Water molecule nucleophilically attacks the oxazolinium anomeric carbon atom to open the oxazolinium ring, resulting in the formation of the hydrolysis products of a shortened HA molecule, a GlcAβ(1→3)GlcNAc molecule and/or shortened HA chains with GlcA and GlcNAc end structures (Figure 3C).

In the polymerization, the oxazoline monomer is readily recognized by the enzyme and is activated by protonation at the donor site (Figure 3D), because the protonated monomer structure is very close to that of the oxazolinium transition state (Figure 3B). Therefore, the monomer can be regarded as a TSAS monomer in an activated form. This structure facilitates recognition and further activation by the enzyme, with lowering the activation energy for the subsequent reactions. The 4-hydroxy group of GlcA in another molecule of the monomer, or in the non-reducing end of the growing chain placed in the acceptor site, regioselectively adds to the anomeric carbon atom of the oxazolinium ion from the β-side with ring-opening to form a β(1→4) glycosidic linkage between GlcNAc and GlcA, as shown in Figure 3E. Repetition of this regio- and stereoselective glycosylation, that is, the ring-opening polyaddition reaction, is catalyzed by the enzyme, giving rise to synthetic HA; thus the monomer formula is the same as that of the product HA.

**HAase-catalyzed synthesis of hyaluronan**: A TSAS monomer (**1a**) which is a high energy form of GlcNAc was synthesized by conventional chemical methods.[24] Under the conditions with HAase, two kinds of the reactions of monomer **1a** can occur: 1) enzymatic polymerization of **1a** to **3a** and 2) hydrolysis of **1a** enzymatically and/or non-enzymatically resulting in the oxazoline ring-opened disaccharide (Scheme 2).

Figure 4 shows the reaction time-courses of **1a** with HAase from ovine testes (OTH; ▲) and without enzyme (△) at pH 7.5 and 30 °C. Monomer consumption was greatly accelerated by the enzyme addition; **1a** was completely consumed within 20 h, providing synthetic HA (**3a**). In contrast, **1a** remained in 70 % without enzyme after 20 h.

Bovine testicular HAase (BTH) also catalyzed polymerization of **1a** to **3a**. However, the reaction with bee venom HAase was not successful (Table 1), probably due to the rel-

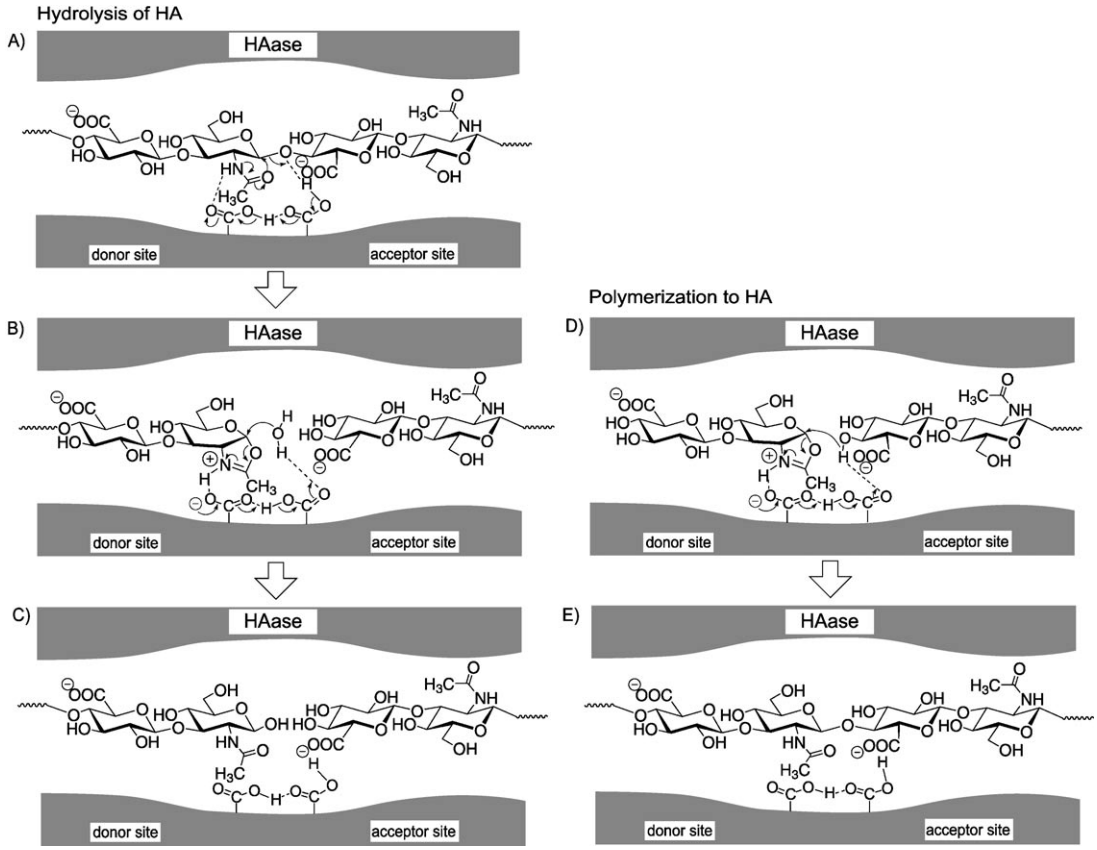
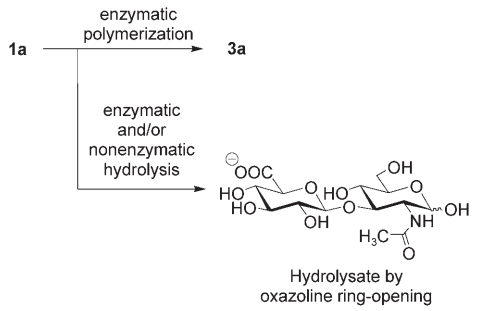



Figure 3. Postulated reaction mechanism catalyzed by HAase.




Scheme 2. Possible reactions of monomer **1a** with the enzyme.

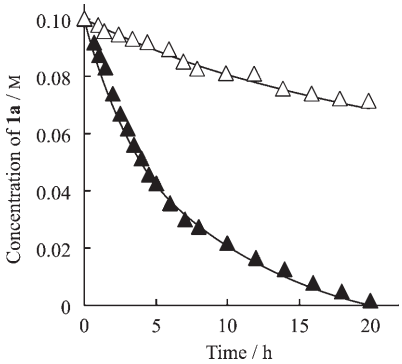



Figure 4. Reaction-time courses of **1a** with OTH (▲) and without enzyme (△). The reaction was carried out in a carbonate buffer at pH 7.5 and 30 °C.

atively lower amino acid sequence identity to mammalian PH-20 HAase. Molecular weights ($M_n$) of **3a** reached 17700, which corresponds to 88 saccharide units.

**HAase-catalyzed synthesis of chondroitin**: The oxazoline monomer of *N*-acetylchondrosine (**2a**)[25] is also a high-energy TSAS monomer, therefore, it can also be polymerized enzymatically in addition to being hydrolyzed enzymatically and non-enzymatically through enzyme catalysis in aqueous media (Scheme 3).

With the catalysis of H-OTH, monomer **2a** disappeared within 23 h at pH 7.5 and 30 °C (●) to produce synthetic Ch (**4a**), whereas it remained in 40 % without enzyme (○) after 23 h accompanying the corresponding hydrolysate (Figure 5).

Polymerization of **2a** was also catalyzed by BTH, providing polymer **4a** in a lower yield (Table 2). Similar to the polymerization of **1a**, bee venom HAase was not effective for the polymerization. Notably, H-OTH produced **4a** in a higher yield and with a higher molecular weight ($M_n$ = 4,600) within 2 h. This molecular weight value corresponds to that of natural Ch.

Table 1. Enzymatic polymerization of **1a**.

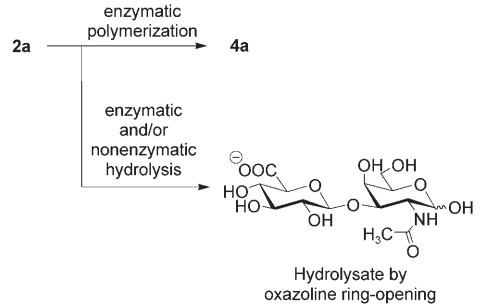
| Enzyme[b] | Polymerization[a] pH | Enzyme [wt%] | $T$ [°C] | $t$[c] [h] | Polymer (**3a**) Yield[d] [%] | $M_n$[e] | $M_w$[e] |
|---|---|---|---|---|---|---|---|
| OTH | 7.5 | 10 | 30 | 52 | 78 | 5500 | 13800 |
| | | | | | 53[f] | 13300 | 22000 |
| BTH | 7.5 | 10 | 30 | 60 | 53 | 7800 | 17600 |
| | | | | | 34[f] | 17700 | 25000 |
| bee venom | 7.5 | 10 | 30 | 72 | 0 | – | – |

[a] In a carbonate buffer (50 mM); initial concentration of **1a**, 0.1 M. [b] OTH (560 units $mg^{-1}$ from ICN Biochemicals); BTH, (500 units $mg^{-1}$ from SIGMA). [c] Indicating the time for complete consumption of **1a**. [d] Determined by HPLC containing products with molecular weight higher than tetrasaccharides unless otherwise indicated. [e] Determined by SEC calibrated with hyaluronan standards. [f] Isolated yields after purification.

Table 2. Enzymatic polymerization of **2a**.

| Enzyme[b] | Polymerization[a] pH | Enzyme [wt%] | $T$ [°C] | $t$[c] [h] | Polymer (**4a**) Yield[d] [%] | $M_n$[e] | $M_w$[e] |
|---|---|---|---|---|---|---|---|
| OTH | 7.5 | 10 | 30 | 23 | 35 | 2500 | 3200 |
| BTH | 7.5 | 10 | 30 | 40 | 10 | 2800 | 3600 |
| bee venom | 7.5 | 10 | 30 | 40 | trace | – | – |
| H-OTH | 7.5 | 10 | 30 | 23 | 50 | 2100 | 2500 |
| H-OTH | 7.5 | 10 | 30 | 2[f] | 19 | 4600 | 6800 |

[a] In a phosphate buffer: 50 mM. [b] OTH, (560 units $mg^{-1}$ from ICN Biochemicals); BTH (330 units $mg^{-1}$ from SIGMA); H-OTH (2160 units $mg^{-1}$ from ICN Biochemicals). [c] Indicating the time for disappearance of **2a** unless otherwise stated. [d] Determined by HPLC containing products with molecular weight higher than tetrasaccharides. [e] Determined by SEC calibrated with hyaluronan standards. [f] The reaction was terminated at the indicated time.




Scheme 3. Two kinds of possible reactions of **2a** during enzymatic reaction.

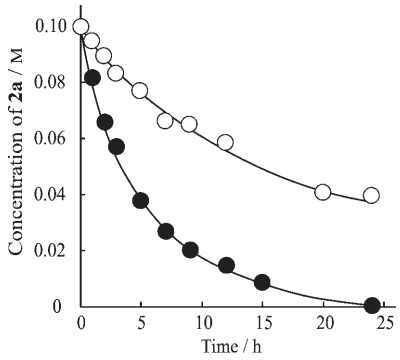
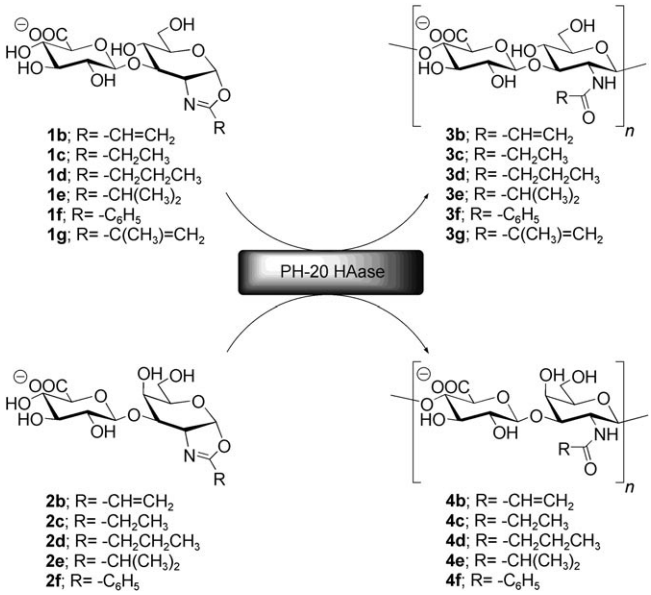



Figure 5. Time dependence of the concentration of monomer **2a** with (●) and without (○) H-OTH. The reaction was carried out in a phosphate buffer at pH 7.5 and 30 °C.




Scheme 4. Enzymatic polymerization to unnatural GAGs catalyzed by PH-20 HAase.

**Synthesis of unnatural glycosaminoglycans**: The enzyme PH-20 produced synthetic HA (**3a**) and Ch (**4a**) from **1a** and **2a**, respectively, both of which are natural-type GAGs. These results motivated us to synthesize unnatural GAGs with various *N*-acyl groups in their hexosamine units by polymerization of 2-substituted oxazoline derivatives as TSAS monomers (Scheme 4).[25,27]

Monomers **1b–g** and **2b–f** bearing various substituents at the 2-position were prepared for the production of unnatural HAs and Chs bearing the corresponding *N*-acyl groups. Polymerization of monomers **1b–d** proceeded successfully with the catalysis of HAase to the corresponding *N*-acryloyl (**3b**), *N*-propionyl (**3c**), and *N*-butylyl (**3d**) HA derivatives, respectively (see Table 3). Furthermore, monomers **2b** and **2c** were also polymerized by the enzyme catalysis, providing *N*-acryloyl (**4b**) and *N*-propionyl (**4c**) Ch derivatives, respectively. These unnatural GAGs have perfectly substituted *N*-acyl groups instead of *N*-acetyl groups found in natural GAGs. Monomers **1e**, **2d**, and **2e** were recognized and consumed by the enzyme catalysis; however, the corresponding polymers were difficult to obtain. Other oxazoline derivatives of **1f**, **1g**, and **2f** were not catalyzed by HAase, which have bulkier substituents at the 2-position. It is to be noted that *N*-acryloyl HA (**3b**) and Ch (**4b**) are functional polymers, such as macromonomers and telechelic polymers, both of which are difficult to obtain by other methods. Thus, HAase-catalyzed polymerization of 2-substituted oxazoline derivative monomers provided us not only the easy access

Table 3. Enzymatic polymerization of **1b–f** and **2b–f**.

| Polymerization[a] | | | Polymer | | | |
|---|---|---|---|---|---|---|
| Monomer | Enzyme | $t$[e] [h] | Product | Yield[g] [%] | $M_n$[h] | $M_w$[h] |
| **1b** | OTH[b] | 48 | **3b** | 50 | 5900 | 16800 |
| **1c** | OTH[b] | 48 | **3c** | 65 | 6900 | 17500 |
| **1d** | OTH[b] | 60 | **3d** | 47 | 4500 | 13500 |
| **1e** | OTH[b] | 96 | **3e** | trace | | |
| **1f** | OTH[b] | 168[f] | **3f** | 0 | | |
| **1g** | OTH[b] | 168[f] | **3g** | 0 | | |
| **2b** | H-OTH[c] | 24 | **4b** | 19 | 3400 | 4600 |
| **2c** | H-OTH[d] | 35 | **4c** | 46 | 2700 | 3600 |
| **2d** | H-OTH[d] | 122 | **4d** | trace | | |
| **2e** | H-OTH[d] | 168 | **4e** | trace | | |
| **2f** | H-OTH[c] | 239[f] | **4f** | 0 | | |

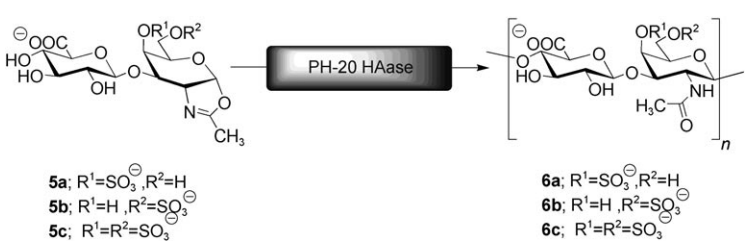
[a] In a phosphate buffer at pH 7.5, 50 mM (for monomers **1b–g**) or in a carbonate buffer at pH 7.5, 50 mM (for monomers **2b–f**); monomer concentration: 0.1 M, amount of enzyme, 10 wt% for monomer; reaction at 30°C. [b] OTH (560 unit mg$^{-1}$ from ICN Biochemicals). [c] H-OTH (1870 unit mg$^{-1}$ from SIGMA). [d] H-OTH (2502 unit mg$^{-1}$ from ICN Biochemicals). [e] Indicating the time for complete consumption of monomer unless otherwise stated. [f] Reaction was terminated at the indicated time. [g] Determined by HPLC containing products with molecular weight higher than tetrasaccharides. [h] Determined by SEC calibrated with hyaluronan standards.

to unnatural GAGs with various *N*-acyl groups, but also new insights for substrate recognition by HAase.

**Synthesis of chondroitin sulfate with uniform structure**: Ch is normally sulfated in vivo after and/or during the chain elongation by the action of particular sulfotransferases. This modification process modifies Ch to ChS to give a number of biological functions. During this process, Ch produces many ChS variants with substantial structural diversity and this leads to difficulty in studying the various functions of ChS at a molecular level. We synthesized a ChS polymer with a uniform structure by means of HAase-catalyzed polymerization of a TSAS monomer.

Newly synthesized TSAS monomers of chondrosine bearing sulfate groups at the C4 (**5a**), C6 (**5b**), and both C4 and C6 (**5c**) atoms were subjected to enzymatic polymerization catalyzed by PH-20 HAase (Scheme 5).[26]

Monomer **5a** disappeared with the addition of H-OTH within 1 h, whereas it remained in 97% without enzyme (Figure 6A). Consumption of monomer **5b** was accelerated with the enzyme catalysis (Figure 6B). It was completely consumed within 18 h; **5b** was gradually decomposed without enzyme and remained in 37% after 18 h. Monomer **5c** was slightly recognized and consumed by the enzyme; it disappeared within 35 h (Figure 6C). Without enzyme, it remained in 15% after 35 h.




Scheme 5. Synthesis of ChS with well-defined structure by PH-20 HAase-catalyzed polymerization.

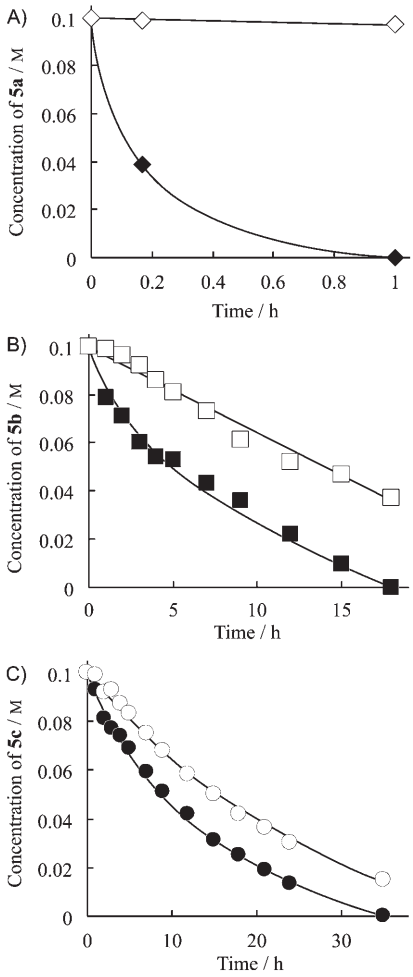



Figure 6. Reaction-time courses of monomers (A) **5a**, (B) **5b** and (C) **5c** with H-OTH (black symbols) and without enzyme (white symbols). The reactions were performed in a phosphate buffer at pH 7.5 and 30°C.

Table 4 indicates the polymerization results of monomers **5a–c** with HAase. H-OTH-catalyzed polymerization of **5a**

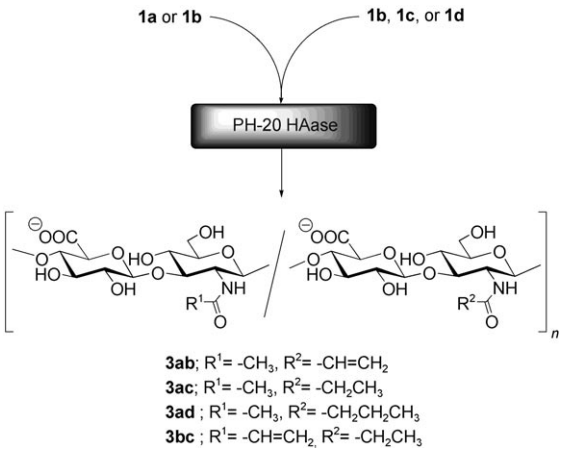
Table 4. Enzymatic polymerization of monomers **5a–c** with HAase.

| Polymerization[a] | | | Polymer | | | |
|---|---|---|---|---|---|---|
| Monomer | Enzyme[b] | $t$[c] [h] | Product | Yield[d] [%] | $M_n$[e] | $M_w$[e] |
| **5a** | H-OTH | 1.5 | **6a** | 75 | 11700 | 21000 |
| **5a** | H-BTH | 48 | **6a** | 20 | 8500 | 11600 |
| **5a** | bee venom | 48 | **6a** | 0 | | |
| **5b** | H-OTH | 18 | **6b** | 0 | | |
| **5c** | H-OTH | 35 | **6c** | 0 | | |

[a] In a phosphate buffer (50 mM, pH 7.5) at 30°C. Initial concentration of each monomer was 0.10 M. Amount of enzyme was 10 wt% for each monomer. [b] H-OTH (3720 units mg$^{-1}$ from SIGMA); H-BTH (1010 units mg$^{-1}$ from SIGMA). [c] Indicating the time for complete consumption of monomer. [d] Determined by HPLC containing products with molecular weight more than tetrasaccharide. [e] Determined by SEC calibrated with hyaluronan standards.

produced the corresponding Ch4S (**6a**), bearing sulfate groups exclusively at the C4 atom of GalNAc units, with $M_n = 11\,700$ in good yields. H-BTH also polymerized **5a**, giving rise to **6a** in lower yields of 20%. However, bee venom did not exhibit the polymerization activity for **5a**, yet it did afford the corresponding hydrolyzed disaccharide through oxazoline ring-opening. It should be noted that monomers **5b** and **5c** were not polymerized by the catalysis of H-OTH at all, providing only the corresponding hydrolysates with an oxazoline ring-opened structure. These results suggest that H-OTH recognizes and catalyzes reactions of monomers **5a–c** only at the donor site, but it polymerizes **5a**, which is able to locate also at the acceptor site.

**Copolymerization with the sugar oxazoline monomers**: The use of PH-20 HAase enabled the production of various GAGs with well-defined structure through the homopolymerization of sugar oxazoline monomers. These results imply the possibility of cross reactions, that is, copolymerization of the monomers. First, we performed copolymerization of monomers **1a–d** to the corresponding HA derivatives (Scheme 6).[57]




Scheme 6. Enzymatic copolymerizations to HA derivatives with using monomers **1a–d** by PH-20 HAase.

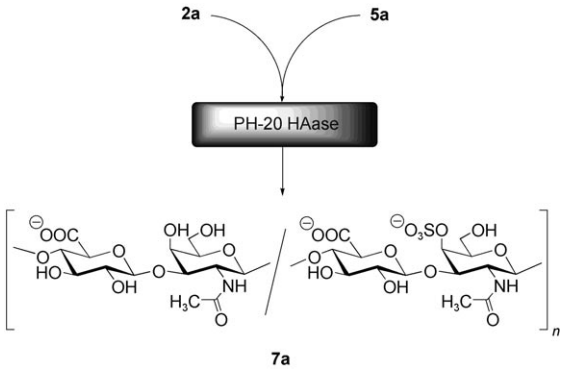
Table 5 shows the copolymerization results of **1a**/**1b**, **1a**/**1c**, **1a**/**1d**, and **1b**/**1c** with equimolar amounts of monomers. All reactions proceeded successfully, providing the corresponding copolymers **3ab**, **3ac**, **3ad**, and **3bc** with relatively high $M_n$ values in good yields. Composition of the copolymer was close to the comonomer feed ratio, except for copolymer **3ad**, in which **1a** has a higher polymerizability than **1d**.

Further, copolymerization of monomers **2a** and **5a** was investigated by varying the comonomer feed ratio (Scheme 7).[58,59] All of the reactions progressed rapidly, giving rise to copolymer **7a** within a short reaction time (2.0–2.5 h) in good yields. Comonomers **2a** and **5a** were incorporated in **7a** in a ratio close to that of the comonomer feed. For example, with an equimolar feed ratio a copolymer

Table 5. Enzymatic copolymerization of **1a**/**1b**, **1a**/**1c**, **1a**/**1d**, and **1b**/**1c** with HAase.

| Copolymerization[a] | | | | Copolymer | | | | |
|---|---|---|---|---|---|---|---|---|
| Comonomer[b] | | $t$ | Product | Composition | | Yield[e] | $M_n$[f] | $M_w$[f] |
| **I** | **II** | [h][c] | | **I** | **II**[d] | [%] | | |
| **1a** | **1b** | 27 | **3ab** | 0.52 | 0.48 | 43 | 6700 | 12100 |
| **1a** | **1c** | 48 | **3ac** | 0.50 | 0.50 | 49 | 10200 | 18700 |
| **1a** | **1d** | 60 | **3ad** | 0.78 | 0.22 | 32 | 8100 | 17400 |
| **1b** | **1c** | 48 | **3bc** | 0.51 | 0.49 | 40 | 9200 | 17100 |

[a] In a carbonate buffer at pH 7.5, 50 mM in $H_2O$; total monomer concentration, 0.10 M; enzyme, OTH (560 unit mg$^{-1}$), 10 wt% for the total amount of comonomers; reaction at 30 °C. [b] Each comonomer was mixed as equimolar amount for the other comonomer. [c] Indicating the time for complete consumption of both monomers. [d] Determined by $^1$H NMR measurements. [e] Isolated yields after purification ((weight of the isolated copolymer/weight of the feed comonomers) × 100). [f] Determined by size-exclusion chromatography (SEC) calibrated with hyaluronan standards.

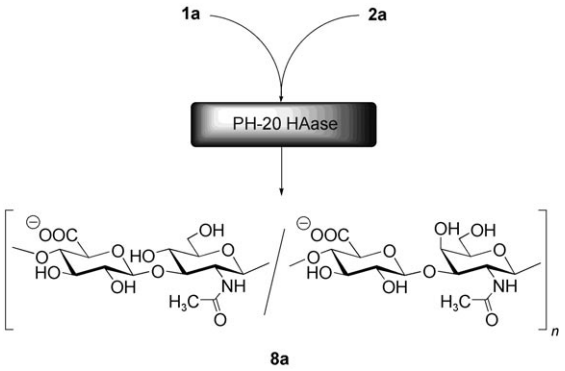



Scheme 7. Synthesis of ChS with controlled degree of sulfation via enzymatic copolymerization of **2a** and **5a** catalyzed by PH-20 HAase.

with a composition of 0.56/0.44 and a molecular weight ($M_n$) of 5200 was produced. These results indicate that synthetic ChS with a controlled degree of sulfation can be prepared through copolymerization by varying the comonomer feed ratio.

Finally, it is surprising that monomers **1a** and **2a**, which have completely different structures, were copolymerized by HAase catalysis to give copolymer **8a**, which is an HA–Ch hybrid polysaccharide (Scheme 8).[60,61] Naturally occurring HA and Ch usually coexist in the ECMs and cooperatively work there. Therefore, the hybrid is a novel potential biomaterial with character of both HA and Ch. Copolymer **8a** with different compositions was successfully produced in good yield through the copolymerization of **1a** and **2a** with varied feed ratios. Both monomers showed a similar copolymerizability. For example, an equimolar mixture of **1a** and **2a** was copolymerized after 48 h to give **8a** with a composition of 0.51/0.49, which has a satisfactory high $M_n$ value of 6000 ($M_w = 16\,000$). The comonomer composition in **8a** was controllable.




Scheme 8. Synthesis of an HA-Ch hybrid through PH-20 HAase-catalyzed copolymerization of **1a** and **2a**.

## Conclusion

In this paper we report the multiple production of natural and unnatural GAGs by the catalysis of a single hydrolase enzyme, a hyaluronidase supercatalyst. The enzyme recognized various kinds of sugar oxazoline monomers prepared on the basis of our concept of TSAS and catalyzed their polymerization under total control of regioselectivity and stereochemistry. Copolymerizations between substrate monomers with different structure occurred smoothly by the enzyme catalysis, leading to new GAGs. These results have led us to further explore the design and synthesize novel polysaccharides through a single-step reaction; such polysaccharides have been very difficult to obtain by means of conventional synthetic methods.

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Published online: June 29, 2006

  