Rapid Chemoenzymatic Synthesis of Monodisperse Hyaluronan Oligosaccharides with Immobilized Enzyme Reactors*

Paul L. DeAngelis‡, Leonard C. Oatman, and Daniel F. Gay

From the Department of Biochemistry and Molecular Biology, Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

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We describe the chemoenzymatic synthesis of a variety of monodisperse hyaluronan (β4-glucuronic acid-β3-N-acetylglucosamine (HA)) oligosaccharides. Potential medical applications for HA oligosaccharides (10–20 sugars in length) include killing cancerous tumors and enhancing wound vascularization. Previously, the lack of defined oligosaccharides has limited the exploration of these sugars as components of new therapeutics. The Pasteurella multocida (HA synthase, pmHAS, a polymerizing enzyme that normally elongates HA chains rapidly (1–100 sugars/s), was converted by mutagenesis into two single-action glycosyltransferases (glucuronic acid transferase and N-acetylgalactosamine transferase). The two resulting enzymes were purified and immobilized individually onto solid supports. The two types of enzymes were used in an alternating fashion to produce extremely rich sugar polymers of a single length (up to HA20) in a controlled, stepwise fashion without purification of the intermediates. These molecules are the longest, non-block, monodisperse synthetic oligosaccharides hitherto reported. This technology platform is also amenable to the synthesis of medicated- or radioactive oligosaccharides for biomedical testing. Furthermore, these experiments with immobilized mutant enzymes prove both that pmHAS-catalyzed polymerization is non-processive and that a monomer of enzyme is the functional catalytic unit.

Complex carbohydrates play many essential roles in vertebrates, but these molecules are often difficult to isolate or to synthesize in pure form and in large amounts for study. HA, a member of the glycosaminoglycan polysaccharide family that includes heparin and chondroitin, is a repeating β4-glucuronic acid-β3-N-acetylglucosamine polymer that is prevalent in the vertebrate body. HA has numerous essential roles in mammals, including modulation of cellular adhesion, signaling, and motility (1, 2). The polymer is thought to be synthesized initially as a chain of ~10^5–6 Da (10^3–4 sugars), which then degrades during turnover in the body to generate a variety of smaller polymers. Although the mechanisms are not completely understood, it has been observed that the size of the HA polymer dictates its effect on cells. Various cell-surface receptors or binding proteins, termed hyaladherins, are postulated to sense the presence and the size of HA (3–5). Hyaladherins thought to play important roles include CD44 and RHAMM. Multivalent binding and/or multimerization of receptors may potentially occur with large polymers but not with small chains, resulting in the alteration of various signaling events.

Recently, several groups have reported that HA oligosaccharides (~2–3.5 kDa or ~10–17 sugars) have extremely interesting effects on cellular behavior. These small sugar molecules induce a variety of cancer cells to undergo programmed cell death or apoptosis (6, 7). This intriguing finding immediately suggests the use of HA oligosaccharides as an aid to chemotherapy or radiation treatments to reduce tumor load or limit spread (metastasis). HA oligosaccharides were also shown to induce angiogenesis of normal endothelia in several model systems (8–10). Such activity may be of utility for enhancing healing of chronic wounds, ulcers, and burns in the future. For these initial anticancer and neovascularization experiments, mixtures of HA oligosaccharides were isolated from partial enzymatic digests of native high molecular weight HA polymer. Unfortunately, the low yields have limited animal studies, and the size(s) of the most potent molecule(s) in the mixture of sugars is not yet known.

We describe a new chemoenzymatic synthesis methodology for the preparation of desirable monodisperse HA oligosaccharides of any desired size. Our catalysts are recombinant derivatives of an enzyme that catalyzes the polymerization of the HA polysaccharide, the HA synthase. Naturally occurring HA synthases are dual-action glycosyltransferases that add two different sugars, GlcUA and GlcNAc, to form a polymer chain (11, 12), as in Equation 1.

\[
n\text{UDP-GlcUA} + \text{UDP-GlcNAc} \rightarrow 2\text{UDP} + \text{GlcUA-GlcNAc},
\]

In contrast, the vast majority of the other known glycosyltransferases transfer only one type of monosaccharide to an acceptor molecule. Various HA synthases have been identified and molecularly cloned from vertebrates, Gram-positive Groups A and C Streptococcus bacteria and Gram-negative Type A Pasteurella multocida bacteria. The pathogenic bacteria form extracellular coatings of HA, called capsules, that serve roles including camouflaging the microbe from host defenses and potentially hijacking normal host HA recognition or signaling functions (13).

HA synthases will transfer sugars as in Equation 1 at ~1–100 monosaccharides/s in vitro to form 10^4- to 10^6-Da polymer chains. The native forms of the known HA synthases are thus...
unsuitable for production of short defined oligosaccharides; it is too difficult to control enzyme activity precisely and reproducibly through kinetic means (e.g. reducing substrate concentration, temperature, and/or reaction time).

The *Pasteurella* HA synthase, pmHAS, sequentially adds single monosaccharides to the nonreducing terminus of the nascent HA chain (14). This enzyme possesses two independent transferase (Tase) activities in a single polypeptide as shown by mutational analyses of recombinant *Escherichia coli*-derived pmHAS (15, 16). A lesion in one active site leaves the activity in the other unmutated active site relatively unaffected. Therefore, the separate reactions in Equations 2 and 3 are now possible.

\[
\text{UDP-GlcNac} + [\text{GlcUA-GlcNac}] \rightarrow \text{UDP + GlcNac-GlcUA-GlcNac} \tag{Eq. 2}
\]

\[
\text{UDP-GlcUA} + [\text{GlcNac-GlcUA}] \rightarrow \text{UDP + GlcNac-GlcUA-GlcUA} \tag{Eq. 3}
\]

In this report, we harnessed the two component enzyme activities of the HA synthase, a βGlcNac-Tase and a βGlcUA-Tase, for oligosaccharide synthesis in an immobilized enzyme reactor format. This is the first demonstration of the controlled chemoenzymatic synthesis of any glycosaminoglycan oligosaccharide. Furthermore, we have utilized our strategy to synthesize the longest monodisperse oligosaccharides of any composition hitherto reported without block addition or dendritic schemes.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Immobilization**—The soluble, truncated dual-action wild-type pmHAS<sup>703</sup> enzyme was mutagenized with the QuikChange system (Stratagene) to produce a pair of single-action enzymes: the GlcNac-Tase pmHAS<sup>703</sup>[D527N,D529N] and the GlcUA-Tase pmHAS<sup>703</sup>[D247N,D249N]. The mutant enzymes in the bacterial lysates (15) were purified by chromatography on Toyopearl red AF resin (Tosoh) using salt elution (50 mM HEPEs, pH 7.2, 15 mM ethylene glycol with 0–1.5 mM NaCl gradient in 1 h). The fractions containing the mutant protein (~90% pure by SDS-PAGE/Coomassie staining) were immobilized by means of their free amino groups to N-hydroxysuccinimide agarose beads (Sigma). Typically, ~95% of the protein was coupled to the beads after mixing for 4–6 h at 4 °C. Residual activated esters were quenched with TEG buffer (50 mM Tris, pH 7.2, 15 mM ethylene glycol) for 2 h at 4 °C before washing the beads extensively with more TEG. The enzyme reactors (~18 mg of protein in 4 ml of packed beads in a small glass column) were catalytically active for at least 8 months with storage at 4 °C in TEG buffer with 0.05% sodium azide preservative.

**Chemoenzymatic Synthesis**—In the typical oligosaccharide synthesis, 90 μmol of acceptor oligosaccharide and 110–135 μmol (1.2–1.5 equivalents) of UDP-sugar (final ~15 mM) in reaction buffer (TEG buffer plus 17 mM MnCl<sub>2</sub>) were circulated over an enzyme reactor at room temperature. The tetrasaccharide HA4, the starting acceptor for the synthesis of longer oligosaccharides, was generated by exhaustive deglycosylation of streptococcal HA polymer (Sigma) with ovine testicular hyaluronidase Type V (Sigma) and purified by extensive chloroform radiation of streptococcal HA polymer (Sigma) with ovine testicular hyaluronidase Type V (Sigma) and purified by extensive chloroform extraction, ultrafiltration, and gel filtration chromatography on P2 resin (Bio-Rad). For converting HA4 starting material (with a GlcUA at the nonreducing terminus) into the pentasaccharide HA5, the GlcNac from UDP-GlcNac was transferred with the GlcNac-Tase reactor as in Equation 2.

The reactions were monitored by TLC (silica plates developed with 1-butanol/acetic acid/H<sub>2</sub>O, 15:5:1 for HA4 to HA8 or 1:1:1 for HA8 to HA14) and naphthoresorcinol staining (dipped in 0.2% w/v reagent in 96% ethanol, 4% sulfuric acid and followed by heating at 100 °C). Typically, each step of the 90-ml scale reactions was judged to be complete by TLC within 1 or 2 passes of the mixture through the reactor (~5–10 min contact time), but the reaction mixture was further recirculated for a total of 12 passes (~1–2 h) to ensure virtually complete oligosaccharide conversion. After the reaction mixture was harvested, the enzyme reactor was washed with a column volume of TEG buffer, and this washing was added to the reaction mixture. A small amount of MnCl<sub>2</sub> was added to compensate for the volume increase because of the wash step (final ~17 mM).

The next UDP-sugar (in this specific case, UDP-GlcUA) was added to the reaction mixture and then applied to the next reactor (converting HA5 into the hexasaccharide HA6 with immobilized GlcUA-Tase) to perform the reaction of Equation 3. This repetitive synthesis was continued by adding the next appropriate UDP-sugar and switching enzyme reactors. Between each step, the reactors were washed extensively with TEG to remove any residual reaction products retained on the column from the previous step.

At the end of the desired synthesis, the reaction mixtures were lyophilized, and the oligosaccharides were desalted by gel filtration on P4 resin (Bio-Rad) eluted with 0.2 M ammonium formate. The major sugar peak was harvested, and the volatile residual salts were removed by lyophilization from water three times.

HA20 was prepared starting with purified HA13 from the synthesis above. In this synthesis, for proof of principle and for convenience, all of the required UDP-sugars for the complete synthesis were added at the first step.

**Oligosaccharide Analyses**—For MALDI-TOF MS, the matrix solution (50 mg/ml 6-aza-2-thiothymine in 50% acetonitrile, 49.9% water, 0.1% trifluoroacetic acid, 10 mM ammonium citrate) was mixed 1:1 with the samples containing ~0.1 μg/μl oligosaccharide in water, spotted onto the target plate, and vacuum-dried. The samples were analyzed in the negative ion, reflectron mode on a Voyager Elite DE mass spectrometer (acceleration, 20 kV; low mass gate, 500 Da; delayed extraction, 150 ns). The oligosaccharides were also analyzed by 20% polyacrylamide gel electrophoresis with acridine orange staining, as described previously (17).

**RESULTS**

**Generation of Immobilized Enzyme Reactors**—Previously, we produced a soluble form of pmHAS by removing a portion of the carboxyl terminus (residues 704–972), which encodes the putative membrane association region (15). The good solubility and higher yields allow for the purification of active HA synthase. Mutation of a predicted UDP-sugar substrate-binding amino acid motif, DXD (16), in either of the two enzyme active sites into NXX converts the dual-action HA synthase into essentially a single-action glycosyltransferase. Mutation of the A1 domain yields a βGlcUA-Tase, whereas mutation of the A2 domain yields a β3GlcNac-Tase (Fig. 1A). The pmHAS mutants that contained only a single change in a DXD motif (e.g. DXN or NXD) reported earlier were not suitable for preparative scale synthesis because their HA polymerizing activity could be rescued partially by the high UDP-sugar concentration utilized (16). On the other hand, the NXX double mutants were virtu-
the next required UDP-sugar was added, and the reaction mixture was recirculated on the next enzyme reactor. No significant runaway polymerization (i.e., multiple sugar additions on a single reactor) was noted with these NNX mutant enzyme reactors even in the presence of both UDP-sugars. No intermediate purification measures were performed during the 8, 9, or 10 sugar addition steps to produce HA12, HA13, or HA14, respectively. The total synthesis time was about 2 days. Cycling the desalted tridecasaccharide HA13 through seven more enzyme reactor steps created a longer oligosaccharide, the 20-mer HA20.

Fig. 1. Schematic of catalyst generation and dual-enzyme reactor scheme. A, mutagenesis was used to transform the dual-action HA synthase into two single-action catalysts, GlcNAc-transferase (GN-T) and GlcUA-transferase (GA-T). The resulting enzymes were purified and immobilized onto agarose beads. B, a starting acceptor (e.g., tetrasaccharide HA4) was combined with the UDP-GlcNAc precursor and circulated through the GlcNAc-transferase reactor (white circle, GlcNAc, black circle, GlcUA). After coupling, UDP-GlcUA precursor was added to the mixture and circulated through the GlcUA-transferase reactor. This stepwise synthesis can be repeated as desired (dashed line) until the target oligosaccharide size is reached. In this study, a total of 16 addition steps were performed to produce HA20.

DISCUSSION

The recombinant Pasteurella enzyme, designated a Class II HA synthase, has several unique intrinsic properties that allow chemoenzymatic synthesis of desirable short oligosaccharides. In contrast, all the known Class I HA synthases (streptococcal, viral, and vertebral) are relatively unsuitable for this synthetic task. Only pmHAS will elongate in vitro exogenously supplied oligosaccharides (e.g., HA4) (14). The Class I HAS are not as well understood as pmHAS, and the two component sugar transferase activities have not been separated in a practical fashion by molecular genetic means.

In our dual-enzyme reactor strategy, the final size of the oligosaccharide depends on the number of sugar addition steps employed. Substantial benefits of this scheme are that purification of intermediates is not needed after every step and that high stepwise yields are possible by recirculating the reaction mixture over a given enzyme reactor. An added benefit of utilizing pmHAS derivatives for multistep syntheses is that these enzymes are relatively insensitive to the UDP by-product of the transferase reaction (~60% inhibition at 15 mM UDP with 1 mM substrates). In contrast, the class I HAS enzymes are greatly inhibited by relatively low concentrations of UDP (>90% inhibition at 0.5 mM UDP with 1 mM substrates). Indeed, the pmHAS mutants are efficient catalysts, as judged by swift reaction times utilizing only 1.2–1.5 molar eq of UDP-sugar per sugar addition step.

Other methods for the production of HA oligosaccharides have been reported, but they have shortcomings. Chemical synthesis of carbohydrates is difficult because of the demands of stereoselective (i.e., α versus β glycosidic linkages) and regioselective (i.e., only one of the multiple functionalities per sugar ring) coupling of sugars. State of the art synthetic strategies utilize multiple protection/deprotection cycles in a variety of toxic and/or flammable solvents with often less than quantitative yields. In contrast, the enzyme is the “perfect” carbohydrate chemist, performing sugar additions with no side-products in aqueous solution. The largest HA oligosaccharide synthesized by chemical means to date was the hexasaccharide (HA6), containing a methoxynaphthyl group at the reducing terminus (18). Although it is a very nice example, this product is too small for the interesting biological activities described ear-
lier. Another major difficulty of organic synthesis is that the reaction rate for longer oligosaccharide formation is significantly slower than that for shorter sugars. In contrast, the pmHAS-catalyzed reaction rate appears to increase for the longer HA oligosaccharide acceptors (not shown).

The cost of UDP-sugars used in chemoenzymatic synthesis, a once ominous barrier, has been significantly lowered recently. Recombinant permeabilized bacterial systems for the production of kilogram quantities of nucleotide-sugars are becoming available (19). Even though the costs of these fine biochemicals may be higher than simpler organic chemicals and synthetic reagents, the reduced number of reaction steps, the higher overall yields, and the avoidance of toxic materials lowers the overall economic differential between a “standard” and a chemoenzymatic carbohydrate synthesis.

As noted earlier, the initial discovery experiments implying that small HA chains had interesting biological properties utilized mixtures of oligosaccharides prepared by partial digestion of high molecular weight HA polysaccharide with degradative enzymes. Such protocols typically suffer from poor reproducibility and low yields of the target species (e.g., one length in the range of HA10–HA20). Some HA chains are cleaved too much (the limit digest is HA4), resulting in inactive fragments, whereas other HA chains are not sufficiently fragmented, resulting in longer molecules which might possibly counteract the desirable effect of the shorter target HA oligosaccharides. Recently, two groups have reported anion-exchange chromatography purification schemes to separate desirable HA oligosaccharides from partial digests (20, 21). However, in these reports, only HA-derived materials were isolated, and the processes relied on chromatographic separations which may be difficult to scale up.

In addition to being an advance in carbohydrate synthesis, this work also yields basic science knowledge with respect to elucidating the mechanism of GAG synthesis in Pasteurella. Two modes of polymer synthesis are possible: (i) processive (i.e., nascent polymer is retained by the glycosyltransferase until the chain is completed), or (ii) nonprocessive (i.e., nascent polymer is repetitively bound and released by the glycosyltransferase). In our immobilized reactor format, the HA oligosaccharide must be bound transiently to a mutant synthase, extended by one sugar, and released before the oligosaccharide is acted on by a second mutant synthase. The rapidity and the efficiency of our chemoenzymatic synthesis implies that the pmHAS catalyst elongates the HA polymer in a nonprocessive fashion. To form the long HA polysaccharide chains (~10^5 sugars) observed in the Pasteurella bacterial capsule, other proteins or components of the polymer transport apparatus probably assist in vivo with chain retention, because this property does not seem to be an intrinsic characteristic of pmHAS.

Previously, we showed that reactions containing a mixture of two mutant enzymes (i.e., a GlcNAC-Tase and a GlcUA-Tase) formed HA polymers relatively efficiently in comparison to wild type (15, 16). One explanation for this observation is that two pmHAS monomers actually form the active catalytic species, and the two polypeptides cooperate to perform the reaction; a lesion in any one site would be compensated by employing a pair of molecules. However, based on the success of the reactor synthesis, pmHAS must act as a monomer, because the two mutant enzymes are immobilized in separate locations that cannot physically interact.

Our chemoenzymatic route also allows the use of modified acceptor molecules. For example, previously we elongated radiolabeled acceptor (HA4 reduced with borotritide) into longer HA chains (14), but the foreign moiety at the reducing terminus of the HA polymer could instead be a drug or another polymer to enhance therapeutic effect. The pmHAS wild-type enzyme and pmHAS-based transferases described here only transfer authentic HA monosaccharides from UDP-sugars; the C4 epimer analogs (i.e., galactose-based) and UDP-glucose do not substitute (22). In the future, we expect that mutant enzymes suitable for reactors will be developed to catalyze the incorporation of unnatural sugars to form new molecules with altered biological activity and/or useful chemical properties. Overall, our chemoenzymatic synthesis platform opens up a wide spectrum of new biomedical applications. The creation of single molecular entities, HA12 through HA20, is just the tip of the iceberg of possibilities.

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Chemoenzymatic Synthesis of HA

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