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Player Three Has Entered: Discovery of a Novel Biopolymer, Poly-β-1,3-*N*-acetylglucosamine, and Its Companion Glycoside Phosphorylase

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The hunt for unique glycoside phosphorylases identified an enzyme that catalyzes the reversible degradation of a novel *N*-acetylglucosamine biopolymer, acholetin.

While biochemists have access to an ever-expanding database of (meta-)genome sequence data, functional characterization is the rate-limiting step to expand the working repertoire of carbohydrate-active enzymes (CAZymes). In this issue of *ACS Central Science*, the Withers lab addressed these limitations through the design of a substrate-flexible, high-throughput functional screen that enabled the identification of a unique enzyme capable of both synthesizing and degrading a novel β-1,3 linked poly-*N*-acetylglucosamine biopolymer[1] (Figure 1).

Carbohydrates are essential macromolecules which are required for many biological functions across all three domains of life, including their role as structural or energy storage biopolymers and as cellular ligands. The diversity of carbohydrate structures in nature is immense, owing to the various monosaccharide structures and modifications, each of which can be bound in a variety of glycosidic bond linkages. Conversely, the sequence space of CAZymes is equally immense, with tens of thousands of genes annotated from (meta-)genomic sequence data.[2] Because carbohydrates are not synthesized in a template-based manner like peptides, predictions with regard to product formation or biosynthetic pathways are difficult without prior characterization of similar products or enzymes. Despite these challenges, enzymatic synthesis of carbohydrates is an attractive alternative to chemical synthesis, since the use of enzymes results in a more uniform product with fewer catalytic steps.

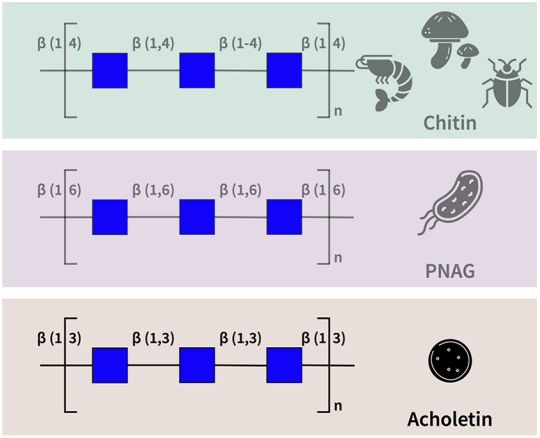

Figure 1. Complete set of β-linked *N*-acetylglucosamine polysaccharides. (top) Poly-β-1,4-*N*-acetylglucosamine (chitin), which is produced by crustaceans, fungi, insects, and mollusks. (middle) Poly-β-1,6-*N*-acetylglucosamine (PNAG), which is produced by select bacteria. (bottom) Poly-β-1,3-*N*-acetylglucosamine (acholetin), which is hypothesized to be produced by the mycoplasma *Acholeplasma laidlawii*.[1] *N*-Acetylglucosamine monosaccharides are represented as blue squares following the symbol nomenclature for glycans.

Continued characterization of the CAZyme sequence space is necessary to identify enzymes with unique degradation or synthesis activities. CAZymes with new activities can be integrated into enzymatic synthesis pathways to produce carbohydrates for use in various chemical industries, including food, specialty chemical, and pharmaceutical industries.

While carbohydrate bioprocessing has focused heavily on cellulose or starch, both abundant polymers built from

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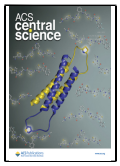

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glucose, homopolymers of *N*-acetylglucosamine (GlcNAc) are also widely available. Homopolymers of GlcNAc include chitin, the second most abundant polysaccharide on Earth, and poly-β-1,6-*N*-acetyglucosamine (PNAG), a soluble polymer incorporated into bacterial biofilms (Figure 1). Distinct classes of CAZymes are required to synthesize, disrupt, or modify poly-*N*-acetylglucosamines. Of interest for the enzymatic synthesis of oligosaccharides are glycosyltransferases (GTs) that form glycosidic bonds between activated monosaccharides and an acceptor sugar[3] and glycoside phosphorylases (GPs) that also form glycosidic bonds through reverse phosphorolysis.[4] However, the availability of low-cost substrates for reverse phosphorolysis makes GPs an attractive catalyst for the enzymatic synthesis of oligosaccharides, in addition to their relative stability when compared to membrane-bound GTs.

In this latest work from the Withers lab,[1] the initial goal was to identify GPs with expanded activities for chitin waste valorization. On the basis of structural and sequence similarity, many GPs of interest for this study are classified into a single glycoside hydrolase (GH) family (GH94).[5] The sequence space for GH family 94 is large, with over 4000 genes identified; however, only 36 enzymes with functional characterization are currently reported in the CAZy database.[2] This largely untapped reservoir of glycoside phosphorylases likely contains unique activities that, if identified, would further increase the attractiveness of this class of CAZymes for enzymatic syntheses of poly- and oligosaccharides.

Previously, the Withers lab had designed a high-throughput GP functional screen that detected phosphorolysis of the glycosidic bond in the presence of inorganic phosphate and an activated β-glucoside.[6] However, for their work reported here, the authors used a functional screening method capable of detecting the formation of glycosidic bonds through reverse phosphorolysis. This chromogenic assay was developed initially to support GH94 enzyme engineering[7] and was further adapted for a rational exploration of the GH94 sequence space,[8] or high-throughput functional screening of metagenomic libraries by the Withers lab.[9] A large benefit to the detection of reverse phosphorolysis is that an unlimited number of substrates can be screened since color development depends on the liberation of a free inorganic phosphate group that is present on every donor monosaccharide.

This flexibility of the high-throughput chromogenic assay allows for any combination of donors and acceptors to be tested with potential GP enzymes. When the authors tested *N*-acetamido sugars, they detected activity from an enzyme that preferentially uses GlcNAc1-P as a donor and multiple *N*-acetylated mono- or oligosaccharides as an acceptor. Surprisingly, while this enzyme, named acholetin phosphorylase (AchP), could use *N*,*N*′-diacetylchitobiose as an acceptor for reverse phosphorolysis, the enzyme was incapable of using this disaccharide for phosphorolysis, suggesting the formation of glycosidic bonds other than β-1,4 linkages. The greater significance of AchP using a broad range of acceptor sugars is the potential to synthesize diverse oligosaccharides that currently have no enzymatic synthesis route or those that require unstable enzyme catalysts. Although the functional screen was high-throughput, NMR was required to identify the glycosidic bond linkages in the AchP synthesized polymer. This new β-1,3 linked polysaccharide was named acholetin, after the source mycoplasma species *Acholeplasma laidlawii* ("achole-") and "-tin" from chitin.

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Aside from identifying a novel biopolymer, one of the more exciting aspects of this paper is that the authors also demonstrated that AchP could be used to synthesize acholetin and other β-1,3 linked oligosaccharides. First, they designed a two-pot enzymatic synthesis method to produce pure acholetin, with a yield of 0.34 g of acholetin per g of GlcNAc (Figure 2). In the first reaction, a bacterial kinase was used to generate GlcNAc1-P, which was precipitated and then used in the second reaction with GlcNAc as an acceptor and AchP to synthesize acholetin. On a smaller scale, the authors also demonstrated that AchP could be used to synthesize a core glycan moiety, by synthesizing the core GlcNAc-β-1,3-GalNAc-α-OR glycan present in mucin O-glycans.

A lack of high-throughput methods to identify unique polysaccharide compositions or glycosidic linkages is a significant bottleneck in glycoscience. Now, with the identification

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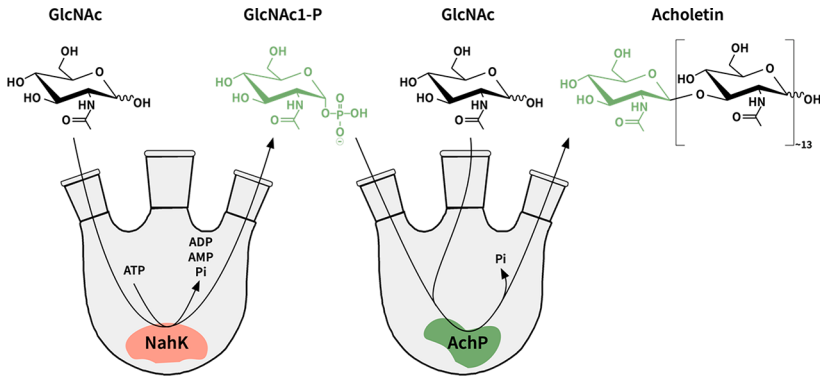

Figure 2. Two-pot synthesis method for the enzymatic production of acholetin. (left) Phosphorylation of *N*-acetylglucosamine (GlcNAc) via an *N*-acetylhexosamine-1-kinase from the bacterium *Bifidobacterium longum* (NahK). (right) Reverse phosphorylation using acholetin phosphorylase (AchP) with *N*-acetylglucosamine-1-P (GlcNAc1-P) as the donor and GlcNAc as the acceptor.

and synthesis of acholetin, this unique biopolymer can be integrated into glycan arrays to identify acholetin-binding proteins, which would represent a new type of glycan-binding protein. Alternatively, catalytic null AchP could be conjugated to a fluorophore to detect β-1,3 linked GlcNAc polysaccharides. Most importantly, the identification of AchP with activity on β-1,3-*N*-acetylglucosamines opens the door for both new enzymatic synthesis pathways and the potential development of *A. laidlawii* antagonists. Novel oligosaccharide synthesis should be possible, given the broad activity of AchP using various *N*-acetamido monosaccharides as donors or acceptors, including the sialic acid precursor *N*-acetylmannosamine. Catalytic efficiency of AchP on these alternative *N*-acetamido sugars could be further improved through enzyme engineering. Lastly, as this study has demonstrated, the untapped potential of the glycoside phosphorylase sequence−function space likely harbors other enzymes with unusual activities that can be harnessed for natural and novel oligosaccharide synthesis.

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