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Highly Efficient Chemoenzymatic Synthesis of β 1–4-Linked Galactosides with Promiscuous Bacterial β 1–4-Galactosyltransferases

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Abstract

An efficient one-pot multienzyme approach has been developed for the synthesis of structural diverse LacNAc, lactose, and their derivatives including those containing negatively charged 6-*O*-sulfated GlcNAc and C2-substituted GlcNAc or Glc analogs. Two bacterial β 1–4-galactosyltransferases, NmLgtB and Hp1–4GalT, exhibits promiscuous and complementary acceptor substrate specificity. The application of these enzymes in the one-pot multienzyme system allows the access to complex disaccharides with diverse structural modifications from monosaccharide derivatives and inexpensive Glc-1-P without using expensive sugar nucleotide. Bacterial carbohydrate-biosynthetic enzymes have been proven as efficient tools in novel synthesis of diverse and complex carbohydrates.

β 1–4-Linked galactosides such as Gal β 1–4GlcNAc (*N*-acetyllactosamine or LacNAc) and Gal β 1–4Glc (lactose or Lac) are common glycan motifs in nature. The former has been found broadly in oligosaccharides, *N*- and *O*-linked glycoproteins, and glycolipids; while the latter has been found in oligosaccharides and glycolipids. Quite often, naturally occurring modifications can take place on the Gal and/or GlcNAc after the formation of oligosaccharides and glycoconjugates,¹ resulting in diverse carbohydrate post-glycosylational modifications (PGMs).² Common natural modifications are 6-*O*-sulfation or 3-*O*-sulfation on the Gal,³ and/or 6-*O*-sulfation on the GlcNAc.⁴ For example, keratan sulfate (KS) [3Gal β 1–4GlcNAc β 1–]_n is a poly-*O*-sulfated polysaccharide with numerous 6-*O*-sulfation on some of the Gal and GlcNAc residues⁵. 6-*O*-Sulfation on Gal or GlcNAc is also common in Lewis x and sialyl Lewis x structures⁶ and they have been shown to affect the binding affinity of the related carbohydrate-binding proteins (e.g. Selectins⁷ and Siglecs⁸). In addition, *N*-sulfation can sometimes take place on the *N*-deacetylated GlcNAc to give *N*-sulfo-glucosamine (GlcNS)-containing structures.⁹ Although enzymatic synthesis of Gal β 1–4GlcNAc has been successfully achieved using β 1–4-galactosyltransferases with or without the generation or regeneration of sugar nucleotide donor UDP-Gal,¹⁰ the investigation of the enzymatic systems in synthesizing structures with modified galactosyltransferase acceptors, especially those with naturally occurring modifications including *N*- and *O*-sulfation, has been limited. Here, we report the promiscuous acceptor substrate specificity of two bacterial β 1–4-galactosyltransferases and their application in efficient one-pot multienzyme chemoenzymatic synthesis of β 1–4-linked galactosides

containing sulfated GlcNAc. The method can be used as a general approach to add a β 1–4-linked galactose residue to the terminal GlcNAc, Glc, and their modified derivatives on oligosaccharides and glycoconjugates.

β 1–4-Galactosyltransferases (β 1–4GalTs) are enzymes that catalyze the transfer of galactose (Gal) from sugar nucleotide UDP-Gal to *N*-acetylglucosamine (GlcNAc) or glucose (Glc) for the formation of β 1–4-galactosyl linkage in LacNAc and lactose. Bovine β 1–4-galactosyltransferase T1 (β 1–4GalT1) is one of the most well studied mammalian glycosyltransferases.¹¹ It uses GlcNAc as an acceptor in the absence of α -lactalbumin (LA), a calcium binding protein. In the presence of α -lactalbumin, the formation of β 1–4GalT1-LA complex or lactose synthase alters the acceptor specificity of β 1–4GalT1 from GlcNAc to glucose.¹² The protein has been applied in the synthesis of LacNAc-containing structures.^{10a} Acceptor substrate specificity assays using derivatives of methyl 2-acetamido-2-deoxy- β -D-glucopyranoside (GlcNAc β OME) in which one of the hydrogen in the CH₃ at the C-2 *N*-acetyl group and the hydrogen in the –OH at different positions of GlcNAc was systematically substituting with a methyl group indicated that 3-methyl ether was a much poorer acceptor substrate but 6-methyl ether can be well tolerated by the enzyme.¹³ The enzyme can tolerate GlcNAc with an α 1–6-linked L-fucose or an α 2–6-linked methyl ester of *N*-acetylneuraminic acid (Neu5Ac) but not free Neu5Ac as acceptors.¹³ GlcNAc derivatives with different C2 *N*-acyl groups from propionyl to dodecanoyl and other *N*-acyl groups have been shown to be tolerable acceptors.¹⁴ *N*-Acetyl-D-muramic acid, the 3-*O*-lactyl ether of GlcNAc, was also shown to be an acceptor for the enzyme.^{13,15}

Many bacteria express β 1–4GalT for the formation of capsular polysaccharides (CPS) and lipopolysaccharides (LPS).¹⁶ In fact, β 1–4GalTs encoded by the *lgtB* gene of *Neisseria meningitidis* and *Neisseria gonorrhoeae* have been cloned and expressed in *Escherichia coli*.^{16a–b} A β 1–4GalT encoded by *HP0826* gene of *Helicobacter pylori* (Hp1–4GalT) has also been identified.^{16c,17} The LgtB from *Neisseria meningitidis* serogroup B strain and the Hp1–4GalT were shown to use *para*-nitrophenyl- β -D-mannopyranoside (Man β pNP) as an acceptor for the formation of Gal β 1–4Man β pNP. The Hp1–4GalT can also catalyze the efficient synthesis of a thio-linked LacNAc derivative Gal β 1-S-4GlcNAc β pNP.¹⁸

Nonetheless, bacterial β 1–4GalTs have not been used in synthesizing LacNAc and lactose derivatives containing naturally existing negatively charged *O*- or *N*-sulfated GlcNAc. Here, we report the cloning of two β 1–4GalTs, from *Neisseria meningitidis* serogroup B strain MC58 genomic DNA (ATCC BAA-335D) (GenBank accession number AE002098) and *Helicobacter pylori* strain J99 genomic DNA (ATCC 700824D) (GenBank accession number AB035971) respectively, and their application in one-pot multienzyme synthesis of LacNAc and Lac derivatives with natural and non-natural modifications.

Neisseria meningitidis β 1–4GalT (NmLgtB) was cloned into pET15b vector and expressed in *Escherichia coli* BL21 (DE3) cells as *N*-terminal His₆-tagged fusion proteins. Under common expression conditions (20°C for 20 h with shaking at 250 rpm in LB medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl), only a low amount (0.7–0.8 mg/L culture) of soluble active enzyme was obtained. The expression level was improved 15-fold to 12.5 mg/L culture under osmotic stress condition using LB medium supplemented with 0.2% glucose and a higher concentration of NaCl (total NaCl concentration was 0.5 M).¹⁹ NmLgtB was not stable when stored at 4°C and was degraded into fragments in 1–2 days. Adding 10% glycerol in the storage buffer and storing at –20°C improved its stability significantly. Under these conditions, the enzyme remained stable with no activity lost for up to 14 months. *Helicobacter pylori* β 1–4GalT (Hp1–4GalT) was initially cloned as N- and C-His₆-tagged fusion proteins but the expression level was low (<0.4 mg/L culture) for either constructs. It was subsequently cloned into pMAL-c4X vector

and expressed in *Escherichia coli* Origami B (DE3) cells as an *N*-maltose binding protein (MBP)-tagged and C-His₆-tagged fusion protein. The C-His₆-tag was introduced during cloning to facilitate easy purification of the protein using Ni²⁺-affinity columns. The resulted Hp1-4GalT was routinely expressed at a level of ~5 mg/L culture in LB medium by incubating at 25°C for 18 h with shaking at 250 rpm after induction with 0.3 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG).

Biochemical characterization of NmLgtB and Hp1-4GalT using 4-methylumbelliferyl-β-D-GlcNAc (GlcNAcβMU) and 4-methylumbelliferyl β-D-Glc (GlcβMU) indicates that while Hp1-4GalT specifically recognizes GlcNAc-terminated glycans as acceptors, NmLgtB can use both GlcNAc- and Glc-terminated glycans as acceptor substrates. Unlike bovine β1-4GalT1, the tolerance of Glc as an acceptor substrate by NmLgtB does not require the presence of α-lactalbumin.

To decrease the cost and increase the efficiency for β1-4GalT-catalyzed preparative-scale synthesis of LacNAc, Lac, and their derivatives with various modifications, a simple one-pot multienzyme system (Scheme 1) was used to generate UDP-Gal (the sugar nucleotide donor of β1-4GalTs) *in situ* from inexpensive glucose-1-phosphate (Glc-1-P). In this system, an *E. coli* K-12 Glc-1-P uridylyltransferase (GalU)²⁰ was used to produce UDP-glucose (UDP-Glc) which was epimerized by an *E. coli* UDP-galactose-4-epimerase (GalE)²¹ to form UDP-Gal which was then used by the NmLgtB or Hp1-4GalT to synthesize disaccharide products. A C-terminal His₆-tagged inorganic pyrophosphatase (PmPpA-His₆) cloned from *Pasteurella multocida* (See Supporting Information) was used to hydrolyze the pyrophosphate formed in GalU-catalyzed reaction to improve the yield of the synthesis.

Both NmLgtB and Hp1-4GalT exhibited substrate promiscuity in preparative-scale synthesis of β1-4-linked galactosides using the one-pot four-enzyme system shown in Scheme 1. More interestingly, they showed complementary acceptor substrate specificities. As shown in Table 1, both NmLgtB and Hp1-4GalT can use free GlcNAc (**1**) and its corresponding β-glycosides (**2** and **4**) as acceptor substrates (64–85% yields). Interestingly, GlcNAc α-glycoside (**3**) was a substrate for NmLgtB, but not for Hp1-4GalT. On the other hand, 6-*O*-sulfated GlcNAc (**5**) and its β-glycoside (**6**) were acceptable substrates for Hp1-4GalT, but not for NmLgtB. In contrast to that reported,¹⁸ however, Glc (**7**) and β-D-glucoside (GlcβOBn **8**) as well as *N*-modified GlcNAc derivatives (**9–11**) including *N*-sulfated glucosamine (**10**) were not acceptors for Hp1-4GalT. On the contrary, they (**7–11**) were perfectly good acceptors for NmLgtB. The Galβ1-4-linked disaccharides (**18–22**) were synthesized in 68–83% yields. The *N*-TFA group and the N₃ group in disaccharide products **20** and **22**, respectively, can be readily converted to a free amine, allowing further derivatization to generate more diverse *N*-substituted GlcNAc analogs.²² Precautions were taken in characterizing the structures of the products using NMR. Different from LgtC (an *Neisseria meningitidis* α1-4-galactosyltransferase)-catalyzed reactions which resulted in altered regio-selectivity when different acceptors were used,²³ all of the glycosidic linkages formed by the NmLgtB and Hp1-4GalT here were confirmed to be β1-4-type.

In summary, NmLgtB can use Glc, both α- and β-linked GlcNAc, and *N*-modified but not 6-*O*-sulfated GlcNAc as acceptors. Quite interestingly, while Hp1-4GalT can not use Glc, α-linked GlcNAc, and *N*-modified GlcNAc as acceptors, it can use 6-*O*-sulfated GlcNAc such as GlcNAc6S (**5**) and GlcNAc6SβProN₃ (**6**) as suitable acceptor substrates. Therefore, NmLgtB and Hp1-4GalT complement each other in efficient synthesis of a broad range of LacNAc, Lac, and their derivatives with charged and non-charged substitutions, many of which are naturally existing glycans with post-glycosylational modification (PGMs).² The promiscuous bacterial enzymes and the one-pot multi-enzyme approach allow the access to diverse structural modified oligosaccharides from monosaccharide derivatives which are

readily accessible by simple chemical synthesis (e.g., 6-*O*-sulfated GlcNAc **5–6** and *N*-sulfated glucosamine **10** were obtained by one-step selective sulfation from the corresponding unprotected glycans, see Supporting Information). We show here once again the power of promiscuous bacterial carbohydrate-biosynthetic enzymes in novel synthesis of diverse and complex carbohydrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

One-pot multienzyme synthesis of β 1-4-linked galactosides with *in situ* generation of UDP-Gal. GalU, glucose-1-phosphate uridylyltransferase (or UDP-glucose pyrophosphorylase); GalE, UDP-galactose C4-epimerase; PpA, *Pasteurella multocida* inorganic pyrophosphatase. NmLgtB, *Neisseria meningitidis* β 1-4-galactosyltransferase; Hp1-4GalT, *Helicobacter pylori* β 1-4-galactosyltransferase.

Table 1

One-pot four-enzyme synthesis of galactosides from Glc-1-P, UTP, and glucose or *N*-acetyl glucosamine derivatives using GalU, PmPpA, GalE, and NmLgtB or Hp1-4GalT.

Acceptors	Products	Yields (%)	
		NmLgtB	Hp1-4GalT
<p>1 GlcNAc</p>	<p>12 Galβ1-4GlcNAc</p>	67
<p>2 GlcNAcβProN₃</p>	<p>13 Galβ1-4GlcNAcβProN₃</p>	85	70
<p>3 GlcNAcαProN₃</p>	<p>14 Galβ1-4GlcNAcαProN₃</p>	51	No Rxn
<p>4 GlcNAcβMU</p>	<p>15 Galβ1-4GlcNAcβMU</p>	68	66

Acceptors	Products	Yields (%)	
		NmLgtB	Hp1-4GalT
<p>5 GlcNAc6S</p>	<p>16 Galβ1-4GlcNAc6S</p>	No Rxn	46
<p>6 GlcNAc6SβProN₃</p>	<p>17 Galβ1-4GlcNAc6SβProN₃</p>	No Rxn	70
<p>7 Glc</p>	<p>18 Galβ1-4Glc</p>	78	No Rxn
<p>8 GlcβOBn</p>	<p>19 Galβ1-4GlcβOBn</p>	83	No Rxn
<p>9 GlcNHTFA</p>	<p>20 Galβ1-4GlcNHTFA</p>	68	No Rxn

Acceptors	Products	Yields (%)	
		NmLgtB	Hp1-4GalT
10 GlcNS	21 Galβ1-4GlcNS	74	No Rxn
11 GlcN ₃	22 Galβ1-4GlcN ₃	81	No Rxn