Enzymatic Polymerization to Artificial Hyaluronan: A Novel Method to Synthesize a Glycosaminoglycan Using a Transition State Analogue Monomer

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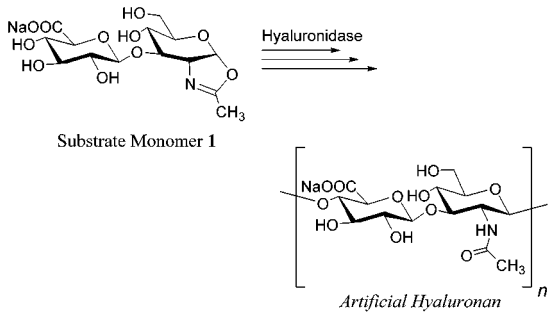
Received September 18, 2001

Hyaluronan (hyaluronic acid, HA) is one of natural polysaccharides classified into glycosaminoglycans (GAGs) that is widely found in living cells and extracellular matrix (ECM), in particular, smooth muscle cells,[1] fibroblasts,[1] vitreous of the human eye,[2] synovial joint fluid,[2] and rooster comb.[2] As a member of ECM, HA plays important functions in vivo, for example, tissue proliferation, regeneration, wound healing, etc.[3] HA is a typical heteropolysaccharide consisting of two kinds of sugar units, glucuronic acid (GlcA) and 2-acetamido-2-deoxy-D-glucose (GlcNAc), and has a structure of a GlcAβ(1→3)GlcNAc disaccharide repeating unit connecting through a β(1→4) glycosidic linkage. This biologically important HA is industrially prepared by extraction from a rooster comb or bacterial culture fluid of *Streptococcus*.[4] Other syntheses of HA reported are the in vitro method with HA synthase via biosynthetic pathways[5] and a transglycosylation catalyzed by hyaluronidase.[6]

Enzymatic polymerization with a glycosyl hydrolase as catalyst has been demonstrated as 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 β(1→4) glycosidic structure.[11] Hyaluronidases,[12] endo-type hydrolysis enzymes of HA (EC 3.2.1.35), hydrolyze HA at the β(1→4) glycosidic linkages between GlcNAc and GlcA residues randomly[13] and thus the hydrolysis product of the GlcAβ-(1→3)GlcNAc disaccharide unit from HA is released. These observations allowed us to employ an oxazoline derivative of an activated GlcNAc form as a donor side. Here, we designed and synthesized a novel GlcAβ(1→3)GlcNAc disaccharide oxazoline derivative on the hypothesis that the oxazoline derivative serves as a transition state analogue substrate monomer for hyaluronidase catalysis, giving rise to artificial HA. This is the first successful synthesis of HA via nonbiosynthetic pathways (Scheme 1).

Scheme 1



The novel GlcAβ(1→3)GlcNAc oxazoline monomer (**1**) was chemically prepared from methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate as donor and benzyl 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-β-D-glucopyranoside as acceptor via five-step reactions. The catalytic behavior of **1** was investigated by employing bovine testicular hyaluronidase (BTH) or ovine testicular hyaluronidase (OTH) (Figure 1). The reaction (0.1 M of **1** in D_2O, carbonate buffer, pH 7.1, BTH or OTH 10 wt % for **1**) was carried out in an NMR probe tube at 30 °C. The reaction proceeded homogeneously. The consumption of **1** was followed by ^{1}H NMR spectroscopy. It became significantly faster by addition of the enzymes at 3.5 h compared with the reaction without enzyme (control).

After the reaction, the enzyme was 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 39% yield with BTH after 64 h and in 52% yield with OTH after 36 h, respectively.[14] It should be noted that the product polysaccharide was not further subjected to hydrolysis under the reaction conditions of pH 7.1 or higher. This irreversible behavior of hyaluronidases catalysis is probably due to the optimal pH of the enzyme hydrolysis being reported as pH 4.0−6.0.[12] ^{1}H and ^{13}C NMR spectra of the polysaccharide obtained were similar to those of natural HA,[15] strongly supporting that the product is "artificial HA" (Figure 2). The molecular weight was measured by size-exclusion chromatography (SEC) by using HA samples having different molecular weight for calibration standards; molecular weight values (M_n) of two artificial HAs were determined as 1.74×10^4 and 1.35×10^4, respectively.[16] Circular dichroism (CD) behavior further supported the structure of artificial HA.[17]

The followings are to be pointed out for the present reaction. 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,B).

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(4) In vivo, HA is synthesized with catalysis of HA synthase contained in cell surface membranes as a transmembrane protein, via alternating addition of GlcA and GlcNAc to the growing chain using their activated nucleotide sugars as substrates, UDP-GlcA and UDP-GlcNAc, respectively.
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(14) Without enzyme, no precipitate was obtained; 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 artificial HA but in little smaller yields.
(15) Specific signals (Figure 2A) from two kinds of anomeric protons were observed at 4.55 and 4.47 ppm with the coupling constants of 7.48 and 6.96 Hz, respectively. The former is derived from GlcNAc residue, and the latter from GlcA residue, showing the formation of β-glycosidic linkage. In the ^{13}C NMR spectrum (Figure 2B), 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, which were confirmed by ^{13}C NMR of natural HA.
(16) M_w values were 6.69×10^4 and 4.12×10^4, respectively.
(17) The CD of artificial and natural HAs were observed around 208 nm derived from amide bonds, and the intensity increased in proportion to the molecular weight of HA.

10.1021/ja017104+ CCC: $20.00
Published on Web 11/06/2001

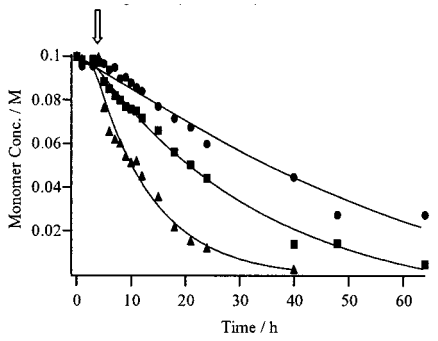

Figure 1. The reaction time-courses with no enzyme (●), BTH (■), and OTH (▲). The arrow shows the addition of the enzyme (after 3.5 h).

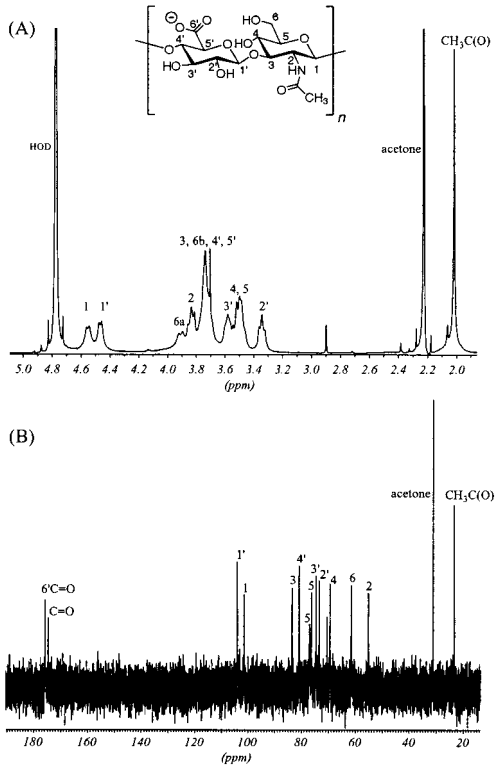

Figure 2. (A) ^{1}H and (B) ^{13}C NMR spectra of artificial HA.

The oxazolinium intermediate in part B structurally corresponds to the protonated species of monomer **1** in the active site as shown in part C. Thus, **1** can be regarded 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. The 4-hydroxyl group in GlcA of another molecule of **1** or of the growing chain end located at the acceptor site nucleophilically attacks the oxazolinium of **1** from the β-side to form the $\beta(1\rightarrow4)$ glycosidic linkage between GlcNAc and GlcA as given in Figure 3C. 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, whereas it catalyzed the $\beta(1\rightarrow4)$ bond formation to lead to HA in vitro.[11d]

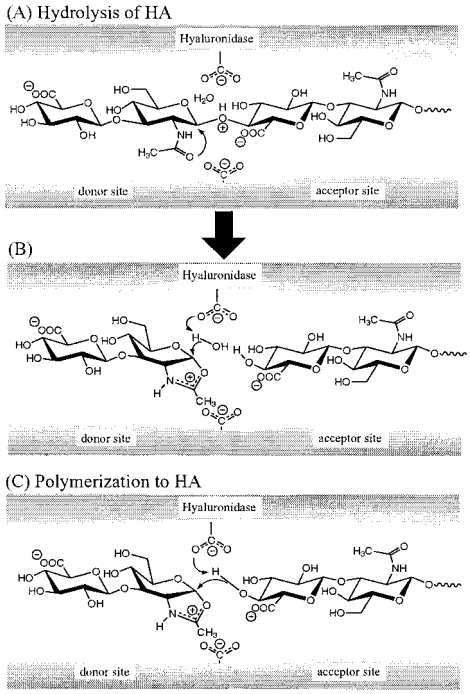

Figure 3. A postulated transition state of the hyaluronidases for the hydrolysis of natural HA (B) and for the polymerization of the monomer **1** to HA (C).

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 the preparation of such HA samples, which are expected to be a useful class of tools for exploring in vivo functions of HA.[18] 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 adition, this reaction strongly suggests the mechanism of in vivo HA synthesis where the hydroxyl group at the growing chain end of the nonreduced terminal attacks the anomeric carbon of UDP-GlcA or UDP-GlcNAc.

Acknowledgment. This work was partially supported by the Mitsubishi Foundation. Hyaluronan samples for calibration standards were gratefully supplied by Denki Kagaku Kogyo Kabushiki Kaisha, Co., Ltd.

Supporting Information Available: Scheme for the synthesis of monomer **1**; text describing synthesis procedures, characterization data of product compounds, and conditions of polymerization reactions; a table of ^{13}C NMR chemical shift values of artificial and natural HA; and a figure giving CD for artificial and natural HA (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA017104+

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