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Single-Molecular Enzymatic Elongation of Hyaluronan Polymers Visualized by High-Speed Atomic Force Microscopy

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Supporting Information

ABSTRACT: Using high-speed scanning atomic force microscopy, we directly observed single-molecular enzymatic elongation of hyaluronan polymer chains at intervals of 10 s on a mica or lipid bilayer surface, on which *Pasteurella multocida* hyaluronic acid synthase (pmHAS) was immobilized. The reaction was started by the addition of both UDP-glucuronic acid and UDP-*N*-acetylglucosamine monomers. The average catalytic elongation rate constant (k_{cat}) was found to be 1.8 mer s^{-1} from one active enzyme physically adsorbed on a mica surface. When pmHAS was immobilized by inserting its hydrophobic tail part into lipid bilayers, most of the enzymes retained their activity, and the k_{cat} values were found to be in the range 1−10 mer s^{-1} for 29 enzymes (average was k_{cat} = 2−4 mer s^{-1}). These k_{cat} values were lowest level of k_{cat} = 1−100 s^{-1} obtained in bulk solution by radioisotope methods.

Carbohydrate-active enzymes that degrade or create glycosidic bonds are being widely studied from the postgenomic point of view.[1,2] Their structures, functions, and families are gradually being stored in databases (http://www.cazy.org/). The kinetic parameters of these enzymes have usually been studied using the Michaelis−Menten equation, employing scintillation counting techniques,[3,4] colorimetric methods,[5,6] and stopped-flow fluorescence.[7] We have used a quartz crystal microbalance (QCM) technique to follow the enzyme−substrate complex intermediate of the enzyme reaction as the mass changes and obtained kinetic parameters for both enzyme-binding and catalytic processes for glycosidase[8] and glycosyltransferase.[9] Glycosaminoglycans play an important role as extracellular matrices in living systems, and there are many unsolved questions concerning their production processes and the mechanisms of the related emzymes.[10,11] We elucidated the mechanisms of glycosyltransferases by determining the kinetic parameters for enzymatic elongation of a chondroitin polymer, using a flow-type high-sensitivity QCM.[12] As another example, hyaluronan, is an important glycosaminoglycan involved as a biological lubricant and in phylaxis of bacteria. However, kinetic studies of hyaluronan synthases are still vague, and k_{cat} values varying from 1 to 100 s^{-1} have been reported.[13−15]

In this communication, we visualized the elongation of hyaluronan polymers catalyzed by *Pasteurella multocida* hyaluronic acid synthase (pmHAS) at the single-molecular level, using high-speed scanning atomic force microscopy (AFM), which was recently developed for the observation of biomolecules by Hansma et al.[16] and Ando et al.[17] pmHAS enzymes (108 kDa, E.C. 2.4.1.212) are known to elongate hyaluronan by alternating polymerization of UDP-glucuronic acid (UDP-GlcA) and UDP-*N*-acetylglucosamine (UDP-GlcNAc) as monomers from the primer hyaluronan oligomer (see Figure 1).[13−15] pmHAS is known to have a hydrophobic tail and to exist on the membrane surface by inserting its hydrophobic tail into lipid bilayers.[18] AFM provides nanometer-scale visualization of biomacromolecules in aqueous solution.[19] We used single-molecule force spectroscopy to obtain the kinetic parameters for enzymatic dextran elongation.[20] Recently, Ando et al. observed rotary behavior of the

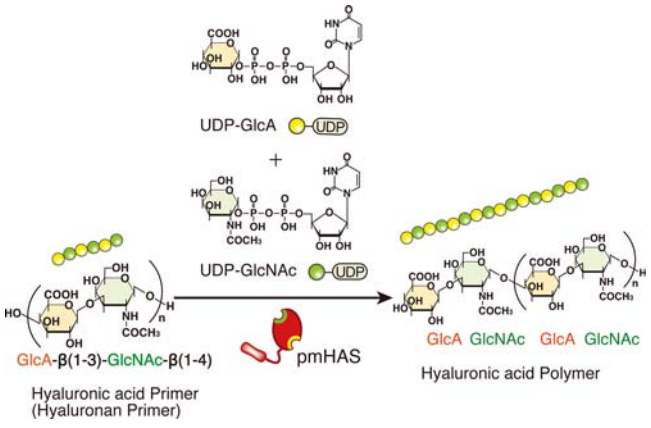

Figure 1. Schematic illustration of elongation of hyaluronan from the primer hyaluronan with UDP-GlcA and UDP-GlcNAc monomers, catalyzed by pmHAS.

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motor protein F1-ATPase using high-speed scanning AFM.[21] In enzymatic reactions, movement of a single cellulase on a crystalline cellulose surface with enzymatic degradation has also been observed.[22] However, the single-molecular level binding of enzymes and the following catalytic reactions have never been imaged. We expect that the high-speed scanning AFM is suitable for the single-molecular detection of the enzymatic elongation of hyaluronan, although the time resolution may be lower than the fluorescent single-molecular enzyme analyses.[23] We succeed in visualizing and kinetically analyzing the enzymatic elongation of a single hyaluronan chain from pmHAS on mica and lipid bilayer surfaces.

First, we physically immobilized pmHAS on a mica surface. An AFM image of pmHAS was obtained using a high-speed scanning AFM system (Nano Live Vision, RIBM, Tsukuba, Japan) equipped with a silicon nitride cantilever (spring constant = 0.1−0.3 N m^{-1}, resonant frequency = 1−2 MHz, tip radius < 15 nm, Olympus BL-AC1-EGS-A2). Two μL of the enzyme solution (30 μg mL^{-1}) were put on a freshly cleaved mica surface. Scanning was consecutively carried out in the same buffer solution, using tapping mode, in one sequential experiment. We could observe ~20 spots of dispersed pmHAS particles, with average height of 4.0 ± 0.5 nm, on the mica surface (see Figure S1A). On addition of 10 μL of the polymer solution of long hyaluronan (average M_w: 200 kDa, Dp = ~1000, 5 μg mL^{-1}) to pmHAS physically adsorbed on the mica surface in the presence of 4 mM $CuCl_2$, hyaluronan polymers were observed individually as single chains of height and length 1 and 400−600 nm, respectively (see Figure S1B). $CuCl_2$ was added as a divalent cation source to immobilize the anionic hyaluronan polymers on the anionic mica surface by acting as chelators. It has been reported that hyaluronan interacted strongly with Cu^{2+}, and the resulting complex was stable.[24,25] We could not observe linear hyaluronan polymers on a mica surface with other divalent cations, such as Mn^{2+}, Mg^{2+}, and Zn^{2+}, or without any divalent cations.

To start enzymatic polymerization on pmHAS physically adsorbed on the mica surface, the short six-mer hyaluronan oligomer was first added as a primer (Figure 2A). In the absence of the primer, it was difficult to observe constantly the elongated hyaluronan chains. It has been reported that the elongation rate of hyaluronan is increased in the presence of the primer in bulk solution, which may supply the starting point of polymerization.[15] We could not observe images of the binding of the six-mer hyaluronan primer to pmHAS because of the short primer chains (~3 nm in length). We then added 10 μL of both UDP-GlcA (10 mM) and UDP-GlcNAc (10 mM) as monomers to the primer-bound pmHAS on the mica surface. We searched the entire area of the mica surface (1.5 mm in diameter) and fortunately could find one hyaluronan polymer chain elongated from pmHAS on the mica surface. We confirmed that the presence of 5−10 mM of Cu^{2+} did not affect and inhibit the elongation reaction from the hyaluronan primer catalyzed by pmHAS in bulk solution. Figure 2B shows the real-time images of the elongation of a single chain (1 nm in height) of hyaluronan polymer from pmHAS (4−5 nm in height) on the mica surface every 10 s after 350 s from injection of the monomers. We could clearly observe that the elongation started from ~80 nm at 350 s to ~160 nm at 420 s after injection of the monomers. The observed length of hyaluronan (80−160 nm) was longer than the persistent length of hyaluronan in bulk solution (~7−10 nm).[26] This is due to the strong interaction between anionic hyaluronan polymers

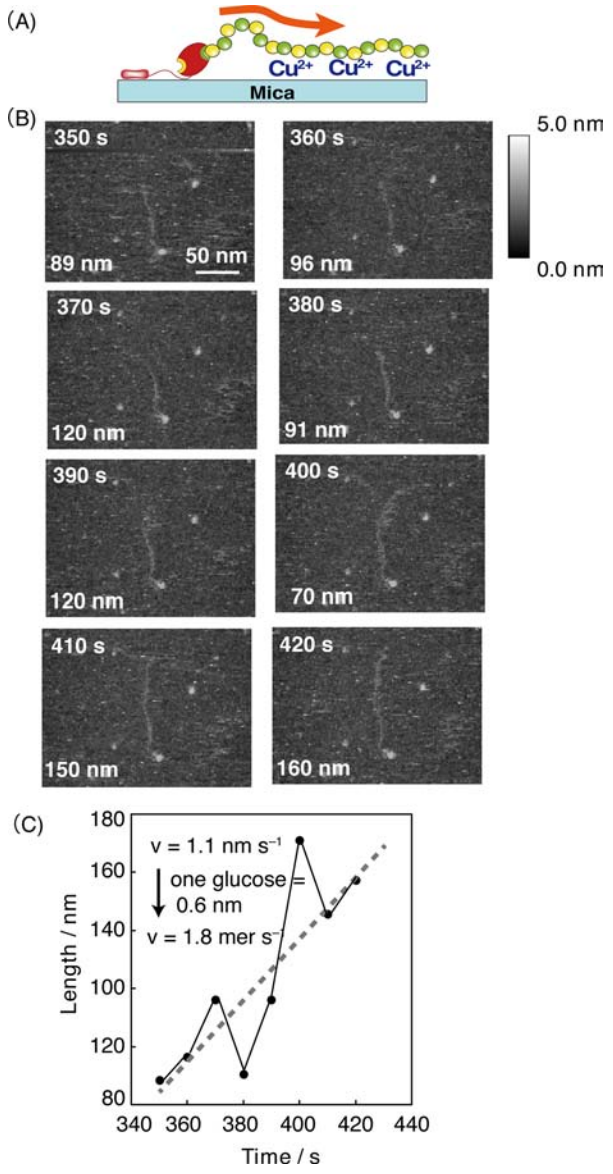

Figure 2. (A) Schematic illustration of elongation of a hyaluronan polymer chain from pmHAS physically adsorbed on a mica surface; the produced anionic hyaluronan polymer is fixed to the anionic mica surface by chelation with divalent Cu^{2+}ions. (B) Real-time observations of one hyaluronan polymer (1 nm in height) from pmHAS (4−5 nm in height) on the mica surface every 10 s after 350 s from the injection of both UDP-GlcA and UDP-GlcNAc monomers. Observation conditions: 20 mM Tris-HCl, pH 7.4, 4 mM $CuCl_2$, 30 μM hyaluronan six-mer, 10 μM UDP-GlcA, 10 μM UDP-GlcNAc, 25 °C, 0.1 flame s^{-1}. (C) Time course of the elongation length of one hyaluronan polymer catalyzed by pmHAS on the mica surface. Average elongation rate (v) was obtained from the apparent slope of a line chart.

and the cationic surface. The reason why the reaction could be observed a few minutes after monomer injection may be the weak interaction of the short polymer chain with the mica surface in the initial stage. The average elongation rate obtained at the initial stage (before the intercept: 80 nm/350 s = 0.2 nm s^{-1}; v = 0.4 mer s^{-1}) was lower than that of the latter stage (after the intercept: v = 1.8 mer s^{-1}) due to the difficulty to obtain constantly the clear images. Actually, we could not observe constantly clear images when the short hyaluronan (40 mer, the calculated length: 15−20 nm) was added on the mica

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surface in the presence of Cu^{2+}. Therefore, we ignored the initial stage of the reaction. In negative control experiments, elongations of hyaluronan were not observed in the absence of the primer and one of two monomers.

Figure 2C shows the time course of the elongation length of the hyaluronan polymer catalyzed by pmHAS. We connect each plot as a line chart, because each molecule will have a different reactivity at each time in the single-molecular reaction. However, we tried to obtain the average elongation rate from a linear slope relation to be 1.1 nm s^{-1}, and this is converted to be 1.8 mer s^{-1} from the length of one glucose unit = 0.6 nm. The k_{cat} value of 1.8 s^{-1} is close to the lowest value of k_{cat} = 1−100 s^{-1} obtained in bulk solution.[13,14] This is explained by the low diffusion of substrates on the surface, the low mobility of the immobilized enzyme, or the low fluidity of the resulting polymers on the mica surface.

When pmHAS was physically adsorbed on the mica surface, the active enzyme was rarely observed, although ∼20 particles of enzymes were observed in an area of diameter 1.5 mm on the mica surface. Next, we tried to immobilize pmHAS in a natural state on membranes by inserting its hydrophobic tail part in lipid bilayers.[18]

Mixed proteoliposomes of diacylphosphatidylcholine from egg yolk (egg-PC) and diacylphosphatidylseline from bovine brain (brain-PS) containing pmHAS were prepared as described in the Supporting Information (SI). The anionic charged proteoliposomes containing egg-PC:brain-PS (1:2) and pmHAS were spread on the anionic mica surface in the presence of 5 mM $MgCl_2$ as chelating divalent cations with the anionic mica surface (Figure 3A). We could observe fairly dispersed particles of height 2 nm on the flat surface (see Figure S2A). By scratching the cantilever to surface in contact mode in AFM, the mica surface was confirmed to be covered by 4 nm-thick lipid bilayers. The anionic proteoliposome was therefore spread as a bilayer on the anionic mica surface as a result of chelation of divalent Mn^{2+} cations, as illustrated in Figures 3A and S2A, on which pmHAS may be immobilized by inserting the hydrophobic tail part (C-terminus) into the lipid bilayers.[18]

To start the elongation reaction, the short six-mer hyaluronan was first injected as a primer for the pmHAS immobilized on the lipid bilayers. Then, 10 μL of both UDP-GlcA (10 mM) and UDP-GlcNAc (10 mM) monomers were added to the primer-bound pmHAS on the lipid bilayers. We also added 4 mM $CuCl_2$ as chelators between the anionic hyaluronan polymers produced and the anionic lipid bilayers (egg-PC:brain-PC = 1:2). We found many enzyme particles (4−5 nm in height), from which hyaluronan polymers elongated as linear polymer chains (1 nm in height), in all areas of the lipid-covered mica surface (1.5 mm in diameter) (Figure 3B). A real-time movie is included as SI.

We also tried to use other positively charged lipid surface, such as synthetic cationic lipids ((+)-*N*,*N*[bis(2-hydroxyethyl)-*N*-methyl-*N*-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide or 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide], instead of the combination of Cu^{2+} chelators and the anionic brain-PS lipid surface. However, it was difficult to obtain clear images of hyaluronan polymers on the synthetic cationic lipid surfaces.

Figure 3B shows time course observations of elongations of hyaluronan polymers from four different pmHAS (enzymes 1−4) as typical examples on lipid bilayers. Figure 3C shows plots of the elongation lengths of four different hyaluronan polymers

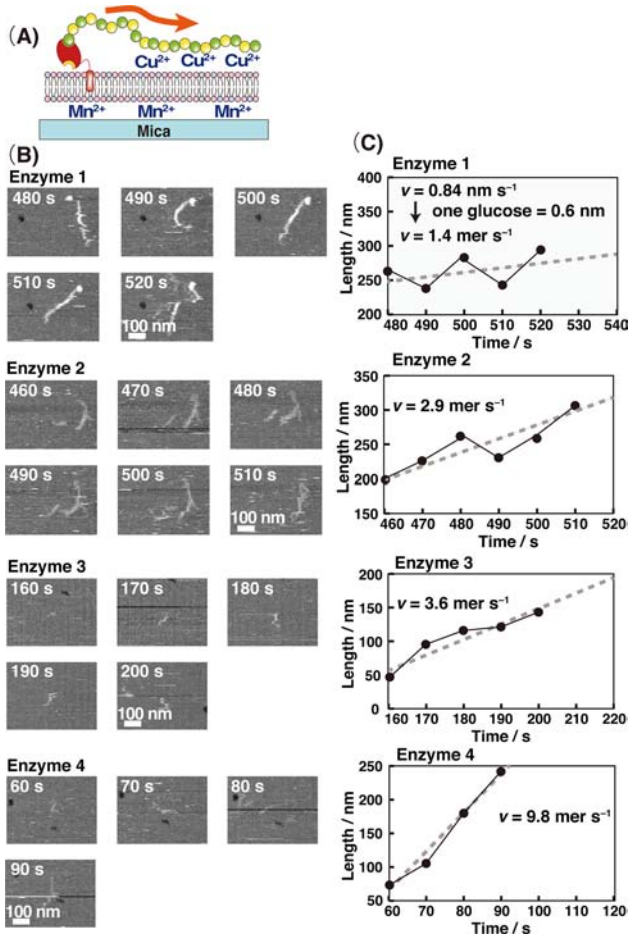

Figure 3. (A) Schematic illustration of elongation of a hyaluronan polymer chain from pmHAS, in which the enzyme is immobilized on an anionic lipid bilayer (egg-PC:brain-PC = 1:2) spread on a mica surface; the produced anionic hyaluronan polymer is fixed to the anionic lipid surface by chelation with divalent Cu^{2+}cations. (B) Typical examples of real-time observations of four different hyaluronan polymers (1 nm in height) from four different pmHAS (enzymes 1−4, 4−5 nm in height) on the lipid surface every 10 s, after 60−480 s from the injection of both UDP-GlcA and UDP-GlcNAc monomers. Observation conditions are the same as in Figure 2. (C) Time courses of elongation lengths of hyaluronan polymers catalyzed by four different pmHAS (enzymes 1−4) on the lipid surface. Average elongation rate (v) was obtained from the apparent slope of each line chart.

from each enzyme against time. The average elongation rates for each enzyme were obtained to be 1.4, 2.9, 3.6, and 9.8 mer s^{-1} from the average linear slope of each line chart. The starting length of the polymer and the starting time were different for each enzyme: 50−260 nm hyaluronan polymers and starting times of 60−480 s. It was also difficult to observe the clear images and to calculate the elongation rate in the initial stage of polymerization due to the week interaction between the short hyaluronan chain and the lipid surface. The length of initial stage also depends on each enzyme and each polymer chain.

We counted 29 enzymes (including enzymes 1−4 in Figure 3) from which hyaluronan polymers were elongated as a single chain, and the obtained elongation rates are summarized in Figure 4. The elongation rates were widely dispersed in the range 1−10 mer s^{-1}, and the maximum counts were observed at around 2−4 mer s^{-1} as the average rate. The range v = 1−10 mer s^{-1} is reasonable because the reaction conditions for each

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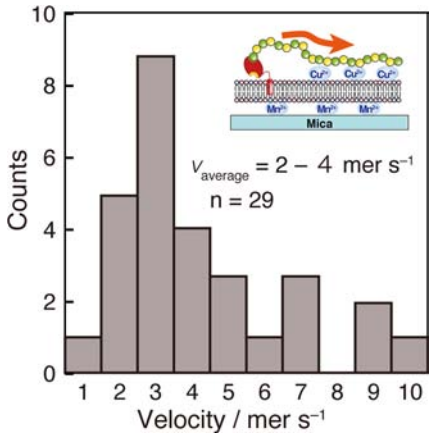

Figure 4. Diversity of elongation rates of hyaluronan polymers catalyzed by 29 different pmHAS immobilized on lipid bilayers.

enzyme will be different on lipid bilayers and the interactions of the produced polymer chains with the lipid surface will be different for each hyaluronan polymer. The average rate of v = 2−4 mer s^{-1} obtained from pmHAS on the lipid bilayers was consistent with that of v = 1.8 mer s^{-1} obtained from pmHAS on the mica surface and was close to the lowest level of the value of k_{cat} = 1−100 s^{-1} obtained in bulk solution by the radioisotope method.[13,14]

In conclusion, we could observe continuous single-molecular elongations catalyzed by a single pmHAS enzyme immobilized on lipid bilayers on a mica surface, using high-speed scanning AFM. This technique can be applied to other enzyme reactions, if the enzymes and the product can be immobilized on the mica surface under suitable conditions.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and an elongation of hyaluronan polymers from pmHAS immobilized on lipid bilayers movie. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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